IMMUNE RESPONSES TO LIPOPOLYSACCHARIDE

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CONTENTS

ABBREVIATIONS

ActD	Actinomycin D
BPI	Bactericidal permeability increasing protein
CARS	Compensatory anti-inflammatory response syndrome
ConA	Concanavalin A
CT	Cocktail
DMEM	Dulbeccos modified eagles medium
DMPC	Dimyristoyl phosphatidyl choline
DMPG	Dimyristoyl phosphatidyl glycerol
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
DTH	Delayed type hypersensitivity
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbant assay
EndoCAb	Endogenous core antibodies
EtBr	Ethidium bromide
EU	Endotoxin units
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
GlcN	Glucosamine
GM-CSF	Granulocyte monocyte colony stimulating factor
HDL	High density lipoprotein
HEK	Human embryonic kidney
HK	Heat killed
HRP	Horse radish peroxidase
HS	Human serum
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRAK	Interleukin-1 receptor associated kinase

IU	International units
Kdo	2-keto-3-deoxyoctonic acid
KLH	Keyhole limpet haemocyanin
LAL	Limulus Amoebocyte Lysate
LBP	Lipopolysaccharide binding protein
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
Mit-C	Mitomycin-C
MOF	Multiple organ failure
MPRL	Microbial Pathogenicity Research Laboratory
MU	Median units
NIC	Nitrocellulose paper
NO	Nitric oxide
NRS	Normal rabbit serum
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCP	Phenol / chloroform / petroleum
PCR	Polymerase chain reaction
PE	Phycoerythrin
PF	Pyrogen free
PMB	Polymyxin B
PSG	Penicillin, Streptomycin, Glutamine
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT	Room temperature
sCD14	Soluble CD14

SDS	Sodium dodecyl sulphate
SI	Stimulation index
SIRS	Systemic inflammatory response syndrome
SOFA	Sepsis - related organ failure assessment
SRBC	Sheep red blood cells
TBS	Tris buffered saline
TE	Trypsin / EDTA
TEA	Triethylamine
TEMED	NNN'N-tetramethyl-1,2-diaminoethane
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TTBS	Tween tris buffered saline

ABSTRACT

For all naturally occurring Gram-negative bacteria, lipopolysaccharide (LPS) is an indispensible component of the outer membrane. As such, LPS has represented a relatively immobile target for the defences of the mammalian innate immune system and, in particular, the cellular LPS receptor Toll-like receptor 4 (TLR-4). Under most circumstances, TLR-4 mediated recognition of LPS provides a strongly protective benefit on the host by initiating a rapid inflammatory response in response to Gram-negative infection. However, systemic inflammation occuring in response to large amounts of LPS can lead to excessive vascular permeability, activation of the clotting cascades, circulatory collapse, shock, multiple organ failure and potentially death. It has been hypothesised that high levels of antibodies towards LPS may provide some protective benefit in patients at risk of endotoxin exposure, and a vaccine based on a liposomal encapsulation of LPS has been proposed as a prophylactic agent to induce such antibodies in high risk groups. This thesis investigates the potential of the vaccine and the role of LPS signaling and T-cell and antibody responses towards LPS in the pathogenesis of sepsis.

The toxicity of LPS is mediated by its capacity to induce pro-inflammatory cytokines in the host. For this reason, the capacity of the vaccine to induce pro-inflammatory cytokines from murine macrophages and human monocytes was assessed. Incorporation of LPS into liposomes was found to reduce its capacity to induce nitric oxide from murine macrophages by approximately 3,200 fold, TNF-α from murine macrophages by approximately 1,000,000 fold and TNF-α from human monocytes by approximately 100,000 fold, suggesting that the vaccine may demonstrate low enough toxicity for use in preliminary clinical trials. The signaling of liposomal LPS was found to be dependent on the serum proteins LBP and CD14.

Two point mutations in the TLR-4 gene were found to be present at a frequency of 10% in a screen of 80 scottish residents. Monocytes derived from individuals heterozygous for the mutations were not found to exhibit any defecit in capacity to recognise LPS of seven different organisms.

T-cell responses to LPS have received very little investigation. The present study investigated LPS induced proliferative responses of T-cells derived from mouse spleen and lymph node and human peripheral blood. Proliferative responses of human peripheral blood mononuclear cells challenged with LPS were seen to peak at seven days and individual responses to particular core types were seen to vary with time. The requirement of the lipid presentation molecule CD1 could not be demonstrated using either CD1 upregulation or antibody blocking experiments. Of 40 healthy volunteers tested, only 50% exhibited T-cells capable of responding to LPS.

In order to investigate the clinical relevance of T-cells reactive to LPS, 27 patients admitted to hospital with inflammatory disease associated with endotoxin exposure were assessed for T-cell and antibody responses to LPS. Patient T-cell responses to most LPS were not significantly lower than those displayed by healthy controls, though responses to *E.coli* R1 LPS were significantly blunted ($p<0.01$). Patient T-cell responses to ConA were also strongly blunted ($p<0.05$), as were levels of IgG antibodies specific for core epitopes of LPS ($p<0.05$). The TLR-4 mutations did not appear at a higher frequency in the patient group than in the healthy population.

The implications of these findings in the context of potential treatments for diseases associated with endotoxin exposure is discussed.

PUBLICATIONS

Erridge C, Stewart J, Bennett-Guerrero E, McIntosh TJ, Poxton IR. The biological activity of a liposomal complete core lipopolysaccharide vaccine. *J. Endotoxin Res.* **8**:39-46 (2002)

Erridge C, Bennett-Guerrero E, Poxton IR. Structure and function of lipopolysaccharides. *Microbes Infect.* **4**:837-851 (2002)

Erridge C, Stewart J, Poxton IR, Currie I, Fearon K. T-cell responses to lipopolysaccharide in gastrointestinal inflammation. Abstract 149 in: *J. Endotoxin Res.* **8**:203 (2002)

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DECLARATION

All of the investigations and procedures presented in this thesis were performed by the author unless otherwise indicated in the text.

CHAPTER 1

INTRODUCTION

1.1 Structure of Lipopolysaccharide

1.1.1 History of LPS structural studies

The history of research into the structure and function of LPS is a complex and intriguing one, beginning more than a hundred years ago. Around the late 1870s, investigators were beginning to realise that some bacteria were capable of excreting toxic compounds. Termed "exotoxins" to describe the fact that they were excreted by the bacteria into their environment, these compounds gained widespread interest when they were shown to be produced by the bacteria responsible for diphtheria, tetanus and botulism (Rietschel et al 1999).

However, it was not until the early 1890s that the first descriptions of endotoxin appear. In 1892 Pfeiffer and Centanni independently described pyrogenic toxins intrinsic to the bacteria *Vibrio cholerae* and *Salmonella typhi*, each capable of eliciting pyrexia and irreversible shock in animal models (reviewed in Rietschel et al 1992). In contrast to the exotoxins previously described, these researchers were able to show that these bacterial products were heat-stable and instead of being actively extruded from the bacteria, remained associated with them. Using the Greek word for within ("endo-") to describe the internal nature of this new class of toxin, the term "endotoxin" was therefore applied to the compound (Pfeiffer 1892).

Efforts to elucidate the chemical nature of the newly discovered endotoxins had to wait another forty years or so, before analytical chemists – working with the relatively impure crude extracts of the time – were able to hypothesise that the heat stable compound consisted of polysaccharide, lipid and protein constituents. However, the protein component described in these early reports could not be detected by Shear *et al* (1943), whose more stringent purifications of *Serratia marcescens* endotoxin allowed him to deduce that this endotoxin

consisted of only lipid and polysaccharide, thereby leading to the coinage of the term lipopolysaccharide.

The idea that protein was not required for the toxic activity of lipopolysaccharide gained further support following the efforts of Luderitz and Westphal in the late 1940s, whose very pure extracts of endotoxin (lacking protein completely), again proved able to retain all biological activity (Westphal et al 1952). By this time, endotoxins from a wide range of different Gram-negative bacteria had been extracted and evidence was beginning to accumulate to suggest that not only were they all nearly identical in basic chemical composition, but each isolated endotoxin led to identical pyrogenic responses when administered to animals (Westphal et al 1977). These findings suggested that endotoxins most likely existed as variants of a common molecule. Insight into this hypothesis came with early attempts at elucidation of the structure of the molecule. The first of these came in the 1950s with the efforts of Luderitz and Westphal, whose acid treatment of LPS preparations yielded the first carbohydrate-free portions of LPS (termed Lipid A), a product which also retained strong biological activity (Westphal et al 1954).

By the 1960s, further evidence came to light to suggest that it was this lipid portion of the molecule that contained the endotoxic activity, as so called 'deep-rough' mutants (lacking the major part of the oligosaccharide portion of the molecule) were found to retain strong biological activity. Other experiments, such as mild alkali removal of fatty acids and protein binding experiments pointed towards the same conclusion (reviewed in Rietschel et al 1994). However, it was not until 1983 that the correct structure of *Salmonella* lipid A was elucidated by Takayama and Raetz in the USA (Takayama et al 1983) and that of *Escherichia coli* by Imoto *et al* in Europe (1983). Following these advances, the chemical synthesis of pure lipid A allowed experimenters to confirm that it was indeed this portion of the molecule that was responsible for

the biological activity of the whole molecule (Galanos et al 1985, Kusumoto et al 1992).

1.1.2 General structure of LPS

Today, with procedures such as NMR, HPLC and epitope studies, the structure of the lipopolysaccharides of many Gram-negative organisms have been characterised, and they have all been seen to conform to a common general architecture (Figure 1.1). Modifications of this basic structure account for the endotoxins of all Gram-negative organisms so far investigated and can be formally classified as containing three separate regions. Lipid A is the highly hydrophobic and endotoxically active part of the molecule. Covalently attached to this is the core section of the molecule which can itself be formally divided into inner and outer core. The inner core is proximal to the lipid A and contains a high proportion of unusual sugars such as 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-manno heptose (Hep). The outer core extends further from the bacterial surface and is more likely to consist of more common sugars such as hexoses and hexosamines. Onto this is attached, in most cases, a polymer of repeating saccharide subunits called the O-polysaccharide, or O-chain, also typically composed of common hexoses. This O-chain is not ubiquitous, however, as it is seen to be truncated or lacking in a number of Gram-negative strains. Bacteria which colonise mucosal surfaces, for example, often express LPS with a truncated non-repeating O-chain known as lipo-oligosaccharide (LOS) (Kosma 1999, Brandtzaeg et al 2001). In addition, certain strains carry mutations in the otherwise well conserved '*rfb*' locus (which contains a selection of genes involved in O-chain synthesis and attachment) and are termed 'Rough mutants' to differentiate them from the wild type 'smooth' strains which express O-chain bearing LPS.

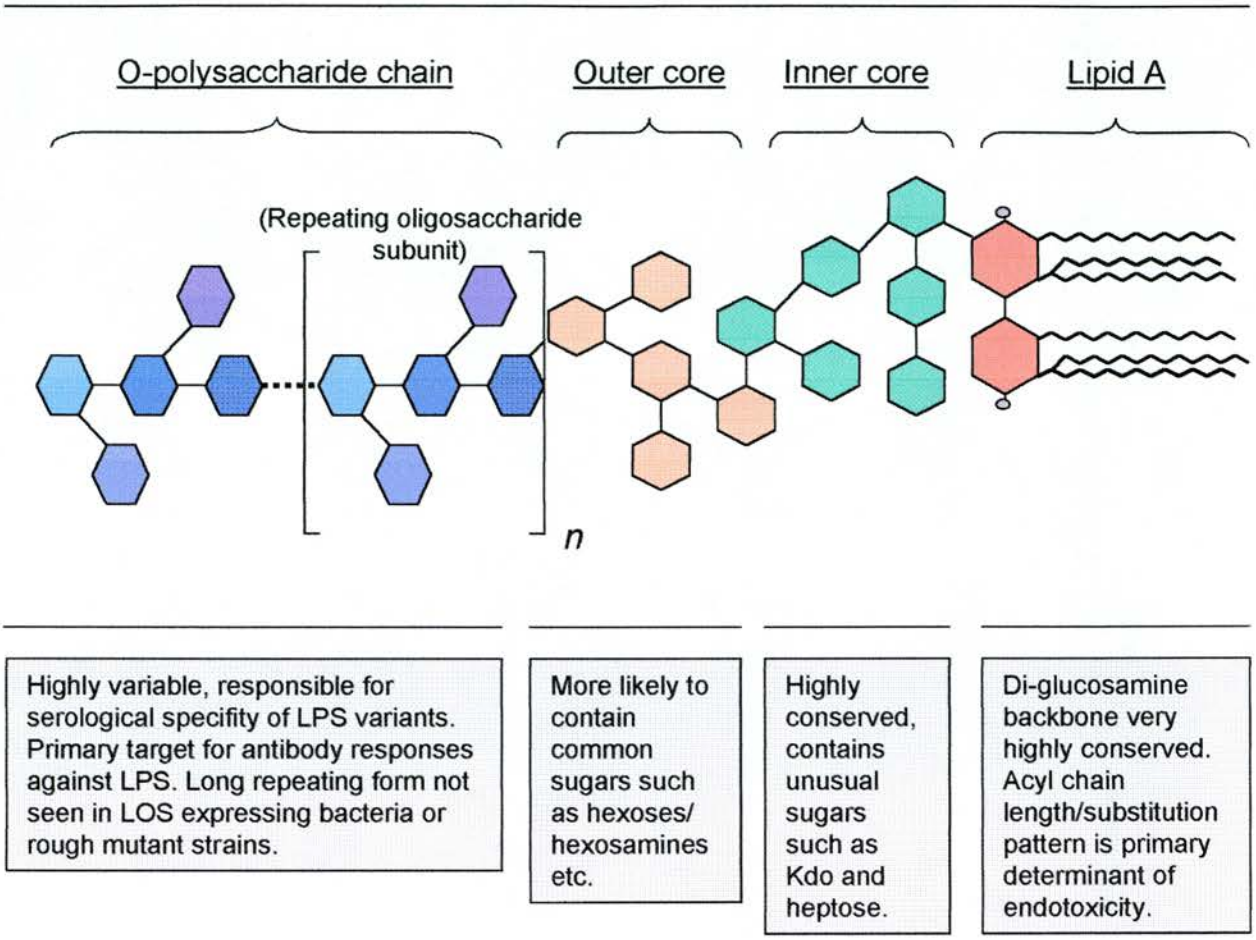

Figure 1.1 General structure of lipopolysaccharides

1.1.3 O-polysaccharide

The repeating units of the O-polysaccharide region consist of between one and eight glycosyl residues and differ between strains by means of the sugars, sequence, chemical linkage, substitution and ring forms utilised. As can be expected, this leads to an almost limitless diversity of O-chain structure and is verified in nature with the observation of hundreds of serotypes for particular Gram-negative species. In addition, the number of units used to complete the chain varies between 0 and ~ 50 and a single organism will produce a wide range of these lengths as a result of incomplete synthesis of the chains. This gives rise to the classical ladder pattern of molecular weights seen when smooth LPS is visualised by SDS-PAGE (Hancock and Poxton 1998).

The O-polysaccharide is also the outermost part of the LPS molecule expressed on bacteria and is therefore the major antigen targeted by host antibody responses. These responses can be highly O-chain specific, and for this reason the O-chain is often also referred to as the O-antigen. As a result of this, serology of O-antigens has become a useful tool in typing strains of organisms and LPS.

The O-polysaccharide region is also recognised by the innate arm of the immune system, playing a role in both the activation and inhibition of complement activation (Joiner et al 1986). For many organisms, eg *Salmonella*, the O-chain is essential for survival in host serum as it prevents penetration of the complement membrane attack complex (Joiner et al 1984).

1.1.4 Core polysaccharide

While the O-polysaccharide region of bacterial endotoxins is extremely variable, the oligosaccharide structures in the core part of the molecule are much more limited, with some regions being highly conserved between different strains and species. As an illustration, while there are now over 160 identified O-serotypes

for *E. coli*, only five unique core structures have so far been determined (R1-R4 and K12) (Rietschel et al 1993). Figure 1.2 shows some examples of the range of core structures seen in a selection of wild type and 'rough' strain Gram-negative bacteria.

The outer core typically consists of common hexose sugars such as glucose, galactose, N-acetyl galactosamine and N-acetyl glucosamine (hence alternatively referred to as the hexose region) and is generally more variable than the inner core.

The inner core is characterised by more unusual sugars, particularly 2-keto-3-deoxyoctonic acid (Kdo) and heptose. The former of these is seen in almost every LPS looked at to date, being α-bound to the carbohydrate backbone of lipid A in every case. The only exceptions yet seen to this rule are *Acinetobacter* and *Burkholderia cepacia* LPS which employ the alternative 2-keto-D-glycero-D-talo-octonic acid (Ko) in its place (Rietschel et al 1994, Cox et al 1995). The bond between the lipid A and this first Kdo residue is normally very acid-labile with moderate pH (<4.4) easily hydrolysing the bond to release the core (and with it the O-antigen) from the lipid A (Morrison et al 1999).

Interestingly, while the O-chain and the majority of the core can be dispensed with in some viable mutants, this Kdo residue is always absolutely required for bacterial viability. Evidence for this is seen in the fact that the smallest saccharide component seen in the LPS of any organism is that of a deep rough mutant of *Haemophilus influenzae* (Helander et al 1988) which has only one Kdo residue attached to its lipid A. Of the naturally-occurring bacteria, the smallest core yet seen is that of *Chlamydia* spp, which consists of only a triplet of Kdo units (Brade et al 1987) (Figure 1.2). This primary Kdo residue always has a negatively charged substituent on it (such as phosphate, typically at position 4) and it appears that this is also essential for viability.

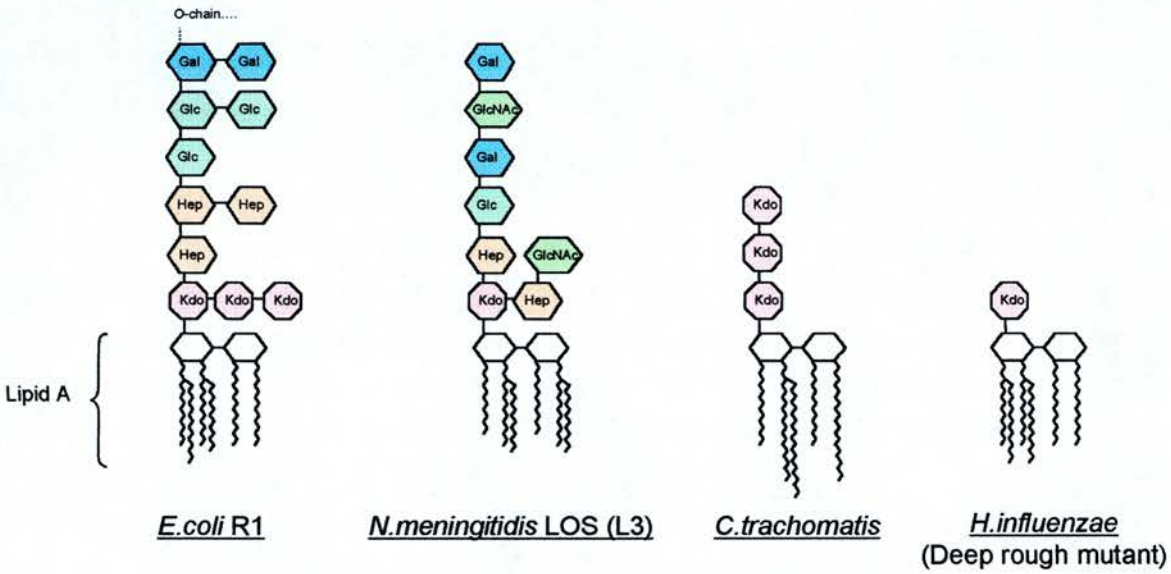

Figure 1.2 Core structures of selected lipopolysaccharides

The fact, therefore, that Kdo synthesis is absolutely essential for Gram-negative bacterial viability presents rational drug designers with a tantalising opportunity. Drugs targeting the Kdo synthesis enzymes would show not only a wide specificity towards Gram-negative organisms but would also represent an entirely new class of antibiotics (Hammond et al 1987).

A second unusual sugar seen in the inner core is heptose, typically in the L-glycero-D-manno configuration, but also occasionally as D-glycero- D-manno heptose. *Yersinia* strains, for example, use this isomer exclusively (Venezia et al 1985).

Both inner and outer core sugar residues can be substituted with charged groups like phosphate, pyrophosphate, 2-aminoethylphosphate and 2-aminoethylpyrophosphate. In the inner core it is thought that these substituents maintain a close association with the Ca^{2+} and Mg^{2+} ions that are required for membrane structure and function (Rietschel et al 1994).

Since the inner core is reasonably well conserved (the structure is identical, for example, for all strains of *E. coli* and *Salmonella*) this provides the possibility of an epitope capable of inducing an antibody response of broad enough specificity to be cross reactive antigenically with the LPS of many different strains of Gram-negative bacteria. This thought has already been applied in the consideration of a new vaccine to elicit such widely cross reactive antibodies in human sera (Bennett-Guerrero et al 2000, Erridge et al 2002b) and in the production of a monoclonal antibody capable of binding to a wide range of *E. coli* and *Salmonella* LPSs (Di Padova et al 1993).

Finally, it should be pointed out that while the endotoxically active part of the molecule is lipid A, the nature and number of attached saccharide residues and

substituents do have considerable impact on modulating this activity. The mechanisms for this will be discussed later.

1.1.5 Lipid A

In 1985 it was shown that synthetic lipid A exhibits identical biological properties to *E. coli* lipid A. (Galanos et al 1985), thus proving that lipid A is the part of the molecule responsible for its endotoxic activity. Structurally, lipid A is typically composed of a β- D-GlcN-(1-6)-α- D -GlcN disaccharide carrying two phosphoryl groups (at positions 1 and 4'). Both phosphates can be further substituted with groups such as phosphate, ethanolamine, ethanolamine phosphate, ethanolamine diphosphate, GlcN, 4-amino-4-deoxy-L-arabino-pyranose and D-arabino-furanose.

To this structure are attached up to 4 acyl chains by ester or amide linkage. These chains can then in turn be substituted by further fatty acids to provide LPS molecules with up to 7 acyl substituents, which vary quite considerably between species in nature, number, length, order and saturation (Kosma 1999, Venezia et al 1985, Goldberg et al 1996). These can be attached to the lipid A either symmetrically (3+3, *e.g. Neisseria meningitidis*) (Brandtzaeg 2001) or asymmetrically (4+2, *e.g. E. coli*) (Alexander et al 2001). Unsaturated fatty acids are rarely seen in lipid A, but have been reported in *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* LPS and in Enterobacteriaceae grown at low temperature. It is possible though not yet proven that this helps the bacteria regulate membrane fluidity.

The polysaccharide portion of the molecule is almost invariably linked (see above) to the lipid A by means of a linking Kdo residue at position 6'. This portion of the molecule therefore constitutes the most highly conserved region seen between LPS molecules: a phosphorylated di-glucosamine backbone attached to at least one Kdo (or Ko) residue. (*Campylobacter jejuni* LPS is the

notable exception to this rule, in which GlcN II is replaced by the related molecule 2,3-diamino-2,3-dideoxy- D-glucose (GlcN3N) – Moran et al 1997).

Some examples of lipid As from selected human pathogens and chemically synthesised derivatives are depicted in Figure 1.3. These are the chemically-elucidated complete structures of lipid A, though it should be borne in mind that like O-chain, extracted lipid A often possesses a considerable proportion of partial or incomplete structures as a result of incomplete biosynthesis. In addition to this intrinsic variation, it can be seen that the number, nature and lengths of the acyl chains varies quite substantially between the LPS of different organisms. Of particular significance may be the observation that lipid As substituted with only five acyl chains – such as those of *C.trachomatis*, *B.fragilis*, *P.gingivalis* or *P.aeruginosa* – tend to be the ones expressing low biological activity *in vitro*.

1.2 Innate immune responses to LPS

The innate immune recognition of molecules associated with pathogenic organisms has evolved over the millenia to provide the host with an immediate and tailored defence against microbial invasion. In the main, the targets recognised by these defences tend to be molecules which are absolutely required by the pathogen for virulence or survival but which are also highly conserved among species and strains of those organisms. In the case of Gram-negative bacteria, the most potent and well studied activator of the innate immune response is LPS.

Decades before the chemical structure of LPS was identified, its biological effects in humans or animal models were well established. Administration of even small amounts of LPS was seen to result in fever, tachycardia, shock and potentially multiple organ failure (Redl et al 1993, Martich et al 1993, Glauser et

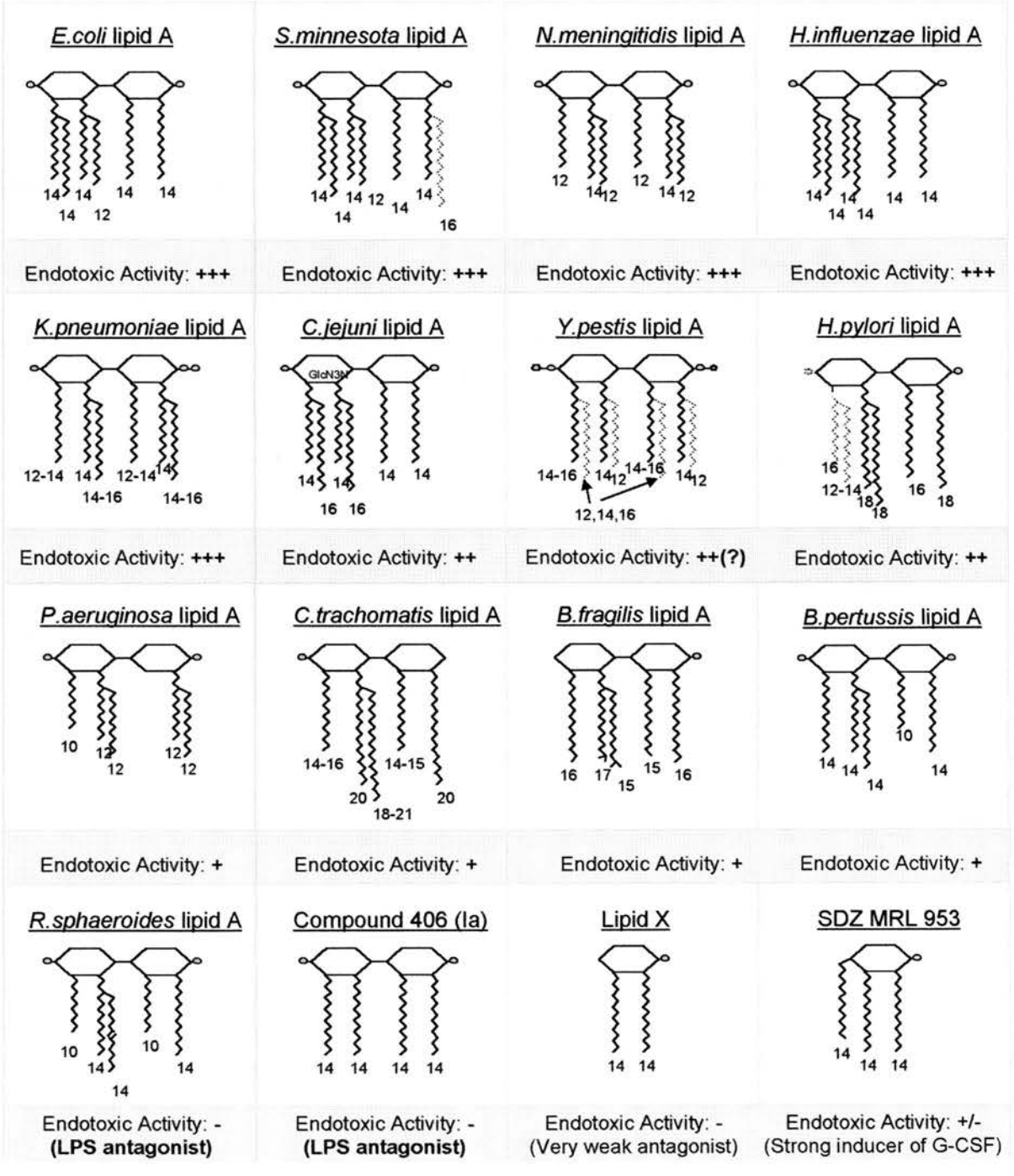

Figure 1.3 Lipid A structures of selected lipopolysaccharides and synthetic analogues

al 1991, Suffredini et al 1989, Galanos et al 1993, Michie et al 1988, Taveira daSilva et al 1993). This reaction towards LPS is now established to be a direct response of the cells of the immune system to the lipid A component of the molecule. Exactly how the recognition of this component of LPS is achieved has proven difficult to decipher, but the last five years or so have shown considerable advancement in our understanding of the receptors and signalling pathways for LPS.

1.2.1 Cellular responses to LPS

LPS in the host is dealt with at the cellular level via two distinct pathways, as summarised in Figure 1.4. In some cell types and via some receptors, it can be merely removed from the circulation and degraded without any stimulatory action. Such non-stimulatory pathways exist in macrophages and Kupffer cells and are present to remove and detoxify LPS from either the local environment or the circulation (Wright 1991). In the main, it is thought that these activities are mediated by the surface proteins CD18 and scavenger receptor, though it should also be pointed out that some lipid-binding proteins in the serum are also capable of binding LPS for direction towards the non-stimulatory degradative pathway (Kohn and Kung 1995).

On the other hand, specific receptors exist on other cell types which are exquisitely sensitive to the presence of LPS and engagement of these receptors results in the eliciting of a strongly pro-inflammatory reaction. Cells of the myeloid lineage in particular are capable of responding to even minute concentrations of LPS with near immediate release of pro-inflammatory cytokines (Martich et al 1993, Pugin et al 1993, Mayeux et al 1997). The role of these in the host is to provide rapid recognition of the presence of Gram-negative bacteria – a situation which requires immediate and effective action by the immune system.

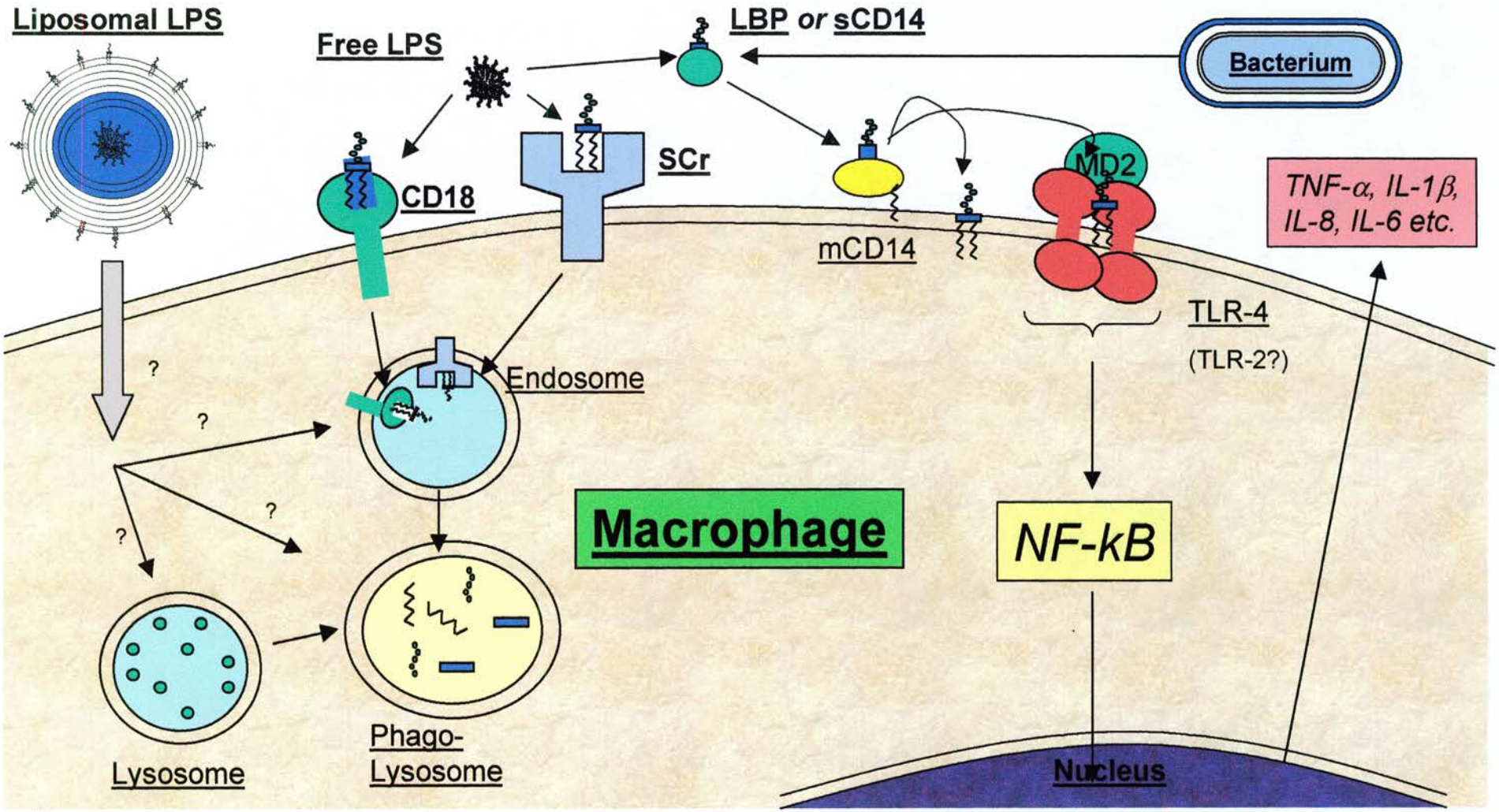

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Figure 1.4 Signalling and non-signalling pathways of lipopolysaccharide

Mediators released by cells such as monocytes in response to recognition of LPS include the pro-inflammatory cytokines IL-1β, TNF-α, IL-8, IL-6, IL-12, IL-15, IL-18; the colony stimulating factors M-CSF, G-CSF and GM-CSF; lipid-derived mediators such as platelet activating factor, prostaglandin E_2 and thromboxane; and several reactive oxygen intermediates such as the superoxide anion (O_2^-) and hydroxyl radicals (Alexander et al 2001). The function of these molecules are to (i) induce local inflammation (ii) recruit further immune cells to the environment (iii) activate nearby cells to take action and (iv) kill the microbes directly. Further to these cellular responses, it should also be pointed out that activation of both the classical and alternative complement pathways can be achieved via direct molecular recognition of lipid A (Galanos et al 1971).

In most situations involving a local point of infection, this rapid and inherent response to LPS is extremely effective, with the invaders being swiftly dealt with as a result of the aggressive inflammatory reaction mounted against them. However, while this reaction is obviously of great benefit to the host under normal conditions, it can become life-threatening when expressed systemically. As will be discussed later, if the body is exposed systemically to LPS the resulting overwhelming pro-inflammatory reaction can have deadly consequences.

1.2.2 Role of LBP and CD14 in LPS recognition

The typical conformation of naturally-occurring LPS presents something of a problem for the receptors of the immune system. Naturally-occurring endotoxin exists as large conglomerates of LPS monomers, either as part of intact bacterial membranes, as part of membrane fragments, or as part of free-floating micelles of LPS shed from dying or dividing bacteria (Manthey and Vogel 1994, Mainous et al 1991). Within these structures, the hydrophobic lipid A components of the LPS monomers are effectively shielded from recognition by

mammalian receptors, so a mechanism is required by the mammalian immune system to retreive monomeric LPS from these hydrophobic domains for presentation to the various cellular receptors for LPS. Without such a mechanism, free LPS monomers would be unable to cross the large thermodynamic barrier of the aqueous environment between receptor and target.

As such, mammalian serum contains many proteins dedicated to the shuttling of lipids from cell to cell. Of these, investigators have shown that while some are able to slightly inhibit or upregulate LPS signalling others appear crucial for LPS recognition.

High density lipoprotein (HDL), for example, is typically associated with so-called reverse cholesterol transport, but it is also a major binding protein for circulating LPS (Wurfel et al 1995) serving to reduce the amount of LPS available in the serum for activation of inflammatory cells. Bactericidal permeability increasing protein (BPI) similarly reduces activity of avilable LPS by binding it and effectively sequestering it away from the serum lipoproteins that stimulate LPS mediated activation – an activity which can also be demonstrated *in vitro* (Weiss et al 1992).

Sharing approximately 45% amino acid homolgy with BPI, however, LPS binding protein (LBP) performs the opposite role. LBP acts to shuttle LPS monomers from bacterial membranes and other multimeric LPS structures to the lipoprotein required for sensitive recognition of LPS, CD14 (Hailman et al 1994, Shumann et al 1990). LBP knockout mice are only slightly hyporesponsive to LPS however (Wurfel et al 1997), suggesting that it does not play an entirely essential role in sensitive recognition of LPS.

CD14, on the other hand has been shown to be absolutely required for sensitive recognition of LPS (Wright et al 1990). CD14 is expressed primarily on monocytes and neutrophils as a phosphatidyl-inositol anchored cell surface membrane protein. However, enzymic cleavage of the lipid anchor results in the release of an LPS binding soluble CD14 (sCD14) into the serum. Here, sCD14 acts to shuttle monomeric LPS from bacterial membranes and from LBP to the cell surface of other cell types. It has been shown capable of inserting LPS monomers directly into the plasma membrane of host cells (a function it shares with LBP) (Wurfel et al 1997). Cells from CD14 knockout mice show greatly blunted responses to LPS, though it should be pointed out that they can still respond to very high concentrations of LPS (Haziot et al 1996), suggesting that presence of CD14 is not an absolutely necessary component of the cellular LPS receptor.

1.2.3 Receptors for LPS

While it had been recognised for some time that CD14 was required by cells to confer sensitive responses to LPS, it was reasoned that it could not function as the sole receptor molecule for LPS. Being a phosphatidyl-inositol anchored protein, CD14 lacks a transmembrane domain with which to facilitate intracellular signalling. In addition to this, it was noted that CD14 deficient human cells could still respond to LPS even in serum-free conditions lacking soluble CD14 (Akashi et al 2000).

This mystery remained unsolved until 1999, when the *lps* gene responsible for the LPS hyporesponsive phenotype of C3H/HeJ mouse was at last identified and shown to code for a mutated version of the protein TLR-4 (Toll-like receptor, so called because of its homology with the *Drosophila* Toll protein) (Qureshi et al 1999, Poltorak et al 1998). Examination of the TLR-4 knockout mouse generated the same year revealed a phenotype similar to that of the C3H/HeJ

mouse, and thus the function of TLR-4 as an essential component of the LPS receptor was confirmed (Hoshino et al 1999).

However, it was soon realised that this was not yet the complete picture. Transfection of some TLR-4 deficient cell lines with TLR-4 was not in itself sufficient to render them sensitive to LPS (Qureshi et al 1999, Hoshino et al 1999). Thus at least one further molecule was seen to be required to complete the LPS receptor complex. This was discovered shortly afterwards to be the protein MD-2 when it was seen that co-transfection of these cells with MD-2 restored LPS sensitivity (Shimazu et al 1999). An extracellular protein lacking a transmembrane domain, MD-2 remains attached to the cell surface via its interaction with TLR-4.

However, this hypothesis of the TLR-4/MD-2 complex being the sole signalling receptor for LPS went through a period of some controversy. Many groups were able to show responses to their LPS preparations that were independent of TLR-4. Moreover, it appeared that another Toll like receptor, TLR-2, was critical in these responses. Only stringent purification protocols capable of providing LPS extracts free from protein contaminants were finally able to reveal that the majority of LPSs signal solely through TLR4, while the TLR-2 signalling previously seen appeared to be due to lipoprotein contamination of those preparations (Shimazu et al 1999).

Nevertheless, two very interesting exceptions to this rule have recently been described. The LPSs from *Porphyromonas gingivalis* and *Leptospira interrogans* have both been shown to be recognised not by TLR-4, but by TLR-2 (Hirschfield et al 2001, Werts et al 2001). Interestingly, the five branched acyl chains and mono-phosphorylated lipid-A of *P. gingivalis* differs substantially from the typical hexa-acyl di-phosporylated *E.coli* lipid-A like template recognised by TLR-4 (Kumada et al 1995). While the structure of *Leptospira interrogans* lipid-A has

yet to be determined, it is tempting to speculate that it may share structural features with *P. gingivalis* lipid A. Further, it is interesting to note that LPS extracted from *Bacteroides fragilis* has already been shown capable of activating TLR-4 mutant mouse (C3H/HeJ) cells and shares a lipid A of striking resemblance (see Figure 1.3) to that of *P. gingivalis* lipid A (Delahooke et al 1996). Determining the chemical structures of lipid A from other organisms with LPS capable of stimulating TLR-4 deficient cells (such as that of *Treponema denticola* - Rosen et al 1990) may well shed some light on the matter of which domains or particular three dimensional arrangements of LPS are differentiated by TLR-2 and TLR-4.

1.2.4 Mechanisms for recognition of LPS by TLR-4/MD-2

Much evidence has recently emerged to shed some light on the mechanisms by which the TLR-4/MD-2 complex might recognise LPS. Firstly, it appears that the main role of LPS binding protein (LBP) in the serum is to deliver LPS to either soluble or membrane bound CD14 (Hailman et al 1994). Then, as it is now becoming clearer that CD14 does not play an essential role in the TLR-4/MD-2 receptor complex (see above), it seems that the likely role of CD14 is to catalyse insertion of LPS molecules into either the plasma membrane (a function of CD14 demonstrated by Wurfel *et al* 1997) or directly into the receptor complex. In support of this hypothesis, Ulevitch and co-workers have used photo-activated cross-linking to reveal LPS in close proximity with both TLR-4 and MD-2, but only when in the presence of CD14 (da Silva Correia et al 2001). In addition, it has been shown that LPS is removed from mCD14 before it is internalised (Vasselon 1999).

As for which of TLR-4 and MD-2 participate in the discrimination of active lipid As, evidence is now emerging to suggest a role for both of these proteins in this function. For example, it has been shown that expression of murine TLR-4 in human cells renders them sensitive to the partial structure lipid IVa, whereas

expression of human TLR-4 in unresponsive C3H/HeJ mouse cells does not (Lien et al 2000, Poltorak et al 2000). On the other hand, Akashi *et al* have shown that while transfection of Ba/F3 cells with murine TLR-4 and MD-2 allows them to respond to the partial structure lipid IVa, replacement of the murine MD-2 with the human equivalent removes this ability (Akashi et al 2001). In addition, this chimaeric complex was seen to confer an antagonistic effect on lipid IVa, while it remains an agonist in cells expressing the purely murine complex (Akashi et al 2001). MD-2 has also been shown to have a direct effect on the discrimination of another TLR-4 ligand, the anti-mitotic compound Taxol. Signalling in response to Taxol has been shown to be ablated following the mutation of a single glycine residue in MD-2, whereas LPS signalling remains unaffected (Kawasaki et al 2001). Further, distinct differences have been observed in the abilities of human and mouse MD-2 molecules to recognise Taxol (Kawasaki et al 2000).

Nevertheless, it seems clear that dimerisation of TLR-4 appears to be the key to activation of the complex, as chimaeric constructs of TLR-4/CD4 have been shown to dimerise at the cytosolic domains and induce cellular activation (Medzhitov et al 1997). However, the question of how LPS is able to induce dimerisation of TLR-4 remains as yet unclear. Depicted in Figure 1.5 are three models which suggest how particular types of LPS may result in dimerisation of TLR4 monomers and ultimately cellular activation.

In the first model (Figure 1.5a), LPS is recognised within the complex of MD-2 and TLR-4. MD-2 has been shown to have a high affinity for LPS (Kd = 65 nM, similar to that of CD14 - Viriyakosul et al, 2001) and may function to hold LPS molecules in place for TLR-4 mediated pattern recognition. Alternatively, the incorporation of the LPS into either molecule may induce a conformational change which then promotes dimerisation of TLR-4. In this model, LPS molecules are delivered directy to the complex by CD14.

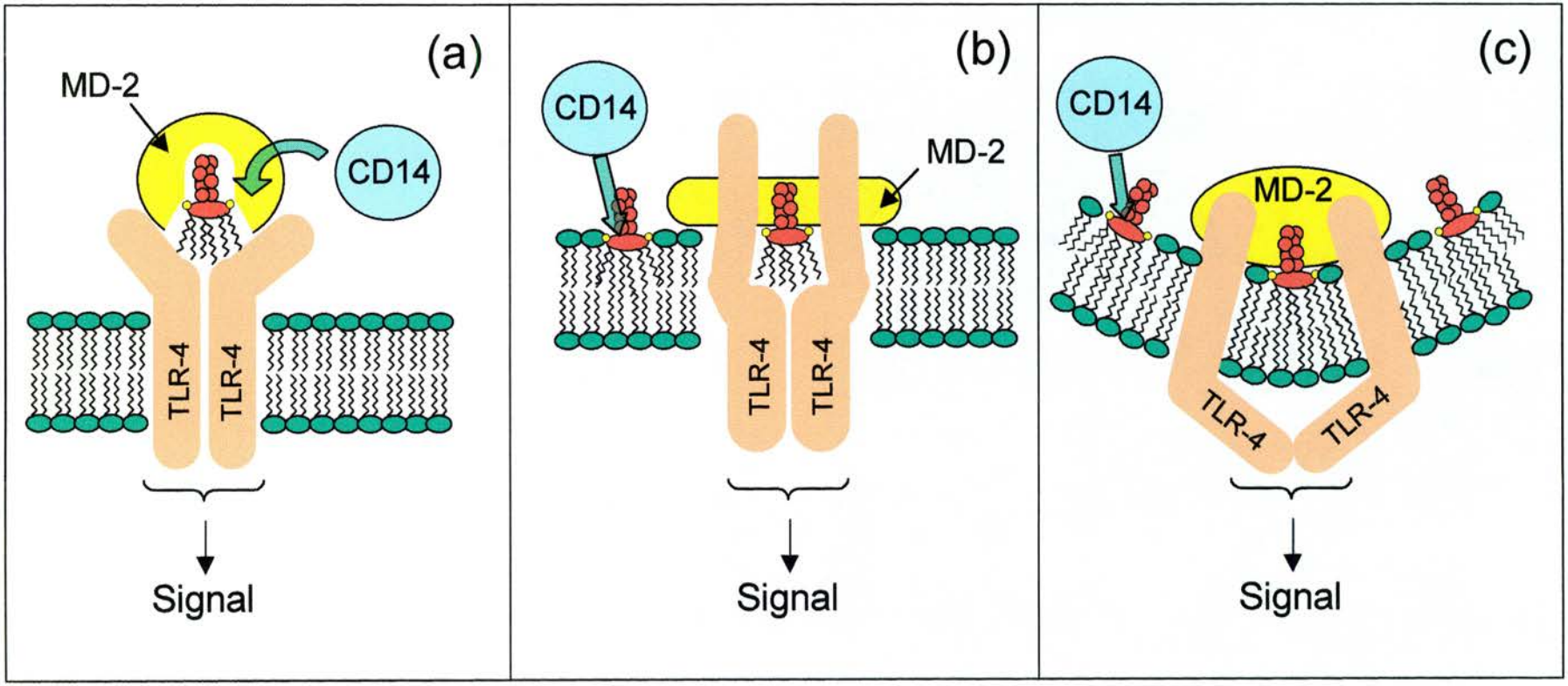


Figure 1.5 Proposed models for mechanism of TLR4/MD2 mediated recognition of LPS
(a) CD14 transfers LPS to the TLR4/MD2 complex (b) CD14 catalyses insertion of LPS into the plasma membrane - TLR4 recognises acyl component, MD2 recognises polar component (c) CD14 catalyses insertion of LPS into the plasma membrane - TLR4/MD2 complex recognises resulting alteration of membrane architecture.

Alternatively, since it has been shown that CD14 rapidly catalyses the insertion of LPS into the plasma membrane of host cells (Wurfel et al 1997), it may be possible that this is where TLR-4 and MD-2 encounter the LPS (Figure 1.5b). In this scenario, the shape of the lipid A is recognised by the transmembrane domains of TLR-4, while the (exposed) charged head group may be recognised by MD-2.

Finally, it is possible that disruptions to the physical properties of the host cell membrane occurring as a result of the incorporation of the unusual lipid-A are recognised by TLR-4/MD-2, as first suggested by Samuel Wright (Wright 1999). Evidence for this include the striking correlation between lipid A phase transition temperature (Tc) and toxicity (Seydel et al 1993) and the fact that the membrane active agent chlorpromazine is able to render cells sensitive to the inactive lipid IVa (Thiemblemont et al 1998). The effects of lipid A antagonists can also be explained in this model by functioning to counteract the physical changes in the membrane caused by lipid A. In this model, local membrane architecture is altered in such a way as to dimerise the TLR subunits with the assistance of MD-2 resulting in signalling (Figure 1.5c).

1.2.5 Intracellular signalling of the TLR-4/MD-2 complex

The TLR-4 signalling pathway shows striking similarities to that employed by the IL-1 receptor (IL-1R). In both pathways, the adaptor protein MyD88 (myeloid differentiation factor 88) is thought to be recruited to activated (i.e. dimerised) IL-1R or TLR-4 cytosolic domains where it catalyses the activation of serine/threonine kinases of the IRAK family. These then act through TNF-receptor associated factor 6 (TRAF-6) to promote both MAP kinase cascades and the NF-κB inducing cascade (reviewed in Underhill et al 2002).

Recently a protein has also been identified that interacts both with the cytoplasmic tail of the IL1 receptor and with IRAKs. Termed Tollip this protein

has also been shown to co-precipitate with TLR-2 and TLR-4 (Bulut et al 2001), and is also likely to play a role in recruiting IRAKs to the dimerised Toll receptors (as depicted in Figure 1.6).

Evidence is accumulating, however, to suggest that LPS signalling may proceed along alternative parallel pathways. For example, certain cellular responses towards LPS do not require MyD88, and while the activation of NF-κB in response to LPS is merely delayed in MyD88 deficient cells, it is completely abolished in TLR-4 deficient cells (Kawei 1999). Another recently discovered protein TIRAP (or MAL) has been shown to interact with both TLR-4 and IRAK-2 and on over-expression also activates NF-κB (Fitzgerald et al 2001, Horng et al 2001). It is therefore possible that this adaptor protein is responsible for at least one MyD88-independent signalling pathway, though its full function remains to be determined.

1.2.6 Roles of other Toll like receptors

At least ten Toll-like receptors have now been seen to be expressed in human cells, and appear to be used to recognise particular "pathogen associated molecular patterns" (PAMPs) contained in highly conserved molecules. Table 1.1 shows some of the molecules recently shown to be recognised by the various Toll-like receptors. Interestingly, it has recently been shown that recognition of some molecules is achieved via heterodimers of different Toll-like receptors. For example, TLR-2 has been shown to co-operate with at least TLR-6, and possibly other TLRs in the recognition of molecules (Ozinsky et al 2000). Indeed, it has even been shown that dimerisation of TLR-2 as a homodimer does not result in cellular activation (Ozinsky et al 2000). If this co-operation between receptor molecules turns out to be more widespread among the TLRs, this may have significance for the number and type of pathogen associated molecules recognised.

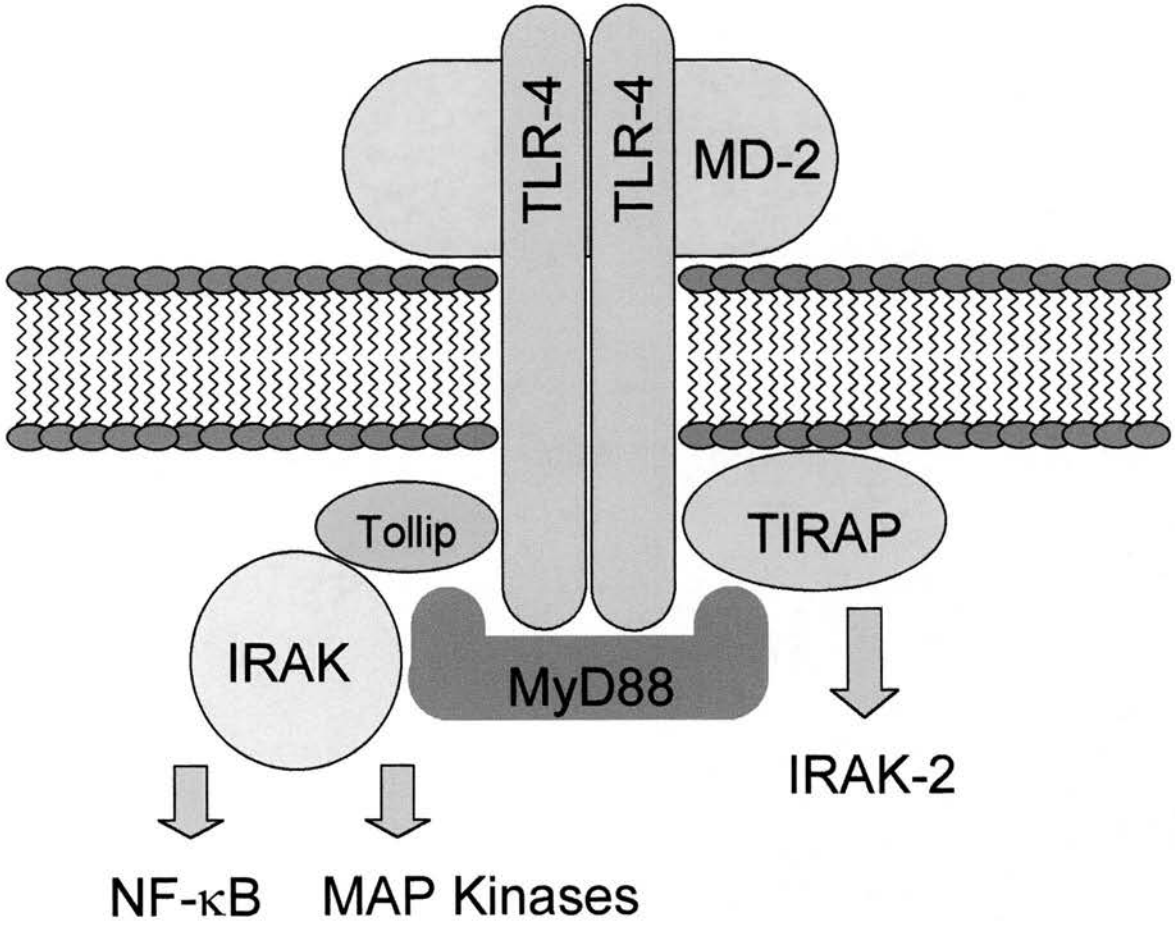


Figure 1.6 Proposed components of TLR4/MD2 intracellular signalling complex
Tollip - Toll interacting protein, TIRAP - Toll/Interleukin-1 receptor associated protein, IRAK - Interleukin-1 receptor associated kinase, MAP kinase - mitogen activated protein kinase, NF-κB - nuclear factor κB.

However, of more interest is the fact that the signalling via different TLRs can result in differential gene expression. For example, macrophages stimulated via TLR-2 can be triggered to release TNF-α, IL-1β and MIP-2, whereas macrophages stimulated via TLR-4 are triggered to release all of these cytokines, but also IL-12 p40, IFN-γ and macrophage chemotactic protein 5 (Hirschfield et al 2001). If this observation transpires to be relevant for all the TLRs, or even possible pairings of the TLRs, it may turn out that the innate immune response is capable of producing very specialised actions tailored to each individual pathogen.

Table 1.1: Identified ligands of the Toll-like receptors

Toll receptor	Ligand
TLR-1	*Unknown*
TLR-2 (+TLR-6 / unidentified TLR)	Peptidoglycan, bacterial lipoproteins, *Leptospira* and *Porphyromonas* LPS, *S. cerevisiae* zymosan, *T.cruzii* GPI anchor, *M. tuberculosis* lipoarabinomannan and phosphatidylinositol dimannoside
TLR-3	Double stranded (ds) RNA
TLR-4	Most LPS, Taxol, HSP-60 (human and chlamydial), fibronectin EDA, F-protein of respiratory syncitial virus
TLR-5	Flagellin
TLR-6	*Unknown*
TLR-7	*Unknown*
TLR-8	*Unknown*
TLR-9	CpG DNA
TLR-10	*Unknown*

1.2.7 Signaling of liposomal LPS

The idea of using LPS as either an adjuvant or a vaccine has over the years received a great deal of interest (Cryz et al 1987, Barclay et al 1986, Cryz et al 1991, Bhattercharjee et al 1996, McCabe et al 1972, McCabe et al 1977,

Bhattacharjee et al 1999). That the O-chain and core epitopes of LPS are highly immunoreactive and capable of eliciting both specific and broadly cross-reactive antibody responses are undoubted (Ziegler et al 1979, Teng et al 1985, Ziegler et al 1982, Scott and Barclay 1987, Di Padova et al 1993) – only the inherent toxicity of LPS (as discussed above) precludes its widespread use as an agent for vaccination. For this reason, ways of reducing the biological activity of LPS by avoiding the TLR-mediated pro-inflammatory response have deservedly received much attention.

Encouraged by the reduction in activity seen when LPS was allowed to integrate into the plasma membrane of sheep red blood cells (Galanos et al 1971, Pollack et al 1989), people sought other ways of reducing the activity of LPS. Dijkstra was the first to show that incorporation of LPS into liposomal vesicles was able to mask the toxic lipid A and reduce its biological activity by up to a thousand-fold (Dijkstra et al 1987).

Since that initial observation, considerable interest has been shown in liposomal LPS, and each study has shown a significant reduction in toxicity. For example, studies of *S. minnesota* rough (Re) and wild-type LPS showed that incorporation of LPS into liposomes according to the method of Dijkstra *et al* (1988) results in the reduction of IL-1β secretion from RAW 264.7 macrophages by between 100- and 1000- fold, (Dijkstra et al 1987) and TNF-α release of 100-1000- fold (Dijkstra et al 1988b). A reduction in activity as measured by limulus amoebocyte lysate assay has been observed to be in the order of between 100- and 1000- fold (Dijkstra et al 1988a), and actinomycin D-sensitized mice have been shown to have a 10-fold reduction in lethality when challenged with liposomal lipid A compared with native lipid A (Dijkstra et al 1989). Finally, encapsulation of *Neisseria meningitidis* LPS within liposomes has been shown to reduce the pyrogenicity of LPS in rabbits by up to 1000-fold (Petrov et al

1992). These findings offer promise for the concept of a lipopolysaccharide-based vaccine.

1.3 Antibody responses to LPS

1.3.1 History

While the innate immune response to LPS is focused on the lipid A component of the molecule, it remains for the adaptive immune response to react to the polysaccharide component. As long ago as the 1960s, it was shown by Luderitz and Westphal that strong variation exists between the O-polysaccarides of various bacterial strains and that the antibodies raised against these O-chains are rarely cross-reactive. The early nineteen-seventies saw several groups demonstrating that antibodies raised against particular O-chains could protect animals from infection or endotoxaemia from bacteria of the same O-antigen. However, the early studies of Braude and McCabe and others demonstrated that antibodies raised against the core-structures of LPS could cross-react with LPS from heterologous strains and these antibodies appeared capable of protecting animals from challenge with heterologous Gram-negative bacteria (Braude et al 1972, McCabe et al 1972).

Encouraged by these findings, these researchers and others raced to produce therapeutic preparations of these antibodies for use in human clinical trials. The resulting monoclonals and hyperimmune sera tested revealed ambiguous results in preliminary trials, leading to a great deal of controversy and ultimately the removal of one such drug (HA1A) from the market (Baumgartner 1994).

This section will now discuss the role of anti-LPS antibodies in protection from LPS-mediated pathologies and review the events that led to the removal of one

such preparation from the marketplace and the prospects for a better designed vaccine approach.

1.3.2 Antibodies to O-polysaccharide

The O-polysaccharide of LPS demonstrates incredible diversity. More than a hundred different sugars have so far been found to contribute to their structure, and each of these may combine with several others to create unique O-polysaccharide subunits. Further to this, these sugars can of course be joined in any order and vary considerably in their use of glycosidic linkages. Thus the possibility for variation of the O-antigen tertiary structure is immense, and by direct correlation so must the potential range of antibodies directed against these O-chains. However, since the O-chain is seen to adopt an irregular spiral shape, it is probable that particular O-chains display a particular series of repeating epitopes and one or more terminal epitopes that provide immunodominant motifs for antibody formation and recognition (Barclay 1990).

Around the early seventies, several groups were beginning to show that antibodies directed against the polysaccharide (O-antigen) of LPS were able to protect against infection and shock in animal models when challenged with bacteria of homologous serotype (Kirkland et al 1984, Barclay et al 1986, Cross et al 1988). Unsurprisingly, however, given the vast diversity seen in the O-polysacchirides of different strains and species of Gram-negative bacteria, these antibodies could not be shown to protect against challenge with heterologous O-serotypes (Kirkland et al 1984, Barclay et al 1986, Cross et al 1988).

More recently, direct evidence has emerged to show that O-polysaccharide directed anibodies can decrease the toxicity of LPS. For example, Burd *et al* (1993) have shown that an IgG3 monoclonal antibody directed against the O-chain of *E.coli* O111 is able to enhance uptake of that LPS by macrophages and

also reduces resultant TNF secretion of these cells. These authors concluded that Fc-mediated uptake was bypassing the mechanism of TNF secretion.

In terms of clinical benefit, however, it was realised that to provide humoral immunity against the LPSs from a wide range of LPSs, O-polysaccharide-directed antibodies would not work as they were simply too strain-specific. Instead, a much more widely cross-reactive antibody would have to be found capable of neutralising the LPSs from many different strains and species.

1.3.3 Antibodies to core epitopes

In contrast to O-polysaccharide, the core glycolipid structures of LPS are relatively conserved between different serotypes and species of Gram-negative bacteria (Jansson et al 1981, Westphal et al 1983, Barclay 1990). All strains of *E.coli*, for example, express one of only five distinct core types (R1-4 and K12) (Jansson et al 1981). For this reason, it was suggested by Chedid as far back as 1968 that a small number of antibodies directed against this core structure of the LPS molecule might act as 'masterkeys', being able to react with the LPS from an extensive range of strains (Chedid et al 1968).

Certainly, the structural properties of core LPS lend themselves to this hypothesis. Different core types tend to share more structural features between themselves than the highly diverse O-chains, and the relatively well conserved sugars, phosphoryl residues and other charged substituents (such as ethanolamine) of the inner core therefore represent particularly good targets for potentially cross-reacting antibodies. On the other hand, it is clear that while some antibodies raised against core epitopes may react well with both rough and smooth LPS (Di Padova et al 1993), others may fail to react with complete S-LPS, as evidenced by Western blots (DeJongh-Leuvenink et al 1986, Pollack et al 1989), presumably representing those antibodies directed against core epitopes masked by O-polysaccharide substitution.

Nevertheless, the cross-protection hypothesis gained further credence with the demonstration in 1972 by both Braude and McCabe that antibodies directed against inner core epitopes were able to protect against bacterial challenge from heterologous strains in animal models (Braude et al 1972, McCabe et al 1972).

As most pathogenic bacteria typically express smooth LPS, however, these findings may at first seem contra-intuitive. However, since it has been shown that the LPS extracted from S-strain bacteria contains considerable amounts of unsubstituted core (R-LPS) without polysaccharide (Westphal et al 1983), it is thought that these epitopes are expressed on the surface of all wild type bacteria. Thus, covering a considerable proportion of the bacterial surface, antibodies directed against these core epitopes should be able to bind to all bacteria expressing either that core type or a closely related one, regardless of the O-chain substitution in that strain. Figure 1.7 summarises the potential binding sites for antibodies to LPS molecules.

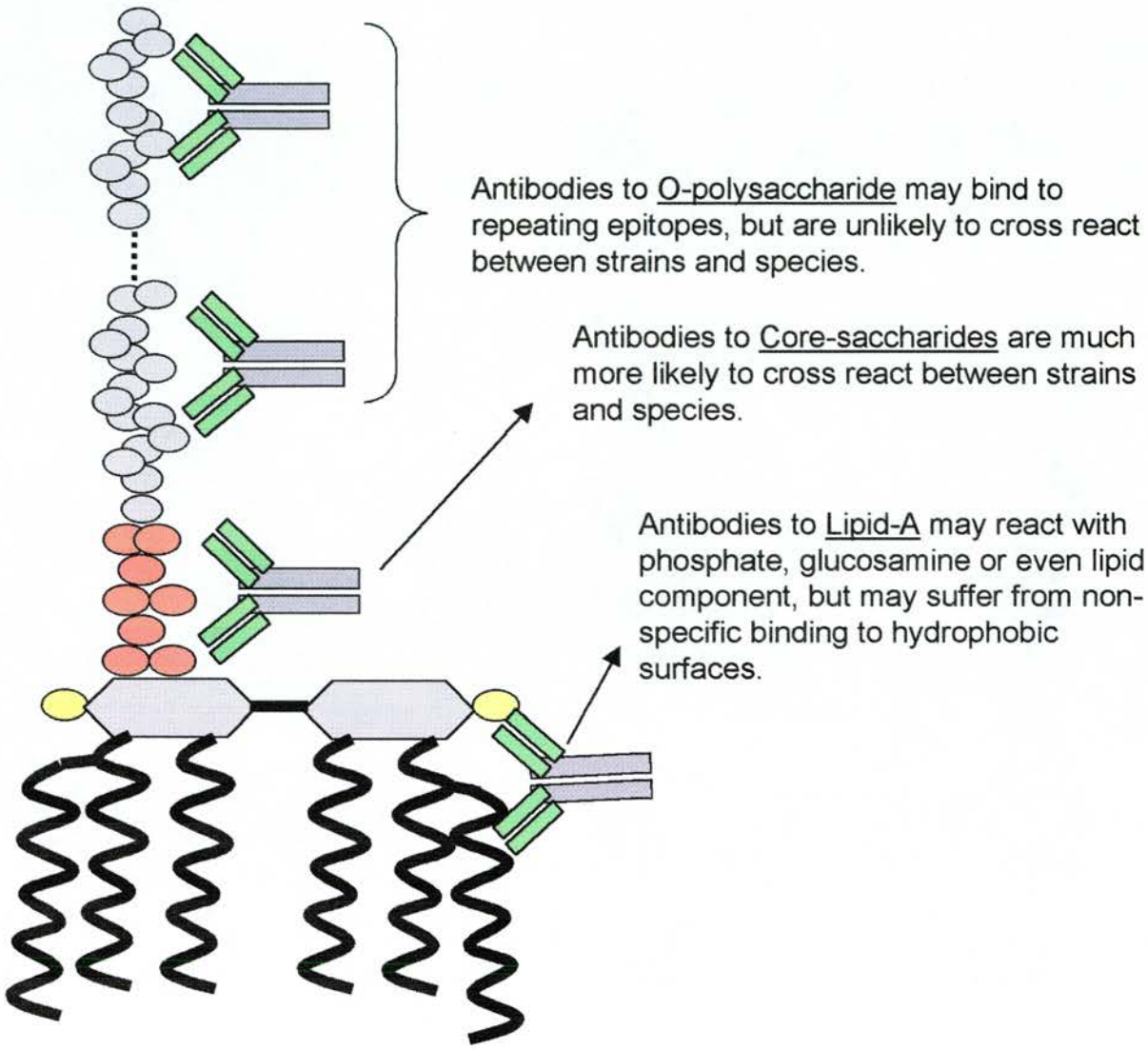


Figure 1.7 Potential binding sites for antibodies directed against LPS

1.3.4 Core antibodies in sepsis

Antibodies to core saccharides are not merely laboratory artefacts. Many studies have been able to show that antibodies directed against core epitopes occur naturally and exist in the sera of healthy subjects (Barclay et al 1987, Scott et al 1987, Barclay et al 1989). Indeed it was the opinion of Barclay (1990) that "there is now significant evidence to suggest that natural LPS-cross-reactive IgG antibodies to common shared epitope structures of the Gram-negative endotoxin core emerge as a result of continuous exposure to endotoxins, and that these antibodies may constitute a significant component of the host defence against Gram-negative bacteraemia and endotoxaemia."

Measurement of anti-core antibodies has been assisted by the development of an ELISA based on four rough mutant LPS (approximately Rb-Rc) from the organisms *Escherichia coli* K12, *Pseudomonas aeruginosa* PAC611, *Klebsiella pneumoniae* KaM10b and *Salmonella typhimurium* 878. (Barclay et al 1987). Developed to provide a measurement of the titre of cross-reacting anti-core antibodies in human subjects, this test has been used as the basis of the investigations into the role played by anti-core antibodies in various disease processes.

For example, anti-core antibodies were seen to be depleted during episodes of endotoxaemia, then restored in 2 of 3 patients studied with septic shock (Barclay et al 1989). The same protocol has been used to measure LPS antibody kinetics in pancreatitis patients (Windsor et al 1993), surgical patients with gut mucosal hypoperfusion (Mythen et al 1993), sepsis patients (Barclay et al 1989) and patients with haemolytic uraemic syndrome (Heydermann et al 1994), with each of these studies suggesting that depletion of anti-core LPS antibodies can occur in these patient groups. Further, recent studies of anti-LPS antibodies in the sera of pre- and post-surgery patients have revealed a correlation between high endogenous levels of anti-core LPS antibodies and protection from adverse

post-operative outcome (Freeman et al 1985, Bennett-Guerrero et al 1997, Hamilton-Davies et al 1997, Bennett-Guerrero et al 2001).

Taken together, the data from these studies provide further support for the hypothesis that augmentation of levels of anti-core antibodies in patients may provide some protective benefit in disease processes involving either Gram-negative infection or endotoxaemia.

1.3.5 Clinical trials of anti-LPS antibodies

The succesful application of anti-LPS antibodies to animal models of disease in the laboratory led to a strong interest among researchers in the clinical application of such antibodies. As it is extremely difficult to condense the sequence of events and reports that accompanied these efforts, I have listed the majority of these in Table 1.2 (at the end of this chapter) with the more significant ones being reviewed as follows.

In the early seventies, two groups in particular focussed their interests on the clinical applications of passive transfer of LPS-reactive antibodies. McCabe and colleagues measured the titres of naturally-occurring antibodies to *S. minnesota* Re chemotype LPS in cases of Gram-negative bacteraemia. They found that high titres of anti-Re-LPS were associated with a decreased frequency of shock or fatal outcome, whereas no protection was observed from O-specific antibodies (McCabe et al 1972). Subsequent animal studies performed by the same group appeared to show that antibodies raised against *E.coli* J5 (Rc chemotype) LPS or *S. minnesota* Re LPS could protect those animals against heterologous Gram-negative bacterial infection (McCabe et al 1977).

Then in 1979, Ziegler and colleagues were able to show that antibodies raised against certain R-mutant LPS (in particular that of *E.coli* J5) were capable of protecting animals against challenge from both infection and endotoxin from

heterologous smooth bacteria (Ziegler et al 1979). This demonstration led the same group to attempt in 1982 the therapeutic passive transfer of plasma from donors immunised with J5 to patients with sepsis (Ziegler et al 1982). Their initial interpretation of the data was that a beneficial effect was observed on treated patients. However, later interpretation of the same data (Baumgartner 1994) questioned the validity of their assumptions. In particular, concerns were voiced over the lack of evaluation of the severity of illnesses among the patients at entry other than by listing underlying diseases, and the unusually high mortality shown in the control group of patients with septic shock (77%). The following year saw the first openly negative result for passive transfer of J5 human antiserum when McCutchan *et al* were unable to show any protective benefit from the administration of a single prophylactic dose of human antiserum to J5 in Gram-negative-infected patients with prolonged neutropenia (McCutchan et al 1983).

Nevertheless, two years later Baumgartner et al attempted prophylactic passive transfer of antibody raised in human volunteers immunised with *E.coli* J5 to high-risk surgery patients (Baumgartner et al 1985). In this small preliminary study, some degree of protection was observed against septic shock, raising hopes that larger trials of the antibody would demonstrate the same benefit (Baumgartner et al 1985). However subsequent re-analysis of the results of this initial study were later questioned by the same authors (Baumgartner et al 1987) – their main concerns being the small sample size and the fact that while the one-tailed statistical test was significant, the more appropriate two-tailed test was not.

In the same year (1985), the study of Teng et al showed protection against Gram-negative bacteraemia and endotoxaemia with the administration of human monoclonal IgM antibodies (Teng et al 1985). However, later analysts suggested that these data should be interpreted with caution, as they were performed with

ascites or hybridoma fluids, not with purified antibodies (Baumgartner 1994). To add to the controversy, Calandra *et al* wrote in 1988 that they had been unable to demonstrate a benefit when anti-J5 intravenous immune globulin was used in the treatment of Gram-negative septic shock (Calandra et al 1988).

Further concerned at the lack of unequivocal evidence as to the efficacy of anti-LPS antibodies in the treatment of these conditions, Baumgartner's group attempted to repeat some of the earlier animal studies. They reported their inability to reproduce the results of the early J5 antiserum cross-protection studies in animals (Baumgartner et al 1990, Baumgartner 1991).

Meanwhile, two large pharmaceutical companies had purchased the rights to two monoclonals derived from *E. coli* J5 immunisations – termed E5 and HA1A. Human IgMs directed against lipid A, both E5 and HA1A had shown initially promising results in animal models (Teng et al 1985, Young et al 1989). Eager to be the first to the marketplace, Centocor rushed HA1A into clinical trials, the first of which showed ambiguous results but which was nevertheless granted approval for sale by a panel of experts in the USA in September 1991. HA1A became available for sale in the USA and in Europe very shortly afterwards.

However, doubts were soon raised as to the validity of the conclusions from this initial trial when Baumgartner showed that HA1A was able to bind non-specifically to hydrophobic surfaces such as Gram-negative and Gram-positive bacterial membranes, yeasts, high density lipoproteins and cardiolipin but only very poorly to its purported ligand, lipid A (Baumgartner et al 1991).

Concerned by this and the inability of others to repeat animal protection studies, a number of authors wrote letters of concern to the New England Journal of Medicine demanding a re-examination of Centocor's initial study (Various authors, 1991). Following this, the US FDA used the freedom of information act

to obtain Centocor's initial data for re-analysis and deduced that the null hypothesis had not been convincingly rejected (Wenzel 1992). However, since it appeared that when compared with the placebo group, a significant decrease in the mortality of 200 patients with Gram-negative bacteraemia in shock at study entry had been seen (Ziegler et al 1991, Warren et al 1992), a second trial was demanded. Interim analysis of the results from this second trial, however, revealed a slightly higher mortality in the HA1A-treated cohort than in the controls and the trial was aborted (Baumgartner 1994). Concurrently, HA1A was also immediately removed from the marketplace.

In parallel, the competing anti lipid-A monoclonal E5 had also entered preliminary clincal trials. No change in mortality in patients with documented Gram-negative infection was observed in the first trial, though it seemed clear that in one subgroup (patients without shock at entry) a decrease in mortality was observed. For this reason a second study of E5 was called for. This second study looked at over 800 patients with sepsis syndrome without shock, but failed to show any reduction in mortality (Wenzel et al 1991), and investigations into the clinical applications of E5 were also ceased.

The failure of these two antibodies has left pharmaceutical companies and researchers an interesting question. Were these failures due to the poor binding/cross reactivity of these antibodies? Or have the protective benefits of anti-LPS antibodies been overstated?

Certainly, some researchers are of the opinion that neither of these two antibodies had been shown capable of binding well enough to their range of target ligands (Baumgertner 1994). One particular criticism has been the assertion that immunoglobulin of the IgM subclass has a natural tendency to bind non-specifically to hydrophobic substances. This binding, they suggest,

may have been misinterpreted as cross-reactivity to lipid A or rough LPS in preliminary investigations of these antibodies (Heumann et al 1991).

If these assertions are assumed to be correct, then it remains to be seen whether or not a broadly cross-reactive monoclonal antibody is capable of providing a benefit in patients at risk of Gram-negative infection or endotoxaemia. One potentially significant newly developed monoclonal antibody, WN1-222.5 has been reported to react with all smooth and most rough LPS from all *E.coli* and *Salmonella* strains so far investigated (Di Padova et al 1993). Immunoblots using this antibody reveal a clear ladder pattern, indicating that it reacts with both substituted and unsubstituted LPS. In bioassays too, this antibody has been shown able to inhibit LPS activity in LAL assay, and TNF and IL-6 release from LPS-challenged murine peritoneal macrophages. In animal models it has been shown to neutralise the pyrogenic response to LPS in rabbits and protects D-galactosamine-treated mice from the lethal effects of both *E.coli* and *S.abortus-equi*. However it has not been shown able to bind LPS from *Enterobacter*, *Klebsiella*, *Serratia*, *Proteus* or *Pseudomonas* spp. Nevertheless, such broad reactivity between strains suggests that administration of a mixture of similarly derived antibodies may provide the broad spectrum of reactivity required to provide protection against infections in the natural setting.

1.3.6 Vaccination for LPS?

An active immunisation strategy has several theoretical advantages over monoclonal antibody-based treatments. Of these, the most pertinent are low production costs and the ability of the vaccine to generate high titre endogenous antibodies in recipients. Indeed it could be argued that such a prophylactic treatment for high risk patients would be more desirable than treatment with passive immunisation, as it is difficult clinically to detect the early signs of endotoxaemia in time to effectively intervene with a specific anti-endotoxin therapy (Cross et al 1994, Opal 1995).

However, since administration of even as little as several nanograms of LPS is enough to induce symptoms of systemic inflammation (Suffredini et al 1989, Martich et al 1993), ways must be sought to reduce the biological activity of LPS-based vaccines for them to work effectively. Further, since it is generally considered that purified LPS is poorly immunogenic, strategies must be devised to enhance the immunogenicity of the LPS used in the vaccine.

In murine systems, one approach is to add pure LPS to sheep red blood cells. Since the lipid A is incorporated into the plasma membrane of the red blood cells, the toxicity of the LPS is reduced while the preparation remains immunogenic (Pollack et al 1989, Heumann et al 1991). However, in terms of human vaccination, approaches to date have typically involved covalent attachment of various O-polysaccharides to carrier proteins. Since these preparations contain no lipid A, the toxicity of the LPS is effectively neutralised and the protein remains available for MHC-mediated induction of T-dependent help. Examples of this approach include *P.aeruginosa* or *E.coli* O18 O-chain linked to toxin A of *P. aeruginosa* (Cryz et al 1987, Schiff et al 1993) and *E.coli* O18 O-chain linked to cholera toxin (Cryz et al 1991). Covalent attachment of *E.coli* J5 LPS to the outer membrane protein from group B meningococcus has resulted in a vaccine that has been shown to be highly immunogenic and well tolerated in experimental animal systems (Bhattercharjee et al 1996). Polycolonal antibodies produced in response to this vaccine appear able to bind to LPS from a number of Gram-negative bacteria and protect animals from lethal endotoxin challenge and infection with Gram-negative bacteria (Bhattercharjee et al 1994).

In summary, these findings suggest that induction of high levels of broadly cross-reactive antibodies towards LPS may potentially be more easily and/or effectively achieved using a vaccine strategy than by the passive immunotherapy alternatives previously attempted.

Table 1.2: History to date of development of LPS antibody therapies

Author (Year)	Outcome*	Study
McCabe (1972)	–	High titres of anti-*S.minnesota* Re-LPS in septic patients is associated with a decreased frequency of shock or fatal outcome, whereas no protection was observed from O-specific antibodies.
McCabe (1977)	Positive	Animal studies show antibodies raised against *E.coli* J5 LPS or *S. minnesota* Re LPS protect those animals against heterologous Gram-negative bacterial infection.
Ziegler (1979)	Positive	Anti- rough mutant LPS (especially against *E.coli* J5) were capable of protecting animals against challenge from both infection and endotoxin of heterologous smooth bacteria.
Ziegler (1982)	Positive?	Therapeutic passive transfer of plasma from donors immunised with J5 had a beneficial effect on patients with sepsis. Later analysis (Baumgartner 1994) revealed that this study was limited because there was no evaluation of the severity of illnesses among the patients at entry other than by listing underlying diseases. Also an unusually high percentage of mortality was observed in the control group of patients with septic shock (77%).
Pollack (1983)	–	High levels of antibodies to endotoxin correlate with improved outcome in sepsis patients.
McCutchan (1983)	Negative	Prophylactic use of single dose human antiserum to J5 in Gram-negative infected patients with prolonged neutropenia is ineffective.
Baumgartner (1985)	Positive?	Prophylactic passive transfer of antibody raised in human volunteers immunised with *E.coli* J5 protects patients from Gram-negative shock and death in surgical patients. Criticisms are that only a small number of patients developed septic shock and the statistics were only significant using the one-tailed statistical test, while the more appropriate two-tailed test was not significant.

Table 1.2 (continued)

Author (Year)	Outcome*	Study
Teng (1985)	Positive?	Protection against Gram-negative bacteraemia and endotoxaemia with human monoclonal anti-LPS IgM antibodies. Should be interpreted with caution however, as they were performed with ascites or hybridoma fluids and not with purified antibodies.
Baumgartner (1987)	Negative	Baumgartner's reanalysis of their own 1985 findings – casts doubt on validity of their earlier deductions.
Barclay (1987)	–	Barclay's EndoCAb ELISA screens blood donors for high levels of cross reactive antibodies for preparation of hyperimmune intravenous IgG.
Calandra (1988)	Negative	No benefit seen when anti-J5 intravenous immune globulin used in treatment of Gram-negative septic shock.
Barclay (1989)	–	Anti-core antibodies seen to be depleted during episodes of endotoxaemia, then restored in 2/3 patients studied with septic shock.
Baumgartner (1990)	Negative	Baumgartner's group failed to reproduce the results of the early J5 antiserum cross-protection studies in animals.
Baumgartner (1991a)	Negative	Unable to reproduce protection seen in early animal studies with antisera against J5 or Re mutants.
Baumgartner (1991b)	Negative	Immunisation of volunteers with *E.coli* J5 vaccine induced an increase in IgG and IgM type specific anti-J5 LPS antibodies, but no increase seen in antibodies directed against other epitopes of the core LPS or against lipid A.
Baumgartner (1991c)	Negative	Baumgartner writes to editor of NEJM to point out that their tests of HA1A shows it binds non-specifically to hydrophobic molecules such as Gram-negative and Gram positive bacterial membranes, yeasts, high density lipoproteins and cardiolipin. It bound only very weakly to lipid A.

Table 1.2 (continued)

Author (Year)	Outcome*	Study
Ziegler (1991)	Equivocal	Clinical trial of treatment of Gram-negative bacteraemia and septic shock with HA1A did not reduce mortality in either overall patients or patients with proven Gram-negative sepsis. However, compared with placebo group, there was a significant decrease in the mortality in 200 patients with Gram-negative bacteraemia. Later analysis (Warren et al 1992) also suggested a protective effect limited to patients who were in shock at study entry.
Wenzel (1991)	Negative	Second study of the human monoclonal IgM E5 (Xoma corp) looked at over 800 patients with sepsis syndrome without shock, but failed to show any reduction in mortality.
Various (1991)	–	A string of letters written by various researchers to *New Eng J Med* urging caution as to the interpretation of data for the anti-LPS trials.
J5 study grp (1992)	Negative	Treatment of severe infectious purpura in children with human *E. coli* J5 antisera reveals no protective benefit.
IVCSG (1992)	Negative	No benefit seen when anti-core LPS intravenous immune globulin administered to patients at high risk of post surgical infection.
Baumgartner (1992)	Negative	Unable to reproduce protection seen in early animal studies with antisera against *E. coli* J5 or Re mutants.
Wenzel (1992)	Negative	Independent re-analysis of the early HA1A data deduced that the null hypothesis had not been convincingly rejected.
Nys (1993)	–	Patients who undergo major surgery were less likely to succumb to sepsis if they maintained high levels of anti-core glycolipid antibodies.
Di Padova (1993)	–	Used cyclic immunisation of heat killed bacteria with different Ra chemotypes to create the widely cross reactive murine monoclonal antibody WN1 222-5.
Windsor (1993)	–	EndoCAb measurement in pancreatitis patients reveals correlation between falling anti-core IgG levels and adverse ouctome.

Table 1.2 (continued)

Author (Year)	Outcome*	Study
Heyderman (1994)	–	EndoCAb measurement in haemolytic uraemic syndrome patients.
Bhatt (1994)	Positive	Affinity purified anti-*E. coli* J5 LPS IgG protects neutropenic rats against Gram-negative spesis.
Baumgartner (1994)	–	Baumgartner's review of failures of clinical trials for anti-LPS.

* For all of the studies which involved human or animal trials of transfer of antibody or hyper-immune plasma, a brief summary of whether a protective benefit was observed is listed in the '*Outcome*' column. For sake of clarity, studies providing indirect evidence of LPS antibody function (*i.e.* those involving measurement of antibodies but not passive transfer) have not been summarised in this column and are indicated by a dash.

1.4 T-cell responses to LPS

1.4.1 Overview of T-cell responses to LPS

In the traditionally accepted view of T-cell immunology, the role of the T-cell is to recognise peptide presented to them by the MHC molecules of presenting cells. Then, depending on the type of T-cell and the secondary signals present at the time of antigen recognition, appropriate cytokines are released to orchestrate an immune response, or the cell is targeted for apoptosis or lysis. This rather narrow view of the T-cells capabilities has been expanded somewhat in the last decade or so by some novel discoveries.

The first of these occurred in 1992 when it was discovered for the first time that protein-derived antigens were not the only type of antigen capable of being presented to and recognised by T-cells. Lipid antigens too, were shown to be a viable T-cell substrate. Mediated by the non-polymorphic MHC-like CD1 molecules, this interaction led to new insights into the role of the T-cell in both innate and acquired immunity. Then in 1994 it was shown for the first time that human T-cells could mount a response towards LPS.

Together these two findings opened up new questions for the role of lipid reactive T-cells in the human host in health and disease.

1.4.2 Early evidence for LPS reactive T-cells

The significance of T-cell responses in human immunity cannot be overestimated. The almost complete lack of capacity to defend against infection seen in nude mice or athymic human patients need only be considered to realise how significant an effect on the immune system a loss of these cells have. Together, the various subsets of T-cells protect against viral challenge, assist in

the class switching and activation of B-cells and produce the cytokines essential to orchestrate the whole immune response.

In terms of LPS recognition, however, while other cell types have been intensively studied, T-cells have remained largely uninvestigated. The responses of monocytes, macrophages, dendritic cells and B-cells in particular to LPS have been very well characterised while surprisingly little is known of the responses made by T-cells to LPS. This lack of interest in LPS-reactive T-cells may have been precipitated by the long held (and false) conception that peripheral T-cells were largely refractory to the effects of LPS. Only a small number of murine studies showed any reaction at all, and none of these were extensively pursued (Tough et al 1997, Vogel et al 1983, Andersson et al 1977). For example, as far back as 1977, the proliferation of a cloned murine IL-2-dependent cytotoxic T cell (line CT6) in response to LPS was demonstrated (Andersson et al 1977). This response turned out to be dose- and time-dependent and blocked by polymyxin B. In addition, stimulation of highly purified murine splenic T-cells showed that 3% of this population were capable of proliferating in response to LPS. However, the same study also revealed that this population was missing in the C3H/HeJ mouse strain, allowing the deduction with hindsight that this T-cell proliferation is dependent on the Toll-like receptor mediated innate recognition of LPS.

Other than infrequent articles like these, very little attention was paid to the study of T-cells responsive to lipid antigens. This remained the case until an interesting discovery was made in 1992.

1.4.3 The CD1 story

For some time, the CD1 molecules had presented something of a mystery to geneticists and immunologists alike. Genomic analysis revealed that they existed in every mammalian species looked at (Calabi et al 1989) while

immunological and sequence analysis revealed striking similarities to the MHC class of molecules (being 30% identical to MHC class I heavy chain and similarly associated with β2-microglobulin) (Park et al 2000). However, unlike MHC molecules, they did not show the high degree of polymorphism observed in MHC molecules to present diverse peptides, nor had they ever been shown capable of presenting peptides to T-cells.

Comprising five distinct genes (isotypes) in humans, the CD1 molecules can be separated into two groups on the basis of homology (Calabi et al 1989). Group 1 CD1 molecules are found in humans but are absent from mouse and rat and comprise CD1a,b,c and e. Group 2 comprises only CD1d and is found in all species studied so far. Human CD1e has been seen to be transcribed but no protein product has yet been seen (Cattoretti et al 1989).

The first direct evidence implicating CD1 in the regulation of T-cell function was provided in 1989 with the observation of a subset of human circulating $CD4^-$ $CD8^-$ T-cell clones expressing either αβ or γδ TCRs which were capable of lysing tumor cells expressing specific isoforms of human CD1 (Porcelli et al 1989). This mystery became all the more tantalising when in 1992 Porcelli *et al* demonstrated a human T-cell clone capable of recognising an antigen from *Mycobacterium tuberculosis* that was restricted not by MHC-I or MHC-II, but by the CD1 molecule CD1b (Porcelli et al 1992). With an immunological function finally suggested for this molecule, the same group set about purifying their mycobacterial extracts to determine what type of antigen was being presented by the CD1 molecule. Proteolysis of the sample had no effect on antigenicity, suggesting that a peptide antigen was not involved. More curiously, the antigen appeared to be eluting within the hydrophobic fractions of their purifications. HPLC analysis was finally able to narrow down the antigen to the mycobacterial lipid mycolic acid (Beckman et al 1994) – a member of the α-branched, β-

hydroxy family of long chain fatty acids expressed by mycobacteria (Minnikin 1982).

This was the first demonstation of any lipid antigen being presented to T-cells and other labs scrambled to discover other lipids capable of being presented by CD1. In 1995, Sieling *et al* managed to show CD1b presentation of another mycobacterial lipid, lipoarabinomannan (LAM) (Sieling et al 1995). Further to this, they were able to show that glutaraldehyde fixation of the monocytes prevented presentation (suggesting that internalisation of antigen was required), as did chloroquine treatment, suggesting that endosomal acidification was also required for antigen loading (Sieling et al 1995). The T-cells responding to this antigen were shown to express $\alpha\beta$ TCRs, produce IFN-γ and mediate cytolytic effects on activation.

In 1997, a further lipid antigen of mycobacterial origin was seen to bind CD1 - glucose monomycolate (GMM). Moody et al investigated the structural requirements for binding of this antigen and found that substantial alteration of its lipid tails did not affect presentation (Moody et al 1997). On the other hand, antigenic recognition appeared to be extremely sensitive to variations in its carbohydrate portion or other polar substituents (Moody et al 1997). At this stage the idea was put forward that the lipid region was simply held in place non-specifically by the CD1 molecule, while the polar headgroup was left to interact with (and be recognised by) the T-cell receptor (Moody et al 1997).

By this stage, and with a handful of further CD1 presented mycobacterium-derived lipids discovered, a pattern was beginning to emerge. It was noted that the general architecture of the CD1 ligand appeared to be a di-acyl lipid attached to a strongly polar headgroup (Park et al 2000). Only CD1c proved capable of binding a mono-acyl template (Moody et al 2000).

Determination of the crystal structure of murine CD1d brought credence to this idea when it emerged that the CD1 molecules contain a long groove, reminiscent of the peptide binding groove of MHC-I, but with a lining from end to end of highly hydrophobic residues. This hydrophobic binding pocket was shown to be approximately 30A long by 10-15A wide, giving further support to the idea that the optimum molecular template structure for potential CD1 antigens is a di-acyl lipid format (Zeng et al 1997). The current model suggests that the structure and position of the polar head group is crucial for recognition by the TCR (Porcelli et al 1997). Closer analysis of where the hydrophilic interactions took place have suggested that the TCR should be optimally exposed to between one and four carbohydrate residues extending from the head of the lipid (Moody et al 1997).

This predominance of mycobacterial lipid antigens continued for some time until recently, when different classes of lipids have been shown capable of binding CD1. Self ceramides such as gangliosides, for example, can also be presented by CD1b (Shamshiev et al 1999), suggesting a role for these antigens in the autoimmune dysfunctions of diseases such as multiple sclerosis.

Of considerable interest is the very unusual lipid α-Galactosylceramide (α-GalCer). This glycolipid has only been seen in extracts from marine sponges (Natori et al 1994), but seems exquisitely capable of stimulating an entire population of human CD1d-restricted T-cells. These T-cells express a semi-invariant TCR which can recognise α-GalCer in even picomolar concentrations and exert potent effector and regulatory functions (Kawano et al 1997).

There is some evidence that hydrophobic peptides might also be presented by CD1 (Castano et al 1997), but given the variability of antigenic peptides and the non-polymorphic nature of CD1, it is more likely that it has evolved to present lipid antigens. In addition, human cellular GPI has been suggested to bind CD1d

(Joyce et al 1998), but this has yet to be confirmed. Finally, a recent study has indicated that a non-protein antigen derived from *Haemophilus influenzae* serotype b is also presented to CD1-restricted T-cells (Fairhurst et al 1998).

It has been suggested that the different CD1 molecules exist to sample different subceullular compartments for microbial lipids. Certainly, the different CD1 molecules are seen to take remarkably defined different routes through the cell. CD1b and CD1d are concentrated mainly in the late endosome or lysosome, CD1c in the late endosome and CD1a in the early or recycling endosome, lending credence to the idea that the different CD1 isotypes have evolved to sample different intracellular compartments (Sugita et al 1999, Schaible et al 2000).

One common event must occur, however, for ligand loading of all four isotypes of expressed CD1. Studies have shown that acidification of the ligand loading compartment is essential for antigen binding, as a low pH is required to open the CD1 ligand binding groove for insertion of antigen. Following this, CD1 binds lipids stably, with half lives ranging from a half hour to over twelve hours, an affinity reminiscent of that observed for MHC presentation of peptides (Naidenko et al 1999, Benlagha et al 2000).

1.4.4 Cellular expression of CD1 isotypes

Perhaps unusually, CD1 genes are poorly expressed in unstimulated human peripheral blood cells. However, stimulation of resting human monocytes with GM-CSF is capable of inducing strong expression of the group 1 CD1 molecules (Porcelli et al 1992, Kasinerk et al 1993), suggesting a role for these tissue macrophages in inflammatory lesions. Further, CD1 isotypes are expressed by antigen presenting cells, such as dendritic cells and macrophages (Porcelli et al 1999, Cattoretti et al 1989, Porcelli et al 1995). A subset of human B-cells also express CD1c (Small et al 1987) which seems to be, for some reason, much

more prominent on the circulating B-cells of infants than adults (Plebani et al 1993). In addition, CD1c has been shown to be upregulated on the marginal zone B-cells of lymphoid follicles (Cattoretti et al 1987).

Several studies have shown expression of CD1d on normal human gastric epithelia (Blumberg et al 1991, Balk et al 1994, Canchis et al 1993). These molecules seem to lack glycosylation and association with β2-microglobulin (Balk et al 1994). Interestingly, no group 1 CD1 molecules (*ie* CD1a,b or c) are expressed here. Human CD1d has also been seen to be expressed at low levels on circulating T- and B- cells and resting monocytes and at moderate levels on Langerhans' cells (Porcelli et al 1999). Murine CD1d, on the other hand, is expressed constitutively at low levels by most B-cells and is upregulated on splenic marginal zone B-cells (Roark et al 1998).

1.4.5 CD1 restricted T-cell phenotypes and function

The first CD1-reactive T-cell clones were seen to express neither CD4 or CD8 molecules (Porcelli et al 1989, Beckman et al 1994, Porcelli et al 1992), but it is now seen that CD1 restriction is broadly distributed among all of the T-cell subsets. In particular, there is evidence for CD1 recognition in both innate and acquired T-cell responses. For example, in their studies of patients recently infected with *M. tuberculosis*, Moody *et al* showed that the T-cells of these patients had enhanced CD1 regulated responses to synthetic mycobacterial lipids, suggesting a role for memory T-cells and acquired immunity in these infections (Moody et al 2000). Further, the T-cell receptors of these T-cells showed a wide diversity of peptide sequence, suggesting a clonal selection origin rather than a germline-encoded innate receptor.

As for innate immune responses, the CD1 work has thrown up some very interesting and unexpected new insights into naturally-occurring T-cell populations in humans that seem to offer a lymphocyte bridge between the

innate and acquired immune systems. CD1c, for example, is recognised by a major subset of cytolytic γδ T-cells, found in high numbers in the gut, that express Vδ1 γδ TCRs of limited diversity (Beldjford et al 1993). The antigens being presented by these CD1c molecules have yet to be discovered, though are considered likely to play a role in mucosal immunity (Spada et al 2000).

Another curious T-cell subtype is the NK-T-cell. It is not clear whether these cells are derived from the NK cell lineage or from the T-cell lineage as they are seen to express cell surface markers from both lineages (Godefroy et al 2000). These cells seem inherently capable of recognising CD1d and express strikingly conserved T-cell receptors (Vα24-JαQ in humans, Exley et al 1997, and Vα14Jα28 in mice, Bendelac et al 1997). Together they represent a not inconsiderable proportion of the T-cell population, accounting for up to 20-30% of T-cells in the liver and bone marrow, and 1% of total splenocytes in mice.

Virtually all NK-T-cells are capable of reacting strongly to the unusual lipid α-GalCer expressed in the context of CD1d. This finding has been particularly unexpected as α-GalCer has only been seen to be expressed in marine sponges. Nevertheless, it is tempting to speculate that a mammalian lipid homologue exists that is either expressed and released by cells under certain pathological or inflammatory conditions or that a breakdown product of some other microbial lipid resembling α-GalCer exists, indicating infection requiring the actions of NK-T-cells.

As for the role of these cells in the wider immune response against pathogens, multiple suggestions have been put forward on the basis of their ability to secrete both IL-4 and IFN-γ upon activation (Godefroy et al 2000). Indeed, recent studies have linked reduction in NK-T-cell numbers and alterations in their cytokine secretion patterns to progression of human autoimmune diabetes

and other disorders (Sumida et al 1995, Hammond et al 1998). A role also seems to be played by these cells in the recognition and rejection of tumours (Smyth et al 2000).

The CD1-restricted T-cells capable of recognising mycobacterial infection, on the other hand, are seen to react differently. CD1-restricted $CD4^-$ $CD8^-$ T-cells have been shown able to induce apoptosis of presenting cells via Fas ligand (Stenger et al 1997) and some $CD8^+$ CD1-restricted cells have been demonstrated to be able to employ perforin and granulysin to induce apoptosis and kill mycobacteria respectively (Park et al 2000, Stenger et al 1997). $CD4^+$, $CD8^+$ and double-negative CD1 restricted cells all appear to produce low levels of IL-4 but high levels of IFN-γ, a significant anti-mycobacterial cytokine which is thought to be required for activation of macrophage-mediated killing of intracellular pathogens and development of effective cell mediated immunity against these organisms (Porcelli et al 1999).

1.4.6 Can LPS bind to CD1?

As mentioned above, most of the lipid ligands so far discovered for the CD1 molecules have been of the format of a diacyl lipid attached to a polar head group (Sieling et al 1995, Shamshiev et al 1999, Naidenko et al 1999, Benlagha et al 2000). CD1c is so far the only exception to this rule, having been proven capable of binding the mono-acyl isporenyl glycolipids of *M. tuberculosis* (Moody et al 2000).

However, while LPS molecules certainly conform to the more general picture of potential CD1 antigens, (a hydrophobic region attached to a polar headgroup), they do not conform to the proposed optimum diacyl structure as a result of their typical substitution of between 5 and 7 acyl chains. Thus the question emerges of whether or not native LPS monomers are too bulky to fit inside the groove of CD1. As mentioned above, the groove of CD1 is approximately 10-15A wide by

30A long (Zeng et al 1997), while the width and length of enterobacterial LPS molecules, for example, have been calculated at 20-24A in width by 12-14A in length respectively (Alexander et al 2001). This suggests that some processing of LPS molecules via degradative enzymes in cells would be required to produce a ligand suitable for presentation by any of the isotypes of CD1.

It has also been suggested, after consideration of the hydrophilic areas of CD1 and the T-cell receptor, that between only one and four carbohydrate residues extending from the head of the CD1 ligand can be recognised by the T-cell receptor (Moody et al 1997). In terms of LPS, this would represent the minimum structure required for bacterial viability (one to three Kdo residues attached to Lipid A), and since only a very small number of bacteria naturally express LPS with carbohydrate portions this small, some enzymatic glycan trimming of most naturally-occurring LPS would also be required to produce a ligand suitable for expression on CD1.

This then leads to the question of whether or not antigen presenting cells express enzymes capable of this type of degradation? And by extrapolation whether or not such degraded LPS molecules pass through an intermediate structural form capable of being presented on CD1? Some lines of evidence exist to suggest that this is possible. Firstly, CD1b, for example, is seen to be strongly expressed in MIICs, the intracellular compartments where MHC-II is loaded with peptide (Sugita et al 1999). Moreover, these compartments are also seen to be lipid-rich (Prigozy et al 1997, Sugita et al 1996) and possess many degradative enzymes capable of glycan trimming etc (Ernst et al 1998). It has also been shown that LPS passes through these vesicles following cellular ingestion (Thiemblemont et al 1998, Detmers et al 1996).

Further, it has been seen that the enzyme acyloxyhydrolase, an enzyme strongly expressed by neutrophils, is capable of trimming the secondary

substituted acyl chains of LPS to provide a tetra-acyl breakdown product (Rietschel et al 1994). This molecule has the interesting property of being an antagonist of native LPS, thereby contributing to LPS tolerance in these cells. However, this precedent suggests that further acyl trimming or other enzymic activity may result in the formation of LPS derived molecules capable of binding to CD1, as depicted in Figure 1.8.

1.4.7 T-cell responses to LPS

Concurrently to the progress being made in the understanding of CD1 restricted T-cells, another group was looking at the possibility of T-cells reactive towards LPS. Mattern *et al*, working in Germany showed in 1994 for the first time that human T-cells can in fact become activated in response to LPS (Mattern et al 1994) – a finding in direct contrast with earlier work suggesting that human peripheral lymphocytes could not proliferate in response to LPS (Peavy et al 1970, Greaves et al 1974, Ellner et al 1989, Fauci et al 1976). Their explanation for this was that while the proliferation in response to LPS they had observed occured after an unusual delay of around seven days, previous investigators had been looking for this proliferation around the more typical period of 3-4 days incubation (Peavy et al 1970, Greaves et al 1974, Ellner et al 1989, Fauci et al 1976).

Further investigation of this response revealed that the proliferation required contact with accessory monocytes, as proliferation was abolished in transwell culture. This proliferation was proven to be due to the lipid component and not some protein or lipoprotein contaminant of their preparations when it was shown that the chemically synthesised compound 506, equivalent to *E.coli* lipid A, stimulated cells as readily as the native LPS of *Salmonella friedenau*. Interestingly, estimates of the frequency of responding T-cells ranged between 1:400 and 1:1100, thereby excluding the possibilty that the LPS was acting merely as a T-cell mitogen. However, both memory and naïve T-cells were

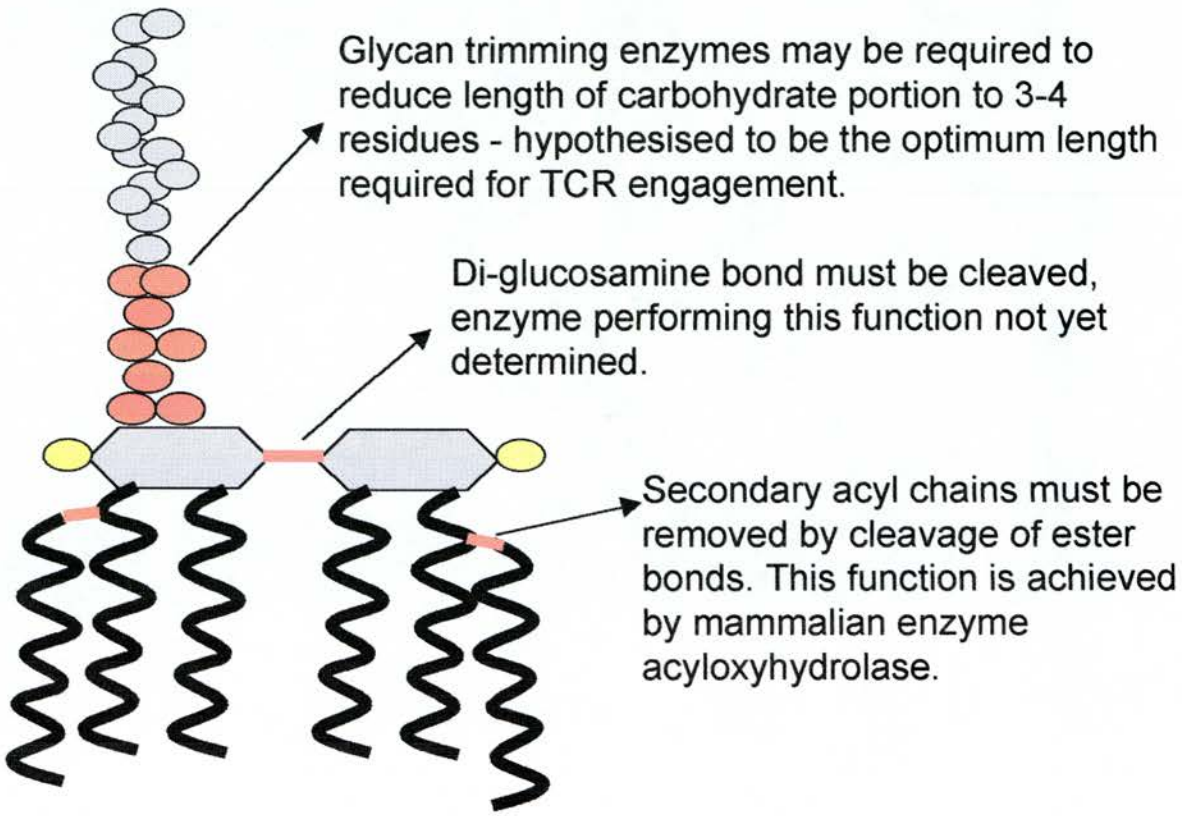


Figure 1.8 Enzymic modifications of LPS required for production of potential CD1 ligands

shown able to proliferate in response to LPS, suggesting a further example of T-cell-mediated crossover between the innate and acquired immune systems.

Of most interest to this group was, however, the fact that only around 50% of the healthy volunteers they looked at demonstrated T-cell responses to LPS – a phenotype which had no correlation with HLA haplotype.

The same group then followed up this line of investigation four years later by reporting that the proliferation is absolutely dependent on the interaction between T-cell CD28 and monocyte CD80 (Mattern et al 1998). Pre-treatment of monocytes with ammonium chloride prevents acidification of vesicles, and hence enzymic antigen processing such as proteolysis (Bauer et al 1988). This treatment abolished LPS-induced proliferation, lending further credence to the idea that the LPS was involved in some kind of presentation pathway.

Both autologous (same donor) and heterlogous monocytes were seen able to induce T-cell proliferation, indicating a likelihood that the response is MHC-independent, and it was noted that T-cells from non-responders could be made to respond if incubated with monocyte preparations from responders (which were shown to have strongly upregulated CD80). Antibody blocking experiments further confirmed that these responses are not MHC-restricted (Mattern et al 1998).

Then the following year, the same group showed that while only around 50% of samples from the peripheral blood of healthy controls responded, 100% of samples of PBMCs extracted from cord blood did. As a result of this finding they were able to pinpoint that the cell type required for the stimulation of the T-cells was not the monocyte, but the $CD34^+$ blood stem cell, a contaminating cell in their original monocyte preparations (Mattern et al 1999). This cell type was deduced to be present in responders and missing in non-responders as antibody

mediated depletion of $CD34^+$ cells abolished T-cell proliferation from responders' PBMC, while addition of $CD34^+$ cells to cell preparations from non-responders allowed them to respond to LPS. Further evidence for the critical role of $CD34^+$ cells in this process became clear when they showed that purified T-cells could be made to proliferate in response to LPS if incubated with irradiated KG-1a cells (a $CD34^+$ cell line).

Despite all of these advances, however, the group did not speculate as to how the LPS was being presented to the T-cells in their experiments other than to rule out the possibility of CD1-mediated presentation on the basis of their belief that CD1 is not expressed on monocytes, $CD34^+$ blood stem cells or KG-1a cells. However, since other studies have shown CD1 to be expressed constitutively at least at low levels on monocytes (Porcelli et al 1999), and CD1a, b and c on cytokine differentiated monocytes (Porcelli et al 1992, Kasinerk et al 1993), the possibility that LPS may interact with CD1 in this proliferation should not be discounted.

1.4.8 Potential implications of LPS-reactive T-cells

Every group working on lipid-reactive T-cells has been quick to speculate on the clinical relevance of their interactions. While the protective value of CD1-restricted antimycobacterial responses remains to be evaluated, evidence certainly exists to suggest that they play a role in natural mycobacterial infection. For example, patients recently infected with *M. tuberculosis* show enhanced responses to synthetic mycobacterial lipids, suggesting a role for CD1-restricted memory T-cells in these infections (Moody et al 2000). Further, the CD1-restricted T-cell clones reactive for mycobacterial lipids have all shown a phenotype capable of mounting a response suitable for the clearance of this type of bacteria (Beckman et al 1994, Porcelli et al 1992, Park et al 2000, Stenger et al 1997).

As for the LPS-reactive T-cells, while the mechanism behind this interaction has not been fully elucidated, it is clear that these T-cells should have some role to play in the immunity against Gram-negative infection. Not only are they activated to proliferate in response to LPS, but they display a clearly Th1 phenotype, quickly expressing and releasing IL-2 and IFN-γ, but not the Th2 cytokines IL-4, IL-5 or IL-10.

If responding populations of these T-cells are eventually shown to confer some benefit on particular patient groups (such as those at risk from Gram-negative infection, sepsis syndrome or endotoxaemia), then vaccination with LPS may become a useful prophylactic option. In terms of vaccines or adjuvants in particular, lipid based antigens should offer a significant advantage over equivalent protein based vaccines. Since the CD1 molecules are shown to be non-polymorphic between individuals, inter-person variablilty in MHC haplotype is avoided, greatly simplifying the selection of suitable lipid vaccine candidates.

Nevertheless, of most interest in the following study is the role of LPS reactive T-cells in endotoxaemia and sepsis syndrome and in particular the relationship between T-cell responses to LPS and clinical outcome.

1.5 Role of LPS in disease

1.5.1 Role of LPS in disease

In addition to their role as essential structural components of the Gram-negative outer membrane, the lipopolysaccharides of many bacteria have been seen to play a distinct role in the pathogenesis of a number of disease processes. Surprisingly diverse applications have been seen for LPS in this capacity, with, for example, some types of LPS acting as cellular adhesins, others protecting against complement-mediated attack or even providing the bacteria with a mechanism for host antigen mimicry in immune evasion (reviewed in Erridge et al 2002a). However, it is the host response to LPS during endotoxaemia that is associated with by far the most morbidity and mortality in human patients (Wenzel et al 1988). For this reason, the following section will discuss the role of LPS in the initiation and resultant pathology of sepsis syndrome.

1.5.2 Incidence of sepsis

Despite ongoing progress in the development of antibiotics and other supportive care therapies, sepsis syndrome continues to carry a high mortality rate (40-50%) (Wakefield et al 1988), which has not seen significant improvement in the past 50 years (Wheeler et al 1999). Reported to affect more than 500,000 patients in the United States annually (Wheeler et al 1999, Various authors 1990), the incidence of sepsis syndrome is, if anything, thought to be becoming yet more frequent (Wheeler et al 1999, Various authors 1990). While it is difficult to place accurate figures on the incidence and outcome of sepsis syndrome, it has been suggested that sepsis syndrome and its related sequelae together account for the thirteenth most common preventable cause of death in the USA (Knuefermann et al 2002).

Patients at risk of sepsis syndrome include those undergoing high risk surgical procedures, trauma patients, those presenting with abdominal complications such as pancreatitis, appendicitis or diverticulitis and those exposed to severe microbial infection, especially of the abdominal cavity (Bahrami et al 1995, Callery et al 1991, Gibb 1993).

1.5.3 Systemic inflammatory response syndrome and sepsis terminology

The history of sepsis syndrome is littered with fairly loose definitions and terms which were used interchangably in the literature and which were not clearly enough defined to allow accurate comparisons between various studies. This led a number of clinicians to call for better definitions of the vaguely used term "sepsis" (Various authors, 1991). Prompted by this concern, Bone and colleagues subsequently proposed a list of clearly defined clinical definitions for the diagnosis of the various conditions seen in patients at risk of sepsis syndrome (Bone 1991, Bone et al 1997).

In brief, these definitions consisted of the following: Systemic inflammatory response syndrome (SIRS) is defined as clinical evidence of a systemic inflammatory response. Sepsis is defined as a SIRS response that can be shown to be derived from an infective source. The term sepsis syndrome (often refered to as 'severe sepsis') is applied to those patients exhibiting sepsis together with evidence of organ hypoperfusion, while septic shock describes sepsis syndrome in conjunction with low blood pressure.

The clinical parameters of these definitions are summarised in Table 1.3 (taken from American College of Chest Physicians, 1992).

Table 1.3: SIRS and sepsis terminology

Bacteraemia: ...is defined as the presence of viable bacteria in the circulating blood, and as such can therefore only be determined by blood culture.

Septicaemia: ...has been used to describe the "systemic disease caused by the spread of microorganisms and their toxins in the circulating blood.". This term is misleading and should therefore no longer be used.

SIRS: ...is defined as the systemic inflammatory response to a wide variety of severe clinical insults (*ie* not necessarily infection). SIRS is characterised by a diagnosis of two or more of the following:

- Core temperature >38.3 or <35.6°C
- Heart rate > 90 beats per minute
- Respiratory rate > 20 breaths per minute *or* $PaCO_2$ < 32mm Hg
- White blood cell count > 12,000/mm^3 or < 4,000/mm^3, or > 10% immature (band) forms.

Sepsis: ...is defined as the systemic inflammatory response *to infection*. Manifestations of sepsis are identical to those listed above for SIRS, but with the addition of clinical evidence of infection.

Severe sepsis: (also frequently refered to as 'Sepsis syndrome') ...is defined as sepsis combined with evidence of altered organ perfusion. Altered organ perfusion may be diagnosed if the patient exhibits the following clinical indicators:

- P_AO_2/F_{IO2} <=280 (without other pulmonary or cardiovascular disease as the cause)
- Elevated lactate level (> upper limits of normal for the laboratory)
- Oliguria (documented urine output < 0.5 ml/kg body weight) for at least one hour (in patients with catheters)
- Acute changes in mental status

Septic shock: ...is defined as *sepsis syndrome* with hypotension: *ie* a systolic blood pressure < 90mm Hg, or decrease from baseline systolic blood pressure > 40mm Hg that is responsive to intravenous fluids or pharmacologic intervention.

MOF / (MODS): Multiple organ failure (multiple organ dysfunction syndrome) is defined as the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention.

1.5.4 Factors involved in the initiation of SIRS and sepsis

The last decade or so has seen considerable advancement in our understanding of the initiation of SIRS and sepsis. Previously considered to be a simple systemic inflammatory response to invading microorganisms, our ideas of how SIRS, sepsis and their associated sequelae occur have been refined to include an understanding of the multi-factorial nature of the inititation of these events (reviewed in Oberholzer et al 2001).

Agents now recognised as being capable of inducing a systemic inflammatory response (SIRS) include not only components from bacteria, yeast and viruses but also host-derived products of necrotic cells and tissue damage. Thus, SIRS and its related sequelae may be initiated not only by almost any kind of infection but also by ischaemia-reperfusion injury, thermal injury, soft tissue injury or haemorrhagic shock (Bone et al 1997, Bone et al 1989). Indeed, studies of SIRS patients in some clinical settings have routinely demonstrated a high percentage of cases in which no obvious source of infection can be found – for example the study of Bone *et al* which demonstrated that only around 45% of patients diagnosed with SIRS could demonstrate a positive blood culture (Bone 1991).

Sepsis, on the other hand, is defined as the subset of SIRS patients whose symptoms can be directly traced to a source of infection (Bone et al 1997). In surgical and burns cases in particular, it is becoming increasingly clear that by far the most common source (>90%) of these infections is the hosts own normal microbial flora (Munford et al 2001). More specifically, evidence is beginning to accumulate to suggest that the most significant source of these commensals is the host gastrointestinal tract, as disruption of the gut barrier has been seen to be readily capable of initiating the septic state (Saadia et al 1990, Gough et al

1992), and is often seen to be compromised in patients suffering from sepsis syndrome (Haglund et al 1975).

The significance of the gut barrier should not be underestimated, as it is this mucosal surface which serves to divide the rest of the body from the large amounts of Gram-positive and Gram-negative bacteria, yeasts, digestive enzymes, lipoproteins, endotoxins and other stongly pro-inflammatory mediators present in the lumen of the gut. Damage of this barrier and release of the contents into the surrounding tissues or circulation is termed 'translocation' and is thought to be initiated following a range of potential insults. These include haemorrhagic shock, thermal injury, malnutrition, jaundice and even simple intestinal obstruction (Saadia et al 1990, Heidecke et al 1999, Baker et al 1988, Bynum et al 1984). The tissues of the gut appear to be particularly sensitive to both ischaemia and reperfusion injuries (Chiu et al 1970, Baker et al 1988, Bynum et al 1984, Haglund et al 1975). For example, a decrease in splanchnic blood has been shown to directly damage the gut mucosa in human patients suffering from shock (Chiu et al 1970, Haglund et al 1975). Further, it has even been suggested that dysregulation or defect of the host immune system may be enough to intitate translocation (Deitch et al 1986). While the exact mechanism for this translocation of bacteria and toxins remains as yet unclear, it has been postulated that tight junctions between the cells of the gut epithelia are critical in maintaining this function (Bounos et al 1977, Rhodes et al 1973).

Finally, it should be pointed out that while gut barrier function clearly has a role to play in sepsis syndrome, breakdown may act as both a cause and as a result of sepsis syndrome.

1.5.5 Pathology of SIRS and sepsis syndrome

Both SIRS and sepsis syndromes are thought to be precipitated by the same basic mechanism.

In the main, it is thought that both conditions are initiated by a systemic release of pro-inflammatory cytokines from the cells of the innate immune system. In particular, it is thought that overproduction of the cytokines TNF-α, IL-1β, IL-8 and IL-6 are the principal mediators of the initital insults of both SIRS and sepsis, though IFN-γ, IL-12 and IL-18 have also been shown to play a role (Oberholzer et al 2001).

These mediators may be elicited in response to any of the infectious agents or products of tissue damage listed above and their effects on the body both locally and systemically are mediated in three main ways:
(i) They induce local inflammation - properties and permeabilities of local blood vessels are changed. Phagocytes, other immune cells and molecules are recruited to the site of infection.
(ii) They induce the production of acute phase proteins by the liver, which bind to bacterial surface molecules enhancing both the activation of complement and phagocytosis.
(iii) They can elevate body temperature, reduce the hosts' spontaneous activity and induce behavioural changes called "sickness syndrome" – all of which are thought to be deleterious for the growth or spread of the organism.

In local foci of infection, these pro-inflammatory mediator-driven responses serve to enhance defence against the organism, limit its spread from that location and ultimately initiate a "clean up" and repair of the damaged area. However, when activation of the innate immune system is severe enough, the host response itself can propel the patient into a systemic inflammatory response syndrome (termed SIRS), or even shock and multiple organ failure (MOF) (Oberholzer et al 2001). These two more serious pathologies are precipitated as a result of the ability of released cytokines to promote: (i) progressive dysfunction of the endothelial surfaces leading to increased microvascular permeability (Tracey et al 1988, Stephens et al 1988), (ii) platelet

sludging leading to blockage of the microcirculation causing maldistribution of blood flow and possibly ischaemia (Sigurdsson et al 1992), which in turn may cause reperfusion injury (Cipolle et al 1993) and induction of heat shock proteins (Rinaldo et al 1990), (iii) massive consumption of clotting proteins, thereby removing the patient's ability to clot properly (Oberholzer et al 2001), (iv) activation of the coagulation system and impairment of the protein C–protein–S inhibitory pathway (Levi et al 1993) and (v) profound vasodilation, fluid transudation and maldistribution of blood flow which may result in profound shock (Gomez-Jiminez et al 1995, Miyauchi et al 1990). These consequences may ultimately lead to organ dysfunction and failure unless homeostasis is quickly restored. Further, approximately 40% of patients with sepsis go on to develop shock, a condition which adversely affects prognosis (Kreger et al 1980).

The critical roles of proinflammatory cytokines in these pathologies are confirmed by studies observing a correlation between the magnitude of plasma IL-1β, IL-6 and TNF-α levels and negative outcome of septic and burns patients (Drost et al 1993, Casey et al 1993). However, it should be pointed out that more recent studies have also shown correlations between adverse outcome and high levels of anti-inflammatory mediators such as IL-10 and TGF-β and poor outcome in adult respiratory distress syndrome (Neidhardt et al 1997) and sepsis (Sherry et al 1996).

1.5.6 Role of LPS in sepsis syndrome

As mentioned above, it appears that the critical event in the initiation of SIRS is the widespread and overwhelming production of pro-inflammatory cytokines. Many agents are capable of eliciting these molecules from the cells of the innate immune system, but by far the most extensively studied in terms of its role in the initiation and pathology of sepsis syndrome is endotoxin. Capable of inducing aggressive release of pro-inflammatory mediators from the cells of the innate

immune system at concentrations as low as several picograms per millilitre, endotoxin has been shown capable of inducing all of the symptoms of sepsis syndrome on administration to human volunteers (Michie et al 1988). Indeed, proof that LPS alone can induce all of the characteristic features of septic shock in humans came from a laboratory worker who self-administered 1mg of purified *Salmonella minnesota* LPS intravenously, resulting in severe shock and organ failure within 3 hours (Taveira daSilva et al 1993). Further evidence for its involvement comes from the observation of its presence (at least transiently) in the plasma of at least 86% of patients with sepsis syndrome (Goldie et al 1995). The main source of endotoxin in these patients is thought to be translocation from the gut (Saadia et al 1990, Wells et al 1986), though it should be borne in mind that it may also occur in conjunction with Gram-negative infection from other routes.

Indirect evidence for the role of LPS in sepsis syndrome also exists. For example, investigations of antibody responses to LPS have demonstrated an association between low preoperative serum anti-endotoxin antibody levels and adverse outcome following surgery (Bennett-Guerrero et al 1997, Freeman and Gould 1985, Gould et al 1989, Hamilton-Davies et al 1997). Low or falling levels of anti-endotoxin have also been shown to correlate with adverse outcome in patients diagnosed with abdominal sepsis (Wakefield et al 1998) and pancreatitis (Windsor et al 1993).

Together these lines of evidence suggest that LPS may have a strong role to play in the initiation of sepsis syndrome.

1.5.7 Sepsis and immunological hyporeactivity

Although most patients survive the initial SIRS insult, these patients remain at increased risk of developing secondary or opportunistic infections because of the frequent onset of a secondary phase of immune hyporeactivity (Oberholzer

et al 2001, Bone et al 1997). This phase is seen to be characterised by a depression mainly in the production of Th1 cytokines (IL-2, IFN-γ) whereas the production of Th2 cytokines (IL-4, IL-10) remains largely unchanged or even upregulated (Cavaillon et al 2001, Oberholzer et al 2002). This was shown after vascular surgery (Berguer et al 1999), cardiopulmonary bypass (Naldini et al 1998), cholecystectomy (Brune et al 1999) and in burn and trauma patients (O'Sullivan et al 1995). Further cellular effects of this phase include a shift of CD8 cells towards the Type 2 phenotype, increased IL-4 production and a decrease in expression of HLA-DR (Kox et al 1997).

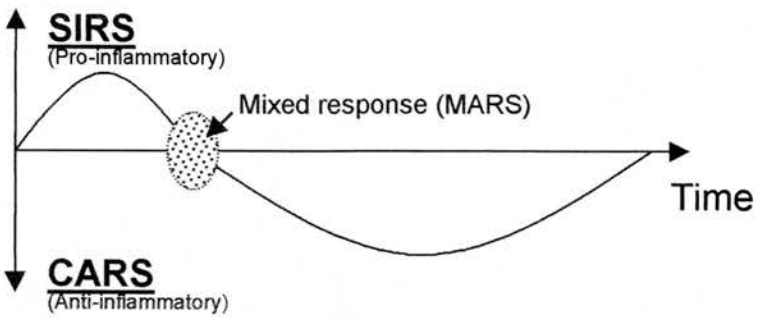
The first observation of PBMC hyporeactivity in sepsis patients came with the study of Wood and colleagues in 1984, showing that PBMC from these patients exhibited decreased IL-2 production in response to PHA stimulation (Wood et al 1984). Subsequent studies by many groups have noted specific defects in every population of immune cells from patients in this hyporeactive state. Monocytes, for example, have been shown to have a diminished capacity to release TNF-α, IL-1α, IL-1β, IL-6, IL-10 and IL-12 in response to LPS (Ertel et al 1987, Munoz et al 1991, Sekatrian et al 1994, Van Deuren et al 1994) whereas this is not the case for IL-1 receptor antagonist (Van Deuren et al 1994). Similar observations were reported in human volunteers after LPS injection: the capacity to produce IL-1, TNF-α and IL-6 were all all significantly reduced independently of the nature of the activating agent (ie LPS, IL-1β, staphylococcal toxic shock syndrome toxin-1) (Granowitz et al 1993). Neutrophils are similarly affected, with those extracted from septic patients demonstrating a reduced capacity to release IL-1β, IL-1ra and IL-8 upon activation with LPS (McCall et al 1993). Aberrant T-cell function in septic patients has also been reported. The study of Heidecke *et al* (1999), for example, looked at T-cell function in patients with post-operative sepsis due to intra-abdominal infection and showed that T-cell proliferation and production of IL-2 were seen to be severely suppressed in the

non-survivors, while survivors were shown to have normal levels of proliferation and IL-2 release. As a result of their findings, they suggest a significant role for T-cells in the response to post-operative infection.

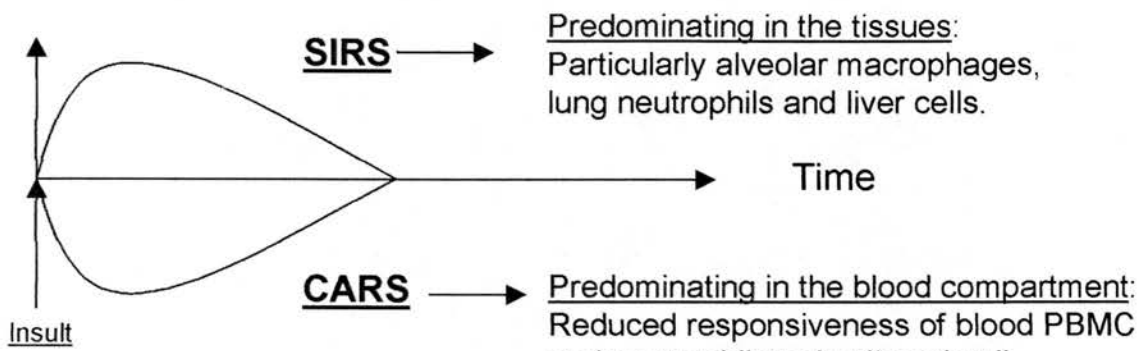
Animal models of sepsis too, often reveal a phase of hyporeactivity similar to that seen in humans. Martineau and colleauges have used the rat infusion model of intra-abdominal infection to show temporary functional defects in B and T-cells consistent with those seen in patients with peritonitis (Martineau et al 2000). Further, Gough *et al* have used the murine model of sepsis (caecal ligation and puncture), to show a systemic immunosuppression that eventually reverts to normal (Gough et al 1992).

1.5.8 The CARS hypothesis

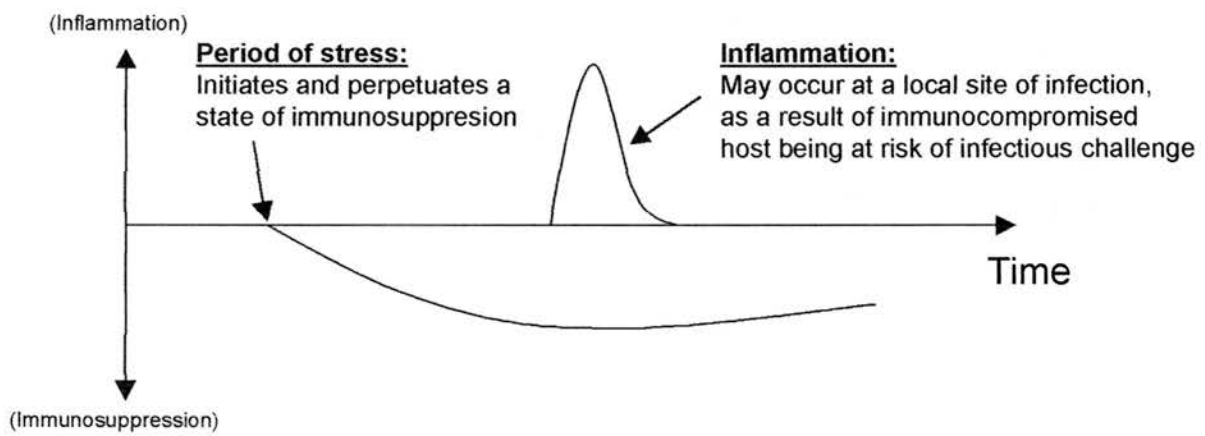
The phenomenon of anergy following a pro-inflammatory episode has led to a number of hypotheses being put forward to explain it. The longest running of these, initially put forward by Bone *et al* (1997), holds that the primary phase of systemic production of pro-inflammatory cytokines is eventually countered and replaced by a systemic compensatory anti-inflammatory response (CARS). In this model, the initial over-zealous activation of the innate immune response is thought be countered with anti-inflammatory and Type 2 cytokines such as IL-10, TGF-β and IL-4 (Cavaillon et al 2001). Widespread upregulation of these secondary cytokines leads to macrophage deactivation, T-cell anergy and the rapid apoptotic loss of many lymphoid tissues throughout the body, all of which contribute to the development of the CARS syndrome and its associated morbidity and mortality (Oberholzer et al 2001). Such a 'two-wave' model is depicted in Figure 1.9a, and Bones proposed list of immunological definitions of the SIRS and CARS phases, based on the cytokine levels observed in patient plasma, is summarised in Table 1.4.



(a) The "Two Wave" model, proposed by Bone *et al* [278]



(b) The"Compartmentalised" model, proposed by Cavaillon *et al*



(c) The"Stress Induced" model, proposed by Munford *et al*

Figure 1.9 Proposed models of immunological status in sepsis patients

Table 1.4: Possible immunological definitions of SIRS and CARS from patient plasma (from Oberholzer et al 2001)

SIRS: IL-6 > 1000pg/ml, IL-1β (detectable), TNF-α (detectable), IL-8 > 100pg/ml, E-selectin > 150 ng/ml.

CARS: IL-10 (detectable), IL-1ra > 1500 pg/ml, sTNF-R I (p55) > 1500 pg/ml, sTNF-R II (p75) > 1500 pg/ml, PBMC HLA-DR < 30%.

However, since its initial proposal in 1997, numerous studies have revealed inconsistencies in the basic assumptions behind this so called 'two wave' model of sepsis (Hassoun et al 2001). In particular, it has been seen that while cells from individual patients may be hyporesponsive to one stimulus, they may be normally reactive to another (Heidecke et al 1999, Cavaillon et al 2001, Zedler et al 1999, Christou et al 1984, Tellado et al 1990). Further, particular cell types purified from sepsis patients may exhibit conflicting inflammatory mediators (Bone et al 1997, Oberholzer et al 2002, Heidecke et al 1999, Cavaillon et al 2001). Such a mixed response has been termed MARS (mixed anti-inflammatory response syndrome) which some hypothesise as a transitional phase between SIRS and CARS phases (Bone et al 1997).

In order to explain these discrepancies, at least two further models of the immunological events precipitating and perpetuating this phase of immunological hyporeactivity have been proposed. The first of these was proposed by Jean-Marc Cavaillon and colleagues (Cavaillon et al 2001) and brings forward the idea of compartmentalisation. In this model, the authors suggest that while cells extracted from the blood of patients may be hyporesponsive to certain stimuli, cells derived from the tissues are normally responsive or even primed to the same stimuli at all stages of sepsis. This partitioning of the two responses, they suggest, allows a vigorous inflammatory

response to remain at the site of infection, while sites distant from the infection are protected from the damaging effects of pro-inflammatory molecules by the concomittant release of systemic anti-inflammatory molecules such as IL-10 and TGF-β. Such a 'biphasic' model of immune hyporeactivity is depicted in Figure 1.9b.

However, recent advances in our understanding of the strong interactions between neurological and immunological functions have led to the proposal of a more radical model. In particular the observation that stress hormones such as catecholamines can suppress the activity of immune cells (Jones et al 1989), inhibiting production of TNF-α (Severn et al 1992) and promoting a state favouring production of the anti-inflammatory IL-10 (Suberville et al 1996, van ser Poll et al 1996), have led Munford and colleagues to suggest a third model of sepsis related hypo-reactivity (Munford and Pugin 2001a, Munford and Pugin 2001b).

In this model, (Figure 1.9c) it is proposed that the systemic anti-inflammatory response seen in patients is not merely a compensatory reaction in response to systemic pro-inflammatory cytokines, but a much more generalised response of the body to stress. Stresses as diverse as cold exposure, strenuous exercise, major injury and even psychological stress is sufficient, they assert, to induce a systemic anti-inflammatory state via cytokines, the HPA axis and the sympathetic-adreno-medullary axis (Munford and Pugin 2001a, Munford and Pugin 2001b).

Such systemic anti-inflammation, they further suggest, would be able to prevent potential damage of uninvolved tissues, while concentrating white blood cells towards sites of infection, and may therefore be considered a normal reaction to microbial challenge. However they also point out that while this state may induce favourable outcome in dealing with a local point of infection, the associated

systemic immunosuppression may also lead to secondary infection from viruses, bacteria or fungi. Thus it is possible, they surmise, that a stress-induced phase of immunosuppression may in fact precede and even precipitate an episode of SIRS.

This idea that a period of immunological hyporeactivity may precede an episode of SIRS or sepsis gains support from the many studies of immunological status in pre-surgical patients. It has often been reported that patients entering hospital may be in an immunologically hyporeactive state *before* they undergo surgery or any SIRS episode. So called 'walk-in anergic' patients are seen to be at considerably more risk from poor outcome when compared to patients exhibiting normal immune function on admission (Christou et al 1984, Christou et al 1989, Johnson et al 1979, Adami et al 1980, Christou et al 1983).

For example, in 1984 Christou *et al* prospectively measured the delayed type hypersensitivity (DTH) reaction of patients admited for elective surgery. Patients were considered anergic if they responded to one or less of five skin test recall antigens. Intriguingly, while no major episodes of sepsis occurred in the control group of immunocompetent patients, 25% of the anergic patients showed evidence of sepsis ($p<0.05$) (Christou et al 1984). Moreover, mortality in the anergic group was seen to be much higher than among the immunocompetent controls (6% vs 40%, $p<0.05$), a correlation which was also borne out in a larger study of the correlation between DTH reaction and clinical outcome (Christou et al 1989).

However, as for which of these three models most accurately reflects the true immunological picture in these patients remains to be verified. Only examination of the cellular and cytokine responses from a wide range of tissue types, taken at a wide range of time points from the many different patient subgroups will begin to shed light on this complex matter.

1.6 Aims of Thesis

The immunological responses of human beings to lipopolysaccharide may confer on the host either beneficial or deleterious effects depending on the context and magnitude of endotoxin challenge. The objective of this thesis was to investigate the role of the different arms of the human immune system in dealing with LPS challenge.

The three main areas of research included:

1) The innate immune responses to LPS and a liposomal LPS-based vaccine were assessed. The kinetics and requirement of serum-based lipid shuttle proteins in the mediation of these responses were also investigated. Capacity of cells carrying a common mutation in the TLR4 gene to mount effective responses to a wide range of LPS types were assessed.

2) The responses of human and murine T-cells to LPS challenge were assessed. The LPS of different species and strains of bacteria were compared in terms of their capacity to activate T-cells. Responses of human T-cells to different types of LPS were assessed over time. The hypothesis that the lipid presentation molecule CD1 may be involved in the T-cell recognition of LPS was tested.

3) T-cell and antibody responses to LPS were compared between healthy volunteers and patients admitted to hospital with suspected endotoxaemia. Clinical scores, TLR4 mutations, white blood cell counts, patient length of stay and outcome were assessed for correlation with these responses.

CHAPTER 2

MATERIAL AND METHODS

2.1 General cells, media and materials

2.1.1 General chemicals

Unless otherwise stated, all chemicals used were of AnalAR grade. Aqueous solutions were made up in either distilled water or pyrogen free water (Millipore, Billerica, MA, USA). Phosphate buffered saline (PBS) used in all experiments was made by dissolving one tablet of PBS (Oxoid, Basingstoke, UK) per 100mls of pyrogen free water and autoclaved before use.

2.1.2 General media

Bacteria were grown in nutrient broth (filter-sterilized, Gibco, Paisley, UK). Mammalian tissue culture cell lines were maintained in either RPMI-1640 (Sigma, Gillingham, UK) supplemented with 1% ready-mixed penicillin / streptomycin / glutamine (PSG, Sigma) and 5% foetal calf serum (Gibco, Sigma or Imperial laboratories, Andover, UK), hereafter refered to as RPMI/5%FCS, or DMEM (Sigma) supplemented with 1% PSG (Sigma) and 5% foetal calf serum (Gibco), hereafter refered to as DMEM/5%FCS. Primary mammalian cell cultures were maintained in either RPMI/5%FCS or RPMI supplemented with 1% readymixed PSG and 10% pooled human AB serum (Sigma), hereafter refered to as RPMI/10%HS.

2.1.3 Bacterial strains

Heat-killed bacteria required for murine immunization protocols were either *Escherichia coli* K12 or *E. coli* O18 K^-. Bacteria were grown from –70°C bead aliquot in 100mls nutrient broth in shaking incubator overnight at 37°C. After harvesting and washing (15 minutes, 500g) three times in normal saline (0.85% NaCl in distilled water) they were resuspended in 10mls normal saline and heat-killed by incubation in a 100°C boiling water bath for 10 mins. Samples was allowed to cool and aliquots plated on blood agar to confirm complete killing.

Cells were counted by haemocytometer microscopy then diluted to 1 x 10^9 per ml in normal saline and stored in glass bijoux at –20°C before use in immunization or antigen challenge protocols.

2.1.4 Mammalian cell lines

Cell lines employed in various assays included the murine macrophage cell line J774 (ECACC no: 85011428), the human monocyte cell line THP-1 (ECACC no: 88081201) and the murine fibroblast cell line L929 (ECACC no: 85011425). J774.1 and THP-1 cells were grown in RPMI/5%FCS. L929 cells were grown in DMEM/5%FCS. Maintenance of all cell lines was as described in section 2.3.1 below.

2.1.5 Lipopolysaccharides

Lipopolysaccharides from a wide variety of species and strains of bacteria were used. Of these, only the smooth LPS of *E.coli* O18 K^- was prepared by the author, all others were made up from aliquots taken from the Microbial Pathogenicity Research Laboratory (MPRL, Edinburgh University Department of Medical Microbiology, Teviot Place, Edinburgh, EH8 9AG) –20°C stocks of LPS, or were kind gifts from colleagues (listed in Table 2.1). Each LPS was resuspended to a final concentration of 1mg/ml in pyrogen-free water supplemented with 0.1% triethylamine (Sigma) to ensure complete solubility.

For murine vaccinations with native LPS, only *E.coli* O18 K^- and *E.coli* K12 were used. For comparisons of LPS and liposomal LPS, native or liposomal preparations of either *E.coli* K12 LPS or "cocktail" LPS (an equimolar mixture of *E.coli* R1, *E.coli* K12, *Bacteroides fragilis* rough LPS and *Pseudomonas aeruginosa* PAC 608 LPS) were employed. For T-cell proliferation experiments, the LPS from *E.coli* R1, *E.coli* R2, *E.coli* R3, *E.coli* R4, *E.coli* K12, *B.fragilis*, *Pseudomonas aeruginosa* PAC 611, *Klebsiella aerogenes*, *K.friedlander*, *K.pneumoniae*, *Salmonella typhimurium* and *S.minnesota* were used. For

measurements of endogenous core antibody (EndoCAb) titre measurements, ELISA plates were coated with LPS from *E.coli* K12, *P.aeruginosa* PAC 611, *K.aerogenes* KaM10b and *S.typhimurium* 878. Finally, Toll-receptor 4 polymorphism experiments included analysis of the responses to LPS from *E.coli* R1, *Neisseria meningitidis, B.fragilis* rough, *Porphyromonas gingivalis*, *Yersinia pestis, Chlamydia trachomatis* and *P.aeruginosa* PAC 611. The exact strain, source and extraction method of each of these LPS is listed in Table 2.1.

Table 2.1 Sources and strains of lipopolysaccharides used in all experiments

Host bacteria	MPRL Ref	Reference/serotype	Extraction	LPS type	Performed by
E.coli O18 K$^-$	-	-	phenol/H_2O	S	C. Erridge
E.coli R1	32	MPRL 2316, NCTC 13114	pcp	R	R. Brown
E.coli R2	17	-	pcp	R	F. Mcloughlan
E.coli R3	18	-	pcp	R	F. Mcloughlan
E.coli R4	20	-	pcp	R	F. Mcloughlan
E.coli K12	26	MPRL 1899, NCTC 13116	pcp	R	R. Brown
B.fragilis R	69	MPRL 1504, NCTC 9343	pcp	R	D. Delahooke
B.fragilis S	68	MPRL 1504, NCTC 9343	phenol/H_2O	S	D. Delahooke
P.aeruginosa	50	PAC 608, NCTC 13124	pcp	R	R. Brown
P.aeruginosa	49	PAC 611, MPRL 1091	pcp	R	C. Currie
S.typhimurium	43	878	pcp	R	F. Mcloughlan
S.minnesota	33	R60	pcp	R	F. Mcloughlan
K.aerogenes	57	KaM10b	pcp	R	F. Erikkson
K.friedlander	60	MPRL 4334	phenol/H_2O	S	L. Millar
K.pneumoniae	58	MPRL 4332	phenol/H_2O	S	L. Millar
N.meningitidis	101	MPRL 2843	Triton/$MgCl_2$	LOS	R. Brown
P.gingivalis	102	MPRL 1675	phenol/H_2O	S	R. Brown
C.trachomatis	-	LGV-1	phenol/H_2O	LOS	A. Eley
Y.pestis	-	-	phenol/H_2O	S	P. Oyston

Notes to Table 2.1: PCP refers to the phenol / chloroform / petroleum extraction technique of Galanos *et al* as described by Hancock and Poxton (1998). LPS extracted by the pcp method is predominantly of the rough form. Triton/$MgCl_2$ refers to the triton detergent based extraction of Uchida *et al* (1987). Phenol/H_2O refers to the phenol water extraction technique as described below. LPS type: R - rough form, S - smooth form, LOS - lipooligosaccharide.

2.2 Preparation of Stimuli

2.2.1 Extraction of LPS

Extraction of smooth LPS from *E.coli* O18K$^-$ was based on the aqueous phenol method of Westphal and Luderitz (1954). Two 2 litre flasks containing 1 litre of nutrient broth (filter sterilised, Gibco) were inoculated with 2.0ml of an overnight preculture grown in nutrient broth. The flasks were then incubated in an orbital incubator (120rpm) at 37°C overnight. Bacterial cells were harvested by centrifugation at 10,000g then washed twice in PBS by centrifugation at 10,000g for 10 minutes. The bacterial pellet was frozen at –20°C, freeze dried and weighed.

This pellet was subsequently ground, resuspended to a concentration of 5% wv in pyrogen free water and heated to 67°C in a water bath in a fume cupboard. Meanwhile 90% w/w aqeuous phenol was prepared by dissolving 90g phenol (BDH, New York, USA) in 10 ml of distilled water at 45°C and then making the volume up to 100ml in distilled water. A volume of phenol solution equal to that of the bacterial suspension was heated to 67°C in a water bath. The prewarmed bacterial suspension and phenol solution were mixed and stirred with a glass rod for 15 minutes at 67°C. Following this, tubes were transferred to an ice bath to allow separation of the phenol and aqueous phases. The tubes were then centrifuged at 10,000g for 15 minutes to complete separation of the phases. The upper (aqueous) phase containing the LPS was carefully removed using a glass pipette and the extraction procedure repeated on the lower phenol phase. The two aqueous phases were pooled, transferred to dialysis tubing (previously washed and boiled for 10 minutes in distilled water) and dialysed against running tapwater overnight, until the smell of phenol could not be detected. Insoluble deposits were then removed by two further centrifugation steps (15

minutes, 10,000g). The dialysed extract was then concentrated by rotary evaporation to approximately one-fifth of its original volume. At this stage LPS should be beginning to precipitate, so the solution was transferred to ultracentrifuge tubes and spun at 10,000g for 3 hours. The gelatinous pellet obtained was resuspended in distilled water using a syringe fitted with a blunted needle and recentrifuged as before. The final pellet was then suspended in a small volume of pyrogen free water, freeze dried and weighed. Glass bijoux were used to store the dried LPS at –20°C until required.

2.2.2 Repurification of LPS

In experiments to determine the TLR usage of different LPS types, removal of all trace of lipoprotein was desired, so the phenol re-extraction method of Hirschfield *et al* (2000) was applied to a selection of LPSs. At room temperature, 5mg of each LPS was individually resuspended in 1ml of endotoxin-free water containing 0.2% tri-ethylamine. Each sample was split into two 500µl aliquots, with one remaining stored at -20°C without further manipulation to serve as an "unextracted" control. Deoxycholate (Sigma) was added to the remaining aliquot to a final concentration of 0.5%, followed by the addition of 500µl of water saturated phenol. The samples were then vortexed intermittently for 5 minutes, and the phases allowed to separate at room temperature for 5 minutes.

Samples were placed on ice for 5 minutes, followed by centrifugation at 4°C for 2 minutes at 13,000x g. The top aqueous layer was transferred to a new tube, and the phenol phase was subjected to re-extraction with 500µl of 0.2% TEA/0.5% DOC. The aqueous phases were pooled and re-extracted with 1ml of water saturated phenol. The pooled aqueous phases were adjusted to 75% ethanol and 30mM sodium acetate and were allowed to precipitate at –20°C for 1 hour.

The precipitates were then centrifuged at 4°C for 10 minutes at 10,000xg, washed in 1ml of cold 100% ethanol, and air dried. Finally, these pellets were

resuspended in the original volume (500μl) of 0.2% TEA without deoxycholate. One hundred percent recovery was assumed for the purified LPS samples.

2.2.3 Preparation of liposomal LPS

Liposomes containing "cocktail" LPS (ie an equimolar mixture of *E.coli* R1, *E.coli* K12, *B.fragilis* rough LPS and *P.aeruginosa* PAC 608 LPS) were kind gift of Drs. E. Guerrero and T. McIntosh (Department of Anesthesiology, Columbia University College of Physicians & Surgeons, New York, USA and Department of Cell Biology, Duke University, Durham, NC, USA). Liposomes containing *E.coli* K12 LPS were prepared by myself with the assistant of honours student Sarah McPhee.

Liposomes were prepared using depyrogenated glassware and reagents according to the method of Dijkstra *et al* (1988) incorporating the modifications of Guerrero *et al* (2000). Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and consisted of dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl glycerol (DMPG) and cholesterol in a 4:1:4 molar ratio. A phospholipid / cholesterol stock was prepared and dissolved in chloroform (55.3mg DMPC + 14.1mg DMPG + 31.6mg Cholesterol in 10mls Chloroform) before being evaporated to dryness at 50°C in a rotary evaporator. 10mg of *E.coli* K12 LPS resuspended in 10mls pyrogen free water supplemented with 0.1% Triethylamine was then added to the dried lipid film. The LPS/lipid suspensions were then rigorously vortexed and sonicated using a bath sonicator for not less than 5 minutes. The preparations were then rotovapped again to dryness and resuspended with vigorous vortexing in PBS. The resulting multilamellar liposomes containing LPS were spun down in a bench centrifuge at 13,000x g, to be stored at –20°C or 4°C.

2.3 Preparation of animals and cells

2.3.1 Maintenance of mammalian cell lines

L929 cells were cultured in RPMI/5%FCS (section 2.1.2) and split every three to four days, or when confluence was reached, using either trypsin/EDTA treatment or mechanical disaggregation. For trypsin/EDTA splits, existing medium was poured off and the adherent cells washed twice in PBS before exposing them to 25mls of proprietary Trypsin/EDTA mix (Gibco) for approximately 5 minutes at 37°C. Disaggregated cells were then washed twice (5 minutes, 300g) in RPMI/5%FCS to remove residual trypsin before plating at a concentration of between 1/5 and 1/20 of the original cellular concentration in standard 75ml tissue culture flasks (Greiner, Longwood, FL, USA). For routine splits (*ie* non-experimental) not requiring fine separation of the cells, cells were scraped from the bottom surface of the flask using sterile plastic scrapers (Nunc) before washing and re-seeding as described above. J774 cells were split in an identical fashion, with the exception that re-seeding occured at a concentration of between 1/3 and 1/7 of the confluent concentration.

THP-1 cells grow in suspension and were split by taking an aliquot of between 1/3 and 1/10 of the confluent population and reseeding into a fresh 75ml tissue culture flask containing RPMI/5%FCS medium.

2.3.2 Separation of peripheral blood mononuclear cells from blood or buffy coats

Venous blood was obtained via venepuncture and collected in sterile heparinised tubes. Buffy coats (a byproduct of the blood transfusion process containing large numbers of white cells) were obtained by arrangement from the Scottish National Blood Transfusion Service (Lauriston Place, Edinburgh). Peripheral blood mononuclear cells (PBMC) were extracted from both samples using the same technique.

10mls of blood or buffy coat was diluted 1:3 in PBS and layered gently onto 10mls of pre-warmed (37°C) Histopaque-1077 (Sigma) in a 50ml Falcon tube, being careful to ensure that the two layers did not mix. Tubes were centrifuged at 300g for 30 minutes to allow red blood cells and granulocytes to separate from the PBMC. Following centrifugation, the layer of cells created at the interface between the two layers was carefully removed by sterile pastette. These PBMC were then washed twice (7 mins, 300g) in PBS or RPMI/5% before being resuspended in appropriate medium at the desired concentration.

2.3.3 Counting of white cells

Pelleted PBMC or splenocytes were resuspended in a small volume of medium (2-10mls). 10μl of this suspension was removed using a sterile tip and carefully mixed with 90μl of white cell diluting fluid (0.01% Gentian violet in 1% acetic acid). A haemocytometer (Hawksley, London) was then assembled and a portion of the sample allowed to fill one chamber. For experiments requiring plating of monocytes, only monocytes (characterised by an irregular nucleus and a prominent extended cytoplasm) were counted.

2.3.4 Separation of monocytes from PBMC

In order to separate monocytes from PBMC, monocytes were counted as described above and the entire population resuspended at $2x10^5$ monocytes per ml. The entire population was then plated at either 100μl per well of a 96 well plate (Iwaki, Stone, UK), 500μl per well of a 24 well plates (Iwaki), or 50mls into a 75ml flat bottomed tissue culture flask (Greiner). Cells were left to adhere for 1 hour at 37°C. Non-adherent cells were then removed with moderate shaking and washing with 3-4 washes of PBS. Remaining adherent cells represent the monocyte fraction of the PBMC.

2.3.5 Thioglycollate stimulation of mice

All animal manipulations were performed by Mr. M. Kerr (Animal House, University of Edinburgh Medical School). A 10% w/v solution of brewers' thioglycollate (Sigma) was prepared in pyrogen free distilled water and autoclaved before storage at 4°C. C3H/HeN mice were then injected with 1ml of this solution into the peritoneal cavity using a 23 gauge needle. Mice were killed after four days and 5mls of sterile PBS injected into the peritoneal cavity using a 25 gauge needle and the area gently palpitated to release cells. Intraperitoneal fluid containing cells was then recovered using a 21 gauge needle.

2.3.6 Vaccination of mice

All animal manipulations were performed by staff of the Animal House, University of Edinburgh Medical School. C3H mice were immunised with either heat killed bacteria (*E.coli* K12 or *E.coli* O18K$^-$), LPS (*E.coli* K12 or *E.coli* O18K$^-$) or sheep red blood cells. LPS samples were adjusted to 0.1mg/ml in normal saline and heat-killed bacteria to $1x10^8$ cells per ml in normal saline. Sheep red blood cells were washed twice in PBS then resuspended in normal saline at a concentration of $2x10^8$ cells/ml before being mixed with an equal amount of Freunds complete adjuvant (Difco, Franklin Lakes, NJ, USA). Mice received duplicate tail base injections of 50μl of each preparation and spleens or lymph nodes were harvested at time points ranging between 3 and 14 days.

2.3.7 Plating of murine peritoneal macrophages

Suspensions of peritoneal cells were washed three times in 25mls of PBS (five minutes, 300g). Cells were then resuspended in a small volume of RPMI/5%FCS, counted and adjusted to $2x10^6$ cells per ml by further addition of RPMI/5%FCS. 1ml of this suspension was then added to each well of a sterile 24 well plate (Greiner) and incubated for one hour (37°C, 5% CO_2) to allow the macrophages to adhere. Following this, the cells were gently washed using sterile pastettes containing PBS to remove non-adherent cells.

2.3.8 Preparation of murine splenocytes

Spleens of naïve or immunised C3H/HeN mice were removed intact and transferred to a sterile universal containing PBS. Sterile agar plates were then opened and a sterile nylon 100μm cell strainer (Falcon, Crowley, UK) placed on top. Spleens were diced with autoclaved scissors and placed on top of the cell strainer membrane. A sterile 200μl pippete tip was then used to diaggregate the cells, while RPMI/5%FCS was constantly washed through to collect cells. The cell strainer and remaining splenic residue were then removed and discarded. Cells washed through to the plate were transferred to a sterile universal for three washes in RPMI/5%FCS at 300g for five minutes before counting and resuspension at the desired concentration.

2.3.9 MACS selection of cells

Antibody selection/depletion of cells from murine derived populations was achieved using the MiniMACS system (Miltenyi Biotech, Bisley, UK). The manufacturers instructions were followed, but in brief a MiniMACS separation column was placed onto the magnet of a MiniMACS separation unit and prepared by passing through 500μl of proprietary buffer. Cells of interest were then stained with antibody (α-murine CD19, Serotec, Oxford, UK, for splenic B-cell depletion experiments) and labeled by incubation with MACS anti-IgG magnetic beads. Following this, a maximum of 10^7 labelled cells in 10^9 unlabelled cells was added to the top of the column and allowed to flow through, followed by three washes of 500μl buffer. Eluant was collected as the depleted cell population. The column was then removed from the magnet and flushed through with 1ml of buffer to elute the positively labelled cells. Cells were counted and washed to resuspend at appropriate conentrations in RPMI/5%FCS.

2.3.10 Creation of CD1a,b,c +ve dendritic cell populations

Human moncytes were allowed to adhere in 75ml tissue culture flasks and washed as described above. Cells were then incubated in 10mls of RPMI/5%FCS containing 200 IU/ml of IL-4 and GM-CSF (Peprotech, Rockyhill, NJ, USA) and harvested after 3 - 4 days incubation at 37°C/5%CO_2.

2.4 Biological Assays

2.4.1 Challenge of cells with LPS/LTA or heat killed bacteria for cytokine measurement

Ten fold dilutions of LPS or *Staphylococcus aureus* lipoteichoic acid (Sigma) were created by adding 450μl of RPMI/10%HS or RPMI to each well of a 24 well plate (Greiner). 4.5μl of defrosted stock (1mg/ml) samples of LPS or LTA were then added to the first well and mixed thoroughly with a fresh pipette tip to provide a concentration of 10μg/ml. A further fresh tip was used to transfer 50μl of this dilution to the next well. It was found that in order to obtain reliable results, a fresh tip must be used for every mixing and transfer step.

For experiments investigating responses to heat killed bacteria, stock samples of heat killed bacteria were diluted to a primary concentration of $1x10^6$ bacterial cells per ml in RPMI/5%FCS and subsequent dilutions made as described above.

Human monocytes were resuspended at $2x10^6$ monocytes/ml in RPMI/10%HS or RPMI without serum and plated at 100μl per well in a 96 well plate (Iwaki). After one hours incubation at 37°C, non-adherent cells were carefully washed off and the medium replaced with 100μl of the LPS dilutions described above. Murine peritoneal macrophages were plated as described (section 2.3.7) and the medium replaced with 500μl of appropriate dilutions of LPS or heat killed bacteria. In all experiments other than those investigating signalling kinetics,

supernatants were measured for TNF-α production at 4 hours, IL-1β production at 18 hours and nitric oxide production at 24 hours, as described below. These timepoints were chosen because peak TNF-α release is seen to occur at around 4 hours (Delahooke et al 1996) while IL-1β and nitric oxide remain clearly detectable in supernatants following an overnight incubation.

For experiments investigating the role of the serum proteins LBP and sCD14 in LPS signalling, fixed concentrations of 100ng/ml LPS or 10μg/ml liposomal LPS were used. Serum free RPMI was supplemented with 1μg/ml purified human plasma lipopolysaccharide binding protein (HBT, Uden, Netherlands) or 1μg/ml recombinant human soluble CD14 (R&D).

For experiments investigating the capacity of the monoclonal antibodies TL2.1 (Cascade bioscience, Winchester, MA, USA) and HTA125 (Serotec) to block TLR signalling, 96 well plates were seeded at $1x10^5$ monocytes per well. 50μl of RPMI/10%HS supplemented with 20μg/ml antibody was added to the cells and allowed to block at room temperature for one hour. 50μl of double concentrated stimulus was then added to each well and incubated overnight at 37°C. Supernatants were harvested and assayed for IL-1β content 18 hours later.

2.4.2 Griess assays for nitric oxide

24 or 96 well plates containing challenged cells were spun at 300g for 7 minutes before carefully decanting 100μl volumes of culture supernates from each well (taken 24 hours after stimulation of cells) into a fresh 96 well plate. A standard curve was prepared using doubling dilutions of a stock solution of Sodium Nitrite (128μM) in blank culture medium. 100μl of Griess reagent (Sigma) was then added to each well and incubated at room temperature for 15 minutes. Griess reagent consisted of sulphanilamide and N-1-napthylethylenediame dihydrochloride. Absorbance was read on an Anthos 2001 plate reader at 540nm.

2.4.3 TNF-α bio-assay

A bioassay was chosen over ELISA based methods of TNF-α measurement on the basis of cost and the ability to measure functional TNF-α. A modified version of the technique of Delahooke *et al* (1996) was employed. L929 cells were disaggregated using the trypsin/EDTA method as described above (mechanical disaggregation was seem to lead to poor reproducibility) and resuspended at $4x10^6$ cells/ml in DMEM/5%FCS. 100μl of this suspension was then added to each well of a 96 well tissue culture plate and left for 3-4 hours to adhere.

Supernatants were then carefully removed from each well and replaced with 90μl (sample wells) or 100μl (standard curve wells) of DMEM/5%FCS containing 1μg/ml Actinomycin-D. To create the standard curve, 10μl of medium was removed from well number 1 and replaced with 10μl of 10,000 IU/ml recombinant TNF-α in PBS (Peprotech). From this well, 3.2 fold dilutions were performed along the standard curve row by taking 45μl along and mixing into the 100μl already present. The final 45μl was discarded from the last well in the row. For sample measurement, 10μl of fresh supernatant from challenged cells was added to the 90μl of DMEM/5%FCS/ActD already present in each sample measurement well. Plates were then incubated at 37°C, 5% CO_2 overnight.

The following day, plates were inverted vigorously to remove medium from the cells. 50μl of crystal violet (0.5% Crystal violet in 20% methanol in dH_2O, filtered through Whatman no1 paper) was added to each well and left to stain for 3-4 minutes. Each well was washed thoroughly under the tap. Following a brief drying spell on tissues to remove the excess water, 50μl of acetic acid (20% Acetic acid in dH_2O) was added to each well to solubilise the cells. Absorbance was read at at 540nm.

2.4.4 Limulus Amoebocyte Lysate (LAL) assays

The Pyrochrome LAL kit (Associates of Cape Cod, Falmouth, MA, USA) was used according to manufacturers instructions. In brief however, Pyrochrome reagent was reconstituted in 3.2mls of pyrochrome buffer and kept on ice. A vial containing 0.2ng of control endotoxin was reconstitued in 2mls of Pyrogen free water to achieve a 1.0 EU/ml concentration. A seven point standard curve was created using doubling dilutions from 1 EU/ml (0.1ng/ml) downwards.

Samples of LPS (10pg/ml) or liposomal LPS (1ng/ml) resuspended in pyrogen-free water (PBS interferes with the assay) were added at 50μl per well in a 96 well plate. Pyrogen-free water alone was included as a control. 50μl of reconstituted pyrochrome reagent was then added to each sample or control and the plate was shaken gently before incubating at 37°C for 30 minutes. To stop the reaction, 25μl of 50% acetic acid in pyrogen free water was added to each well. Absorbance values were read at 405nm on an Anthos 2001 automated plate reader.

2.4.5 Measurement of human IL-1β secretion

Supernatant levels of the human cytokine IL-1β were measured using the Duoset ELISA development system (R&D) according to the manufacturers instructions. Briefly, high-binding ELISA strips (Maxisorp type, Nunc, Rochester, NY, USA) were assembled in ELISA frames and 100μl of capture antibody diluted 1:180 in PBS was added to each well. Plates were sealed and incubated overnight at room temperature. The following day, wells were washed three times in ELISA wash buffer (PBS containing 0.05% Tween-20) using an automated ELISA plate washer (Dynatech, Chantilly, UA, USA) and the last remaining liquid removed by blotting on paper towels.

Wells were then blocked with 300μl of blocking solution (PBS supplemented with 1% BSA, 5% sucrose and 0.05% NaN_3) at room temperature for one hour before washing as described above. Plates were then dried thoroughly by blotting and either used immediately or stored at 4°C for later use.

50-100μl of tissue culture supernatant was added to each well, with one strip providing a dilution curve created by doubling dilutions of 250pg/ml recombinant IL-1β in blank tissue culture medium. Plates were incubated at room temperature for 2 hours before washing (as above). 100μl of detection antibody diluted 180 fold in reagent diluent (PBS supplemented with 1% BSA, sterile filtered at 0.2μm) was then added to each well and the plate allowed to incubate at room temperature for a further 2 hours. Plates were washed as above before adding 100μl of streptavidin conjugated to horseradish peroxidase diluted 200 fold in reagent diluent. Plates were incubated for 20 minutes at room temperature and washed as described. 100μl of substrate solution (one tablet of Tetramethylbenzadine (Sigma) dissolved in 10mls 0.05M phosphate/citrate buffer, pH 5.0 (308mg citric acid and 482mg Na_2HPO_4 dissolved in 100mls distilled water), supplemented with 2μl of fresh 30% hydrogen peroxide) was then added to each well and the plate covered and incubated at room temperature for a further 20 minutes. Finally, 50μl of stop solution (2N H_2SO_4) was added to each well and the plates gently tapped to encourage complete mixing. Optical densities of each well were read at 450nm using an automated plate reader. The blank well OD was subtracted from each value and a standard curve created from the recombinant cytokine values to provide a measure of the amount of cytokine in each sample.

2.4.6 Preparation of endogenous core antibody (EndoCAb) ELISA plates

Stock 1mg/ml (approximately 0.2mM) solutions of LPS from the following strains were prepared using pyrogen free water:

(i) *E.coli* K12 (ii) *P.aeruginosa* PAC611 (iii) *K.aerogenes* KaM10b (iv) *S.typhimurium* 878.

Each solution was sonicated for 30s at 5 microns amplitude using a Sanyo Soniprep 150 sonicator. A 1mg/ml solution of polymyxin-B sulphate (~1mM) in pyrogen free water was then prepared. 1ml of each LPS solution was aliquoted to a new bijoux and mixed with 1ml of 1mM polymyxin-B before sonicating as before for another 30s. The resulting four mixtures were then transferred to cellulose dialysis tubing with a 2000 mwt cut off (Spectrum Medical Industries Inc, Los Angeles, USA) and dialysed against distilled water overnight to remove unbound polymyxin.

Dialysis tubes were then carefully cut and the floccular LPS/polymyxin-B complexes resuspended thoroughly before removing with pipette and storing in fresh glass bijoux at –20°C.

To coat ELISA plates or strips with complex, 100μl of each of the four complexes was combined in a glass universal and then diluted 1:50 by addition of 20mls of coating buffer (see below). After vortexing, this mixture was rapidly plated at 100μl per well of 96 well ELISA plate strips (Nunc, Intermed, Kaastrup, Roskilde, Denmark). Strips were left to coat overnight at 37°C, then washed four times with wash buffer (see below) using an ELISA plate washer (Dynatech). Wells were then post-coated with 100μl of post-coat buffer (see below) and left to incubate at 37°C overnight. After 4 further washes with wash buffer, strips were rinsed with distilled water and allowed to dry thoroughly before storing in foil at –20°C.

Coating buffer (pH=9.6): 0.05M carbonate/bicarbonate buffer (6.2g/l $Na_2CO_3.H_2O$, 4.2g/l $NaHCO_3$) plus 0.05% w/v sodium azide.

Post coat buffer: PBS containing 2% w/v BSA and 0.05% sodium azide.

Wash buffer: PBS containing 0.05% v/v Tween-20 (Sigma).
Dilution buffer: PBS containing 0.05% v/v Tween-20, 4% w/v polyethylene glycol 6000 (Sigma) and 0.05% sodium azide.
Alkaline phosphatase substrate solution: 0.05M sodium carbonate buffer (pH=9.8), 1.0mM Magnesium chloride, made up as follows: Solution A=4.24g Na_2CO_3 in 200mls dH_2O, Solution B=3.36g $NaHCO_3$ in 200mls dH_2O. Mix 55mls of A with 70mls of B and 375mls of dH_2O. Check pH is 9.8. Add 102mg of $MgCl_2$, and dispense into 50ml screw capped bottles. All solutions are stored at 4°C.

2.4.7 Endogenous core antibody (EndoCAb) ELISA

Sufficient strips for a complete experiment were taken from –20°C storage and placed in a frame with dummy strips to complete the plate. Plasma samples were diluted 1:400 in dilution buffer (section 2.4.6) and added at 100µl per well in triplicate. A standard curve was created using doubling dilutions of a stock sample of serum found in a previous study (Barclay *et al*, 1987) to contain a high titre of anti-core LPS antibodies. A well containing dilution buffer only was also included as a negative control. Plates were then wrapped in foil and incubated at 37°C for 90 minutes before washing 4 times with wash buffer (section 2.4.6). Monoclonal antibodies against heavy chains of human IgG or IgM (Sigma) conjugated to alkaline phosphatase were diluted 1:2000 in dilution buffer and added at 100µl per well before incubating for a further 90 minutes at 37°C.

The plates were then washed four times with wash buffer before addition of 100µl alkaline phosphatase substrate to each well made up by the addition of one alkaline phosphatase substrate tablet (Sigma) per 5mls of the buffer described above. Plates were then left to develop at room temperature before reading at 405nm on an Anthos 2001 automated plate reader every 5 minutes or so. The final reading was recorded when the highest optical density value on the plate approached a value of 1.0.

2.4.8 T-cell proliferation assays

Splenocytes extracted from mice or PBMC purified from human blood or buffy coats (section 2.3.4) were resuspended at a concentration of $1x10^6$ cells per ml in RPMI/5%FCS or RPMI supplemented with 20% autologous plasma in some human PBMC experiments. 200μl of this suspension was added to each well of a 96 well plate and 100μl of each LPS dilution (prepared as described in section 2.4.1) or 1μg/ml Concanavalin A (Sigma) added directly on top. Cells were then incubated for 2-14 days at 37°C. 18 hours before harvesting, 10μl of PBS containing 1μCi of tritium labelled thymidine (Amersham, Piscataway, NJ, USA) was added to each well being careful not to cross-contaminate wells. Cells were then harvested onto filter mats (Camo, Newmarket, Suffolk), and thymidine incorporation measured by scintillation in a Packard 1900CA β-counter.

In experiments to block proliferation with antibodies against CD1a,b or c (Serotec), azide was removed from stock antibodies by dialysis in proprietary dialysis cassettes (Serotec) against a large volume of PBS in a stirred container overnight. Final concentrations of anti-CD1a and CD1b were 0.05mg/ml, while the concentration of anti-CD1c was 1mg/ml. Following plating of PBMC and LPS as described above, 10μl of undiluted stock azide free antibody was added to each culture (final concentration of antibodies: 2.5μg/ml α-CD1a,b,d or 50μg/ml α-CD1c).

2.4.9 Staining of cells for flow cytometry

Cells were washed and adjusted to ~$5x10^6$ cells per ml. 100μl was then added to each well of a V-bottom 96 well plate, supplemented with 25μl of neat normal rabbit serum and placed on ice for 20 minutes. Meanwhile, 20mls of PBS was freshly supplemented with 2% normal rabbit serum (Serotec) and used to dilute

primary antibodies to working concentrations according to manufacturers instructions (typically 1:100).

The plate was then centrifuged at 300g for 7 minutes to pellet the cells and thrown over a sink to expel the supernatant. Cells were resuspended in 200μl PBS/NRS and washed twice further in this fashion before resuspension in 10-20μl of each antibody and incubation on ice for a further 45 minutes. Plates were washed a further three times. Cells were then resuspended in 25μl of secondary antibody (conjugated to PE or FITC) diluted according to the manufacturers instructions. Following a final 30 minute incubation on ice in the dark, plates were washed three times in 200μl PBS/NRS and resuspended in PBS supplemented with 1% paraformaldehyde (BDH). Fixed cells were stored at 4°C in the dark until the flow cytometer became available.

2.5 Protein and DNA analysis

2.5.1 Immunoprecipitation

The Mammalian SiezeX immunoprecipitation kit (Pierce, Rockford, IL) was used to immunoprecipitate proteins from cellular preparations. Manufacturers instructions were adhered to, though a brief description of the protocol used follows. Immobilised antibody columns were created using protein-G conjugated resin cross linked to user supplied antibody (CD1a,b,c or d) using disuccinimidyl suberate. Mammalian cells were then lysed using kit supplied lysis reagent, mixed gently for 10 minutes and transferred to the column for a ten minute incubation. Columns were then washed through with proprietary buffer before elution of purified proteins using proprietary elution buffer. For PAGE analysis of lysate protein content, 20μl of eluant was mixed with 5μl of proprietary sample buffer, boiled for 5 minutes and allowed to cool before loading on a PAGE gel.

2.5.2 Preparation of samples for polyacrylamide gel electrophoresis (PAGE)

Protein samples were prepared as described in section 2.5.1. LPS samples were adjusted to 1.0mg/ml and 10μl of sample added to 10μl of single strength PAGE sample buffer (below) and heated for 10 minutes in a 100°C water bath. 20μl of this mixture (10μg total LPS) was run on each track of the gel. Single strength PAGE sample buffer: 0.0625M Tris (BDH) pH 6.8 supplemented with 2.0% w/v SDS (BDH), 10% v/v glycerol (BDH), 1% v/v 2-mercaptoethanol (BDH) and 0.001% bromophenol blue (BDH)

2.5.3 PAGE

PAGE of protein and LPS samples was performed according to the method of Laemmli (1970). PAGE of samples for subsequent immunoblotting was carried out as described below with the exception that SDS was omitted from each of the buffers. The following buffers and solutions were used:

1) Electrode buffer (pH8.3) consisted of 0.025M Tris (BDH), 0.192M glycine (BDH chromatographically homogeneous) and 0.1% w/v SDS (BDH).
2) Separating gel buffer (double strength, pH 8.8) consisted of 0.75M Tris-HCl at pH 8.8 to which 0.2% w/v SDS was added (omitted for immunoblots).
3) Stacking gel buffer (double strength, pH 6.8) consisted of 0.25M Tris-HCl at pH6.8 to which 0.2% w/v SDS was added (omitted for immunoblots).
4) Acrylamide stock solution (40% w/v) contained 100g acrylamide (BDH) and 2.7g methylene bis acrylamide (BDH) and made up to 250ml with distilled water.
5) Ammonium persulphate (15mg/ml) prepared by addition of 300mg ammonium persulphate (BDH) to 20 mls pyrogen free water. Stable for 14 days at 4°C.

The separating gel was prepared according to the proportions described in Table 2.2 and degassed under vacuum prior to addition of 50μl TEMED (NNN'N-tetramethyl-1,2-diaminoethane, BDH) and 1.75 ml ammonium persulphate (BDH). The gel solution was poured between two assembled gel electrophoresis glass plates (160mmx125mmx1.5mm) previously cleaned with methanol and sealed at sides and bottom with vaseline. The gel was overlayed with water saturated butan-2-ol (BDH) and allowed to set. The butan-2-ol was then discarded and the stacking gel, prepared according to the proportions described in Table 2.2, was poured onto the separating gel. A comb was inserted and the gel allowed to set. On removing the comb the gel was fitted into an electrophoresis tank (Jencons Scientific Ltd, Beds.) and electrode buffer added to brim the wells of the gel.

Each well was then loaded with either sample (prepared as described in sections 2.5.1 and 2.5.2) or an equivalent volume of single strength sample buffer. The gel was then run at an initial voltage of 60V, before switching to run at 150V as the samples passed into the separating gel. After electrophoresis, LPS samples were analysed by silver staining (section 2.5.4) and protein samples by coomassie blue staining (section 2.5.5) or by immunoblotting after transfer to nitrocellulose (section 2.5.6).

Table 2.2 Reagents required for preparation of PAGE gels

Reagent:	*12% Separating gel*	*4% Stacking gel*
Distilled Water	5.2 ml	3.5 ml
Separating buffer	17.5 ml	-
Stacking buffer	-	5.0 ml
Acrylamide solution (40%)	10.5 ml	1.0 ml
TEMED	50 μl	20 μl
Ammonium persulphate (15mg/ml)	1.75 ml	0.5 ml

2.5.4 Silver staining of PAGE gels for LPS

An adaptation of the methods of Tsai & Frasch (1982) and Hitchcock & Brown (1983) was used to visualise LPS in PAGE gels. The following reagents and solutions were used in the silver staining procedure:

1) Fixative consisted of 7% v/v acetic acid (BDH) and 25% propan-2-ol (BDH) in distilled water.
2) Oxidising solution consisted of 1.05g periodic acid (BDH) made up in 150ml of distilled water and 4ml of the above fixative solution.
3) Ammoniacal silver nitrate solution was prepared freshly by placing 1.4ml of ammonia solution (BDH, specific gravity 0.88) and 21 mls of 0.36% NaOH in a dedicated 100ml flask and adding in a dropwise fashion with continual mixing 4mls of 19.4% silver nitrate solution. Volume was made up to 100ml with distilled water.
4) Developing solution, prepared just before use, consisted of a 0.019% v/v formaldehyde (BDH) solution containing 0.005% w/v citric acid.

PAGE gels were placed in fixative and left overnight at room temperature on a slowly shaking platform (used for all of the following steps). Fixative was poured off and the gel oxidised for 5 minutes with a freshly prepared solution of periodic acid. The gel was then washed in at least four changes of 200ml distilled water over 4 hours. Ammoniacal silver nitrate solution, freshly prepared as above, was then added to the gel. After staining for 10 minutes, the gel was washed with at least four changes of distilled water (200ml) over 40 minutes. Samples were visualised with the addition of 200ml of freshly made developing solution. Once the desired staining intensity had been obtained (5-10 minutes) the gel was washed repeatedly in large volumes of distilled water and stored in water in the dark before digital scanning and recording.

2.5.5 Coomassie blue staining of PAGE gels for protein

The coomassie blue stain described by Hancock and Poxton (1988) was used. The solutions used were made up in distilled water and included:

Solution (1) - 25% v/v propan-2-ol (BDH), 10% v/v acetic acid (BDH) and 0.05% w/v Coomassie brilliant blue R-250 (BDH).

Solution (2) - 10% v/v propan-2-ol, 10% v/v acetic acid and 0.005% w/v Coomassie blue.

Solution (3) - 10% v/v acetic acid and 0.0025% w/v Coomassie blue.

Solution (4) - 40% v/v methanol (BDH) and 10% v/v acetic acid.

Solution (5) - 10% v/v acetic acid.

PAGE gels were placed in solution 1 overnight and then sequentially through solutions 2-5, for 45-60 minutes each at room temperature with gentle shaking throughout, before digital scanning and recording.

2.5.6 Immunoblotting

Cellular protein extracts (prepared as described in section 2.5.1) were separated by PAGE and transferred to nitrocellulose (NIC) paper for immunochemical analysis. A modification of the method of Towbin *et al* (1979) was followed.

The following reagents and solutions were used:

1) Electrode transfer buffer (pH8.3) - contained 6.1g Tris (BDH), 28.8g glycine (BDH chromatographically homogeneous) and 400mls methanol (BDH) made up to 2000mls with distilled water.
2) Tris buffered saline (pH7.5) (TBS) – consisted of 4.84g Tris (BDH) and 58.48g sodium chloride (BDH) made up to 2 litres with distilled water.
3) Tween-Tris-buffered saline (pH 7.5) (TTBS) – was made as TBS with the addition of 0.025% v/v Tween-20 (Sigma).
4) Blocking solution – was made as for TBS with the addition of 3% w/v gelatin (BioRad EIA grade).

5) Antibody/conjugate diluent – was made as for TBS with the addition of 1% w/v gelatin (BioRad EIA grade).
6) Horse radish peroxidase (HRP) colour developer was made just before use by the mixing of two solutions. Solution A: 60mg 4-chloro-1-napthol (BioRad HRP colour reagent) dissolved in 20mls methanol. Solution B: 100ml TBS containing 60μl hydrogen peroxide (BDH, 30% w/v).

Transfer of antigens from PAGE gels to nitrocellulose

PAGE gels were removed from the electrophoresis apparatus and placed into a Scotchbrite blotting cassette. The gel was then covered with a sheet of NIC presoaked in electrode transfer buffer, then two sheets (either side) of presoaked blotting paper. The cassette was then closed sandwiching the gel, NIC paper and blotting paper between the two Scotchbrite porous foam pads. The assembly was then placed into the immunoblotting tank containing electrode transfer buffer, ensuring that the gel was placed towards the cathode and the NIC towards the anode. A constant current of 40mA was applied overnight at 4°C.

Visualisation of antibody-antigen reactions

After electrophoretic transfer of antigens the NIC was removed and washed in TBS for 10 minutes before placing in blocking solution for 45 minutes (with gentle shaking, as used for all subsequent steps). Once blocked, the NIC was transferred into antibody dilution buffer containing 1μg/ml of antibody (α-CD1a,b,c or d, Serotec) and incubated for 3 hours at room temperature. The NIC was then rinsed briefly in distilled water and washed for two 10 minute periods in TTBS, following which it was incubated with HRP-conjugated anti-mouse IgG (Serotec) diluted in antibody diluent for 60 minutes followed by rinsing in distilled water and washing in TTBS (as above). The binding of antibody to separated antigenic determinants was visualised by addition of HRP colour developing solution to the NIC. The colour was allowed to develop for up

to 30 minutes and then stopped by washing in several changes of distilled water. The blot was dried before digital scanning and recording.

Dot-blot protocol

In order to determine if a particular antibody/protein interaction could be detected in the context of NIC fixation, dot blots were performed. Small sections of NIC (1cm x 4cm) were prepared using a sharp scalpel and washed in TBS with gentle shaking (employed for all subsequent steps) for 10 minutes before drying thoroughly. 1 to 2 μl of protein extract was then placed on the NIC and allowed to dry completely at 37°C. Samples were then washed in TBS for 10 minutes as before. Blocking solution (prepared as above) was then applied for 45 minutes followed by a 2μg/ml solution of antibody (α-MHC-II, CD3 or CD1a,b,c or d, Serotec) diluted in antibody diluent for 3 hours. The NIC was washed twice in Tween-TBS for 10 minutes each then incubated with HRP-conjugated anti-mouse IgG (Serotec) diluted in antibody diluent for 1 hour. NIC were washed two further times in Tween-TBS as before then rinsed with three brief changes of tap water. Developing solution was freshly prepared (as above) and applied to the NIC. Development was stopped by washing the NIC several times in tap water. The NIC was then dried before digital scanning and recording.

2.5.7 Preparation of genomic DNA for polymerase chain reaction (PCR)

The ReadyAmp genomic DNA purification system (Promega, Southampton, UK) was used to extract genomic DNA from whole blood or stored PBMC samples. Manufacturers instructions were followed but in brief consisted of the following. 50μl of stored PBMCs ($2x10^6$/ml) or 100μl of heparinised blood was made up to 1ml with nuclease free water and vortexed occasionally for 10 minutes. Cells were pelleted by centrifugation at 13,000g for 2 minutes, then 200μl of kit supplied resin was added and vortexed. Suspensions were then incubated at 56°C for 20 minutes, followed by 8 minutes at 100°C in a water bath. Samples

were vortexed, then centrifuged and the supernatant containing the isolated single stranded DNA carefully decanted for storage in fresh tubes at –20°C.

2.5.8 PCR of Toll receptor mutations

Patient and volunteer genomic DNA samples were screened for the presence of two mutations in the Toll like receptor 4 gene (Asp299Gly and Thr399Ile) according to the method of Lorenz *et al* (2001). Briefly, 5μl of genomic DNA was added to 25μl of ReadyTaq PCR mix (Sigma) supplemented with 0.2μl (20pmol) of each primer and 19.6μl of PCR grade nuclease free water. Thermal cycling was applied according to the following protocol: 95°C for 4 minutes, then 30 cycles of 95°C for 30s, 55°C for 30s and 72°C for 30s, followed by a final completion stage of 72°C for 4 minutes and a 4°C hold phase.

Primers were designed to incorporate a missense mutation to provide a restriction enzyme site (underlined bases). The positions of the primers in relation to the mutations in the cDNA sequence of TLR-4 are shown in Figure 2.1. The mutation sites are indicated by capital letters and occur immediately following the 299For and 399For primers.

Asp299Gly forward primer: 5'-GATTAGCATACTTAGACTACTACCTCCATG-3'

Asp299Gly reverse primer: 5'-GATCAACTTCTGAAAAAGCATTCCCAC-3'

Thr399Ile forward primer: 5'-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3'

Thr399Ile reverse primer: 5'-ACCTGAAGACTGGAGAGTGAGTTAAATGCT-3'

PCR products were visualised by running on a 1.5% ethidium-stained agarose gel against 2μl of 100 base pair ladder (Promega).

Figure 2.1 cDNA sequence of TLR-4

```
   1 cctctcaccc tttagcccag aactgctttg aatacaccaa ttgctgtggg gcggctcgag
  61 gaagagaaga caccagtgcc tcagaaactg ctcggtcaga cggtgatagc gagccacgca
 121 ttcacagggc cactgctgct cacagaagca gtgaggatga tgccaggatg atgtctgcct
 181 cgcgcctggc tgggactctg atcccagcca tggccttcct ctcctgcgtg agaccagaaa
 241 gctgggagcc ctgcgtggag gtggttccta atattactta tcaatgcatg gagctgaatt
 301 tctacaaaat ccccgacaac ctccccttct caaccaagaa cctggacctg agctttaatc
 361 ccctgaggca tttaggcagc tatagcttct tcagtttccc agaactgcag gtgctggatt
 421 tatccaggtg tgaaatccag acaattgaag atggggcata tcagagccta agccacctct
 481 ctaccttaat attgacagga aaccccatcc agagtttagc cctgggagcc ttttctggac
 541 tatcaagttt acagaagctg gtggctgtgg agacaaatct agcatctcta gagaacttcc
 601 ccattggaca tctcaaaact ttgaaagaac ttaatgtggc tcacaatctt atccaatctt
 661 tcaaattacc tgagtatttt tctaatctga ccaatctaga gcacttggac ctttccagca
 721 acaagattca aagtatttat tgcacagact tgcgggttct acatcaaatg cccctactca
 781 atctctcttt agacctgtcc ctgaacccta tgaactttat ccaaccaggt gcatttaaag
 841 aaattaggct tcataagctg actttaagaa ataattttga tagtttaaat gtaatgaaaa
 901 cttgtattca aggtctggct ggtttagaag tccatcgttt ggttctggga gaatttagaa
 961 atgaaggaaa cttggaaaag tttgacaaat ctgctctaga gggcctgtgc aatttgacca
1021 ttgaagaatt ccgattagca tacttagact actacctcga tgAtattatt gacttattta (299for)
1081 attgtttgac aaatgtttct tcatttcccc tggtgagtgt gactattgaa agggtaaaag
1141 acttttctta taatttcgga tggcaacatt tagaattagt taactgtaaa tttggacagt
1201 ttcccacatt gaaactcaaa tctctcaaaa ggcttacttt cacttccaac aaaggtggga (299rev)
1261 atgctttttc agaagttgat ctaccaagcc ttgagtttct agatctcagt agaaatggct
1321 tgagtttcaa aggttgctgt tctcaaagtg attttgggac aaCcagccta aagtatttag (399for)
1381 atctgagctt caatggtgtt attaccatga gttcaaactt cttgggctta gaacaactag
1441 aacatctgga tttccagcat tccaatttga aacaaatgag tgagttttca gtattcctat
1501 cactcagaaa cctcatttac cttgacattt ctcatactca caccagagtt gctttcaatg
1561 gcatcttcaa tggcttgtcc agtctcgaag tcttgaaaat ggctggcaat tctttccagg
1621 aaaacttcct tccagatatc ttcacagagc tgagaaactt gaccttcctg gacctctctc
1681 agtgtcaact ggagcagttg tctccaacag catttaactc actctccagt cttcaggtac (399rev)
1741 taaatatgag ccacaacaac ttcttttcat tggatacgtt tccttataag tgtctgaact
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2761 aaatgctgcc acatgtcagg ccttatgcta agggtgagta attccatggt gcactagata
2821 tgcagggctg ctaatctcaa ggagcttcca gtgcagaggg aataaatgct agactaaaat
2881 acagagtctt ccaggtgggc atttcaacca actcagtcaa ggaacccatg acaaagaaag
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3121 ttctacttga tgactgcagt cgtcaagggg ctcctgatgc aagatgcccc ttccatttta
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3301 tgatatattt ttatttttat atatccagtt ttcatttttt tacgtcttgc ctataagcta
3361 atatcataaa taaggttgtt taagacgtgc ttcaaatatc catattaacc actatttttc
3421 aaggaagtat ggaaaagtac actctgtcac tttgtcactc gatgtcattc caaagttatt
3481 gcctactaag taatgactgt catgaaagca gcattgaaat aatttgttta aaggggcac
3541 tcttttaaac gggaagaaaa tttccgcttc ctggtcttat catggacaat ttgggctaga
3601 ggcaggaagg aagtgggatg acctcaggag gtcacctttt cttgattcca gaaacatatg
3661 ggctgataaa cccggggtga cctcatgaaa tgagttgcag cagaagttta tttttttcag
3721 aacaagtgat gtttgatgga cctctgaatc tctttaggga gacacagatg gctgggatcc
3781 ctcccctgta cccttctcac tgccaggaga acta
```

2.5.9 Genotyping of Toll receptor mutations

Restriction of PCR products was also achieved according to the method of Lorenz *et al* (2001). Asp299Gly and Thr399Ile products were screened with *Nco*I (restriction site: C/CATGG, Promega, UK) and *Hinf*I (restriction site: G/ANTC, Promega, UK) restriction enzymes respectively. Briefly, 10μl of each PCR product was added to 2μl of appropriate proprietary restriction enzyme buffer, 0.7μl of respective enzyme, 0.2μl of 1mg/ml bovine serum albumin and 7.1μl of nuclease free water, mixed and incubated for 4 hours at 37°C.
Digest products were run on a 3% high resolution agar gel (Nusieve 3.1, Flowgen, Ashby de la Zouch, UK) at low voltage (65volts), alongside 2μl of 100 base pair ladder (Promega). Cut products indicated presence of the mutation.

2.6 Patient recruitment

2.6.1 Patient recruitment

A prospective series of patients presenting with inflammatory disease arising from a suspected infectious origin were asked to participate in the following study. Each diagnosis was made on the basis of judgement of clinical variables by an experienced clinician. Over the course of 14 months, venous blood samples (20mls) were taken from a total of 40 patients enrolled at the Royal Infirmary of Edinburgh Surgical Admissions Unit. Of these patients, six agreed to follow up blood sampling on either one or two occasions. 40 healthy controls were also enrolled.

Patients were enrolled if a clinical diagnosis was confirmed consistent with abdominal inflammation together with a documented source of infection. Written informed consent was obtained from each participant. On enrolement each patient was scored for systemic inflammatory response syndrome (SIRS score, Bone *et al* 1992), sepsis related organ failure assesment (SOFA score, Vincent *et al* 1996) and if presenting with acute pancreatitis or Crohn's disease the

modified Glasgow score (Blamey *et al* 1984) or simple index of Crohn's activity (Harvey *et al* 1980) respectively.

For each sample, PBMC were assessed for capacity to proliferate in response to LPS or LTA (section 2.4.8), or stored at –20°C for subsequent genetic analysis (section 2.5.9). Plasma samples were also retained for each sample for anti-LPS antibody measurements (section 2.4.7).
Approval for the study was granted by the Lothian Research Ethical Committee (LREC No. 2000/5/35). An example consent form is included in Appendix I.

CHAPTER 3

INNATE IMMUNE RESPONSES TO LIPOPOLYSACCHARIDE

3.1 Introduction

A novel liposomal lipopolysaccharide vaccine has been proposed for the prophylactic treatment of pre-surgical patients at risk of endotoxaemia. As one of the key attributes of the vaccine is that it must demonstrate low toxicity, the biological activity of the formulation was compared in the present study with that of native LPS and with heat killed bacteria. Murine macrophages and human monocytes were challenged with various concentrations of each preparation and the resulting release of nitric oxide or TNF-α measured to allow comparison of the different preparations. The mechanism for liposomal LPS signalling was investigated using reconstitution of the serum proteins LBP and sCD14.

Toll receptor usage of different LPS types was investigated using antibody blocking of TLR-2 and TLR-4. Then, as two point mutations in the TLR4 gene have been shown to exist in the Caucasian population at a frequency higher than that expected by chance (6-10%), experiments were performed to determine whether individuals expressing mutant LPS receptors exhibit either more or less sensitive recognition of LPS from particular strains and species of bacteria.

3.2 Results

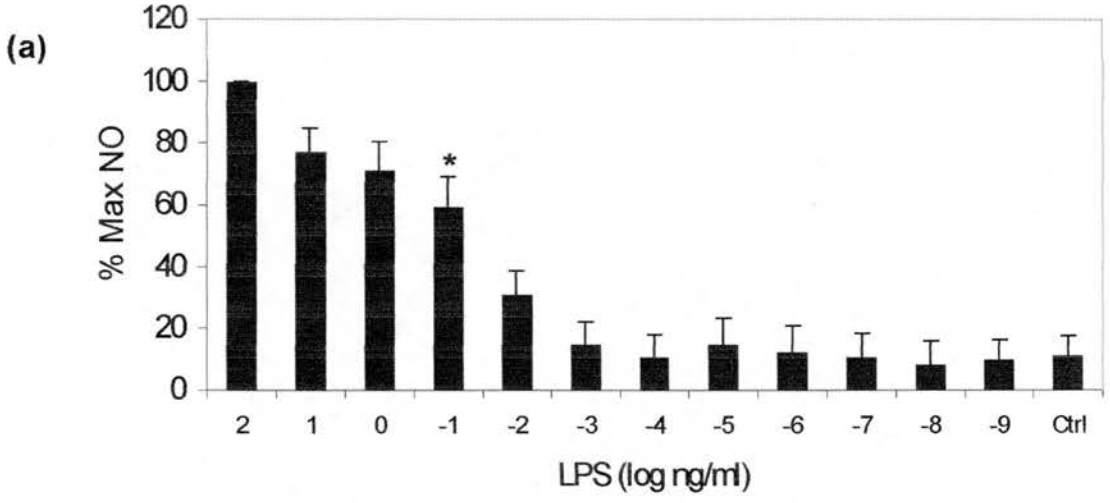
3.2.1 Murine nitric oxide responses to LPS, liposomal LPS and heat killed bacteria

In order to determine the biological activity of a liposomal LPS vaccine, experiments were performed to compare the response of murine thioglycollate elicited peritoneal macrophages to native LPS, liposomal LPS and heat killed bacteria. Peritoneal macrophages were challenged with varying concentrations of either *E.coli* K12 LPS, heat killed *E.coli* K12 bacteria, liposomal *E.coli* K12

LPS or medium alone (control) and supernatants were assayed for production of nitric oxide after a period of 18 hours incubation.

In all experiments, a high level of background expression of nitric oxide was observed. This was initially considered to be as a result of the activated state of the peritoneal macrophages, though alteration of the protocol for thioglycollate stimulation of the mice was found to be unable to reduce this spontaneous activity. As a result, in order to observe a clear point of dilution for the different LPS preparations, the results from many experiments had to be combined. However, since the peak value of nitric oxide produced varied from mouse to mouse (roughly 20 to 125μM), the maximum expression of NO in each experiment was normalised to a value of 100% and all other data points re-scaled proportionally. Collation of normalised data from the many experiments performed was then able to provide an indication of the probable point of dose response for the various stimuli.

Figure 3.1a shows that NO release from murine macrophages challenged with native LPS is maximal in response to 100ng/ml LPS, with this response falling in a dose dependent fashion such that below concentrations of LPS around 0.01ng/ml, no release of NO significantly different from background can be measured. Figure 3.1b shows a similar dose dependent release of NO in response to challenge of cells with liposomal LPS, though the concentration of LPS required to initiate release of NO from cells is 32ng/ml or above. Figure 3.1c shows that the NO response of murine macrophages to heat killed *E.coli* is maximal at 1,000,000 bacteria per ml, reducing in a dose dependent fashion such that concentrations of less than 3,200 bacteria per ml do not stimulate cells to release nitric oxide. These results are summarised in Table 3.1.



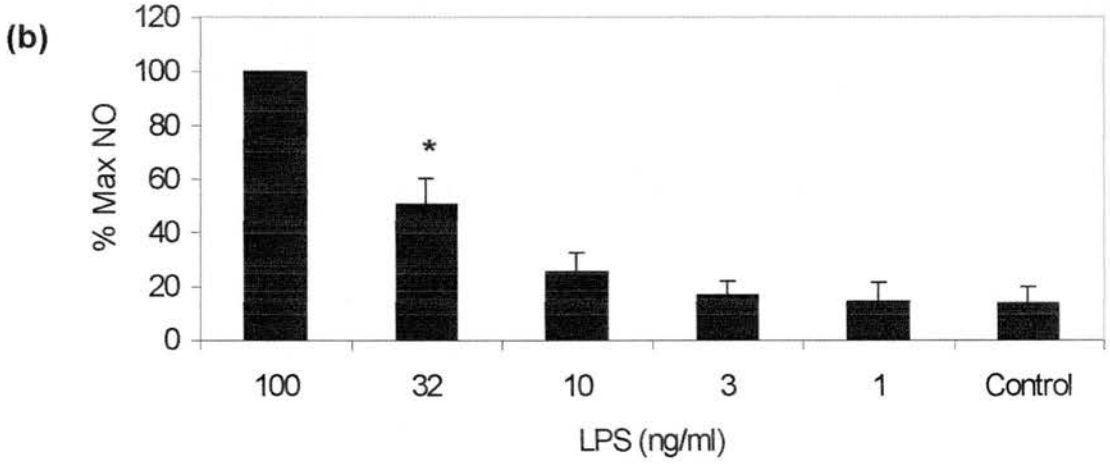


Figure 3.1 Murine macrophage nitric oxide release in response to challenge with (a) native *E.coli* K12 LPS (b) liposomal *E.coli* K12 LPS or (c) heat-killed *E.coli* K12 bacteria

Results are normalized means +/-SEM of 4 independent experiments for LPS, 8 independent experiments for liposomal LPS and 9 independent experiments for heat killed bacteria. * Indicates data point representing lowest concentration at which response is significantly different from control ($p<0.05$). Ctrl represents response of cells incubated in medium alone. Maximum NO production varies between experiments with a range of 20 to 125μM.

(c)

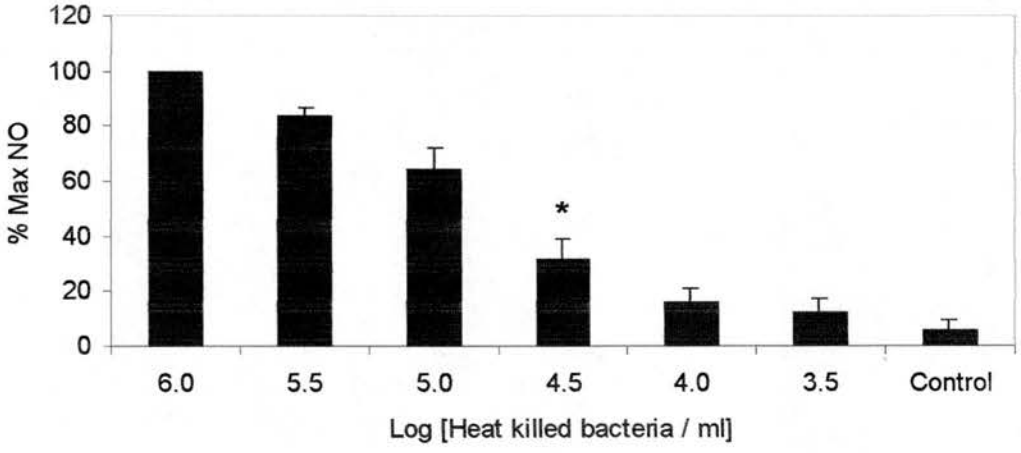


Figure 3.1 Murine macrophage nitric oxide release in response to challenge with (c) heat-killed *E.coli* K12 bacteria

Table 3.1 Comparison of capacity of LPS, liposomal LPS and heat killed bacteria to induce nitric oxide production in murine peritoneal macrophages

Stimulant	Minimum concentration required to stimulate macrophages *
Native *E.coli* K12 LPS	1×10^{-2} ng/ml
Liposomal *E.coli* K12 LPS	32 ng/ml
Heat killed *E.coli* K12 bacteria	3.2×10^{4} bacteria/ml

* Minimum concentration represents the lowest concentrations at which native *E.coli* K12 LPS, liposomal *E.coli* K12 LPS or heat killed *E.coli* K12 bacteria stimulate murine macrophages to release significantly more nitric oxide ($p<0.05$) than medium alone.

The data (Table 3.1) suggest that, weight for weight, LPS contained within liposomes is at least 3,200 fold less capable of inducing a nitric oxide response from murine peritoneal macrophages than native LPS alone. Further, as it has been calculated that 10^6 whole *E.coli* bacteria contain approximately 100ng of membrane bound LPS, it is clear that the liposomal formulation is also 10 times less stimulatory than heat killed bacteria containing the equivalent amount of LPS.

3.2.2 Murine TNF-α responses to LPS, liposomal LPS and heat killed bacteria

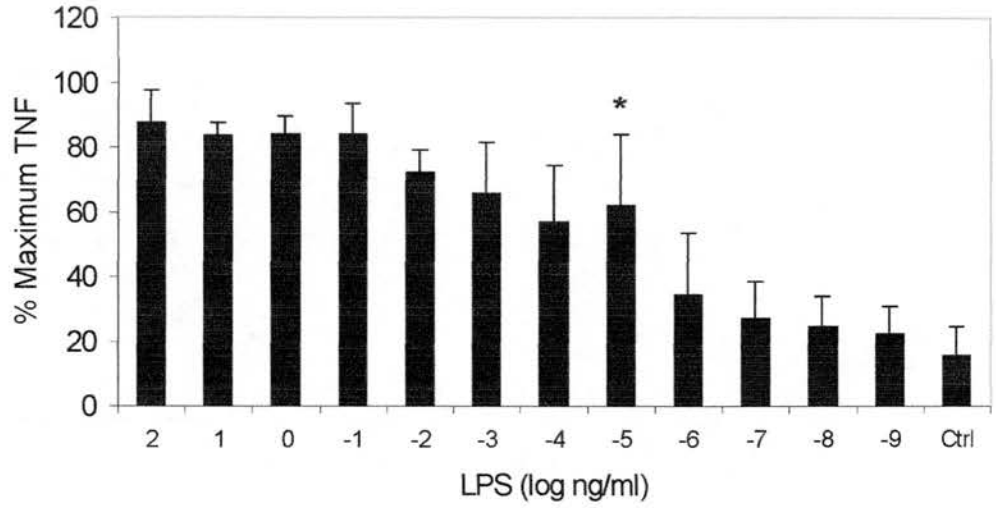
As the cytokine TNF-α has often been implicated in the pathogenesis of severe sepsis, the production of this cytokine in response to LPS, liposomal LPS and heat killed bacteria was investigated. Murine thioglycollate elicited peritoneal

macrophages were challenged with various concentrations of either *E.coli* K12 LPS, heat killed *E.coli* K12 bacteria, liposomal *E.coli* K12 LPS or medium alone (control). Supernatants were assayed for TNF-α production after a 4 hour incubation using a bioassay based on the L929 murine fibroblast cell line.

As the early results generated from this assay demonstrated extreme variablity, the results from a great many experiments had to be discarded. Only extensive modification of the original bioassay protocol was eventually able to result in a relatively reliable protocol (described in section 2.4.3), such that reproducible data could be obtained. Despite these improvements, however, a high background production of TNF-α was observed in all experiments which could not be removed by modulation of the thioglycollate stimulation protocol (a finding mirroring that seen in previous nitric oxide experiments – section 3.2.1).

For this reason, the results from many experiments were combined using the data normalisation procedure previously described for the nitric oxide experiments (section 3.2.1). Figure 3.2a shows that TNF-α release from murine macrophages is maximal in response to concentrations of native LPS ranging from 0.1 to 100ng/ml. Below 0.1ng/ml LPS, TNF-α release is reduced in a dose dependent fashion, until concentrations less than 10^{-5} ng/ml LPS which do not reveal any signalling significantly different from background. Figure 3.2b shows that murine macrophages release TNF-α in response to 100ng/ml liposomal LPS, but no signalling is observed in response to concentrations below 10ng/ml. Figure 3.2c shows that murine macrophage TNF-α release is maximal in response to both 1,000,000 and 320,000 heat killed *E.coli* per ml, then reduces in a dose dependent fashion, such that concentrations below 10,000 bacteria per ml do not stimulate significant release of TNF-α. These results are summarised in Table 3.2.

(a)



(b)

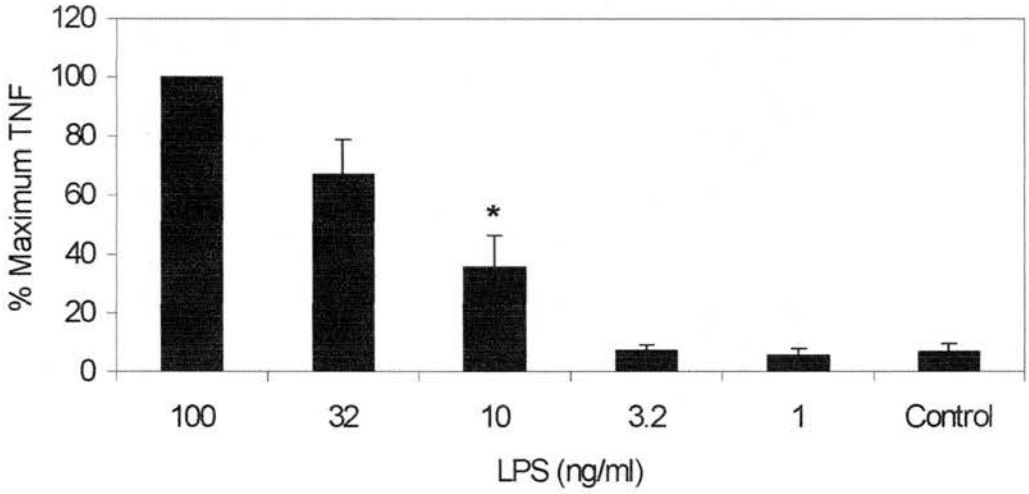


Figure 3.2 Murine macrophage TNF-α release in response to challenge with (a) *E.coli* K12 LPS (b) liposomal *E.coli* K12 LPS or (c) heat-killed *E.coli* K12 bacteria

Results are normalized means +/-SEM of 4 independent experiments for LPS, 5 independent experiments for liposomal LPS and 4 independent experiments for heat killed bacteria. * Indicates data point representing lowest concentration at which response is significantly different from control ($p<0.05$).

Ctrl represents response of cells incubated in medium alone. Peak TNF-α release varied between experiments with a range of 190 to 2070 pg/ml.

(c)

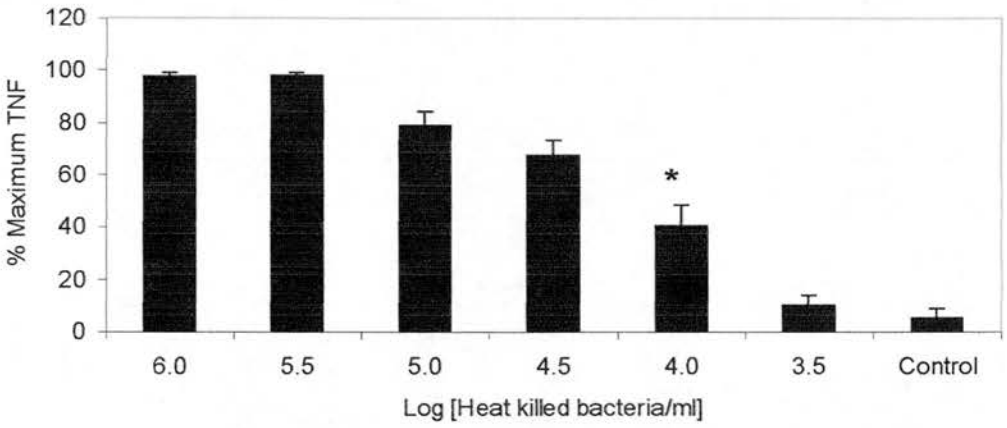


Figure 3.2 Murine macrophage TNF-α release in response to challenge with (a) *E.coli* K12 LPS (b) liposomal *E.coli* K12 LPS or (c) heat-killed *E.coli* K12 bacteria

Table 3.2 Comparison of capacity of LPS, liposomal LPS and heat killed bacteria to induce TNF-α production in murine peritoneal macrophages

Stimulant	Minimum concentration required to stimulate macrophages *
Native *E.coli* K12 LPS	1×10^{-5} ng/ml
Liposomal *E.coli* K12 LPS	10 ng/ml
Heat killed *E.coli* K12 bacteria	1×10^{4} bacteria/ml

* Minimum concentration represents the lowest concentrations at which native *E.coli* K12 LPS, liposomal *E.coli* K12 LPS and heat killed *E.coli* K12 bacteria stimulate murine macrophages to release significantly more TNF-α ($p<0.05$) than medium alone.

The data (Table 3.2) suggest that, weight for weight, LPS contained within liposomes is roughly 1,000,000 fold less capable of inducing a TNF-α response from murine peritoneal macrophages than the equivalent amount of native LPS.

3.2.3 Human TNF-α responses to lipopolysaccharide

The proposed vaccine consists of a liposomal formulation containing equal amounts of complete core LPSs from the following four organisms: *Escherichia coli* K12, *Escherichia coli* R1, *Bacteroides fragilis* and *Pseudomonas aeruginosa* (collectively refered to as 'cocktail' LPS). Experiments were therefore performed to compare the biological activity of each of these native LPS with the liposomal preparation by measuring the release of TNF-α from human monocytes challenged with each preparation.

In a manner similar to that found in preceding murine investigations (sections 3.2.1 and 3.2.2), high backgound levels of TNF-α production from unchallenged monocytes were observed in all early experiments. Much trial and error

eventually traced this problem to the use of foetal calf serum in these experiments since use of serum-free medium, or medium supplemented with human serum ablated all spontaneous TNF-α responses. Foetal calf serum from three different manufacturers (Gibco, Sigma and Imperial laboratories) was tested, but all were found to induce high background production of TNF-α from otherwise unchallenged monocytes. It was later learned that endotoxin contamination of foetal calf serum is a very common occurrence. For this reason, all data generated from experiments using foetal calf serum were abandoned, and experiments were repeated using pooled human AB serum.

Donor to donor variability in the peak value of TNF-α released in the assay was somewhat marked, so normalisation of the data was employed to allow collation of multiple data sets (as described in section 3.2.1).

Figure 3.3 shows the relative amounts of TNF-α released from human monocytes challenged with native *Escherichia coli* K12, *Escherichia coli* R1, *Bacteroides fragilis*, *Pseudomonas aeruginosa* and 'cocktail' LPS. Responses to *Escherichia coli* K12, *Escherichia coli* R1 and cocktail LPS are similar, each stimulating cells maximally at all concentrations of LPS between 1,000 and 1ng/ml, with the response then falling in a dose dependent fashion such that all three preparations fail to stimulate cells at concentrations less than 0.01ng/ml. *B. fragilis* and *P.aeruginosa* LPS are also seen to stimulate cells in a dose-dependant fashion, though *B.fragilis* LPS appears to be approximately 10,000 fold less toxic than the *E. coli* and mixed LPSs while *P. aeruginosa* LPS shows an ability to stimulate monocytes only at concentrations 10^5 - 10^6 times more than that obtained with *E. coli* derived LPS.

Figure 3.4 shows that the liposomal cocktail LPS also stimulates cells in a dose dependent fashion with the minimum signalling concentration being around 1,000ng/ml. This indicates that the vaccine is approximately 10^5 fold less

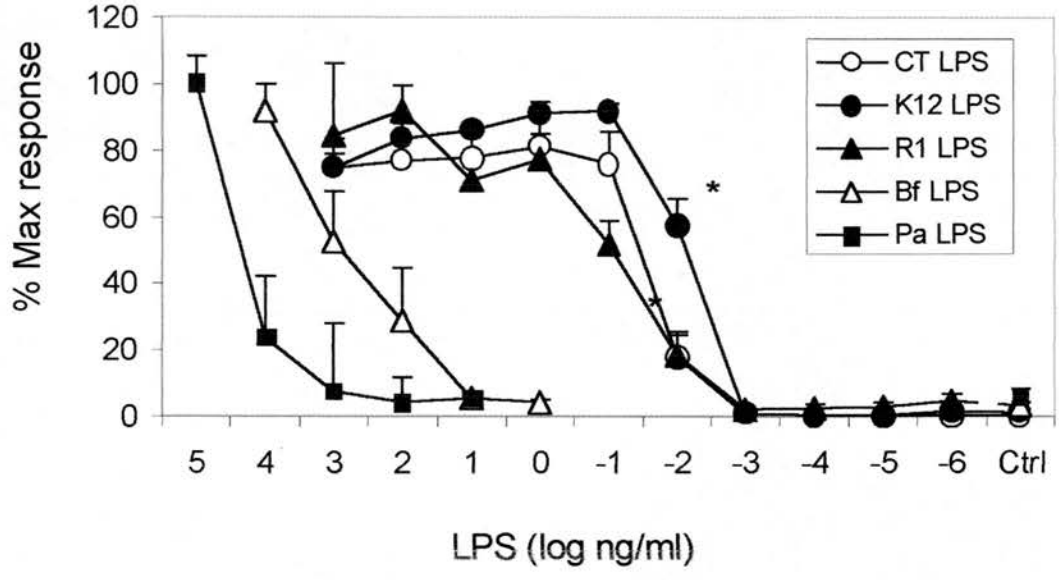


Figure 3.3 TNF-α release from human monocytes challenged with native *Escherichia coli* K12, *Escherichia coli* R1, *Bacteroides fragilis*, *Pseudomonas aeruginosa* or 'cocktail' LPS
Results are normalized means +/-SEM of five independent experiments. * Indicates data point representing lowest concentration at which response is significantly different from control ($p<0.05$). Ctrl represents response of cells incubated in medium alone. CT = cocktail, K12 = *E.coli* K12, R1 = *E.coli* R1, Bf = *B.fragilis*, Pa = *P.aeruginosa*. Peak TNF-α release varied between 52 – 810 pg/ml.

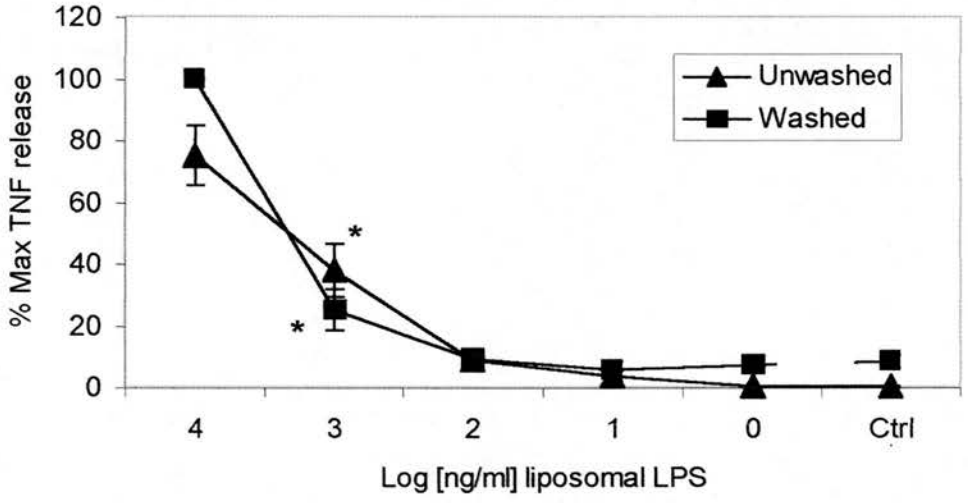


Figure 3.4 TNF-α release from human monocytes challenged with 'cocktail' LPS containing liposomes
Washed liposomes were centrifuged five times further than freshly prepared (unwashed) liposomes. Results are normalized means of four independent experiments +/- SEM. * Indicates lowest concentration at which response is significantly different from control ($p<0.05$). Peak TNF-α release varied between 120 – 435 pg/ml.

stimulatory than the equivalent mixture of unincorporated LPS. Null liposomes (*ie* containing no LPS) did not stimulate production of TNF-α from the monocytes at any concentration (data not shown).

In order to investigate whether or not it was possible to lower the biological activity of the liposomes further, an experiment was performed in which stock LPS containing liposomes were washed five additional times by bench centrifugation and resuspension in PBS, and the resulting activity of this preparation compared with that of unwashed liposomes. Figure 3.4 shows that these additional washing steps were unable to alter the biological activity of the liposomes.

Table 3.3 summarises the lowest concentrations at which the preparations are able to stimulate human peripheral monocytes to release significantly more ($p<0.05$) TNF-α than medium alone.

Table 3.3 Comparison of capacity of native and liposomal LPS preparations to induce TNF-α production from human monocytes

LPS type:	Minimum concentration required to stimulate monocytes *	Activity compared to liposomes (liposomes=1)
E.coli R1	0.01 ng/ml	100,000
E.coli K12	0.01 ng/ml	100,000
B.fragilis	100-1,000 ng/ml **	1 - 10
P.aeruginosa	1,000 – 10,000 ng/ml **	0.1 - 1
Cocktail (CT) LPS	0.01 ng/ml	100,000
Liposomal CT LPS	1,000 ng/ml	1
Washed liposomes	1,000 ng/ml	1
Recovered LPS	0.1 ng/ml	10,000

* Data point taken as lowest value significantly different ($p>0.05$) from control, and hence lowest concentration at which preparation is capable of stimulating cells

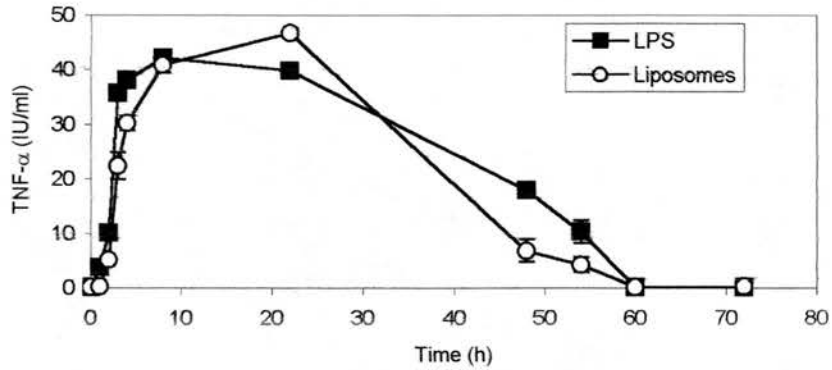
** Monocytes from certain individuals responded to different degrees with *B. fragilis* and *P. aeruginosa* LPS so a range of sensitivities is given.

Then in order to rule out the possibilty that the lowered biological activity of the liposomal formulation of the vaccine was merely due to a delayed release of the LPS slowly over time, an experiment was performed to investigate the timecourse of TNF-α release from the monocytes. Figure 3.5 shows that the kinetics of TNF-α release in response to the liposomal LPS is very similar to that of the native LPS. Production of TNF-α in response to both stimuli increases quickly over the first four hours to reach a peak between 8-24 hours and then diminishes to background levels at around sixty hours.

To rule out the possibility that the liposomes merely contained very low concentrations of LPS due to inefficient incorporation of LPS during manufacture, a sample of liposomal LPS was subjected to phenol re-extraction and the resulting LPS assayed for capacity to induce TNF-α. Figure 3.6 reveals that LPS re-extracted from the liposomes displays greatly more activity (~10,000 fold) than the equivalent amount of unextracted liposomal LPS, indicating that the low biological activity of the vaccine is not due to low LPS content.

In order to investigate further how this reduction in biological activity was achieved, the extracellular signalling pathways of LPS and liposomal LPS were investigated. Figure 3.7 shows the relative dependencies of native LPS and liposomal LPS on the extracellular signalling proteins soluble CD14 (sCD14) and LPS binding protein (LBP). Signalling of 100ng/ml native LPS was seen to be reduced to approximately half of the normal response on removal of serum from the medium. This could be partially restored by addition of purified LBP or completely restored with soluble CD14. Liposomal LPS signalling is completely abolished on removal of serum, yet can also be recovered to approximately two thirds of maximum activity by restoration of either LBP or soluble CD14, with each of these proteins displaying a similar capacity to restore signalling at the concentrations tested.

3.5



3.6

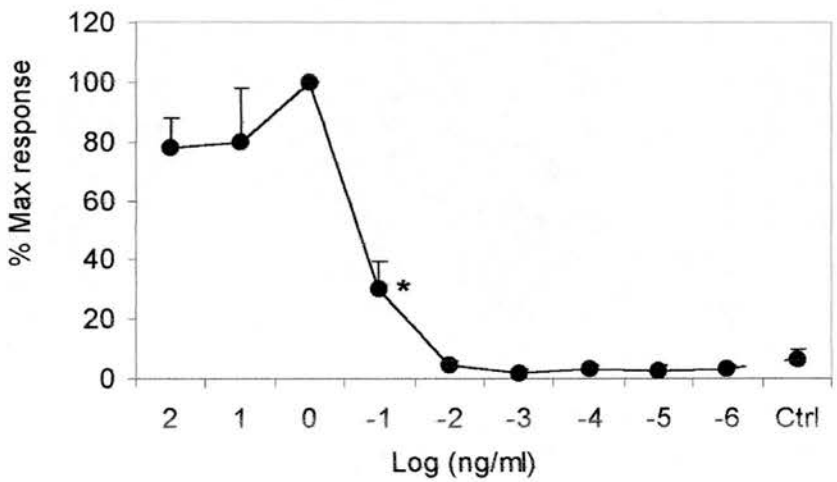


Figure 3.5 Timecourse of TNF-α production from human monocytes exposed to liposomal LPS and native LPS

The amount of TNF-α produced in response to 100ng/ml of pure LPS and 10μg/ml of liposomal LPS was measured at various time points. Results show the mean of triplicate readings ±SEM from one experiment and are representative of three similar experiments.

Figure 3.6 TNF-α production from human monocytes exposed to LPS re-extracted from liposomal LPS

LPS was re-extracted from liposomes by phenol extraction. Results are normalized mean +/- SEM of five independent experiments. * Indicates lowest concentration at which response is significantly different from control ($p<0.05$).

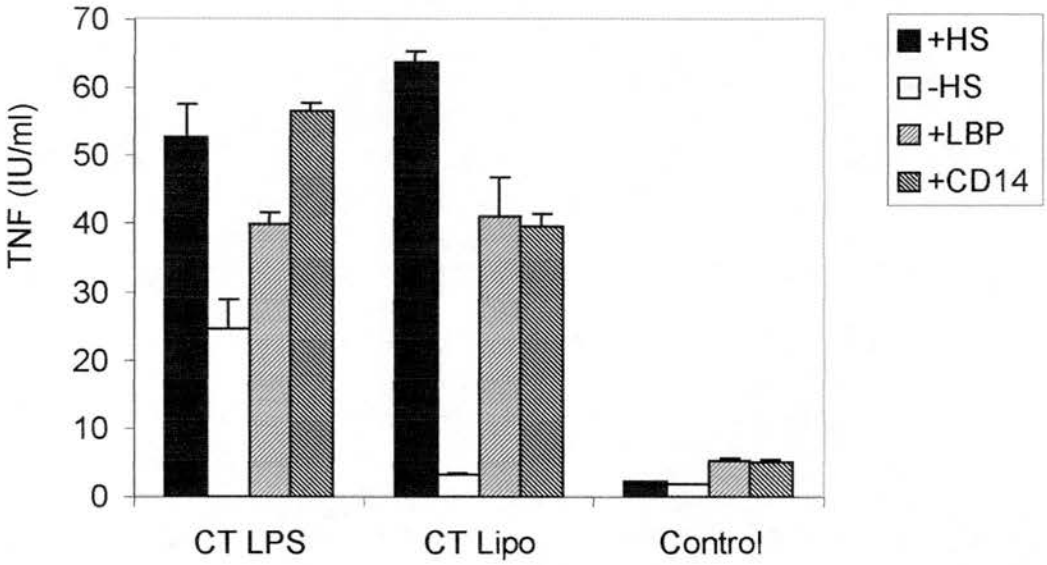


Figure 3.7: Dependence of LPS signalling on serum factors

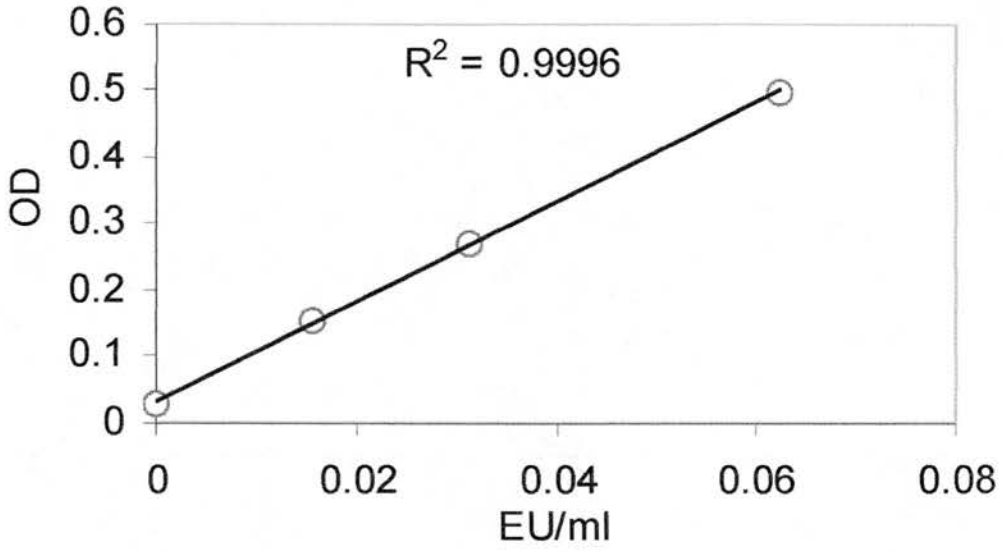
Human peripheral blood monocytes were challenged with 100ng/ml LPS or 10μg/ml liposomal LPS with human serum (+HS), without serum (-HS) and without serum but supplemented with 1μg/ml human LBP (+LBP) or 1μg/ml soluble CD14 (+CD14). Results are representative of three similar experiments, error bars are SEM. CT LPS = 100ng/ml cocktail LPS. CT Lipo = Liposomes containing 10μg/ml cocktail LPS.

3.2.4 Limulus amoebocyte lysate assay of LPS and liposomes

To further clarify the reduced biological activity of the liposomal LPS, the limulus amoebocyte lysis (LAL) assay was employed. As this is an extremely sensitive assay, concentrations of LPS and liposomal LPS were chosen to represent a point in between saturation and background levels from the dilution curves extrapolated from the human TNF-α assays. As a result, the concentrations chosen were 1ng/ml for the liposomal LPS and 0.01ng/ml for the native LPS. Figure 3.8 shows a typical standard curve produced by the kit created by measuring serial dilutions of kit supplied standard (8EU/ng) *E.coli* O111 LPS. The high coefficient of correlation between optical density and LPS concentration indicates a satisfactory accuracy for the assay.

The capacity to induce LAL activation of 0.01ng/ml *E.coli* R1, *E.coli* K12, *B.fragilis*, *P.aeruginosa* and cocktail native LPS and 1ng/ml liposomal cocktail LPS are shown in Figure 3.9. At these concentrations, both *E.coli* R1 and *E.coli* K12 are seen to elicit a very similar LAL response. However, neither *B.fragilis* nor *P.aeruginosa* LPS are able to stimulate the LAL reaction at this concentration. The equimolar cocktail of all four LPSs exhibits a LAL activity approximately half that of the *E.coli* R1 and *E.coli* K12 LPS. Interestingly, 1ng/ml liposomal cocktail LPS is seen to exhibit a LAL activity approximately three times that of the 0.01ng/ml native cocktail LPS, suggesting that at least in this assay, the liposomal formulation is only ~30 fold less active than the equivalent amount of native LPS.

3.8



3.9

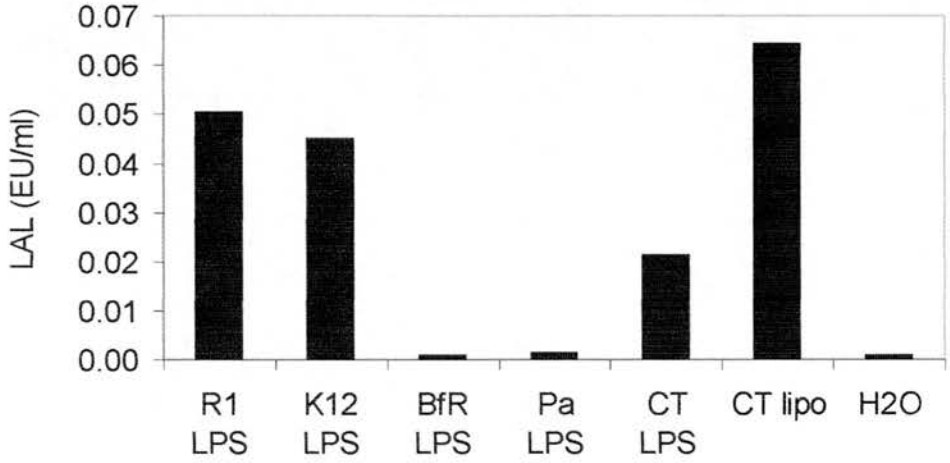


Figure 3.8 LAL assay calibration curve
Typical response of LAL assay standard curve showing linear relationship between LAL assay activity and LPS concentration (OD=optical density). R^2 = coefficient of correlation.

Figure 3.9 LAL assay measurement of biological activity of *E.coli* R1, *E.coli* K12, *B.fragilis*, *P.aeruginosa* and 'cocktail' native LPS and liposomal cocktail LPS
LAL assay activity (EU/ml) of 0.01ng/ml native LPS and 1ng/ml liposomal cocktail LPS. R1= *E.coli* R1 LPS, K12 = *E.coli* K12 LPS, Bf = *B.fragilis* LPS, Pa = *P.aeruginosa* LPS, CT = cocktail LPS. CT lipo = liposomal cocktail LPS. H20 = Pyrogen-free distilled water (control).

3.2.5 Toll receptor utilisation of LPS and LTA

As some LPS are capable of signalling via TLR-2 instead of TLR-4, experiments were performed to determine if antibody blocking of TLR-2 or TLR-4 was capable of reducing signalling in response to further LPS types. Two monoclonal antibodies proposed to be able to block the signalling function of TLR2 or TLR4 are currently commercially available. HTA125 is sold as a TLR4 blocking reagent and TL2.1 as a TLR2 blocking reagent.

However, three separate experiments in this laboratory failed to demonstrate the capacity of either antibody to block human monocyte recognition of either LPS or LTA. Figure 3.10 shows the typical IL-1β responses seen when challenging human monocytes with *E.coli* LPS, *P.gingivalis* LPS and *S.aureus* LTA while blocking with 20μg/ml of either TL2.1, HTA125 or the isotype matched control antibody anti-keyhole limpet haemocyanin (α-KLH). In these experiments, HTA125 does not significantly reduce monocyte signalling in response to any stimulus. Moreover, TL2.1 not only fails to block signalling in response to any of these stimuli, it also proves capable of stimulating cells to produce large amounts of IL-β independently of any other stimulus. The isotype matched control antibody anti-keyhole limpet haemocyanin (α-KLH) had no effect on signalling.

3.2.6 Comparison of wild type and mutant TLR-4 recognition of LPS

Two mutations in the TLR4 gene (Asp299Gly and Ile399Thr) have been shown to exist at a relatively high frequency (at least 6%) in the Caucasian population. This high frequency of TLR4 mutation in the general population prompted an investigation to determine whether individuals expressing these genotypes exhibit either more or less sensitive recognition of LPS from particular strains and species of bacteria.

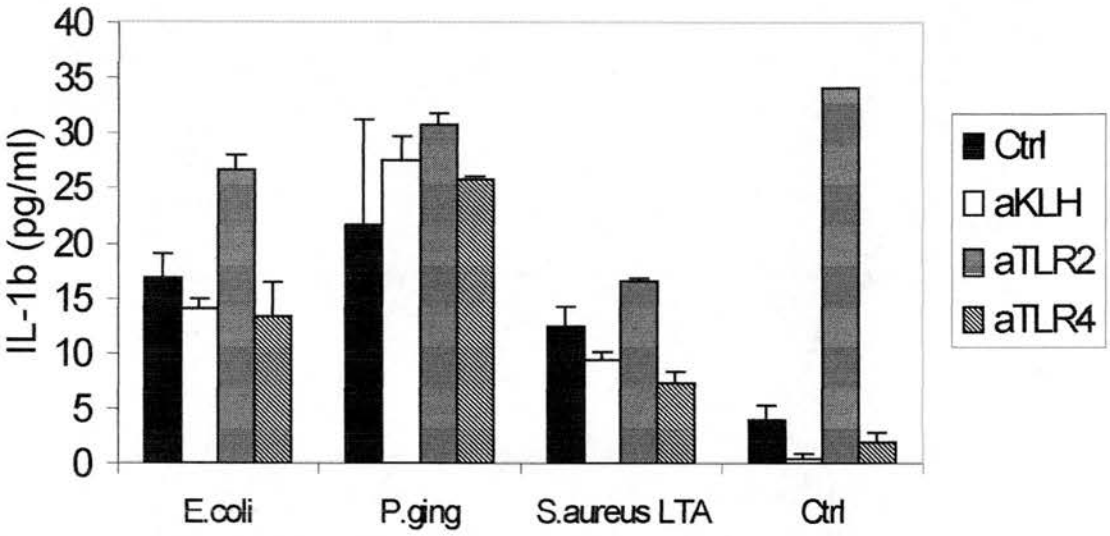


Figure 3.10 Effect of anti-TLR -2 and TLR-4 antibodies on human monocyte recognition of *E.coli* LPS, *P.gingivalis* LPS and *S.aureus* LTA

IL-1β secretion of monocytes incubated with 20μg/ml antibodies (aKLH = isotype matched control anti-keyhole limpet heamocyannin, aTLR2 = TL2.1, aTLR4 = HTA125, Ctrl = medium alone) and 100ng/ml *E.coli* LPS, *P.gingivalis* LPS, *S.aureus* LTA or medium alone (Ctrl).

PCR and restriction fragment length polymorphism (RFLP) analysis of the Toll-receptor 4 gene of 40 healthy volunteers and 40 patients presenting with abdominal inflammation revealed the presence of 8 individuals heterozygous for both mutations (sections 2.5.8 and 2.5.9). Interestingly, every individual carrying the Asp299Gly mutation was also seen to carry the Ile399Thr mutation. No individual was found to carry only one of the mutations and no individuals were found to be homozygous for either mutation.

Figure 3.11a shows an example gel of the PCR products created from the amplification of the two sections of the TLR-4 gene investigated. Figure 3.11b shows the results of restriction analysis of the PCR products. The higher molecular weight (upper) band represents the presence of the wild type allele, while double bands indicate individuals heterozygous for the mutation at that locus. Three wild type and three heterozgous individuals are shown.

Then in order to determine whether or not these mutations altered the ability of these individuals to recognise particular types of LPS, a panel of LPS representing a wide range of Lipid-A structures was investigated. Figures 3.12 a - f show the mean IL-1β responses of three wild type and three heterozyogous mutant volunteers to LPS of *E.coli*, *N.meningitidis*, *Y.pestis*, *C.trachomatis*, *B.fragilis*, *P.aeruginosa*, *P.gingivalis* and *S.aureus* LTA respectively. It can be seen that the responses of both wild type and mutant monocytes to all stimuli tested are remarkably similar. All LPS show dose dependent curves, though the minimum concentration of LPS required to stimulate cells varied between the different LPS types. Responses to *E.coli* LPS occur at a minimum concentration of around 0.01ng/ml, while those to *N.meningitidis* LPS occur at around 0.1ng/ml. Responses to *B.fragilis* LPS occur at a minimum concentration of around 1ng/ml. Responses to *P.gingivalis* and *Y.pestis* LPS occur at a minimum concentration of around 10ng/ml. *C.trachomatis* and *P.aeruginosa* LPS and *S.aureus* LTA are recognised only at concentrations equal to or 100ng/ml.

Slightly more inter-individual variability is seen in the responses to LPS of *B.fragilis*, and *Y.pestis*, though these are not statistically significant. These results are summarised in Table 3.4.

Table 3.4 Comparison of wild type and mutant TLR4 bearing monocyte recognition of various LPS

Stimulus	Minimum concentration activating wild type monocytes [ng/ml] *	Minimum concentration activating TLR4 +/- monocytes [ng/ml] *
E.coli LPS	0.01	0.01
N.meningitidis LPS	0.1	0.1
B.fragilis LPS	1	1
P.gingivalis LPS	10	10
Y.pestis LPS	10	10
C.trachomatis LPS	100	100
P.aeruginosa LPS	100	100
S.aureus LTA	100	100

* Minimum concentration represents the lowest concentration at which the individual stimuli induce production of significantly more IL-1β ($p<0.05$) than cells incubated with medium alone.

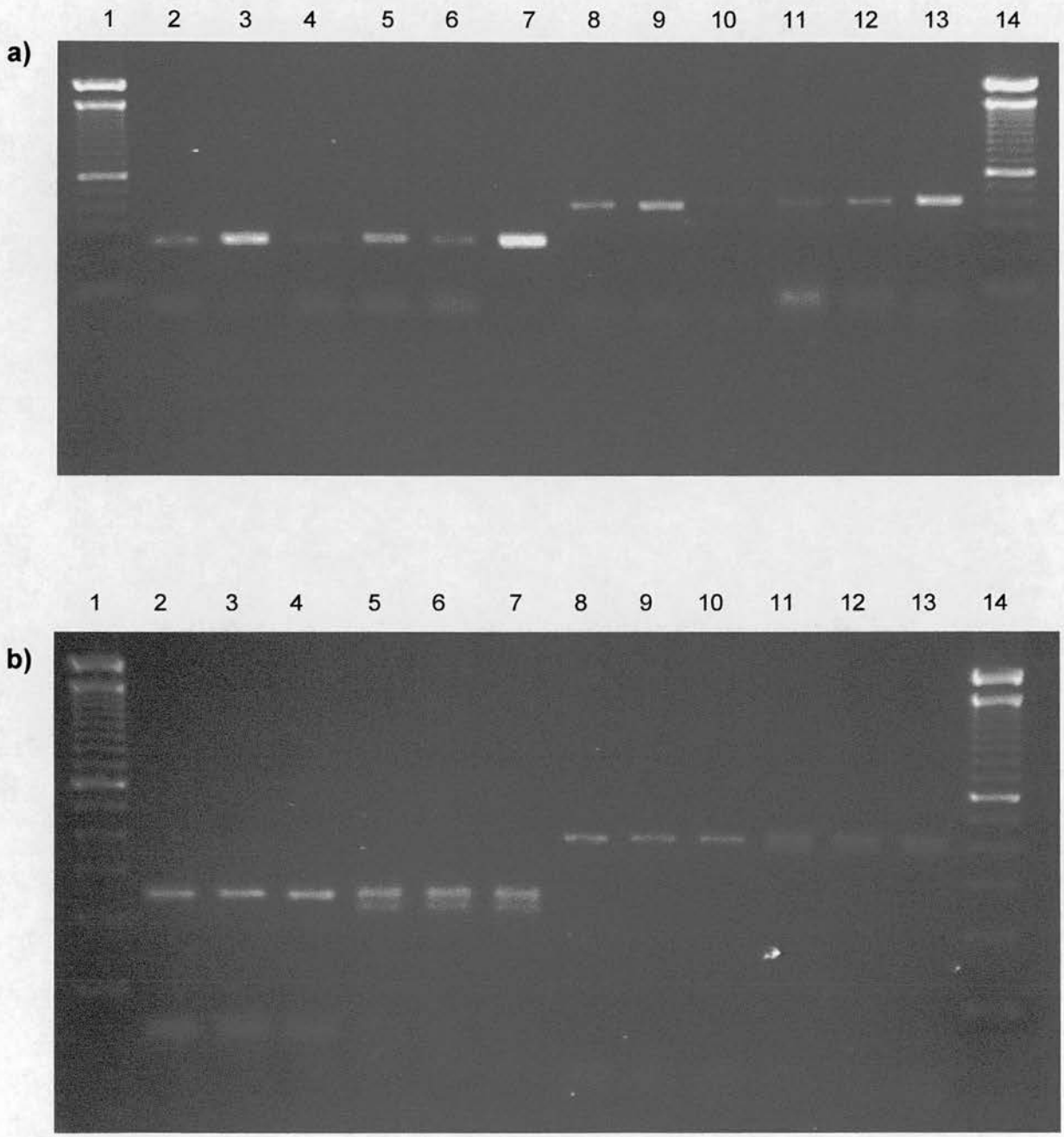


Figure 3.11 PCR products of amplification of two sections of the TLR-4 gene and digest products following RFLP analysis of wild type and mutant TLR-4 alleles
(a) Lanes 1+14, 100 base pair marker. Lanes 2-7, PCR of 299 section of TLR gene from three wild type donors and three heterozygotes. Lanes 8-13, PCR of 399 section of TLR gene from three wild type donors and three heterozygotes. (b) Restriction products following digest with the enzymes *Nco*I (299) or *Hinf*I (399). Lanes 1+14, 100 base pair marker. Lanes 2-7, RFLP of 299 section of TLR gene from three wild type donors and three heterozygotes. Lanes 8-13, RFLP of 399 section of TLR gene from three wild type donors and three heterozygotes. Upper band represents presence of wild type allele, lower band indicates presence of mutant allele.

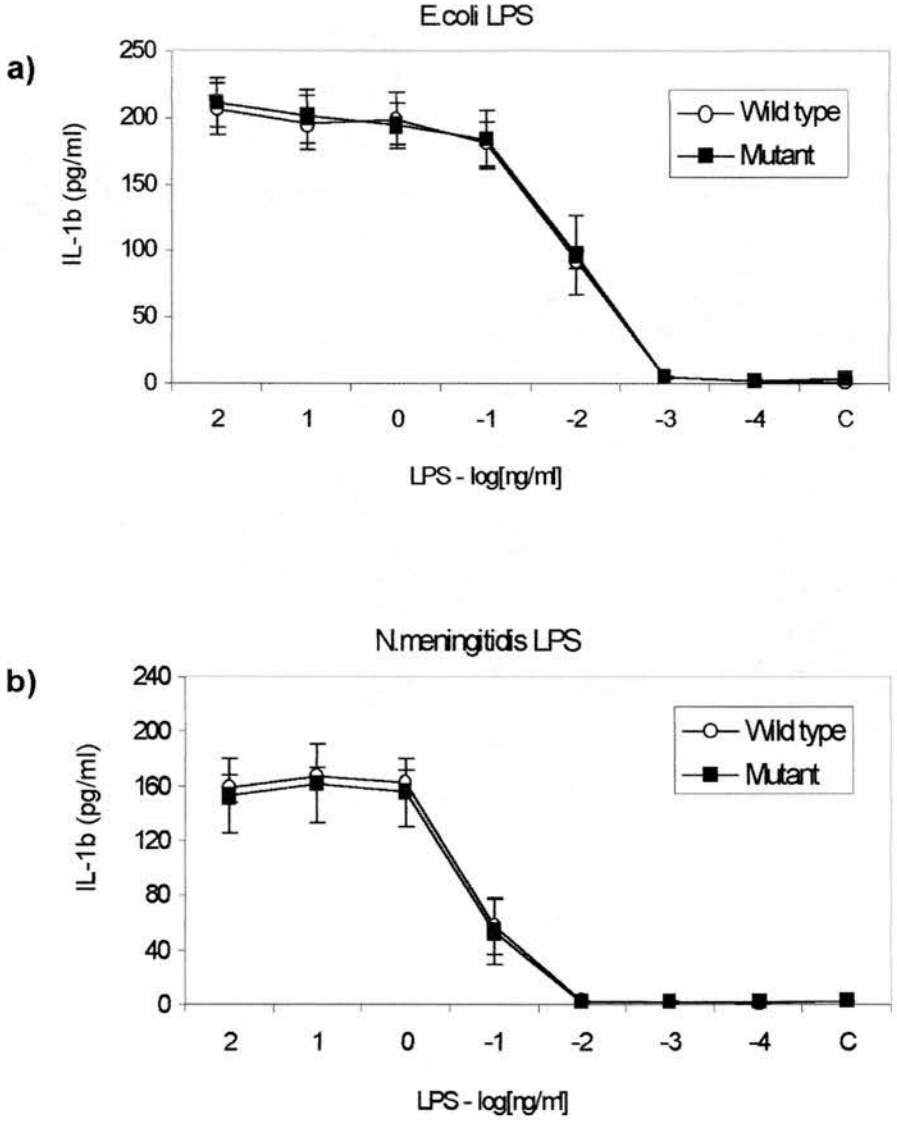


Figure 3.12 Comparison of wild type and mutant TLR-4 recognition of LPS and LTA
(a-h) IL-1β production from human monocytes ($1x10^5$ per well) challenged with varying concentrations of *E.coli*, *N.meningitidis*, *Y.pestis*, *C.trachomatis*, *B.fragilis*, *P.aeruginosa*, *P.gingivalis* LPS or *S.aureus* LTA respectively. Open circles represent mean results +/- SEM from three wild type donors. Filled squares represent mean results +/- SEM from three donors heterozygous for the TLR4 mutation.

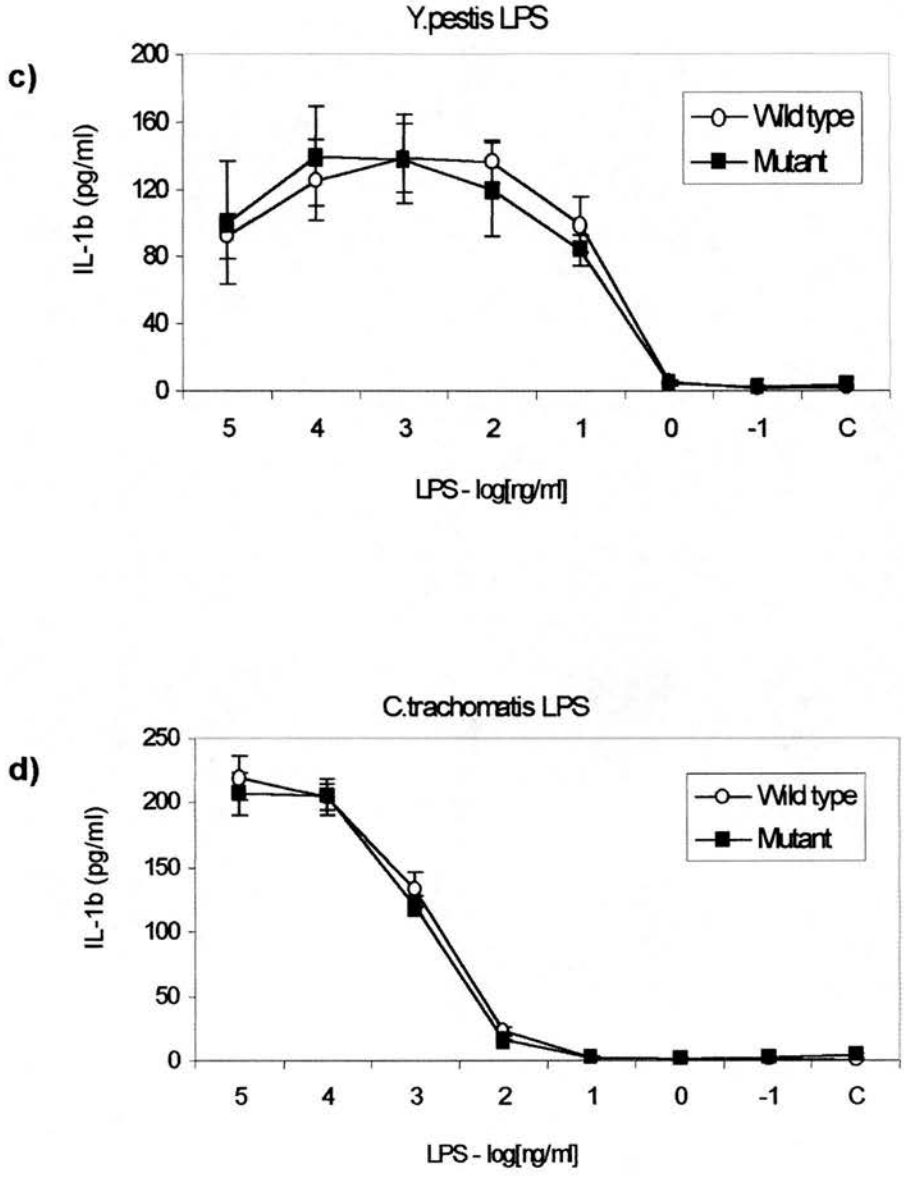


Figure 3.12 Comparison of wild type and mutant TLR-4 recognition of LPS and LTA

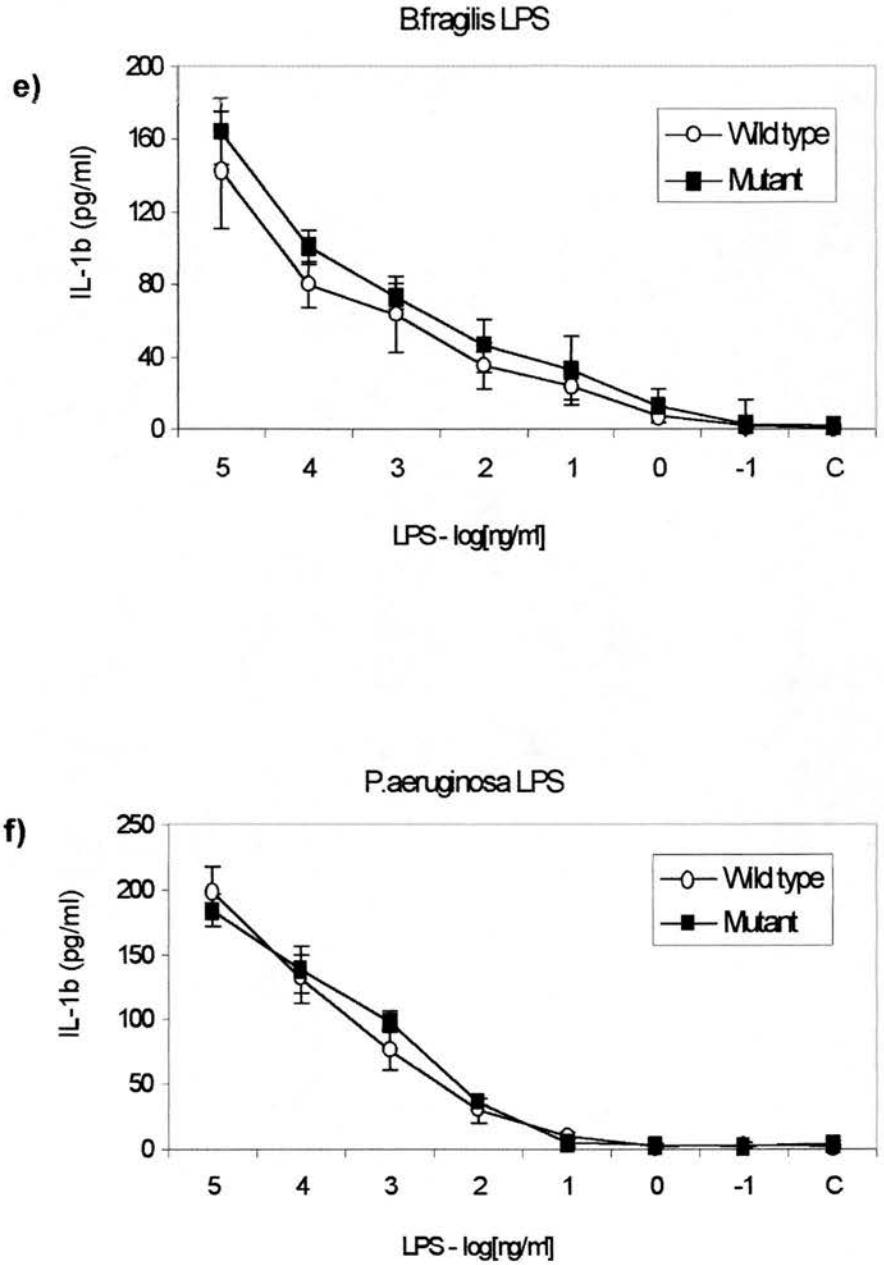


Figure 3.12 Comparison of wild type and mutant TLR-4 recognition of LPS and LTA

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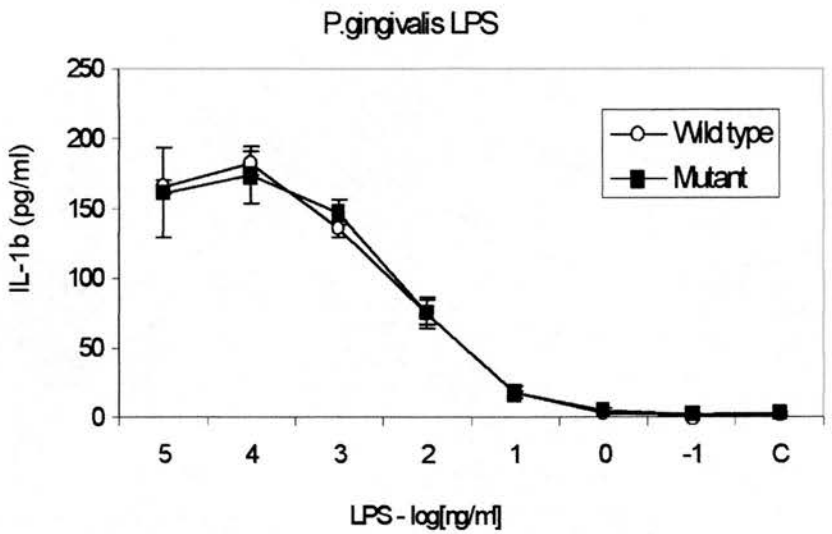

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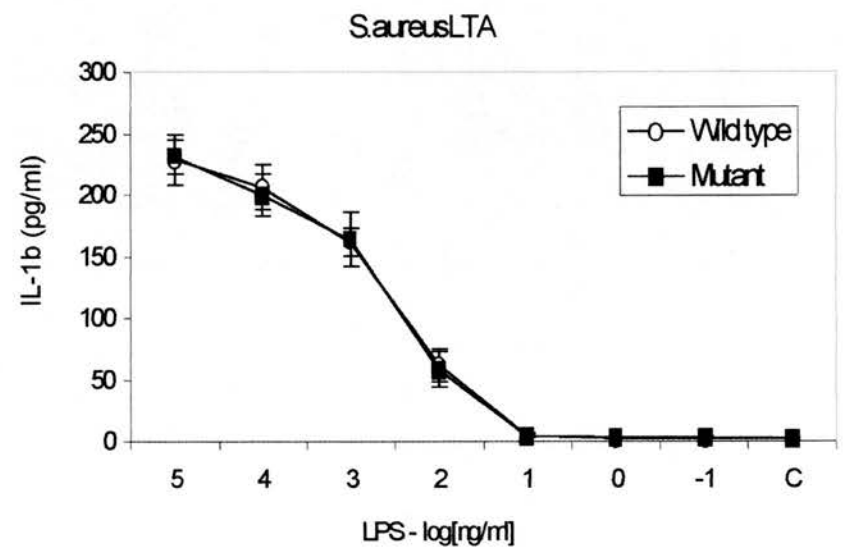


Figure 3.12 Comparison of wild type and mutant TLR-4 recognition of LPS and LTA

3.3 Discussion

3.3.1 Potential benefits of lipopolysaccharide based vaccines

Several studies have suggested that high titres of anti-core LPS antibodies provide protection from adverse outcome following a number of disease processes or surgical procedures (Freeman et al 1985, Bennett-Guerrero et al 1997, Hamilton-Davies et al 1997, Bennett-Guerrero et al 2001). For this reason, it has been suggested by several authors that pre-surgical vaccination with LPS might benefit patients by increasing their anti-LPS antibody titre prior to surgery (Bennett-Guerrero et al 2000, Campbell et al 1996, DeMaria et al 1988, Baumgartner et al 1991b). To avoid repetition, the wider impact of these studies and previous proposals for the designs of such vaccines will be discussed in further detail in section 6.2.

In brief, however, there are two main priorities that must be borne in mind in the design of any lipopolysaccharide based vaccine. The first is that the vaccine formulation must display a greatly reduced toxicity when compared to that of naturally occuring LPS, as administration of as little as several nanograms of LPS is enough, when administered to a human subject, to induce all of the symptoms of systemic inflammation (Suffredini et al 1989, Martich et al 1993, Taveira daSilva et al 1993). The second is that the vaccine must also remain highly immunogenic, as induction of an antibody response to the widest possible range of LPS core types has been shown to offer the best protection from Gram-negative bacterial challenge (McCabe et al 1977, Ziegler et al 1979, Heumann et al 1991, Cross et al 1988).

In order to fulfill these two briefs, a novel liposomal complete core lipopolysaccharide vaccine has been described (Bennett-Guerrero et al 2000) which, it is hoped, will display both low toxicity and high immunogenicity in human clinical trials.

3.3.2 Previously reported properties of the vaccine

In order to induce a broadly cross reactive antibody response, the vaccine contains a mixture of the complete core (Ra-chemotype) LPSs from four different Gram-negative bacteria – *Escherichia coli* K12, *E. coli* R1, *Pseudomonas aeruginosa* PAC 608 and *Bacteroides fragilis*. In rabbits that displayed little or no detectable antibodies to LPS by ELISA or dot-blotting prior to vaccination, the vaccine was shown capable of eliciting an antibody response towards a wide range of LPS types. These included LPS of *E.coli* serotypes O1, O4, O6, O8, O15, O75, O86 and O111; *P.aeruginosa* Fischer types 1, 2 and 3, *K.pneumoniae* serotypes O1, O2ab and O3, *B.fragilis* and *B.vulgatis* (Bennett-Guerrero et al 2000).

Vaccination with the complex was also seen to provide significant protection in a mouse lethality model of endotoxaemia. Galactosamine-treated mice challenged with a lethal dose of *E.coli* O18 LPS showed 70% survival in the pre-vaccinated cohort, compared with only 9% survival in the non-vaccinated cohort (Bennett-Guerrero et al 2000).

The toxicity of the vaccine was also investigated using the rabbit pyrogenicity assay. While administration of free LPS was seen to induce a pronounced pyrogenic response in the rabbits (weight loss, lethargy, altered breathing, increased core body temperature), administration of up to 500μg of liposomal LPS did not display significant pyrogenicity (Bennett-Guerrero et al 2000).

3.3.3 Murine nitric oxide responses to LPS and liposomal LPS

In order to further understand how the reduced biological activity of liposomal LPS is achieved, the present study began by looking at the response of murine peritoneal macrophages to liposomal *E.coli* K12 LPS. As it has already been proposed that heat killed bacteria could also be used as a vaccine for the

production of anti-LPS antibodies (Ziegler et al 1982, Baumgartner et al 1991b, DeMaria et al 1988), the responses of the cells to this antigen were compared with those of both native and liposomal LPS.

Due to its ease of measurement, the effector molecule nitric oxide was chosen as the marker of cellular activation in early experiments. However, two problems hampered initial attempts to compare the biological activities of LPS preparations. The first of these was that in every experiment performed, a high background level of nitric oxide production was seen to be produced from unchallenged cells. It was initially thought that the highly activated state of the peritoneal macrophages was responsible for this, though alteration of the protocol for thioglycollate stimulation of the mice was found to be unable to reduce this spontaneous activity (data not shown).

The second problem concerned the mouse to mouse variation seen in the peak level of nitric oxide produced – which at this stage was thought to reflect the high level of activation of the murine cells. For these reasons, the results from many experiments had to be normalised to a percentage of the peak value obtained from each experiment and combined (as described in section 3.2.1).

Figure 3.1a shows that native *E.coli* K12 LPS is capable of inducing release of nitric oxide from murine thioglycollate elicited peritoneal macrophages over a wide range of concentrations, with a strong response to concentrations of LPS around 100ng/ml that diminishes to levels not significantly different from background at concentrations below around 0.01 ng/ml. In contrast, the lowest concentration at which liposomal *E.coli* K12 LPS is able to stimulate a significant release of nitric oxide is 32ng/ml (Figure 3.1b). These data suggest that liposomal LPS is at least 3,200 fold less capable of inducing a nitric oxide response from murine peritoneal macrophages than the equivalent amount of native LPS.

Analysis of the nitric oxide response of these cells to heat killed *E.coli* K12 bacteria revealed that the lowest concentration capable of inducing a response significantly higher than background was 32,000 bacterial cells per ml (Figure 3.1c). Assuming that an average *E.coli* cell weighs 5pg, and 2% of the weight of the bacteria is LPS, it follows that 10^6 bacteria contain approximately 100ng LPS. Using this conversion factor, it appears that cell associated LPS is only able to stimulate cells at a minimum concentration of around 3.2 ng/ml LPS, making it at least 320 fold less biologically active than free LPS.

No other studies of murine nitric oxide release in response to liposomal LPS are available for comparison with these findings.

3.3.4 Murine TNF-α responses to LPS and liposomal LPS

Many studies have implicated the pro-inflammatory cytokine TNF-α as a significant mediator in the ill effects of endotoxin administration (Calendra et al 1990, Glauser et al 1991, Waage et al 1987). For this reason, production of TNF-α from murine peritoneal macrophages in response to native *E.coli* K12 LPS, liposomal *E.coli* K12 LPS and heat killed *E.coli* K12 bacteria was investigated.

Early attempts at these comparisons were impeded by extreme variablity in TNF-α measurements of supernatants both between and within experiments. Reliable data was only obtained following extensive modification of the original bioassay protocol (section 2.4.3) and normalisation and collation of the results from many experiments as previously described (section 3.2.1). Nevertheless, high background production of TNF-α was seen in the control cultures of all experiments which could not be removed. (This was later found to be due to a contaminant of the foetal calf serum used in the preparation of the culture medium – section 3.2.3).

Native LPS is seen to induce maximal production of TNF-α from all concentrations ranging between 0.1ng/ml and 100ng/ml (Figure 3.2a). The lowest concentration at which native *E.coli* K12 LPS is capable of inducing significantly more TNF-α than control cultures is 10^{-5} ng/ml. This concentration is roughly one million fold lower than the concentration of liposomal LPS required to activate cells (Figure 3.2b). Assuming that the molecular weight of complete core LPS is around 5,000, this suggests that the lowest concentration of LPS capable of inducing cellular activation (10^{-5} ng/ml) is equivalent to approximately 600 LPS molecules per cell. The responses of the monocytes to such low concentrations of LPS may be enhanced by the synergistic effects of pro-inflammatory cytokines released from neighbouring macrophages.

Heat killed *E.coli* K12 bacteria induce activation of cells at a minimum concentration of 10,000 cells/ml (Figure 3.2c), which is equivalent (according to the calculation previously described) to approximately 1 ng/ml LPS. This suggests that cell-associated LPS is roughly 100,000 fold less biologically active than free LPS, though still at least ten fold more toxic than liposomal LPS.

Of note is the fact that murine macrophage release of TNF-α is seen to occur in response to lower concentrations of all stimuli tested than those necessary to stimulate release of nitric oxide (*eg.* 10^{-5}ng/ml LPS vs 10^{-2}ng/ml LPS). This may represent the fact that nitric oxide is an effector molecule of the innate immune response, whereas TNF-α is a signalling molecule and may therefore be more readily released by cells in response to lower levels of endotoxin.

3.3.5 Human TNF-α responses to LPS and liposomal LPS

Having established the reduction in activity of a single LPS incorporated into liposomes, the response of human monocytes to the mixed LPS based vaccine were investigated. Cells were challenged with complete core R-form LPS of

Escherichia coli K12, *Escherichia coli* R1, *Bacteroides fragilis*, *Pseudomonas aeruginosa* or with an equimolar mixture of these LPS (refered to as 'cocktail' LPS). Responses of the cells to these individual LPS were then compared with responses to the vaccine - itself a liposomal formulation of the cocktail LPS described.

Figure 3.3 shows that the biological activities of *E.coli* R1, *E.coli* K12 and cocktail LPS are largely similar when considering human TNF- α responses. Each shows a plateau of maximum response from around 1ng/ml up to 1,000 ng/ml and a lowest activating concentration of 0.01 ng/ml. Inter-individual responses to these LPS were closely similar, but responses to *B.fragilis* and *P.aeruginosa* LPS were quite variable between donors (as evidenced by the larger error bars in Figure 3.3). These two LPS types were seen to demonstrate much lower biological activity than the *E.coli* or cocktail LPS, with *B.fragilis* stimulating cells only at concentrations around 100-1,000ng/ml and *P.aeruginosa* around 1,000-10,000ng/ml. The amount of TNF-α produced in response to each of these stimuli varied between 52 and 810 pg/ml, a figure approximately 10 fold higher than the concentration of normal circulating levels of TNF-α but still roughly 25 fold less than the maximum doses of TNF-α used in early trials of TNF-α as an anti-cancer agent (Mittelman A et al 1992).

By comparison, liposomal cocktail LPS is seen to stimulate cells only at concentrations equal to or above 1,000ng/ml. This represents a reduction in biological activity when compared to native cocktail LPS of around 100,000 fold.

Then in order to ascertain whether or not it was possible to lower the biological activity of the liposomes further, stock liposomal cocktail LPS was washed five times by bench centrifugation and resuspension in PBS. Figure 3.4 shows that these additional washing steps were unable to alter the biological activity of the liposomes.

It was suggested that the reduced activity of the vaccine may be due to very low concentrations of LPS being contained within the liposomes due to inefficient incorporation of LPS at manufacture. To rule out this posibility, a sample of liposomal LPS was subjected to re-extraction of incorporated LPS using the aqueous phenol method (section 2.2.2). LPS resulting from this extraction was assayed for capacity to induce TNF-α from human monocytes and shown to exhibit an activity ~10,000 fold more than the equivalent amount of liposomal LPS (Figure 3.6). The low biological activity of the vaccine is therefore not due to low LPS content.

3.3.6 Kinetics of LPS and liposomal LPS signalling

Another possible explanation for the low biological activity of the liposomal formulation was that LPS was merely being released from the liposomes slowly over time, with the possibility that at a later time than the 4 hour timepoint investigated, sufficient amounts of incorporated LPS may have been released to induce a much stronger release of TNF-α.

In order to rule out this possibility, the kinetics of TNF-α release in response to challenge with native and liposomal cocktail LPS were compared. Figure 3.5 shows that both preparations induce production of significant amounts of TNF- α after as little as one hours incubation. Peak production of TNF- α is seen to occur for both preparations between 8 and 24 hours, with supernatant concentrations of TNF- α gradually diminishing to background levels after around 60 hours, indicating that no delayed peak in LPS release from the vaccine occurs *in vitro*. These kinetics show a largely similar pattern to that described by Delahooke *et al* for *E.coli* and *B.fragilis* native LPS (Delahooke et al 1996), and provide further support for the safety of the vaccine for use in human trials.

3.3.7 Role of serum proteins in LPS and liposomal LPS signalling

In order to further investigate how this reduction in biological activity was achieved, the extracellular signalling pathways of LPS and liposomal LPS were investigated. Experiments were performed in which cells were challenged with LPS or liposomal LPS resuspended in serum free medium reconstituted with recombinant LPS binding protein (LBP) or soluble CD14 (sCD14). The relative dependencies of native LPS and liposomal LPS on the extracellular signalling proteins sCD14 and LBP for efficient signalling are depicted in Figure 3.7.

Signalling of native LPS is seen to be reduced to about half the normal output when serum is removed from the medium. Addition of LBP led to partial restoration of this signal, while supplementation with purified sCD14 led to complete restoration of signalling capacity. Signalling of liposomal LPS, on the other hand, was seen to be entirely ablated on removal of serum from the medium, though this was restored to approximately two thirds of maximal activity by supplementation with either LBP or sCD14. Thus at the concentrations tested (100ng/ml native LPS and 10μg/ml liposomal LPS), presence of LBP or sCD14 is absolutely required for liposomal LPS signalling but not for native LPS signalling. This capacity of high concentrations of native LPS to activate cells in the absence of serum-derived lipid shuttling proteins has been documented before (Beckman et al 1994), and is likely to reflect the ability of membrane bound CD14 present on monocytes to catalyse transfer of monomeric LPS from micelles to the LPS receptor. This is supported by the finding that sensitive responses to LPS *in vivo* also require the presence of CD14 (Wurfel et al 1997, Wright et al 1990).

Interestingly, while the CD14 knockout mouse exhibits an almost complete impairment of LPS signalling (Wright et al 1990), the LBP knockout mouse is only mildly hyporesponsive to LPS (Wurfel et al 1997). By contrast, these

experiments have shown that human LBP and sCD14 possess a broadly similar capacity to restore LPS signalling. An explanation for this difference may lie in the concentrations of LBP and sCD14 chosen for use in these experiments. 1μg/ml of each protein was used as this was calculated to be roughly equivalent to the amount present in the 10% human serum-supplemented medium (Pugin et al 1993, Wurfel et al 1997). However, as LBP is present in mouse serum at concentrations ~100 fold lower (~100ng/ml – Jack et al 1997) than that seen in human serum (5-10μg/ml – Pugin et al 1993), it is possible that the murine LPS recognition system is therefore much less dependent on LBP than the human system.

3.3.8 LAL assay of LPS and liposomal LPS

LAL assay of 0.01ng/ml *E.coli* R1 and *E.coli* K12 LPS show a LAL activity of approximately 0.05 EU/ml (Figure 3.9). This is slightly lower than the 0.08 EU/ml demonstrated by the same amount of kit supplied standard *E.coli* O111 LPS, possibly indicating that smooth LPS is a slightly stronger activator of the LAL system than rough LPS. *B.fragilis* and *P.aeruginosa* LPS do not stimulate the LAL reaction at this concentration, reflecting their ~10,000 – 100,000- fold lower capacity to induce the activation of human LPS receptors in monocyte challenge assays (section 3.2.3). Native cocktail LPS exhibits a LAL activity approximately half that of the *E.coli* type LPS, presumably reflecting the fact that it contains (by mass) 50% *E.coli* type LPS, while the remaining (less active) LPS do not provide additional signalling activity at this concentration.

Surprisingly, the liposomal LPS is seen to be only around 30- fold less active than the equivalent amount of native LPS (section 3.2.4). This reduction in activity is starkly lower than that demonstrated in all of the cell based assays and much less marked than that described by other workers measuring the LAL activity of liposomal LPS. For example, Dijkstra *et al* (1988a) and Bennett-Guerrero *et al* (2000) have shown a reduction in LAL activity of between 100

and 1,000 fold when comparing liposomal LPS with native LPS. One explanation for the difference between the present finding and that of previous workers may be the choice of kit used to measure the LPS. Instructions supplied with the kit preclude the use of PBS in the assay as it interferes with the readout. For this reason, all LPS and liposomal LPS were diluted to working concentrations in pyrogen-free distilled water which, while not greatly affecting the activity of native LPS, may have induced lysis of the liposomes as a result of the osmotic effects of distilled water on the PBS based liposomes. Internalised LPS may therefore have been released into the sample medium, resulting in the higher than expected activity seen.

3.3.9 Mechanisms for reduced toxicity of liposomes

Given the great reduction in toxicity of LPS by incorporation into liposomes, it appears that the vast majority of the LPS present in the liposomes must be shielded from both the cellular and humoral proteins responsible for LPS signalling. The results from the serum protein experiments suggest that the liposomal signalling is occuring as a result of removal of LPS monomers from the outer membrane of the vesicles by serum proteins such as LBP and CD14. In this model, all internal LPS, either incorporated into the bilayers of internal lamellae or in solution between the lamellae, is therefore shielded from interaction from these proteins and therefore do not get channelled to cellular LPS receptor complexes.

Further, the timecourse experiments reveal that fusion of the internal lamellae of the liposomes with the internal membranes of the cell are unlikely to lead to much activation of the cells as the kinetic profile of liposomal LPS was seen to be very similar to that of native LPS. Together these experiments suggest that liposomal incorporation is an efficient method of delivering large amounts of LPS to cells without inducing an overtly inflammatory response.

Interestingly, while the biological activity of the LPS has been clearly diminished by incorporation into liposomes, the immunogenicity of the vaccine has not (Bennett-Guerrero et al 2000). This can perhaps be best explained by suggesting that the liposomal LPS is processed in the cell via pathways that are much more likely to result in antibody formation than the pathways used to deal with native LPS (Figure 1.4).

3.3.10 Comparison with earlier descriptions of liposomal LPS

In these experiments, incorporation of LPS into liposomes has demonstrated a reduction in biological activity in the region of 3,200-fold for murine NO release, one million-fold for murine TNF-α release and up to 100,000-fold when considering release of human TNF-α.

These findings indicate a biological activity lower than that described by previous workers studying liposomal LPS. Dijkstra *et al*, for example, showed that incorporation of *S.minnesota* Re LPS into liposomal vesicles was able to reduce the capacity of the LPS to induce IL-1β from a murine macrophage cell line by only 1,000-fold (Dijkstra et al 1987). The same preparation was shown to reduce TNF-α release from murine macrophages by a factor of 100 - 1000- fold (Dijkstra et al 1988b). Actinomycin D sensitized mice have been shown to have only a 10-fold reduction in lethality when challenged with liposomal lipid A compared with native lipid A (Dijkstra et al 1989). Finally, encapsulation of *Neisseria meningitidis* LPS into liposomes has been shown to reduce the pyrogenicity of LPS in rabbits by up to 1,000 fold (Petrov et al 1992).

Quite why the liposomal LPS preparations created in this laboratory exhibit biological activities so much lower than those previously described remains unclear. However, some explanation may lie in the fact that the techniques used to prepare the vaccine are slightly different from those previously attempted. A modified version of the method described by Dijkstra *et al* (1988a) was

employed (Bennett-Guerrero et al 2000). Thus, in our hands, the liposomes may contain more internal lamellae or may be larger than those previously described, or perhaps the distribution of LPS molecules within the liposomes is different.

In summary, however, the data from these studies provide support for the safety of the proposed vaccine, as the greatly reduced capacity of liposomal LPS to induce pro-inflammatory cytokines from myeloid cells *in vitro* suggests that administration of the vaccine to human subjects should result in a much more limited inflammatory response than that generated by native LPS. The wider potential of this vaccine and other lipopolysaccharide based vaccines is discussed in more depth in section 6.4.

3.3.11 Previously described TLR2/4 ligands

As discussed in the introduction (section 1.2), it was thought for some time that the Toll-like receptor critical for the recognition of all types of LPS was TLR4 (Qureshi et al 1999, Poltorak et al 1998, Hoshino et al 1999). This model was questioned, however, when several groups demonstrated responses to their LPS preparations that were independent of TLR4. In these experiments, it appeared that another Toll-like receptor, TLR2, was critical in these responses. In the majority of these cases, stringent purification protocols were able to demonstrate that the LPSs being investigated were signalling solely through TLR4, while the TLR2 signalling previously seen appeared to be due to lipoprotein contamination of those preparations (Hirschfield et al 2000, Shimazu et al 1999).

However, the LPSs from *Porphyromonas gingivalis* and *Leptospira interrogans* have both been proven to signal exclusively through TLR2 and not TLR4 (Hirschfield et al 2001, Werts et al 2001). This differential signalling of the LPS of some bacterial species via alterative Toll-like receptors may have some influence on the progression of disease as it has recently been shown that while

the ten human Toll-like receptors all induce a core set of inflammatory genes, the different members of the family also induce expression of genes unique to stimulation of that receptor (Hirschfield et al 2001). For example, macrophages stimulated via TLR2 can be triggered to release TNF-α, IL-1β and MIP-2, whereas macrophages stimulated via TLR4 are triggered to release all of these cytokines, but also IL-12 p40, IFN-γ and macrophage chemotactic protein 5 (Hirschfield et al 2001). This suggests that Gram-negative bacteria expressing Toll-like receptor 2 agonist LPS may well be recognised and dealt with quite differently in the human host than bacteria expressing LPS recognised by TLR4.

3.3.12 Antibodies for the investigation of TLR2 and TLR4 signalling

In order to determine if the LPSs from other bacterial species were capable of signalling via TLR2 instead of TLR4, experiments were performed to determine if antibody blocking of TLR2 or TLR4 could reduce signalling in response to different types of LPS. Currently, two monoclonal antibodies are commercially available that are proposed to be able to block the signalling function of TLR2 (TL2.1) or TLR4 (HTA125).

However, in contrast to earlier reports of the succesful use of these antibodies in blocking TLR4 (Akashi et al 2000, Tabeta et al 2000) and TLR2 signalling (Flo et al 2000, Lien et al 1999), three separate experiments in this laboratory failed to demonstrate the capacity of either antibody to block human monocyte recognition of either LPS or LTA. Figure 3.10 shows that the IL-1β secretion of human monocytes in response to challenge with *E.coli* LPS, *P.gingivalis* LPS or *S.aureus* LTA cannot be blocked with either TL2.1 or HTA125. Further, at the concentration used (20μg/ml), the antibody TL2.1 is seen to be capable of inducing cells to release IL-β independently of any other stimulus. This seems contra-intuitive, as previous experiments involving homodimerisation of TLR2 have been shown by previous workers to result in no cellular signalling (Ozinsky

et al 2000). Further, TLR2 mediated signalling appears to be dependent on the formation of heterodimers with TLR1 or TLR6 (Ozinsky et al 2000). It is therefore possible that TL2.1 demonstrates a cross reactivity with epitopes on TLR1 or TLR6, thereby causing the heterodimerisation required for signalling of TLR2. Why these antibodies have been unable to block the signalling of their target receptors in this laboratory remains unclear, though their failure has been noted by other laboratories (L. O'Neill, personal communication).

3.3.13 Structural motifs required for the discrimination of TLR2 and TLR4 ligands

The physical mechanisms by which the TLR2 and TLR4 agonist LPSs are discriminated remain to be determined. However, some interesting differences from the typical hexa-acyl bis-phosphorylated structure demonstrated by most TLR4 agonist enterobacterial LPS have been seen in the structure of the TLR2 agonist lipid A of *Porphyromonas gingivalis*. In particular, *P.gingivalis* lipid A contains only five acyl chains and is mono-phosphorylated (Kumada et al 1995). The structure of *Leptospira interrogans* lipid-A has yet to be determined, though it is tempting to speculate that it may share structural features with *P. gingivalis* lipid A.

Circumstantial evidence exists to support the notion that other bacterial species may express LPS capable of recognition by TLR-2. Cells from C3H/HeJ mice are deficient in TLR-4 signalling, yet remain capable of eliciting responses to LPS from both *B.fragilis* (Delahooke et al 1996) and *Treponema denticola* (Rosen et al 1999). Interestingly, the LPS of *B.fragilis* has been shown to express a lipid A of very similar structure to that of *P.gingivalis*, sharing both the 5 acyl chain and monophosphorylation motif (Figure 1.3). This general structure is also present in the lipid A of *C.trachomatis* LPS (Kosma 1999), which may also therefore represent a potential TLR2 agonist. Determination of the structure of further TLR2 agonist LPSs will provide useful insight into which domains or

particular three dimensional arrangements of LPS are differentiated by TLR2 and TLR4.

3.3.14 Wild type and mutant responses to LPS and LTA

Sequencing of the TLR4 gene from a large number of patients and healthy volunteers has revealed a selection of polymorphisms present in the Caucasian population (Smirnova 2001). While some of these were seen to be present only very rarely, two mutations in particular, Asp299Gly and Ile399Thr, have been shown to exist at a relatively high frequency (at least 6%) (Lorenz et al 2001). This high frequency of TLR4 mutation in the general population prompted an investigation to determine whether individuals expressing these genotypes exhibit either more- or less- sensitive responses to the LPS of particular strains and species of bacteria.

In order to investigate this possibility, monocytes of three individuals heterozygous for both mutations and three individuals homozygous for the wild-type allele were challenged with a panel of LPS selected to represent a broad range of lipid A structures. LPS from *E.coli*, *Neisseria meningitidis*, *Bacteroides fragilis*, *Yersinia pestis*, *Chlamydia trachomatis*, *Porphyromonas gingivalis* and *Pseudomonas aeruginosa* and lipoteichoic acid of *Staphylococcus aureus* were applied to monocytes of wild type and heterozygote individuals and production of the pro-inflammatory cytokine interleukin-1β compared.

It was expected that the responses to *S.aureus* LTA and *P.gingivalis* LPS should be broadly similar as the signalling of these two molecules are not TLR-4-dependent (Hirschfield et al 2001, Lien et al 1999). Surprisingly, however, it can be clearly seen that the responses of both wild type and mutant monocytes to all stimuli tested, including all TLR4 agonists, are remarkably similar (Figures 3.12a-h). Slightly more inter-individual variability exists in the response towards LPS of *B.fragilis* and *P.aeruginosa*, though these are not statistically significant.

Further, analysis of the minimum concentration of LPS required to activate mutant and wild type monocytes also reveals near identical sensitivity of the two versions of the receptor (Table 3.4).

3.3.15 Comparison with previous findings on TLR4 mutant phenotypes

These findings contrast with those of Schwartz and co-workers who have reported several examples of associations between the presence of these mutations and either hyporesponsiveness to LPS or increased susceptibility to Gram-negative infection.

The first such study investigated the response of 83 humans to inhaled LPS, and it was shown that presence of the mutation resulted in blunted responses to inhaled LPS in humans (Arbour et al 2000). Further, transfection of THP-1 cells with the Asp299Gly mutation was shown to blunt LPS signalling *in vitro*, while the Thr399Ile mutation was shown to have no effect on signalling (Arbour et al 2000).

Closely following this, another group reported no association between presence of the Asp299Gly mutation and either susceptibility to or severity of meningococcal disease in a study of over 1,000 patients (Read et al 2001).

Schwartz and colleagues continued to test various patient populations for presence of both mutations and have reported the following associations. A study investigating the course of disease in 91 patients with septic shock compared the frequency of the Asp299Gly mutation with that of 73 healthy controls and found the mutation exclusively in the patient group (Lorenz et al 2002a). This study also points out that a higher prevalence of Gram-negative infections occurred in the patients with the Asp299Gly mutation. A study of 237 patients receiving haematopoietic stem cell transplantation were assessed for presence of the mutation and graft-versus-host disease. Those expressing

either of the mutations demonstrated a slightly higher suscepibility to Gram-negative bacteraemia and a reduced risk of developing acute graft-versus-host disease, but these results were not statistically significant (Lorenz et al 2002b).

The same group then compared the effect of the mutation on risk of atherogenesis in an Italian cohort consisting of 810 individuals (Kiechl et al 2002). They found that the 55 individuals harboring the Asp299Gly mutation showed lower serum levels of pro-inflammatory cytokines, acute-phase reactants and fibrinogen. Also, these individuals were found to be more susceptible to severe bacterial infections, but also demonstrated a significantly lower risk of carotid atherosclerosis and a smaller intima-media thickness in the common carotid artery – consistent with the hypothesis that inflammation initiated by the innate immune response has a strong role to play in atherogenesis. Finally, an investigation into the presence of the Asp299Gly mutation in 351 normal term and 440 prematurely born Finnish infants revealed a correlation between presence of the mutation and premature birth ($p=0.024$) (Lorenz et al 2002c). This, the authors claim, is consistent with the current understanding that Gram-negative urinogenital infection is a risk factor in premature term.

Each of the studies reported by Schwartz and colleagues appear to demonstrate that individuals heterozygous for the Asp299Gly mutation show altered responses to LPS or Gram-negative infection. However, the present study of monocytes derived from individuals heterozygous for both the Asp299Gly and Thr399Ile mutations demonstrates that no reduction in ability to detect any of the seven LPS tested can be detected in these subjects. This discrepancy may perhaps be explained as follows.

Firstly, the rationale or interpretation of several of the studies may be criticised. For example, in the study of 91 septic patients, the group note a higher

frequency of the mutation in the patient group than in a group of healthy controls and cite a *p*-value of 0.05 for this comparison. However, they have correlated the frequency of mutation in the patient population with only a small number of healthy controls (73), when the genotypes of much larger numbers of healthy controls were available from separate studies being undertaken at the same time in the same laboratory. Had the allele frequency of this group been used for comparison in the study, any statistical significance would almost certainly have been removed from their finding. In the study of patients receiving haematopoietic stem cell transplantation, the results were not statistically significant and no biological interpretation of how impaired TLR4 signalling might be involved in graft-versus-host rejection was offered. Finally, in the study of the role of the mutation as a risk factor in premature birth, multiple pregnancies were excluded from analysis and the genotype of only 94 mothers was investigated. Further, the statistically significant correlation quoted was obtained from analysis of the genotypes of the infants, and no explanation was given as to how the innate immunity of the fetus should affect bacterial challenge of the parent. Statistical analysis of the genotypes of the mothers, whose Toll-receptors are more likely to be involved in the detection of urinogenital infection, results in no statistically significant association (p=0.580). For these reasons, and the lack of any positive associations originating from other laboratories, the findings from these studies must be interpreted with caution.

It may yet, however, be possible to explain the demonstration that transfection of THP-1 cells with Asp299Gly mutant TLR4 results in blunted LPS responsiveness (Arbour et al 2000). The present study does not rule out the possibility that receptors homozygous for the Asp299Gly mutation may exhibit blunted LPS responses. In the THP-1 transfection experiments it is possible that the vector used in their transfection protocol induced larger than normal levels of mutant TLR4, such that most receptors on the surface were homodimers of TLR4 mutant molecules. Only measurement of responses from individuals

homozygous for this mutation, or transfection of TLR4 null cells (such as the human embryonic kidney derived 293 cell line) with the Asp299Gly TLR4 construct will shed some light on this matter.

CHAPTER 4

T-CELL RESPONSES TO LIPOPOLYSACCHARIDE

4.1 Introduction

In order to investigate the possibility that T-cells reactive towards LPS may exist, a readily available source of large numbers of lymphoid cells was required. Naïve mouse splenocytes were therefore chosen for preliminary experiments as a result of their ease of extraction and availability. Subsequent experiments investigated proliferative responses of murine lymph node cells and human peripheral blood mononuclear cells in response to LPS.

The optimum conditions for proliferation of human LPS-reactive T-cells were determined, then applied to the PBMC of several healthy donors to investigate whether T-cell clones reactive towards particular LPS core epitopes existed in healthy donors. The response to these epitopes was then assessed at several time points in these individuals. Finally, in order to determine whether CD1-mediated presentation of LPS was required for T-cell recognition of LPS, antibody blocking experiments and co-immunoprecipitation was attempted.

4.2 Results

4.2.1 Murine splenocyte response to LPS

The optimum concentration of LPS required to induce proliferation of murine splenocytes was determined by incubating with varying concentrations of *E.coli* R4 LPS for 3 days in culture. The proliferative responses of these splenocytes were measured by radiolabelled thymidine incorporation and are summarised in Figure 4.1. This experiment shows a strong proliferative response to all concentrations of LPS above 100ng/ml, with a linear relationship appearing between LPS concentration and splenocyte proliferation. Concentrations below about 10ng/ml do not appear to stimulate proliferation in these cells. Further, it can be seen that high concentrations of LPS are able to induce a stronger

proliferative response in the splenocyte population than 1μg/ml of the T-cell mitogen Concanavalin A (ConA).

Naïve mouse splenocytes were then extracted and plated with *E.coli* K12 LPS at various concentrations and incubated for between 4 and 7 days to investigate the kinetics of this proliferative response beyond day 3. Figure 4.2 confirms the dose-dependent nature of the proliferation seen in the first experiment, but also reveal that the cells proliferate strongly to both *E.coli* K12 LPS and ConA at day four, with this response diminishing daily such that by day seven practically no proliferation can be seen.

Next, to determine whether there was any effect of LPS core type on the strength of the proliferative response, *E.coli* R1, R2, R3 and R4 and *Bacteroides fragilis* LPSs were added to splenocytes at equal concentrations by mass and the proliferation measured at three days. Figure 4.3 shows that *E.coli* R3 and R4 LPS exhibit the strongest responses at 10,000ng/ml, with the LPS from R1 and R2 core types slightly less active and that of *B.fragilis* much less active.

Next, in order to determine which cell type within the splenocyte population was responsible for this proliferation, antibody conjugated magnetic beads were employed to provide purified cellular sub-populations. Figure 4.4 shows the effect of enrichment for CD19+ve, CD3+ve and CD1d+ve cells on splenocyte proliferation in response to *E.coli* R4 LPS. These data show that while the CD19+ve fraction responds strongly to the LPS, the CD3+ve and CD1d+ve cellular fractions do not.

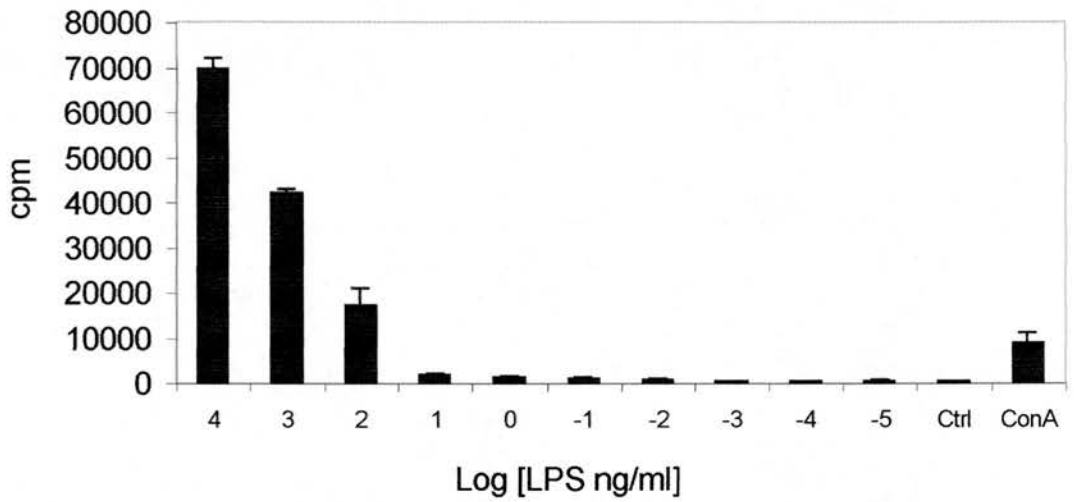


Figure 4.1 Dependence of splenocyte proliferative capacity on LPS concentration
Murine splenocytes were plated at 2 x 10^5 cells per well and challenged with varying concentrations of *E.coli* R4 LPS. Cells were pulsed with ^{3}H radiolabelled thymidine (1μCi per well) 18 hours before harvesting at three days. Control cultures received either medium alone (Ctrl) or 1μg/ml concanavalin A (ConA). cpm = counts per minute.

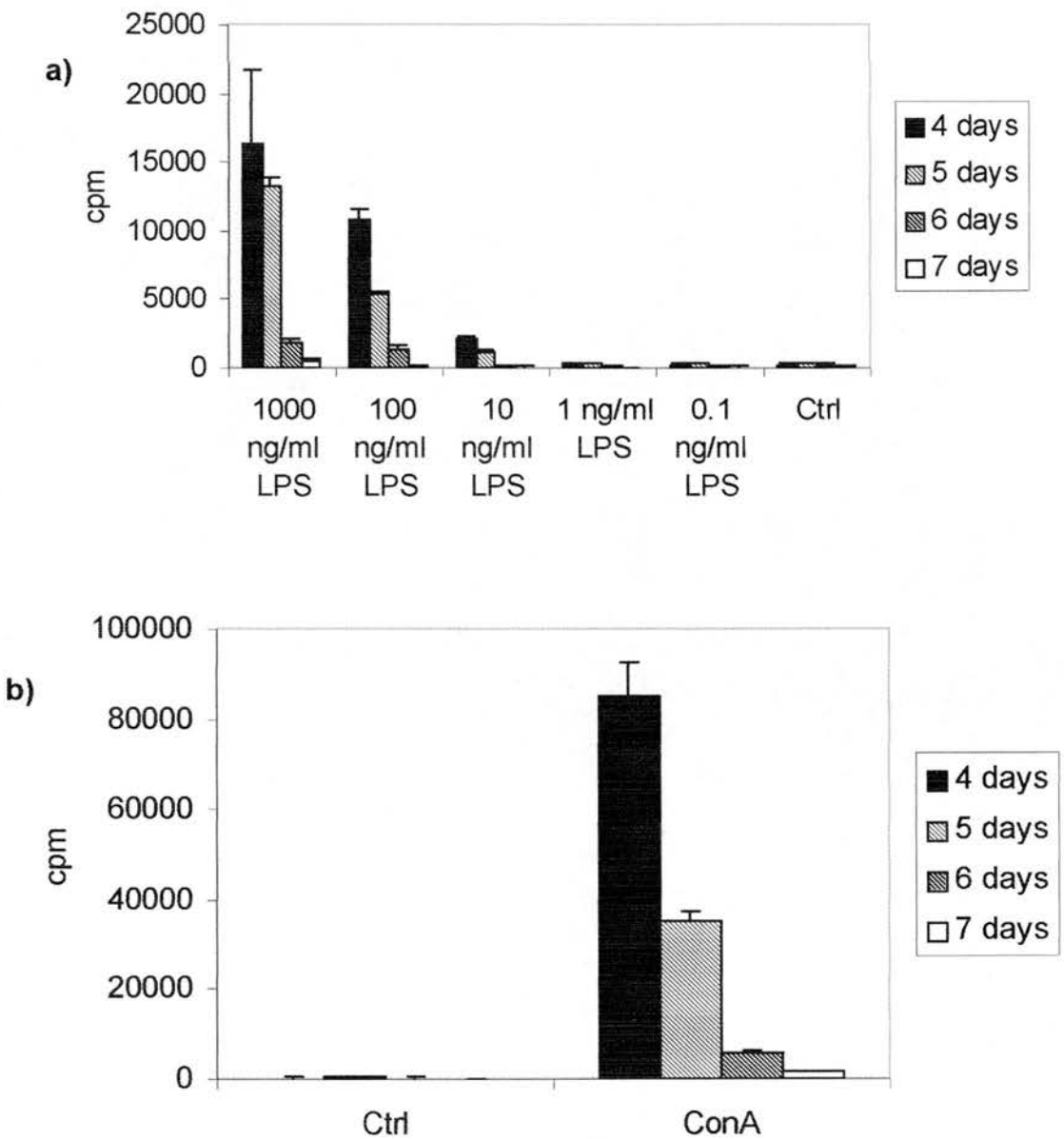


Figure 4.2 Kinetics of murine splenocyte proliferation in response to LPS and ConA
Murine splenocytes were plated at 2 x 10^5 cells per well and challenged with varying concentrations of (a) *E.coli* K12 LPS or (b) 1μg/ml concanavalin A (ConA). Cells were pulsed with ^{3}H-radiolabelled thymidine (1μCi per well) 18 hours before harvesting at four, five, six or seven days. Control cultures (Ctrl) received medium alone. cpm = counts per minute.

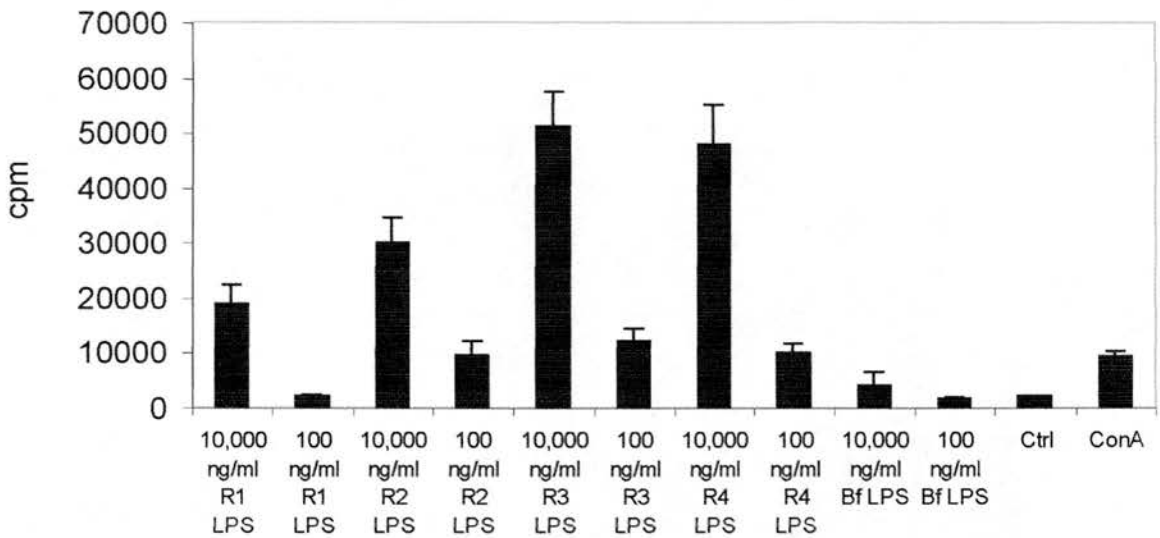


Figure 4.3 Murine splenocyte proliferation in response to different LPS types
Murine splenocytes were plated at 2 x 10^5 cells per well and challenged with either 100ng/ml or 10,000ng/ml of various types of LPS. R1 = *E.coli* R1, R2 = *E.coli* R2, R3 = *E.coli* R3, R4 = *E.coli* R4, Bf = *B.fragilis*, Cells were pulsed with ^{3}H-radiolabelled thymidine (1μCi per well) 18 hours before harvesting at three days. Control cultures received either medium alone (Ctrl) or 1μg/ml concanavalin A (ConA). cpm = counts per minute.

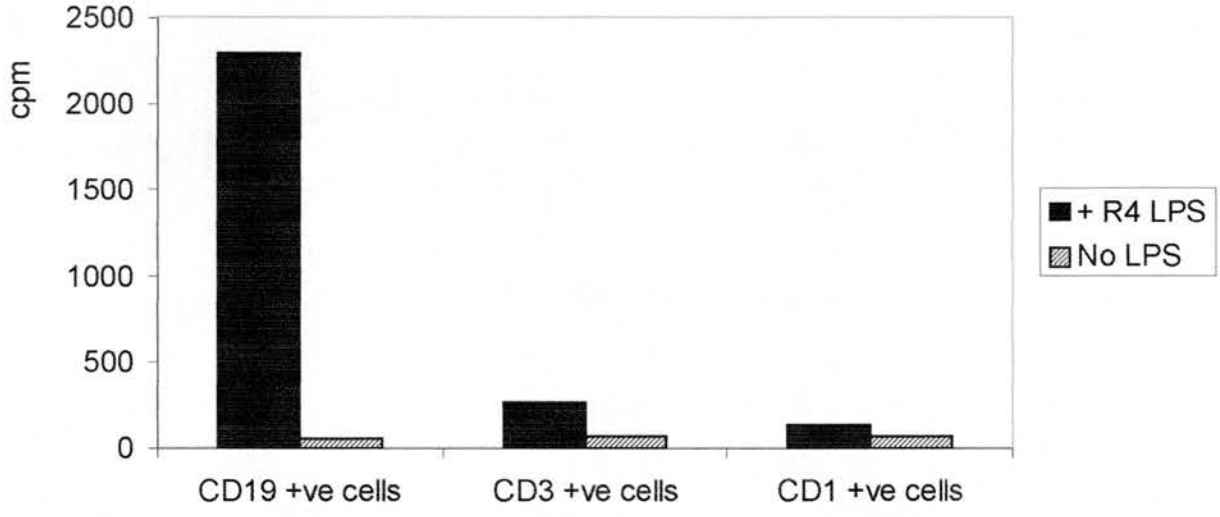


Figure 4.4 Proliferative capacities of CD19+ve, CD3+ve and CD1d+ve cells in response to LPS
Murine splenocytes were sorted into CD19+ve, CD3+ve and CD1d+ve cellular fractions using antibody conjugated magnetic beads. Subpopulations were then cultured with 10μg/ml *E.coli* R4 LPS or medium alone for three days, receiving 1μCi per well ^{3}H-radiolabelled thymidine 18 hours before harvesting. cpm = counts per minute.

To further clarify the role of CD19+ve cells in splenocyte proliferation, the experiment was repeated with the alteration that the CD19 depleted fraction was retained for investigation. Figure 4.5 shows that while non-depleted splenocytes respond strongly to *E.coli* R4 LPS, depletion of CD19+ve cells removes this ability completely. Moreover, the cellular fraction consisting purely of CD19+ve cells is seen to be capable of responding to LPS without help from accessory cells. This fraction does not proliferate in response to ConA, indicating that it is not highly contaminated with T-cells.

These data suggest that the murine splenocyte reactivity towards LPS is mediated primarily by CD19+ve B-cells. Since it appeared that this mitogenic response would probably mask any effect of LPS reactive T-cells present in the splenocyte population, subsequent experiments focused instead on cells extracted from murine lymph nodes.

4.2.2 Murine T-cell response to LPS

In the first instance, cells from the lymph nodes of naïve animals were extracted and incubated with varying concentrations of *E.coli* K12 LPS or the T-cell mitogen ConA. This experiment was repeated three times, each time revealing no proliferative response to LPS from the lymph node cells of naïve animals (Figure 4.6). It was therefore postulated that immunisation of mice with LPS or bacteria prior to lymphectomy may be required in order to create a significant memory T-cell population.

In the first of these experiments, mice were injected at the tail base with 10μg *E.coli* K12 LPS, 10^7 heat killed *E.coli* O18K$^-$ bacteria or 10^7 sheep red blood cells in Freunds complete adjuvant. Inguinal and periaortic lymph nodes were removed 8 days after incubation and the cells extracted. These cells were then exposed to various concentrations of *E.coli* K12 LPS, heat-killed *E.coli* O18K$^-$

bacteria or sheep red blood cells *in vitro* and the proliferation of these cells assessed after two, four or six days using thymidine incorporation. In these and similar experiments using *in vitro* incubation times ranging from 2 days to 6 days, no appreciable proliferative response was seen to occur in response to any of these three antigens, although positive control cultures incubated with ConA always showed proliferation (Figures 4.7a – c).

Suspecting that the lack of proliferation may have had something to do with the length of time between immunisation and harvesting of the lymph nodes, a panel of mice were immunised as before with heat-killed *E.coli* O18K$^-$ bacteria. Lymph nodes were excised from either naïve mice, mice immunised 10 days pre-harvesting or mice immunised 14 days before harvesting. Cells were then incubated with heat killed *E.coli* O18K$^-$ bacteria for four days, with the proliferation being measured using thymidine incorporation as before. Figure 4.8 shows that proliferative responses to the recall antigen were still absent, despite strong responses to the T-cell mitogen ConA. Larger responses to ConA were observed from the later stage mice suggesting that a higher proportion of T-cells exists in these fractions. Since the ConA controls had been working in these experiments, it was assumed that the presence of viable T-cells was not lacking. However, in order to boost the number of antigen presenting cells, splenocytes were chosen as a feeder layer for the T-cells in the next series of experiments.

In order to rule out proliferative effects previously demonstrated to be exhibited by the splenocytes themselves, these cells were treated transiently with the cell division inhibitor mitomycin C and thoroughly washed to provide cells capable of antigen presentation but not division. Figure 4.9 shows the effect of mitomycin-C treatment on the capacity of murine splenocytes to proliferate in response to *E.coli* R4 LPS. This initial experiment appeared to show that while mitomycin C could strongly blunt splenocyte proliferation in response to LPS and ConA, the

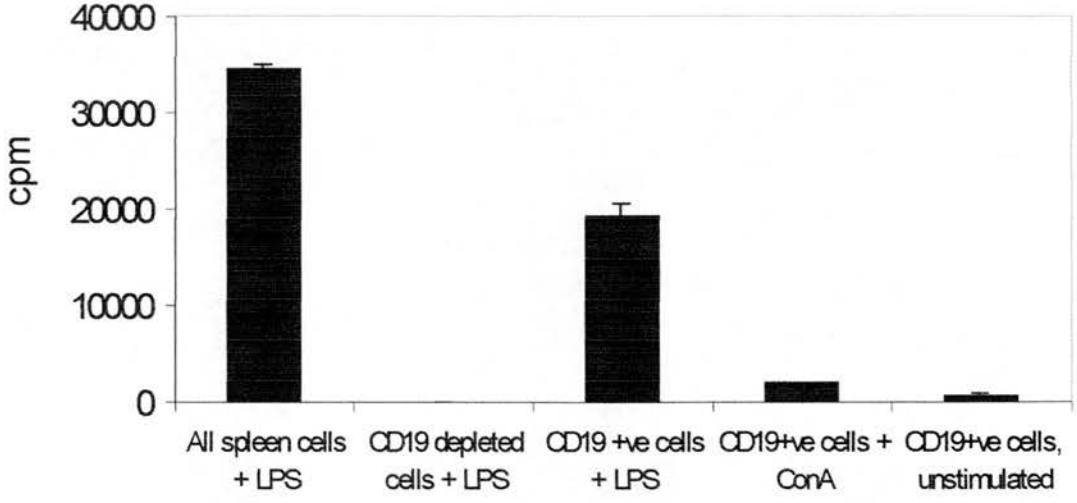


Figure 4.5 Effect of CD19+ve cell depletion/enrichment on splenocyte response to LPS
Murine splenocytes were sorted into CD19+ve and CD19-ve cellular fractions using antibody conjugated magnetic beads. Subpopulations were then cultured with 10μg/ml *E.coli* R4 LPS or 1μg/ml concanavalin A or medium alone for three days, receiving 1μCi per well ^{3}H-radiolabelled thymidine 18 hours before harvesting. cpm = counts per minute.

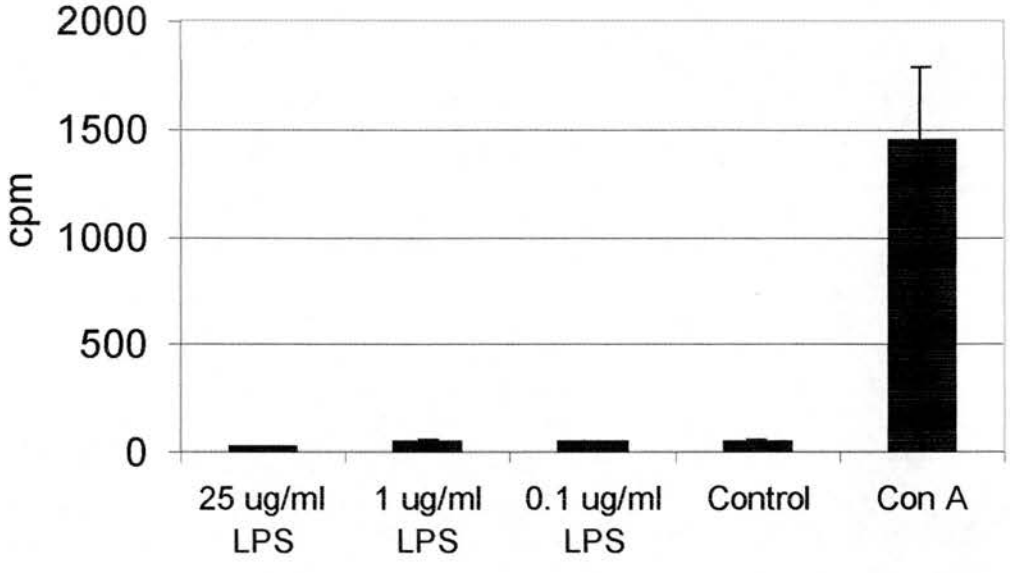
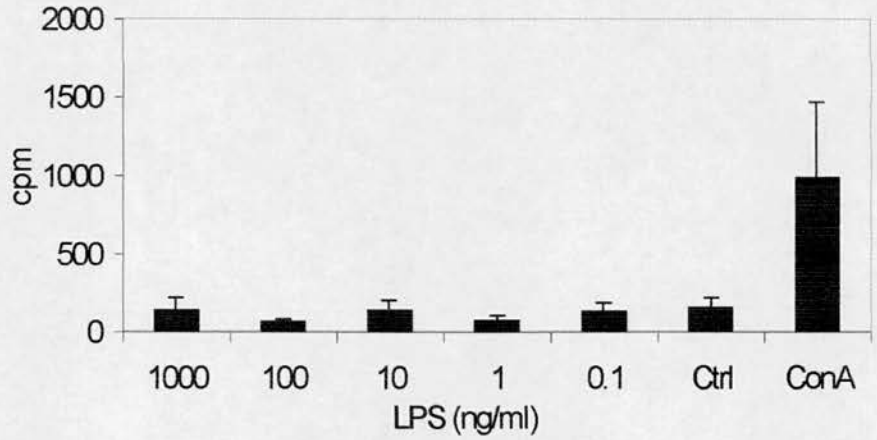


Figure 4.6 Proliferative response to LPS and ConA from naïve mouse lymph node cells
Lymph node cells extracted from naïve mice (2×10^5 per well) were incubated with varying concentrations of *E.coli* K12 LPS or medium alone (control) or 1μg/ml concanavalin A (ConA), pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at five days. Results are representative of three similar experiments. cpm = mean counts per minute of three independent cultures +/- SEM.

a)



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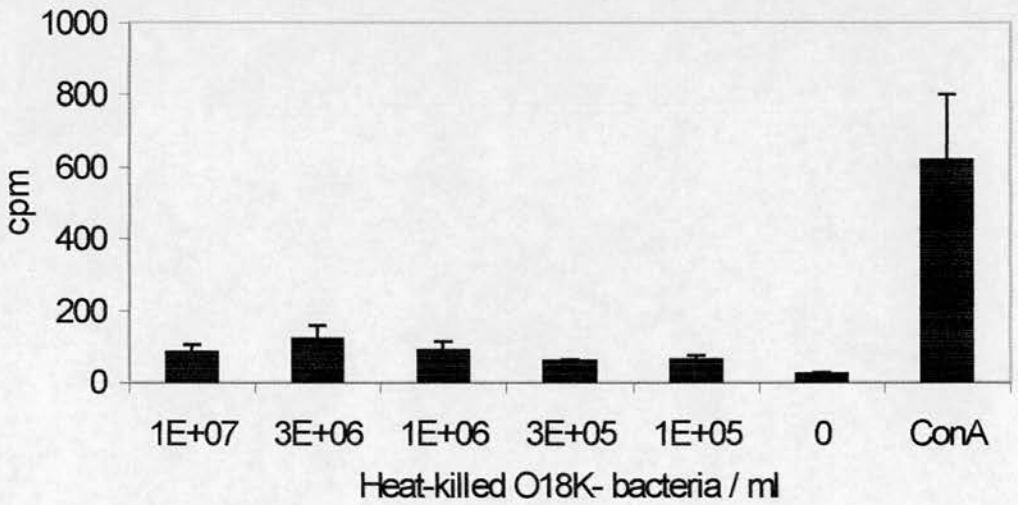


Figure 4.7 Proliferative responses of pre-immunised murine lymph node cells to (a) *E.coli* K12 LPS (b) heat killed *E.coli* O18K- bacteria or (c) sheep red blood cells

Lymph node cells extracted from mice pre-immunised with each antigen were incubated with medium alone (Ctrl), 1μg/ml concanavalin A (ConA) and varying concentrations of (a) *E.coli* K12 LPS (b) heat-killed *E.coli* O18K- bacteria or (c) sheep red blood cells. Cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at six days (LPS and heat killed bacteria) or 2 and 4 days (sheep red blood cells). Results are representative of three similar experiments. cpm = mean counts per minute of three independent cultures +/- SEM.

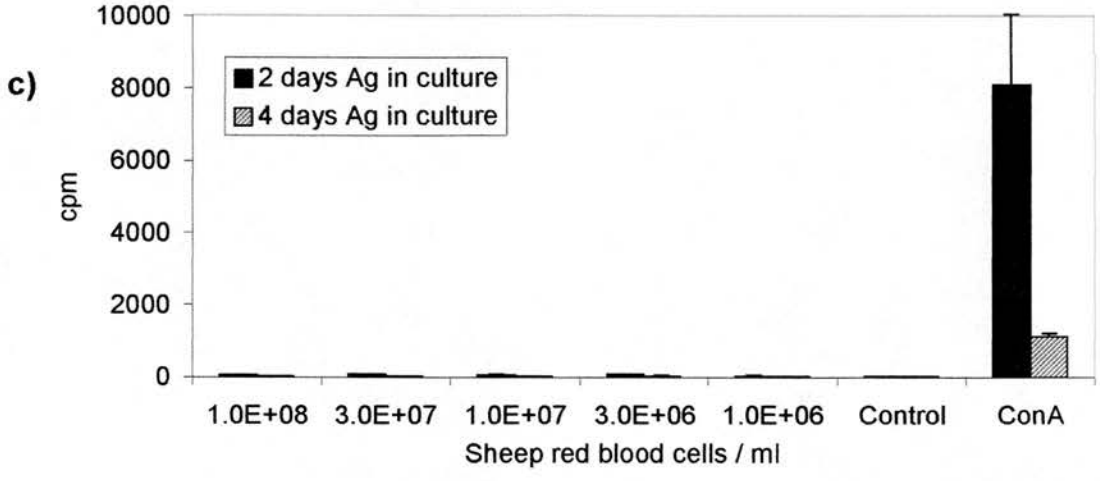


Figure 4.7 Proliferative responses of pre-immunised murine lymph node cells to (a) *E.coli* K12 LPS (b) heat killed *E.coli* O18K- bacteria or (c) sheep red blood cells

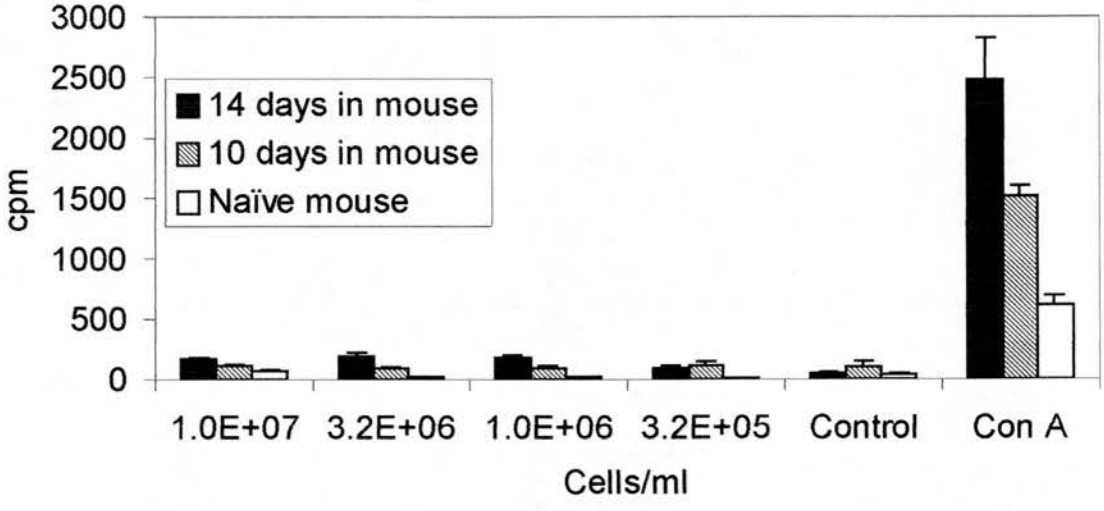


Figure 4.8 Comparison of times between immunisation and harvesting on lymph node cell proliferation in response to antigen

Lymph node cells from either naïve mice, mice immunised 10 days pre-harvesting or mice immunised 14 days before harvesting were challenged with various concentrations of heat-killed *E.coli* O18K- bacteria or medium alone (Ctrl) or 1μg/ml concanavalin A (ConA), pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 4 days. Results are representative of three similar experiments. cpm = mean counts per minute of three independent cultures +/- SEM.

presence of mitomycin-C treated and washed cells did not inhibit the proliferation of fresh splenocytes added on top of them (Figure 4.9).

Mitomycin C- treated splenocytes were then plated into 96 well plates and supplemented with T-cells purified from autologous splenocytes using the MACS magnetic bead purification system (methods, section 2.3.9). Following this, *E.coli* R4 LPS, ConA or medium alone was added to the cells and incubated for 4 days. Figure 4.10 shows that untreated splenocytes proliferate strongly in response to both *E.coli* R4 LPS and ConA, while the response of mitomycin C treated splenocytes is strongly blunted. However, addition of purified naïve T-cells to mitomycin C treated splenocytes does not enhance the proliferative response to either *E.coli* R4 LPS or ConA.

As the yield of T-cells from the MACS purification system was relatively low, passage of murine splenocytes over nylon wool was employed in the next series of experiments to provide larger numbers of purified T-cells. This purification method employs the principle that T-cells exhibit less adhesion to nylon than B-cells or monocytes, and is capable of providing greater numbers of T-cells than the MACS system as it can be easily scaled upwards. Splenocytes were treated with mitomycin C as before, and plated alone or supplemented with nylon wool selected autologous T-cells. Untreated splenocytes were also included in the assay as positive control. Figure 4.11 shows that splenocytes in these experiments respond vigorously to *E.coli* R4 LPS and ConA stimulation, while mitomycin C-treated splenocytes respond only poorly. Purified T-cells exhibit no proliferative response to either stimulus, but, surprisingly, are seen to be able to remove completely the residual proliferative capacity of the mitomycin C-treated splenocytes.

A repeat of the same experiment using longer treatment of the splenocytes with mitomycin C is depicted in Figure 4.12a. The chart reveals that untreated

splenocytes respond with strong proliferation in response to *E.coli* R4 LPS and ConA stimulation. This response to LPS is reduced by the addition of nylon wool purified T-cells, though the ConA response remains unaltered – a finding which can probably be explained due to the fact that the splenocytes are plated at half the usual concentration, with the remainder being made up with T-cells. Mitomycin C-treated splenocytes in this experiment exhibit no proliferative capacity whatsoever. The T-cell fraction retains residual proliferative capacity in response to both LPS and ConA, possibly as a result of B-cell contamination of these relatively crude preparations. However this response is lost when these cells are mixed with the mitomycin C treated splenocytes. It was assumed at this stage that the mitomycin C treated cells were still, despite careful washing, removing the proliferative capacity of T-cells added to the preparation.

Concerned about potential B-cell contamination of the T-cell preparations, the same experiment was repeated using freshly purchased nylon wool. Figure 4.12b shows that normal responses were seen from untreated splenocytes towards both LPS and ConA. Mitomycin C treated splenocytes, purified T-cells and a mixture of the two all failed to proliferate appreciably in response to the two antigens.

As no proliferative T-cell response to LPS could be detected in any of the murine systems investigated, subsequent experiments focused instead on the proliferative response of human peripheral blood mononuclear cells (PBMC) to LPS.

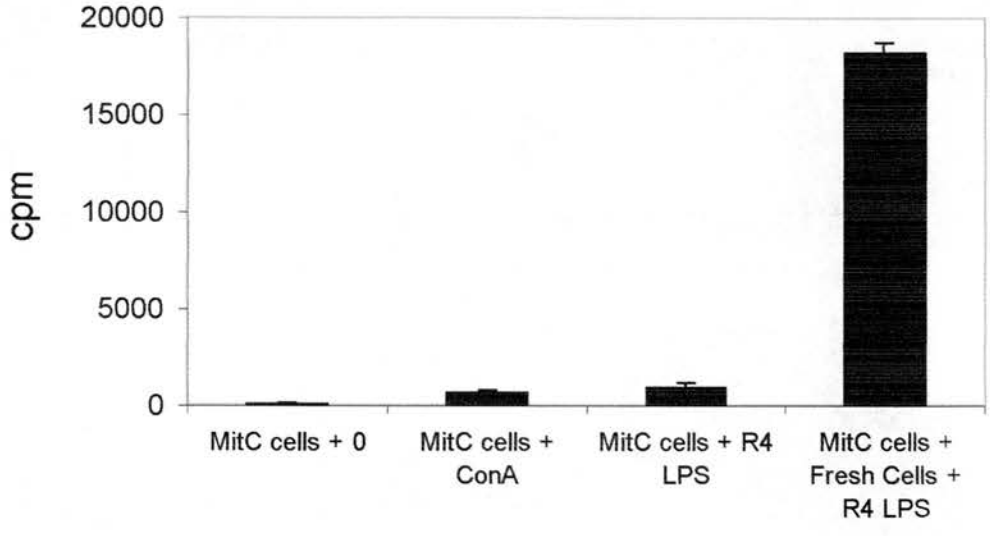


Figure 4.9 Effect of mitomycin-C treatment on the capacity of murine splenocytes to proliferate in response to *E.coli* R4 LPS and ConA

Murine splenocytes were treated with mitomycin-C, washed then plated with medium alone (MitC + 0), 1μg/ml Concanavalin A (MitC + ConA), 10μg/ml *E.coli* R4 LPS (MitC + R4 LPS) or fresh splenocytes plus 10μg/ml *E.coli* R4 LPS (MitC + fresh cells + R4 LPS). Cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 4 days. cpm = mean counts per minute of three independent cultures +/- SEM.

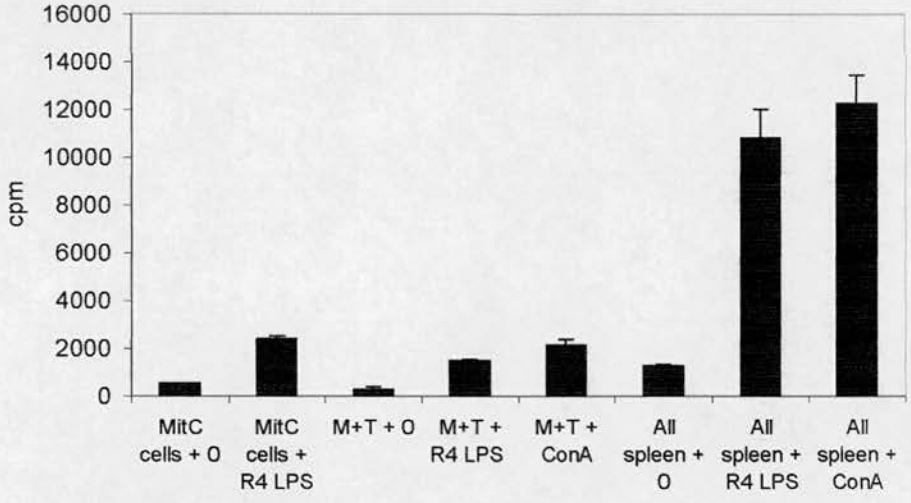


Figure 4.10 Proliferative response of Mitomycin-C treated splenocytes supplemented with MACS purified T-cells to *E.coli* R4 LPS and ConA

Murine splenocytes were treated with mitomycin-C, washed then plated with medium alone (MitC + 0) or 10μg/ml *E.coli* R4 LPS (MitC + R4 LPS). Mixed cultures of mitomycin-C treated splenocytes and MACS purified T-cells were cultured with medium alone (M+T+0),10μg/ml *E.coli* R4 LPS (M+T+R4LPS) or 1μg/ml Concanavalin A (M+T+ConA). Untreated splenocytes (All spleen) were incubated with medium alone (0), 10μg/ml *E.coli* R4 LPS (R4 LPS) or 1μg/ml Concanavalin A (ConA). All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 4 days. cpm = mean counts per minute of three independent cultures +/- SEM.

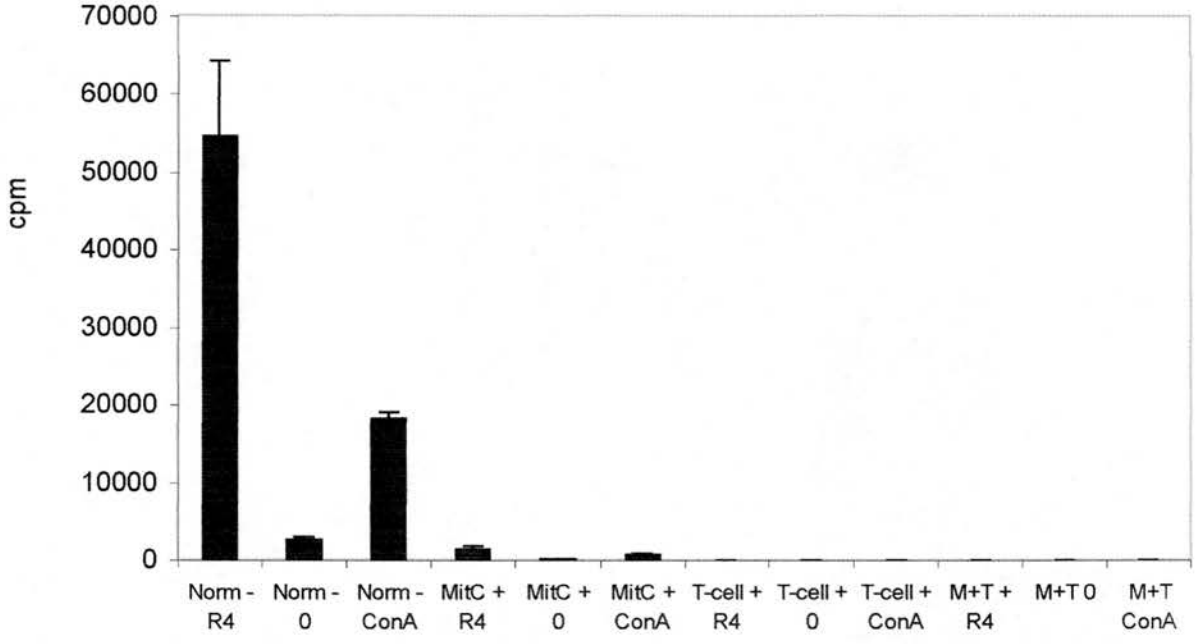
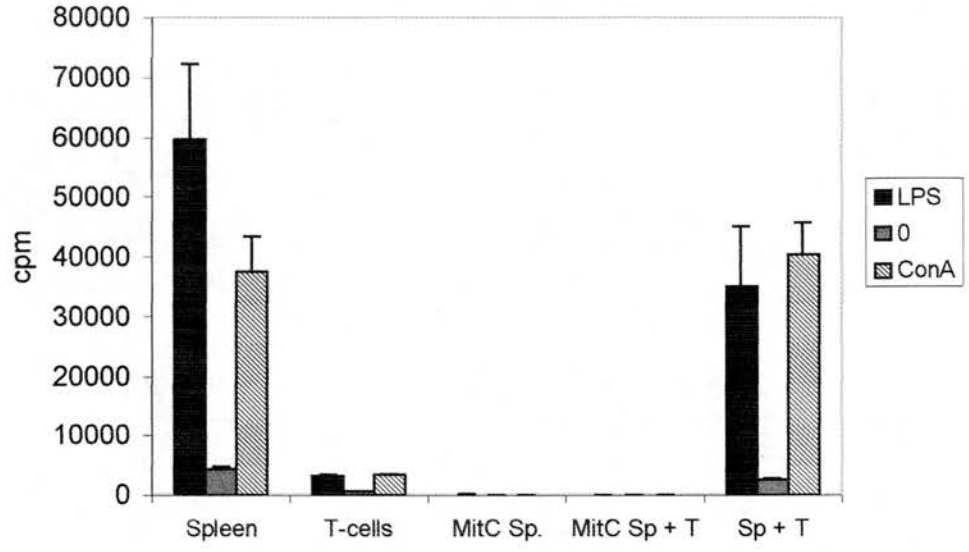


Figure 4.11: Proliferative response of Mitomycin-C treated splenocytes supplemented with nylon wool purified T-cells to *E.coli* R4 LPS and ConA

Untreated murine splenocytes (Norm), mitomycin-C treated splenocytes (MitC), nylon wool purified T-cells (T-cell), or mixed cultures of mitomycin-C treated splenocytes and nylon wool purified T-cells (M+T) were cultured with medium alone (0), 10μg/ml *E.coli* R4 LPS (R4) or 1μg/ml Concanavalin A (ConA). All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 4 days. cpm = mean counts per minute of three independent cultures +/- SEM.

a)



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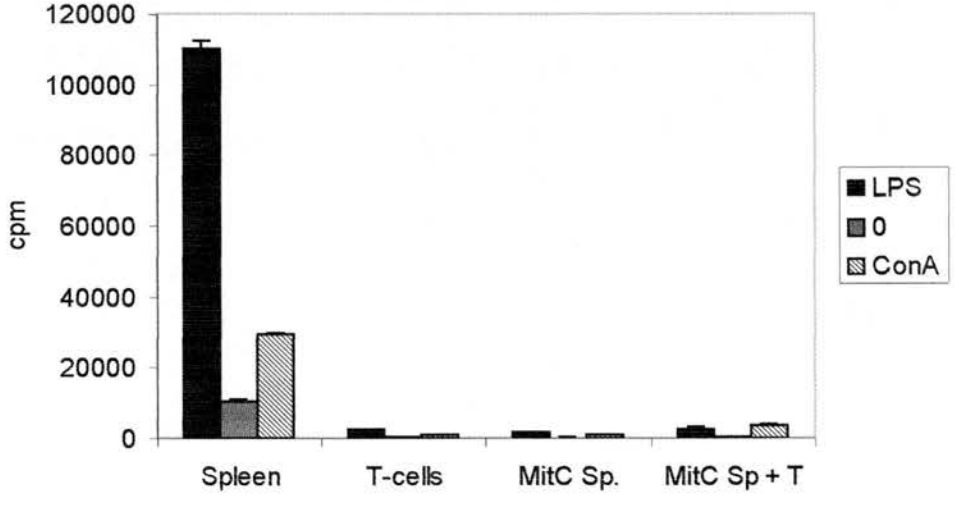


Figure 4.12 Proliferative response of Mitomycin-C treated splenocytes supplemented with nylon wool purified T-cells to *E.coli* R4 LPS and ConA

(a) Untreated murine splenocytes (Spleen), nylon wool purified T-cells (T-cells), mitomycin-C treated splenocytes (MitC Sp), mixed cultures of mitomycin-C treated splenocytes and nylon wool purified T-cells (MitC Sp+T) or mixed cultures of untreated splenocytes and nylon wool purified T-cells (Sp+T) were cultured with medium alone (0), 10μg/ml *E.coli* R4 LPS (LPS) or 1μg/ml Concanavalin A (ConA). All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 4 days. cpm = mean counts per minute of three independent cultures +/- SEM. (b) Repeat of above experiment using fresh nylon wool for T-cell purification.

4.2.3 Human T-cell responses to LPS

In order to investigate the possibility that human PBMCs may be able to proliferate in response to LPS, human PBMCs extracted from buffy coats (methods, section 2.3.2) were incubated with either *E.coli* K12 LPS or heat-killed *E.coli* K12 bacteria for 3 days. Figure 4.13a shows that no proliferation occurred in response to either LPS or heat-killed bacteria, though cultures challenged with ConA exhibited strong proliferation. PBMCs derived from the buffy coats of several different donors revealed the same lack of proliferation, so the same experiment was repeated using a 5 day incubation period. Figure 4.13b shows that proliferative responses were generated to ConA but not to either LPS or heat killed bacteria.

It was considered that perhaps lack of prior exposure to this antigen may be responsible for the lack of response seen. For this reason, the next series of experiments investigated responses to LPS of *E.coli* R1, *E.coli* R2, *E.coli* R3, *E.coli* R4 and *B.fragilis* – all of which may be commonly found in the gut or are associated with infection. The proliferative responses to these antigens from the buffy coats of several dozen individuals were tested using 3 days, 4 days or 6 days incubation. No response was seen to these antigens or to the LPS of *Klebsiella aerogenes*, *K.friedlander*, *K.pneumoniae*, *Salmonella minnesota* or *S.typhimurium* in many similar experiments (typical responses of individual donors are represented in Figures 4.14-4.15).

At this stage it was postulated that perhaps upregulation of the antigen presentation molecule CD1 was required to allow T-cell responses towards LPS. For this reason, the next series of experiments investigated the response of T-cells to LPS in the presence of CD1+ve cells. Figure 4.16a shows the proliferation of buffy coat PBMCs exposed to either LPS alone or LPS supplemented with 200IU/ml of the cytokines IL-4 and GM-CSF, which together induce the expression of CD1a,b and c on human monocytes (Figure 4.29).

Treatment of PBMC in this way did not result in induction of an LPS-specific proliferative response at 4 days (Figure 4.16a) or at 6 days incubation (Figure 4.16b).

In another set of experiments, IL-4/GM-CSF was administered to buffy coat PBMC first (for three days), with LPS being added afterwards (for a further three days). Proliferation was then measured after six days total incubation. Figure 4.17 shows that once again, no difference in proliferation between LPS treated and control cultures could be observed.

Concerned at the large variability seen in proliferation between identically treated adjacent wells in this and previous experiments, an experiment was performed to investigate whether this variability was due to problems in the dispersion of LPS preparations or to an inherent property of the cells used. Buffy coat PBMCs were incubated for 6 days in medium supplemented with 5% foetal calf serum alone but without any other stimulus. Figure 4.18 shows that of the 12 wells, most exhibit a proliferation around 150cpm, while one exhibits a count of around 2800. This inherent variabilty in the unstimulated cell population was assumed to be responsible for the variability seen in previous experiments.

To further clarify the cause of the relatively high background proliferation seen in all previous experiments, the role of the serum was investigated. Buffy coat PBMC were plated with medium containing foetal calf serum or no serum and challenged with LPS as before. Figure 4.19 shows that removal of serum from the medium reduces proliferation of all cultures by a factor of approximately 10. Foetal calf serum from three different manufacturers was tested (Gibco, Sigma and Imperial laboratories) but none could remove the high (>1,000cpm) background proliferation observed in all experiments. For this reason, a series of experiments was performed using buffy coat PBMC supplemented with pooled human serum. While these experiments did display much lower background

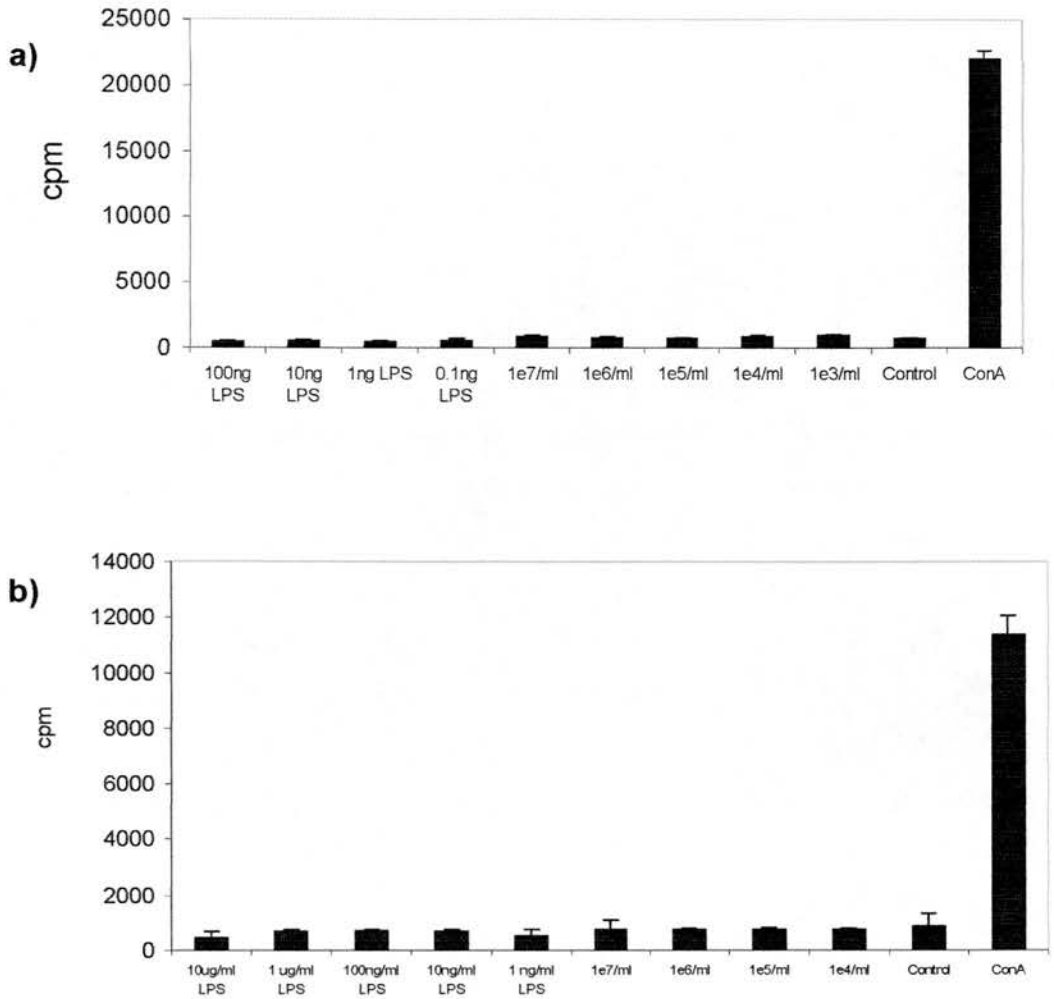
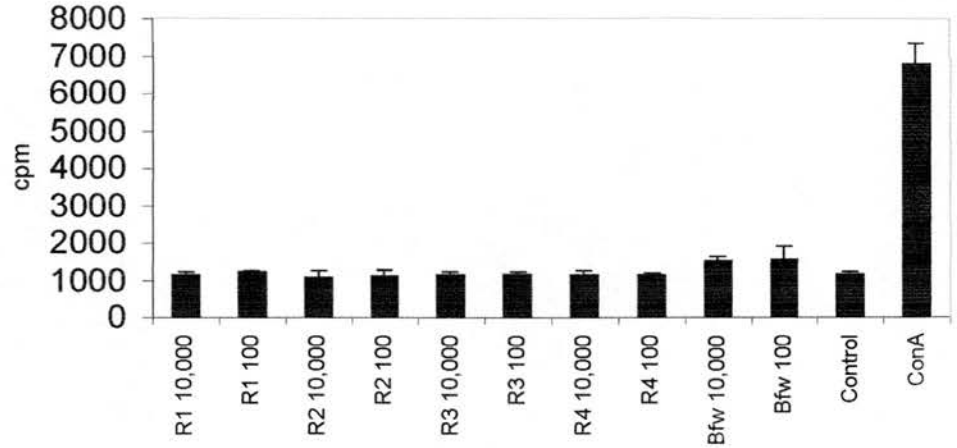


Figure 4.13 Proliferation of human PBMCs exposed to LPS/heat-killed bacteria at (a) 3 days and (b) 5 days

Human PBMC extracted from buffy coats were plated at 2 x 10^5 cells per well and challenged with various concentrations of *E.coli* K12 LPS or heat-killed *E.coli* K12 bacteria (1e7/ml = 1 x 10^7 cells per ml) or medium alone (Control) or 1μg/ml Concanavalin A (ConA). Cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at (a) 3 days or (b) 5 days. cpm = mean counts per minute of three independent cultures +/- SEM.

a)



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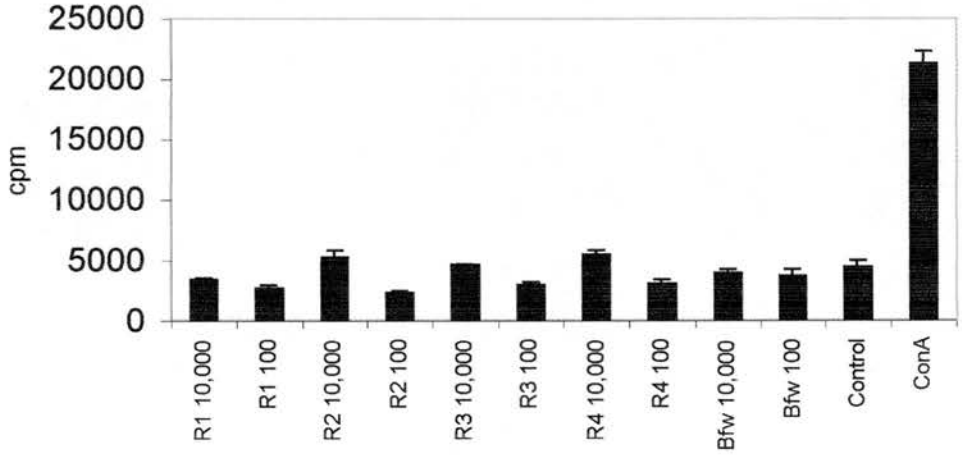
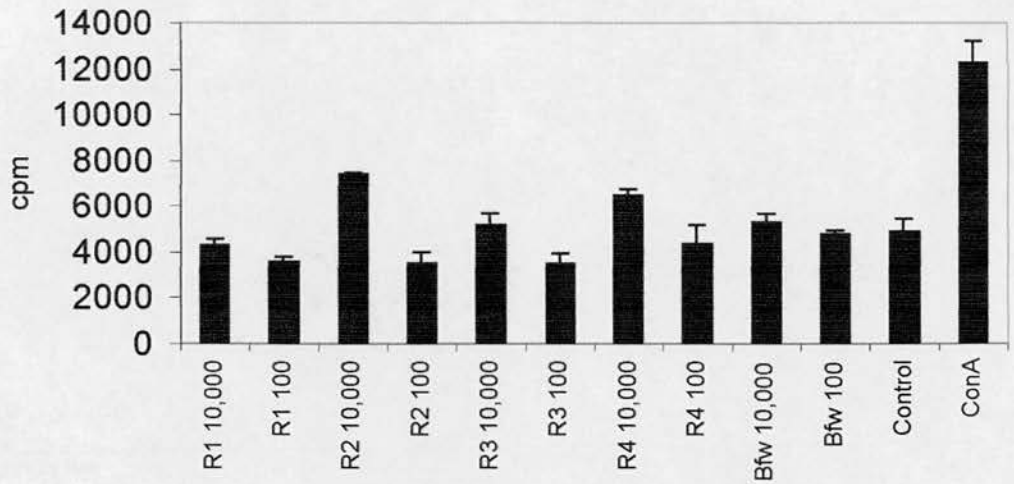


Figure 4.14 Response of human PBMC to various LPS types at (a) 3 days and (b) 4 days incubation

Human PBMC extracted from buffy coats were plated at 2 x 10^5 cells per well and challenged with either 100ng/ml (100) or 10,000ng/ml (10,000) of LPS or medium alone (Control) or 1μg/ml Concanavalin A (ConA). R1 = *E.coli* R1, R2 = *E.coli* R2, R3 = *E.coli* R3, R4 = *E.coli* R4, Bf = *B.fragilis*. All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 3 days. cpm = mean counts per minute of three independent cultures +/- SEM.

a)



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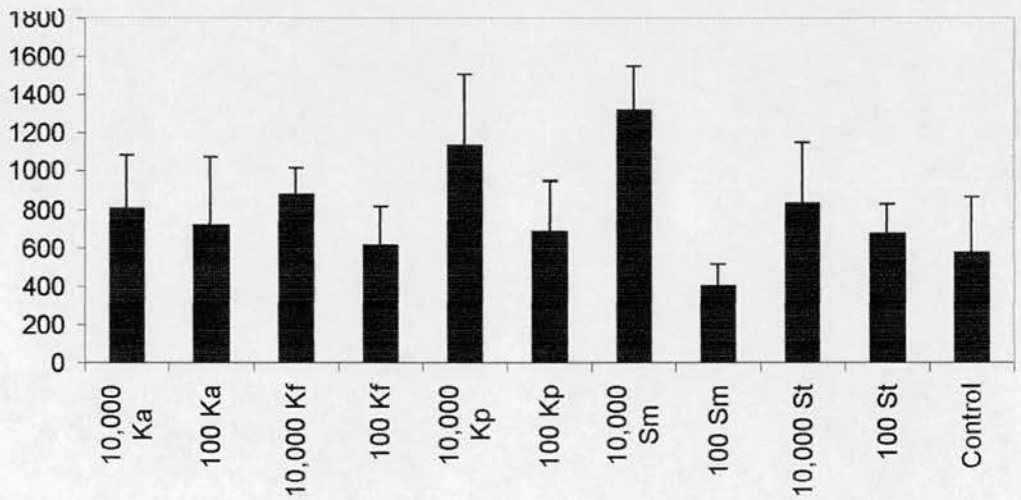


Figure 4.15 Response of human PBMC to various LPS types at 6 days incubation
Human PBMC extracted from buffy coats were plated at 2 x 10^5 cells per well and challenged with either 100ng/ml (100) or 10,000ng/ml (10,000) of LPS or medium alone (Control) or 1μg/ml Concanavalin A (ConA). R1 = *E.coli* R1, R2 = *E.coli* R2, R3 = *E.coli* R3, R4 = *E.coli* R4, Bf = *B.fragilis*, Ka = *Klebsiella aerugines*, Kf = *Klebsiella friedlander*, Kp = *Klebsiella pneumoniae*, Sm = *Salmonella minnesota*, St = *Salmonella typhimurium* LPS. All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 6 days. cpm = mean counts per minute of three independent cultures +/- SEM.

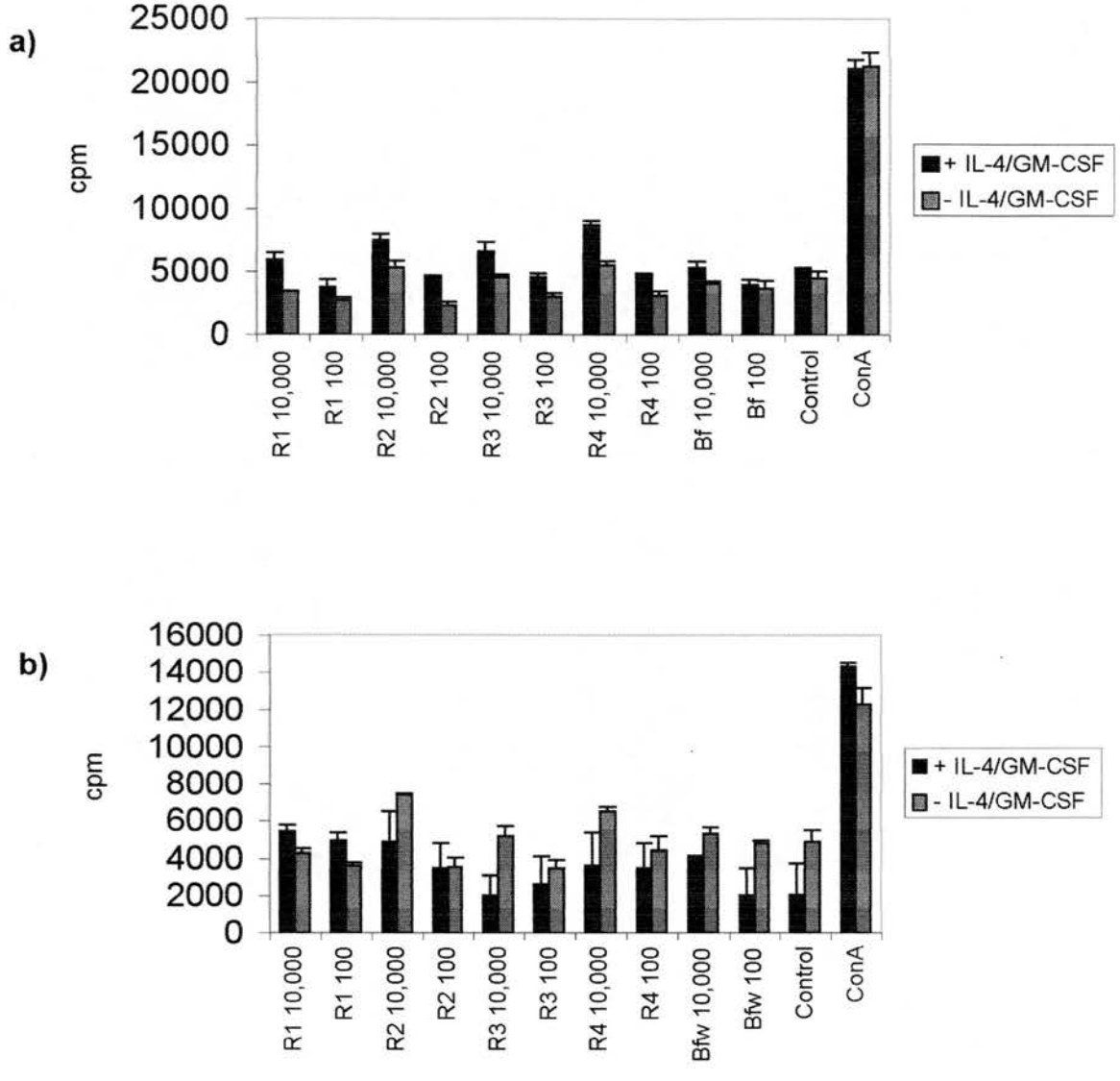


Figure 4.16 Effect of IL-4/GM-CSF treatment on PBMC proliferation in response to LPS at (a) 4 days and (b) 6 days

Human PBMC extracted from buffy coats were plated at 2 x 10^5 cells per well and challenged with either 100ng/ml (100) or 10,000ng/ml (10,000) of LPS or medium alone (Control) or 1μg/ml Concanavalin A (ConA). Culture medium contained either 5%FCS or 5%FCS + 200 IU/ml IL-4 and GM-CSF. R1 = *E.coli* R1, R2 = *E.coli* R2, R3 = *E.coli* R3, R4 = *E.coli* R4, Bf = *B.fragilis* LPS. Cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at (a) 4 days or (b) 6 days. cpm = mean counts per minute of three independent cultures +/- SEM.

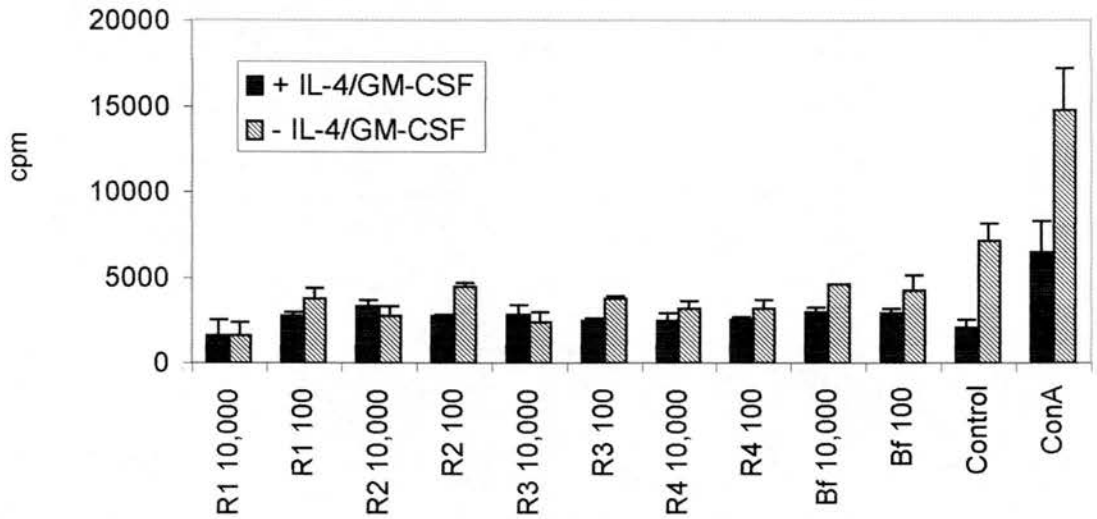


Figure 4.17 Effect of IL-4/GM-CSF pre-treatment on PBMC proliferation in response to LPS
Human PBMC extracted from buffy coats were plated at 2 x 10^5 cells per well and challenged with either 100ng/ml (100) or 10,000ng/ml (10,000) of LPS or medium alone (Control) or 1μg/ml Concanavalin A (ConA). Culture medium contained either 5%FCS or 5% FCS for the first 3 days then 5%FCS + 200 IU/ml IL-4 and GM-CSF for the following 3 days. R1 = *E.coli* R1, R2 = *E.coli* R2, R3 = *E.coli* R3, R4 = *E.coli* R4, Bf = *B.fragilis* LPS. All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 6 days. cpm = mean counts per minute of three independent cultures +/- SEM.

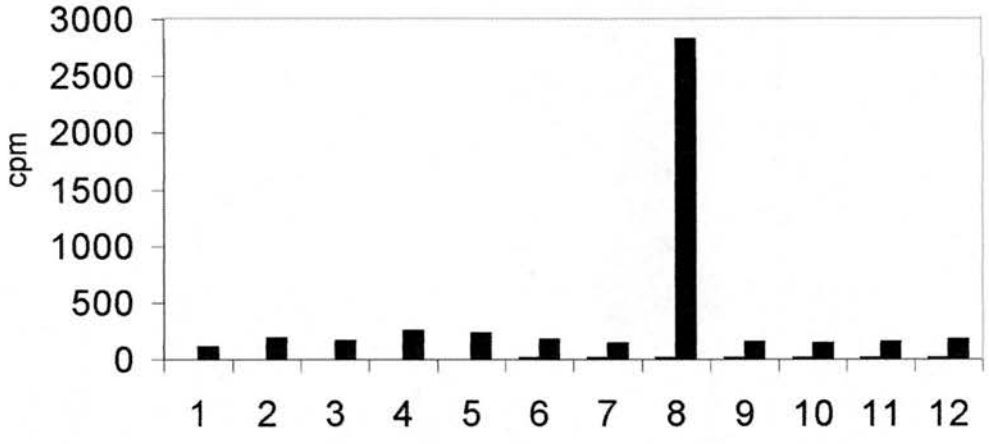


Figure 4.18 Inherent variability in proliferation of unstimulated PBMC
Human PBMC extracted from buffy coats were plated at 2 x 10^5 cells per well in medium alone (RPMI/5%FCS). Twelve identical cultures were were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 6 days. cpm = mean counts per minute of three independent cultures +/- SEM.

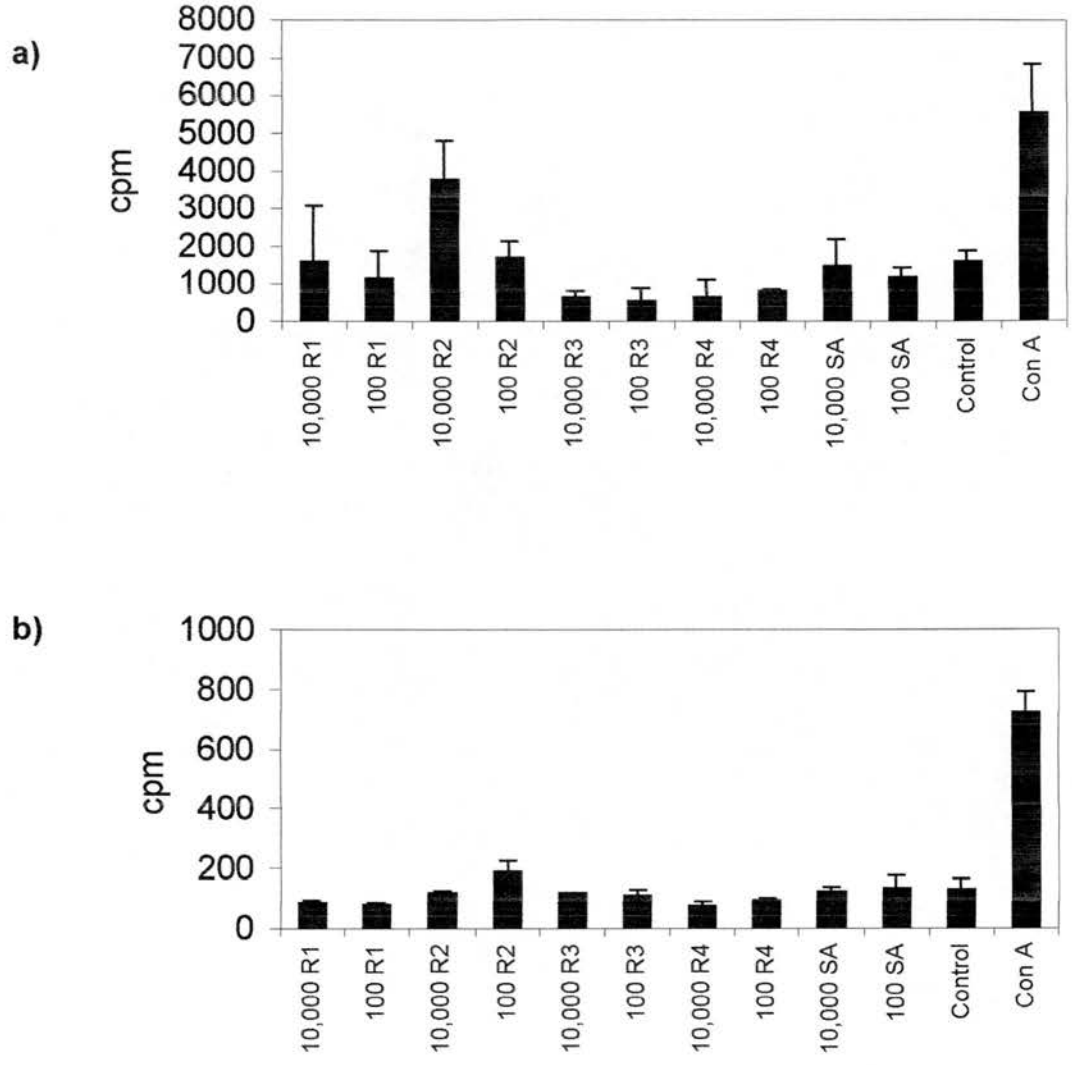


Figure 4.19 Proliferation of PBMCs exposed to LPS or LTA in (a) 5% foetal calf serum or (b) serum free medium

Human PBMC extracted from buffy coats were plated at 2×10^5 cells per well and challenged with either 100ng/ml (100) or 10,000ng/ml (10,000) of LPS or medium alone (Control) or 1μg/ml Concanavalin A (ConA). Culture medium contained (a) 5%fetal calf serum or (b) no serum. R1 = *E.coli* R1, R2 = *E.coli* R2, R3 = *E.coli* R3, R4 = *E.coli* R4, SA = *S.aureus* LTA. All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 6 days. cpm = mean counts per minute of three independent cultures +/- SEM.

counts, they did not reveal any proliferative response to LPS significantly higher than control cultures (data not shown).

To rule out the possibility that there was something wrong with the buffy coats themselves, PBMCs were extracted from fresh venous blood obtained from healthy human volunteers. Figure 4.20 show the results of the first attempt of this experiment. Low background counts are observed and a strong proliferative response is seen towards *E.coli* R1, *E.coli* R2, *E.coli* R3, *E.coli* R4, *Klebsiella aerogenes* and *K.friedlander* LPS. The highest stimulation index of 35.6 is seen in response to LPS of *K. aerogenes*.

Having demonstrated proliferation of human PBMC in response to LPS, a number of experiments were performed in order to determine the optimum experimental conditions required for the detection of LPS-reactive PBMC.

A time course experiment using timepoints of 2, 3, 4, 5, 6, 7, 8, 11, 14 and 17 days was performed to investigate the kinetics of proliferation of PBMC in response to ConA, *S.aureus* lipoteichoic acid and *E.coli* R1 LPS. Figure 4.21a+b show that unstimulated PBMC do not proliferate greatly throughout the period of incubation. Figure 4.21a shows that responses to ConA peak quickly to reach a plateau of strong proliferation between days 3 and 5, before returning quickly to background levels at around day 6. Responses to both LPS and LTA show minimal proliferation before six days, then peak sharply at around day 7 to day 8 before slowly returning to background proliferation after a much longer period than that seen for ConA-treated cultures.

An experiment was then performed to determine the optimum concentration of cells required to detect an LPS specific response. Figure 4.22 shows the response of fresh PBMC plated at 0.1, 0.5, 1, 3 and 5 x 10^5 cells per culture well. Optimum proliferation is clearly obtained when using a cellular

concentration of 3 x 10^5 cells per well, as this provides the highest stimulation index (~18). Cellular concentrations higher than this are seen to result in much higher background proliferation than that found when using lower concentrations.

Then to determine the optimum concentration of LPS to use in subsequent experiments, serial dilution experiments were performed using *E.coli* R1 LPS. Figure 4.23 shows that a relatively linear relationship exists between LPS concentration and proliferative response from human PBMC. The highest response is seen to occur when using the highest dose tested (10μg/ml LPS), with concentrations of LPS below 0.1ng/ml invoking little or no response.

Using the concentrations and times suggested by these experiments, PBMC derived from the blood of six further healthy volunteers were investigated to determine what proportion of people were able to respond to LPS in this way. The responses of donors 2 – 7 to LPS from *E.coli* R1, *E.coli* R2, *E.coli* R3, *E.coli* R4, *B.fragilis*, *S.minnesota*, *S.typhimurium* and LTA of *S.aureus* are shown in Figures 4.24 a – f. It can be seen on comparison of these charts that while the PBMC of some donors respond to most core types of LPS (eg. donor 3, Figure 4.24b), others respond to only a handful (eg. donor 5, Figure 4.24d) and one to none of the LPS tested at all (donor 7, Figure 4.24f). This donor is considered a non-responder because the counts from cultures incubated with LPS are not significantly higher than those of the control cultures, despite the fact that the actual counts for both cultures are quite high.

Then in order to determine which cellular subpopulation within the PBMC was proliferating in these experiments, PBMCs were taken from one volunteer (donor 4), exposed to *E.coli* R3 LPS for 7 days and flow cytometry performed on the markers CD3, CD14 and CD19 (markers for T-cells, monocytes and B-cells respectively). Figure 4.25 shows that a small expansion (~15%) of CD3 and

CD19+ve cells is seen following LPS stimulation, while CD14+ve cells are seen to be slightly reduced (~10%) in number after LPS stimulation.

In order to determine whether the proliferation to different types of LPS varied over time, the 7 volunteers were bled on either one or two further occasions and the PBMC exposed to the same panel of LPS. Figures 4.26 a–f show how the ability to respond to different types of LPS varies in each individual from day to day. It can be seen that while some donors exhibited a very similar profile of response on each occasion (eg. donor 2, Figure 4.26a), others varied considerably (eg. donor 5, fig 4.26d). The non-responder displayed a stimulation index less than 7 in response to LPS on each of the three occasions tested, once again as a result of a high background proliferation, and is therefore classed as a non-responder on each of these three days (Figure 4.26f).

As a more general measure of day to day LPS reactivity, an average score was calculated as the mean proliferative response to the entire panel of LPS on the day tested. This mean value was then plotted against the different days. Figure 4.27 shows that on different days, the overall capacity of some individuals to respond to LPS may rise or fall, with one (donor 1) showing a clear transition from responsiveness to non-responsiveness.

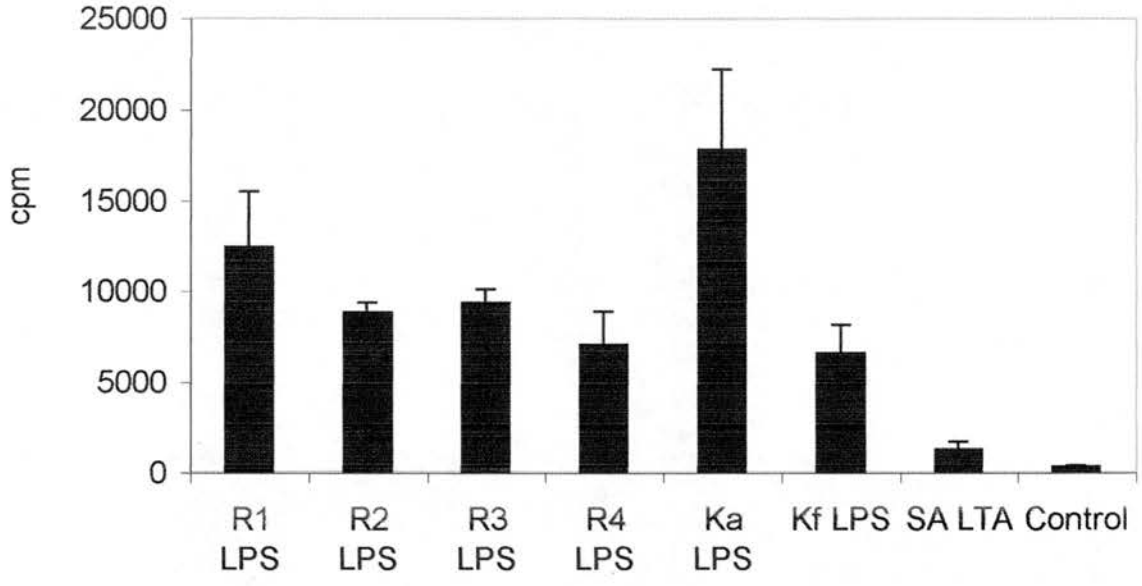


Figure 4.20 Fresh human PBMC proliferation in response to LPS supplemented with human serum
Human PBMC extracted from fresh venous blood (donor 1) were plated at 2 x 10^5 cells per well and challenged with 10μg/ml of LPS or medium alone (Control). Culture medium contained 10% autologous human serum. R1 = *E.coli* R1, R2 = *E.coli* R2, R3 = *E.coli* R3, R4 = *E.coli* R4, Ka = *Klebsiella aerogenes*, Kf = *Klebsiella friedlander*, SA = *S.aureus* LTA. All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 8 days. cpm = mean counts per minute of three independent cultures +/- SEM.

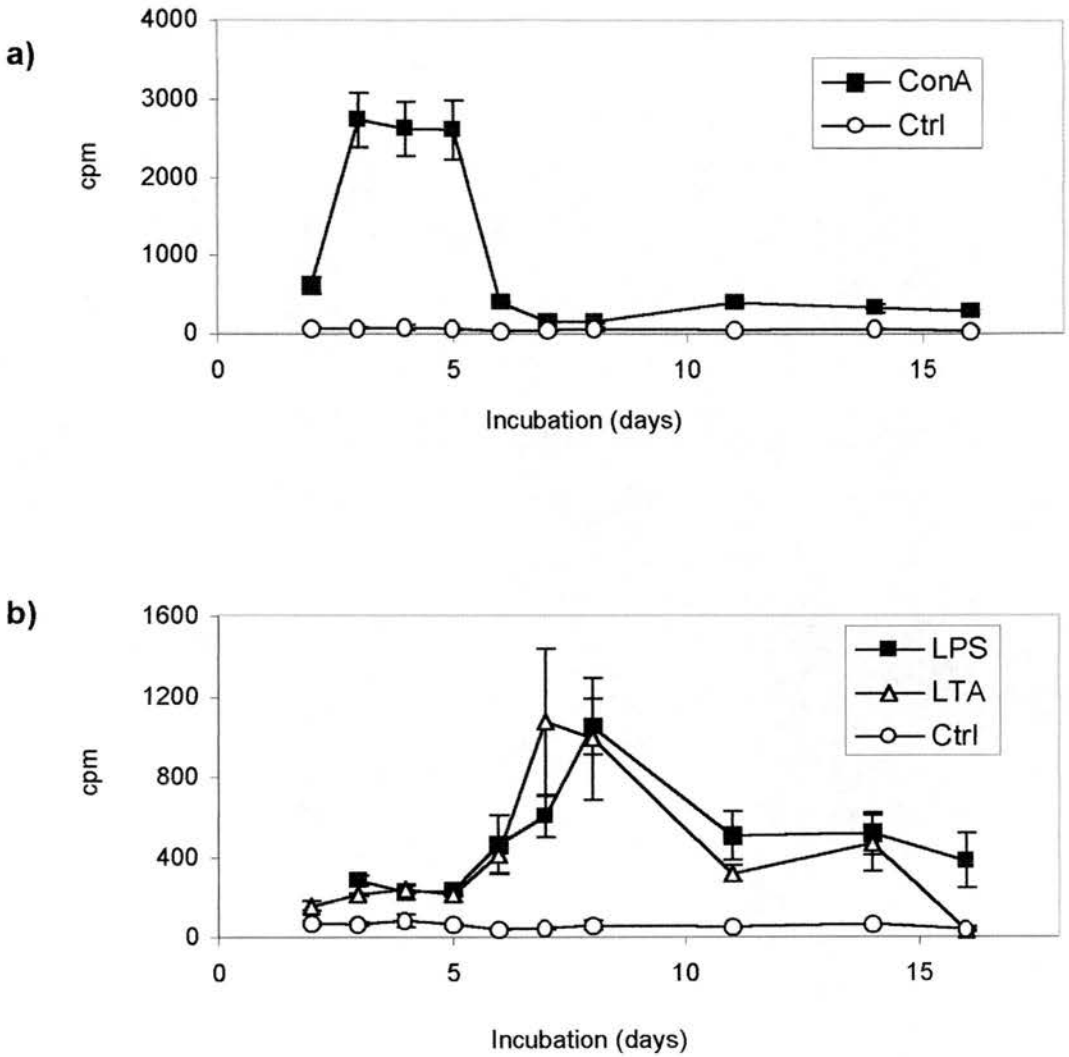


Figure 4.21 Kinetics of proliferation of human PBMC in response to (a) ConA or (b) LPS and LTA
Human PBMC extracted from fresh venous blood (donor 5) were plated at 2 x 10^5 cells per well and challenged with 1μg/ml of Concanavalin-A (ConA) or 10μg/ml *E.coli* R3 LPS or 10μg/ml *S.aureus* LTA or medium alone (Ctrl). All cultures were pulsed for 24hrs with 1μCi ^{3}H-thymidine and harvested at 2,3,4,5,6,7,8,11,14 or 17 days. cpm = mean counts per minute of three independent cultures +/- SEM.

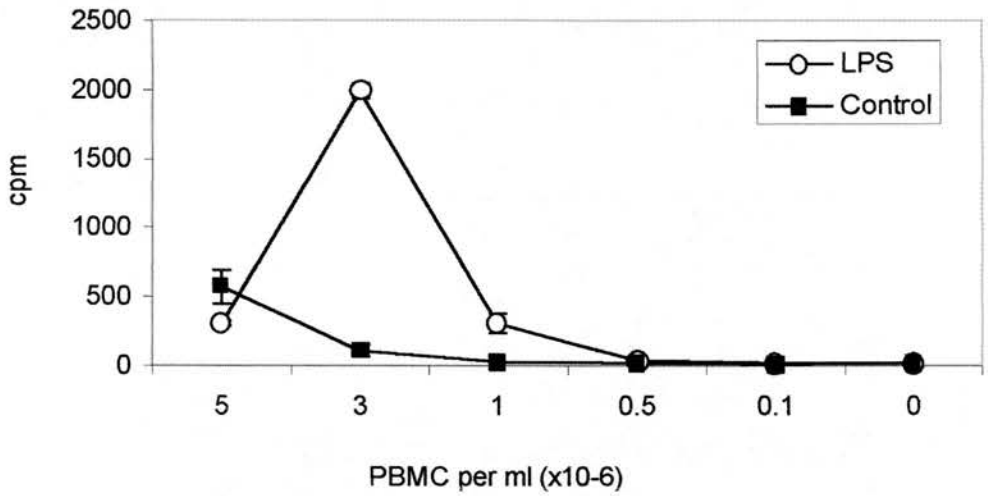


Figure 4.22 Effect of cell number on human PBMC proliferation in response to LPS
Human PBMC extracted from fresh venous blood (donor 5) were plated at 0, 0.1, 0..5, 1, 3 or 5 x 10^5 cells per well and challenged with 10μg/ml *E.coli* R3 LPS (LPS) or medium alone (Ctrl). All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 7 days. (a) cpm = mean counts per minute of three independent cultures +/- SEM. (b) Mean stimulation index of triplicate cultures +/- SEM.

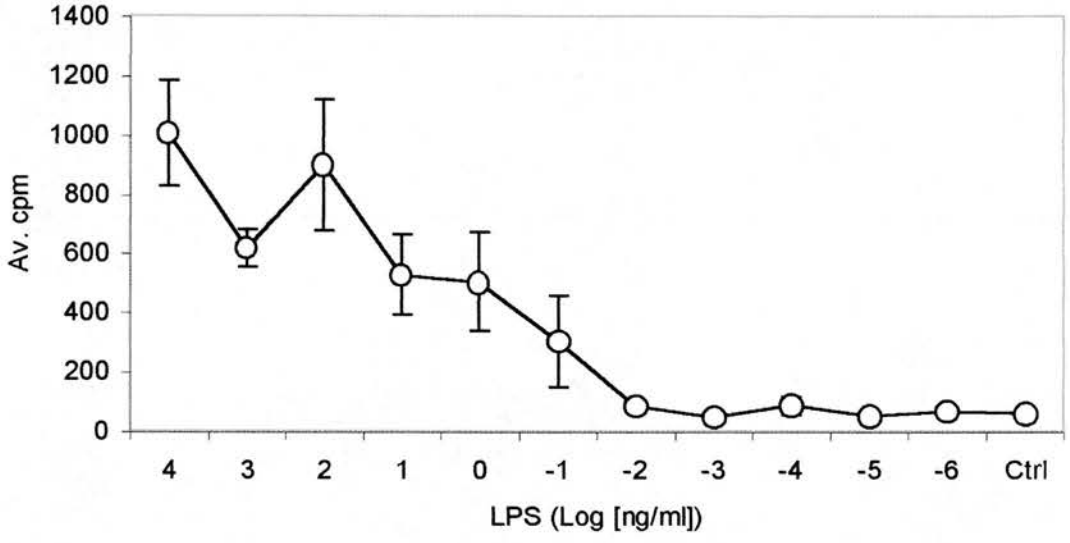
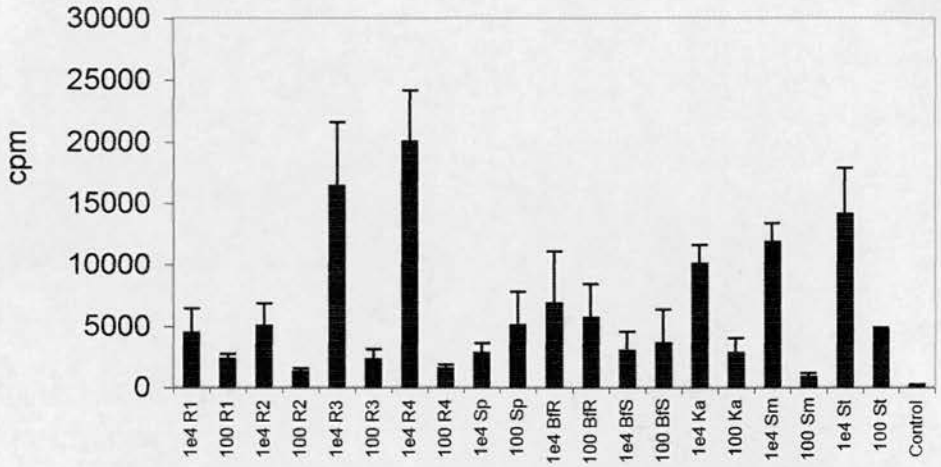


Figure 4.23 Dilution curve for R1 LPS

Human PBMC extracted from fresh venous blood (donor 5) were plated at 2 x 10^5 cells per well and challenged with 10^{-6} to 10^4 ng/ml *E.coli* R3 LPS or medium alone (Ctrl). All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 7 days. cpm = mean counts per minute of three independent cultures +/- SEM.

a)



b)

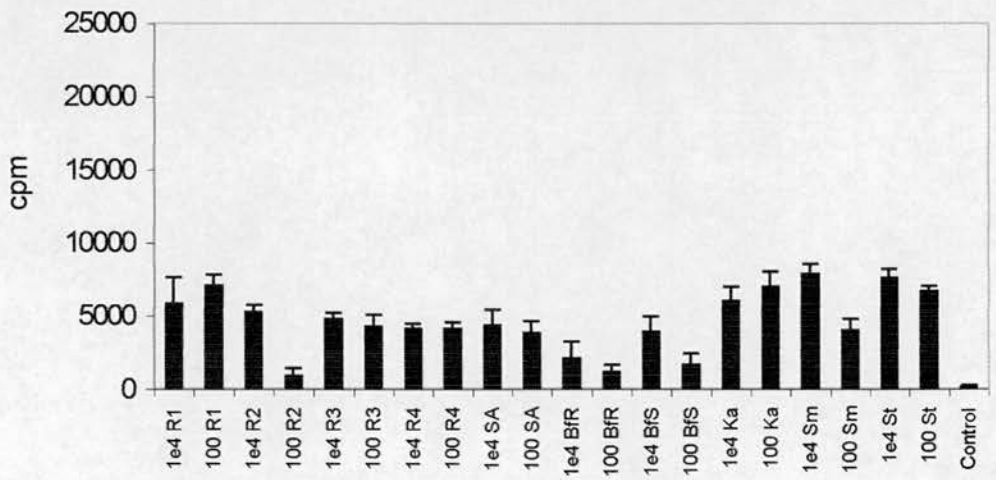
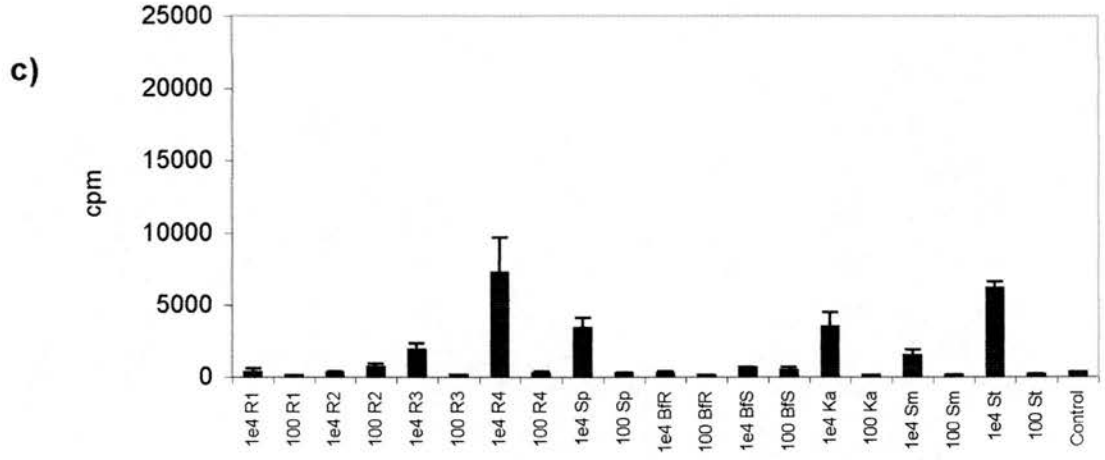


Figure 4.24 Profile of responses of PBMCs from six healthy donors to LPS and LTA
Human PBMC extracted from fresh venous blood were plated at 2 x 10^5 cells per well and challenged with 10μg/ml (1e4) or 100ng/ml (100) of LPS or medium alone (Control). R1 = *E.coli* R1, R2 = *E.coli* R2, R3 = *E.coli* R3, R4 = *E.coli* R4, BfR = *B.fragilis* rough LPS, BfS = *B.fragilis* smooth LPS, Sm = *S.minnesota*, St = *S.typhimurium*, SA = *S.aureus* LTA. All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 7 days. cpm = mean counts per minute of three independent cultures +/- SEM. Figures a – f represent to the responses of donors 2 – 7 respectively.



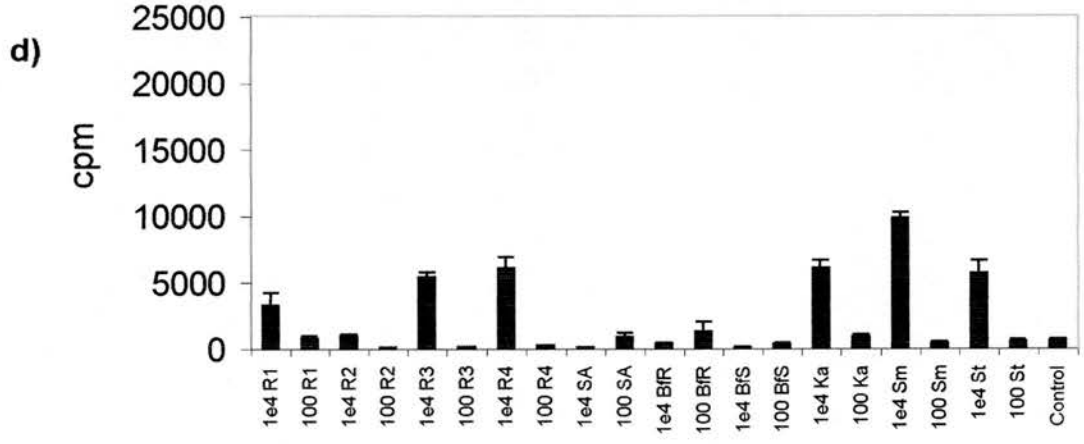
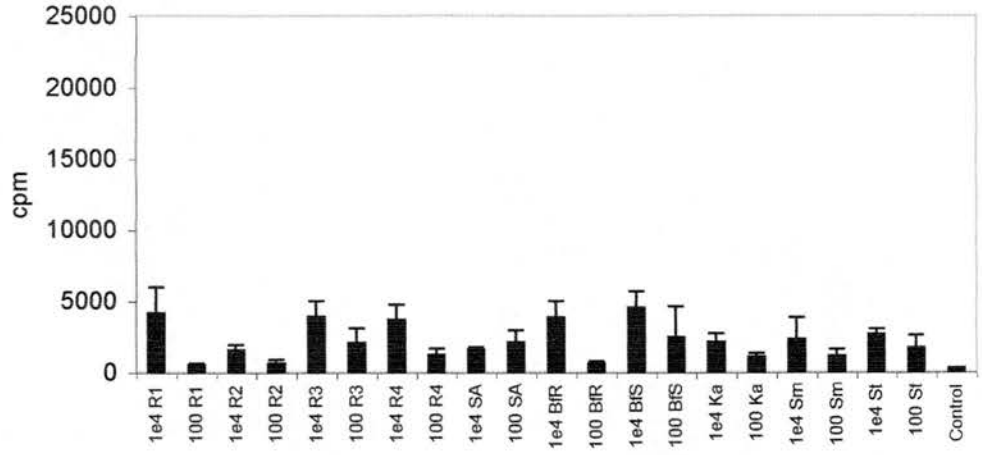


Figure 4.24 Profile of responses of PBMCs from six healthy donors to LPS and LTA
Donors 4 + 5.

e)



f)

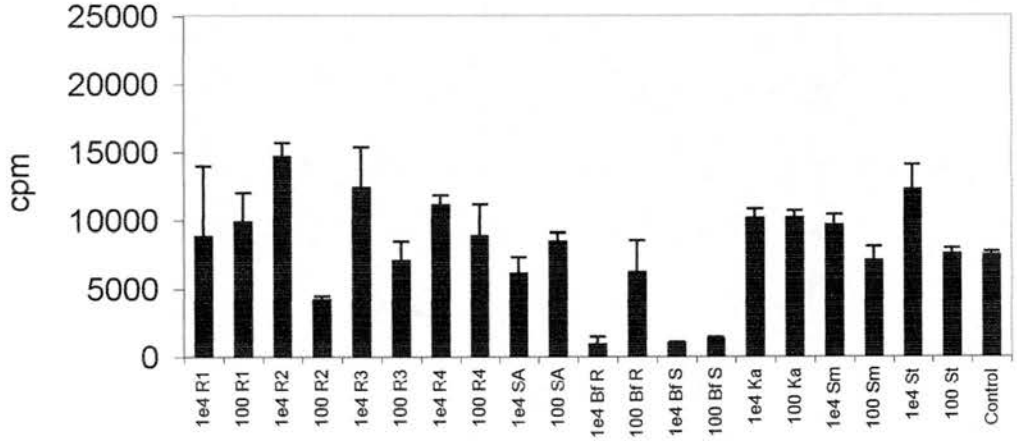


Figure 4.24 Profile of responses of PBMCs from six healthy donors to LPS and LTA
Donors 6 + 7.

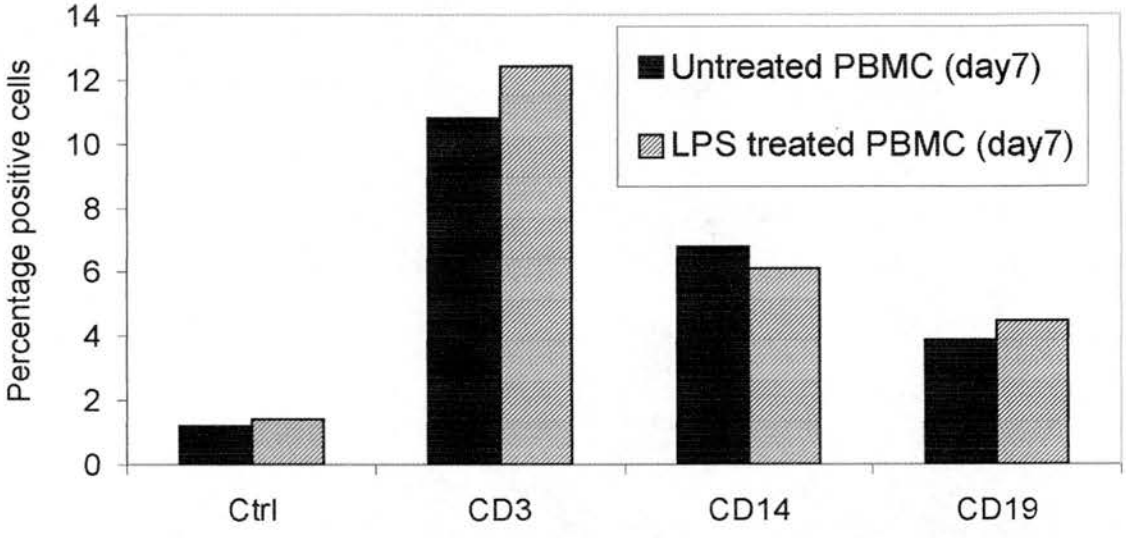
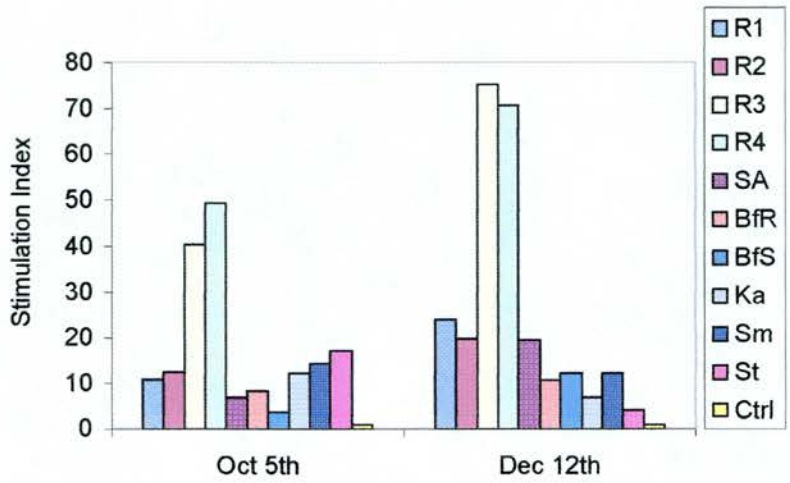


Figure 4.25 Profile of cell subpopulation expansions following LPS stimulation
PBMC extracted from fresh human venous blood (donor 4) were plated in either medium alone (RPMI/10%HS) or medium supplemented with 10μg/ml *E.coli* R3 LPS. Cells were stained for CD3, CD14 and CD19 expression after 7 days incubation and percentage of positively staining cells assessed by flow cytometry.

a)



b)

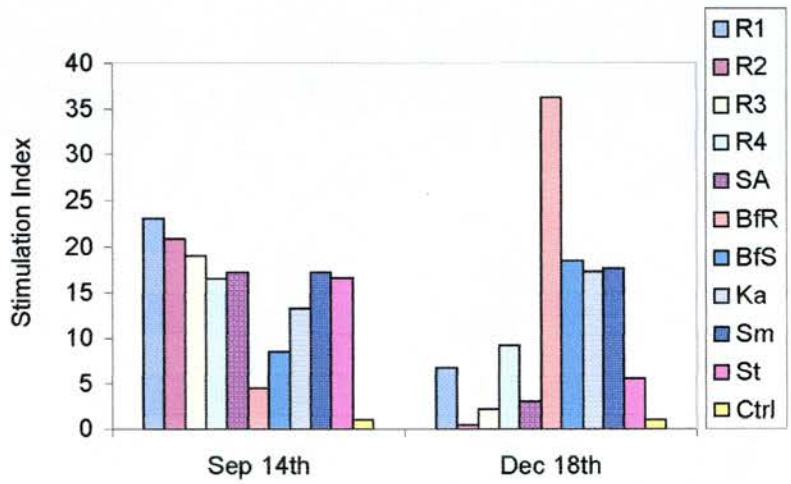
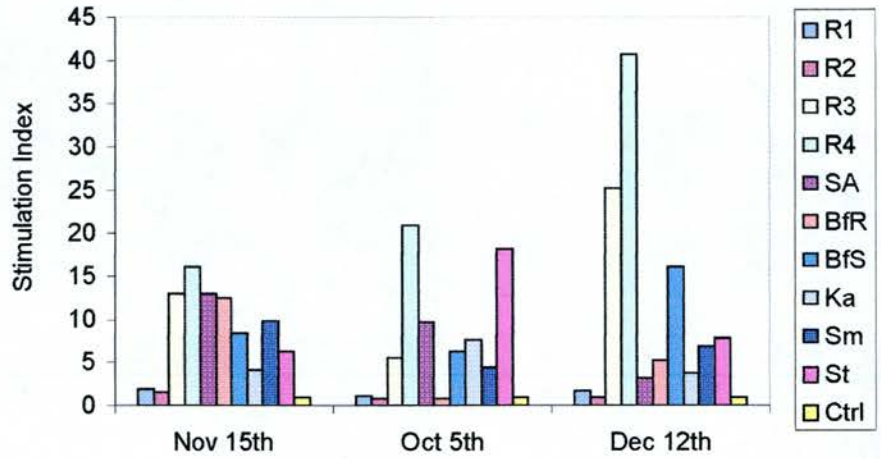


Figure 4.26 Variation of PBMC responses of donors to different LPS types on different days
Human PBMC extracted from fresh venous blood (a–f = donors 2–7 respectively) were plated at 2 x 10^5 cells per well and challenged with 10μg/ml of LPS or medium alone (Ctrl). R1 = *E.coli* R1, R2 = *E.coli* R2, R3 = *E.coli* R3, R4 = *E.coli* R4, SA = S.aureus LTA, BfR = *B.fragilis* rough LPS, BfS = *B.fragilis* smooth LPS, Ka = *K.aerogenes*, Sm = *S.minnesota*, St = *S.typhimurium*. All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 7 days. cpm = mean counts per minute of three independent cultures +/- SEM.

c)



d)

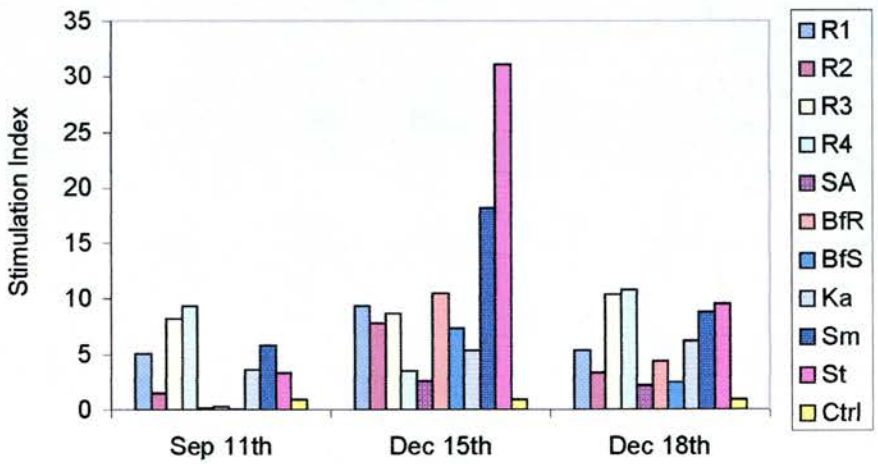
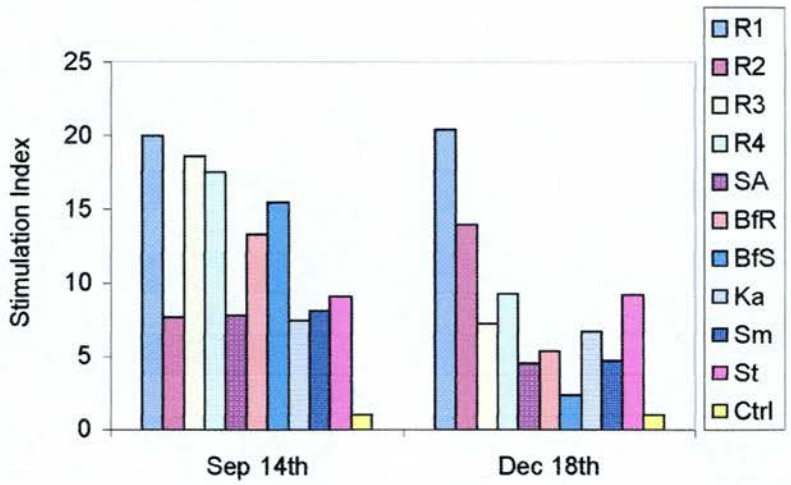


Figure 4.26 Variation of PBMC responses of donors to different LPS types on different days
Donors 4 + 5.

e)



f)

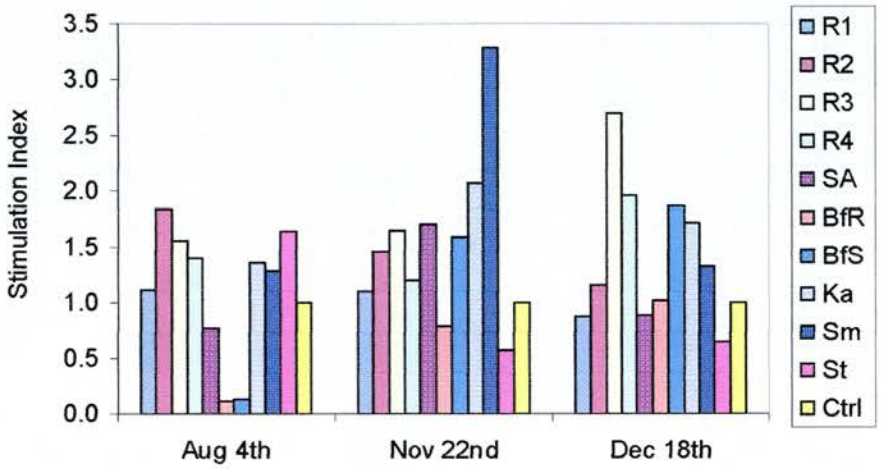


Figure 4.26 Variation of PBMC responses of donors to different LPS types on different days
Donors 6 + 7.

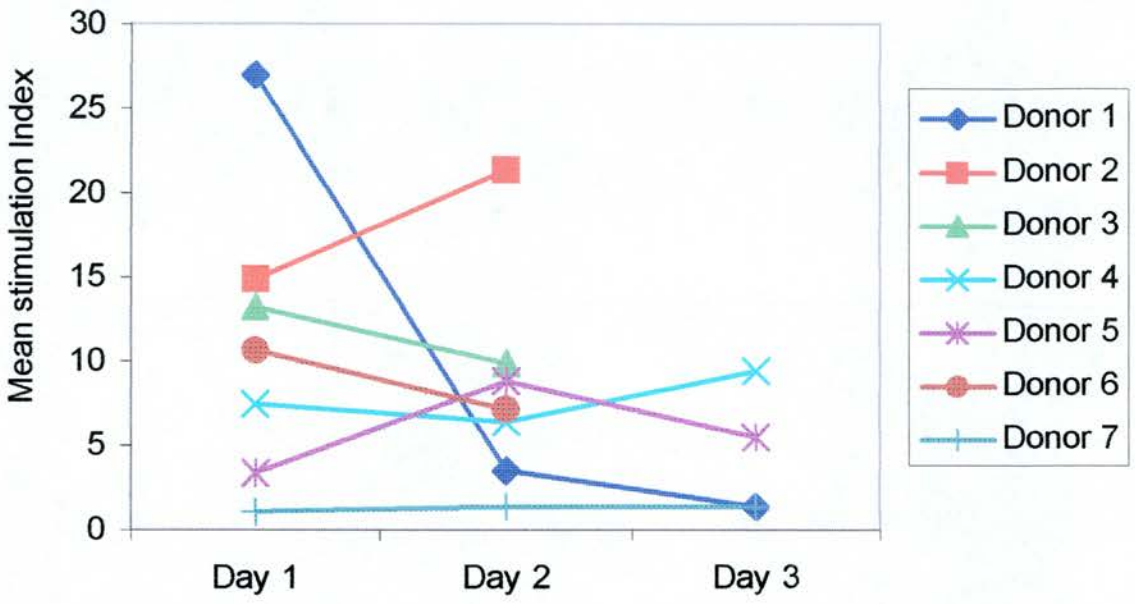


Figure 4.27 Variation of mean PBMC responses to all LPS on different days
Day to day variation in the mean responses of PBMC from 7 healthy donors to panel of LPS. Mean response was calculated as average stimulation index calculated for each individual LPS looked at. Panel included LPS of: *E.coli* R1, *E.coli* R2, *E.coli* R3, *E.coli* R4, *B.fragilis* rough strain, *B.fragilis* smooth strain, *K.aerogenes*, *S.minnesota*, *S.typhimurium*. All responses were measured at 7 days incubation.

4.2.4 Human T-cell responses to liposomal LPS

As an appropriate T-cell response is generally thought to be required to induce class switching for the formation of IgG antibodies, the capacity of the liposomal LPS vaccine described in section 3.2.3 to induce a T-cell response was investigated.

Liposomal cocktail LPS (methods, section 2.1.5) was added to human PBMC and the resulting proliferative response compared with that of native cocktail LPS. Figure 4.28 shows that PBMC proliferation is observed in response to liposomal LPS at all concentrations between 10ng/ml and the highest dose tested (10μg/ml). The proliferative response is seen to be roughly equivalent to that generated towards 0.01ng/ml native cocktail LPS.

4.2.5 Role of CD1 in T-cell responses to LPS

Experiments were performed to investigate the possibility that T-cell proliferation in response to LPS involves presentation of lipid via molecules of the CD1 family. Determination of the expression levels of CD1 a,b,c and d molecules revealed no significant expression of these molecules on freshly isolated untreated PBMC (Figure 4.29). Similarly, monocytes cultured in 10% serum for 7 days do not express any CD1 isotype (Figure 4.29). However, treatment of monocytes with 200IU/ml of each of the cytokines IL-4 and GM-CSF is clearly seen to induce strong expression of the group I CD1 molecules (CD1a,b and c) but not group 2 CD1 molecule CD1d (Figure 4.29). Treatment of monocytes with these cyokines is also seen to have little impact on expression of MHC-I and MHC-II while CD14 expression is significantly reduced (Figure 4.30).

It was then investigated whether expression of CD1 isotypes could be initiated on PBMC by exposure to LPS. Figure 4.31 shows that exposure of PBMC to *E.coli* R3 LPS for seven days results in no upregulation of CD1 expression.

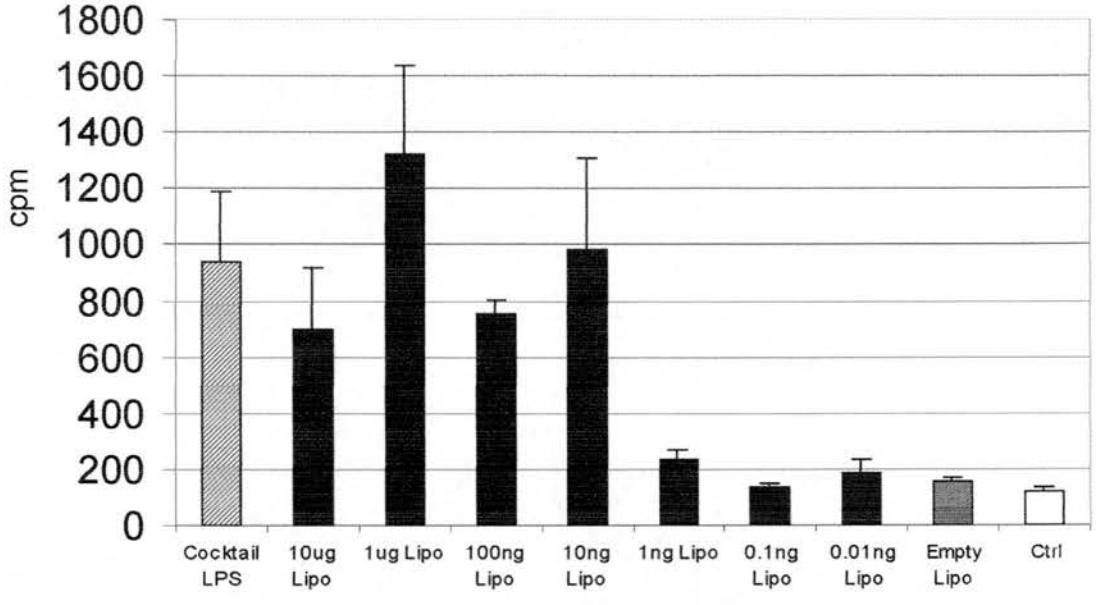


Figure 4.28 Proliferative response of human PBMC to liposomal LPS
Human PBMC extracted from fresh venous blood (donor 1) were plated at 2 x 10^5 cells per well and challenged with 10μg/ml of cocktail LPS or 10μg/ml to 0.01ng/ml cocktail LPS (lipo) containing lipsomes or 10μg/ml empty liposomes or medium alone (Ctrl). All cultures were pulsed for 18hrs with 1μCi ^{3}H-thymidine and harvested at 7 days. cpm = mean counts per minute of three independent cultures +/- SEM.

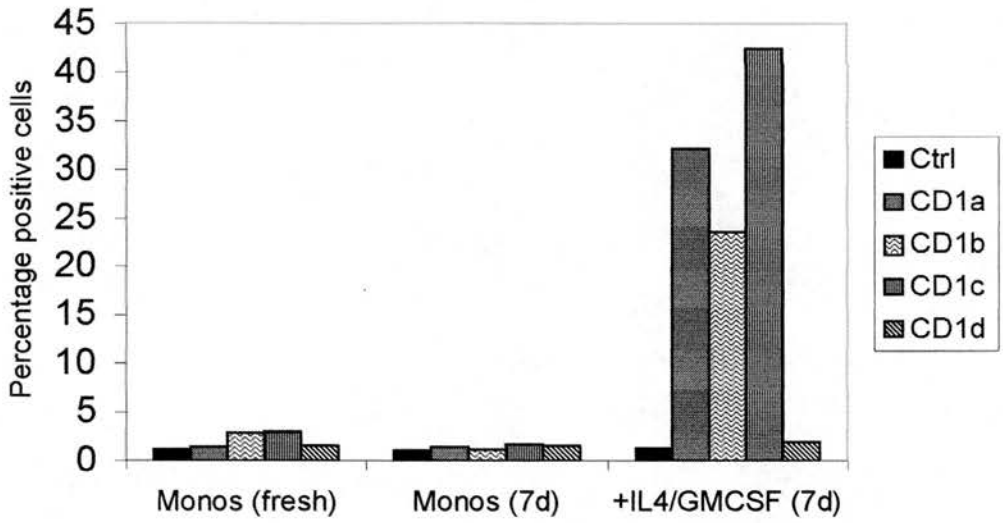


Figure 4.29 Expression of CD1a,b,c and d on fresh monocytes and monocytes cultured for 7 days with or without IL-4/GM-CSF

Monocytes extracted from fresh venous blood (donor 2) were plated in either medium alone (Ctrl) or medium supplemented with 200IU/ml IL-4 and GM-CSF. Cells were stained for CD1a,b,c or d expression immediately (Fresh monos) or after 7 days incubation and percentage of positively staining cells assessed by flow cytometry.

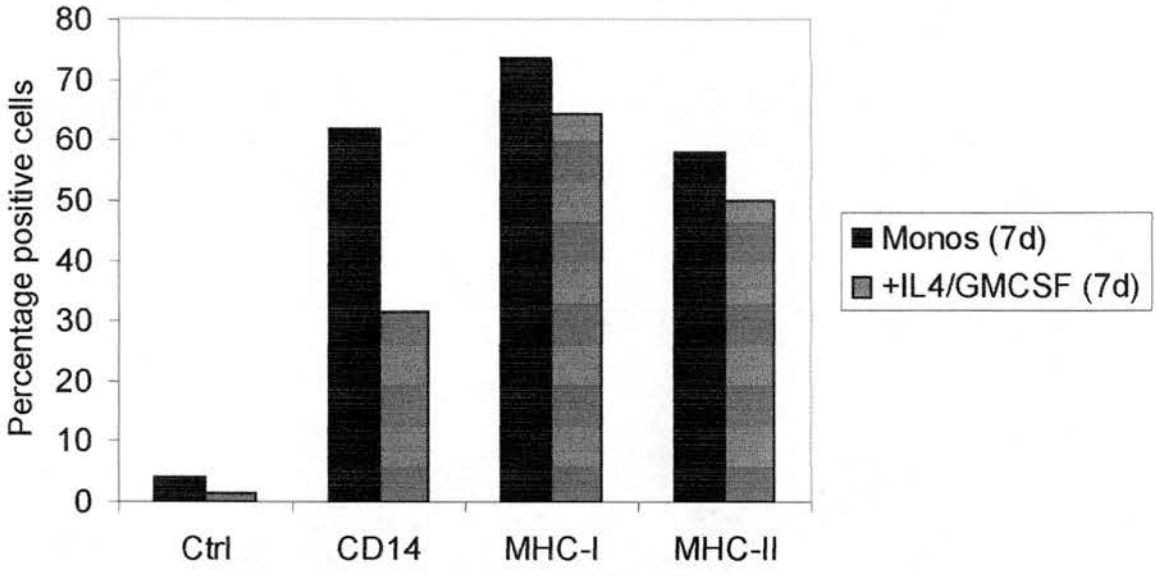


Figure 4.30 Expression of CD14, MHC-I and MHC-II on monocytes treated with IL-4/GM-CSF
Monocytes extracted from fresh venous blood (donor 5) were plated in either medium alone (Ctrl) or medium supplemented with 200IU/ml IL-4 and GM-CSF. Cells were stained for CD14, MHC-I and MHC-II expression after 7 days incubation and percentage of positively staining cells assessed by flow cytometry.

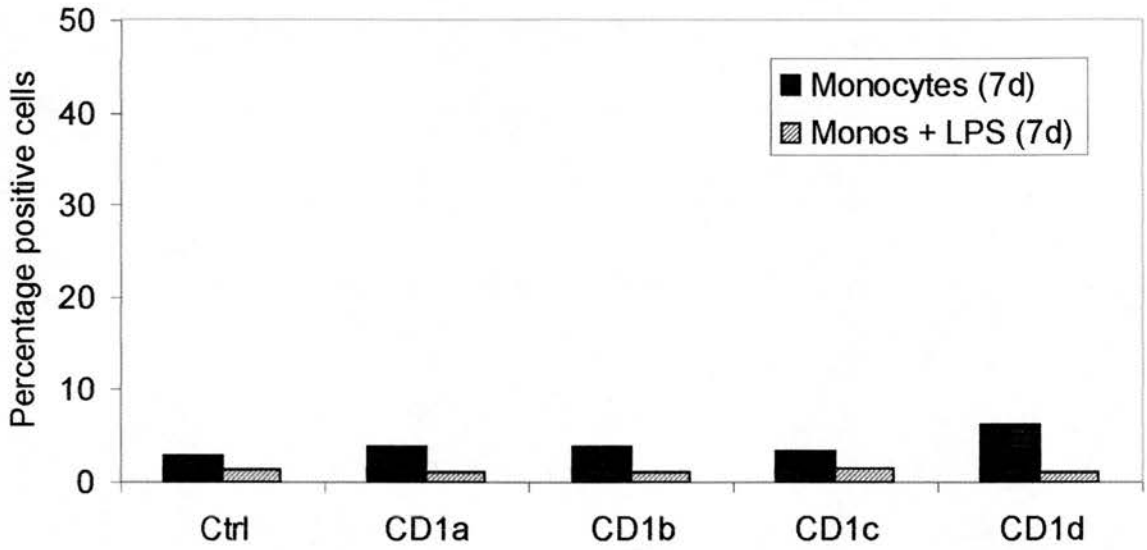


Figure 4.31 CD1 expression on human monocytes exposed to LPS for 7 days
Monocytes extracted from fresh venous blood (donor 1) were plated in either medium alone (Monocytes) or medium supplemented with 10μg/ml *E.coli* R3 LPS (Monocytes + LPS). Cells were stained for CD1a,b,c or d expression after 7 days incubation and percentage of positively staining cells compared with unstained cells (Ctrl) by flow cytometry.

Then to investigate whether antibodies against CD1 could block PBMC proliferation in response to LPS, fresh human PBMC were plated with LPS and antibodies specific for each of the CD1 isotypes. Data from several individuals were combined for each antibody to provide a mean stimulation index. Figure 4.32 shows that blocking with monoclonal antibodies specific for CD1a,b and c has no significant effect on proliferation, while the monoclonal antibody specific for CD1d appears to actually increase the proliferative response of the PBMC to LPS.

Experiments were then performed to determine whether or not LPS could be seen to bind to CD1. In the first series of experiments, expression of exposed presented LPS core structures on the surface of CD1-expressing cells was attempted to be visualised using a monoclonal antibody reactive to the core structures of many types of enterobacterial LPS: Wnt-1. Human monocytes were plated with or without IL-4/GM-CSF treatment to induce CD1a,b and c expression and with or without *E.coli* R1 LPS followed by staining with Wnt-1 at 7 days. Figure 4.33 shows that using this method, no LPS can be seen bound to the cell surface after seven days incubation. Treatment of cells with IL-4/GM-CSF appears capable of inducing some binding of the Wnt-1 antibody regardless of LPS treatment, as evidenced by the relatively high fluoresence displayed by these cultures.

As a result of this failure to detect LPS core structures on the surface of CD1+ve cells, autoradiography of immunoprecipitated CD1 was planned, such that radiolabeled LPS could be used to determine if CD1 could bind LPS.

IL-4/GM-CSF treated human monocytes were pulsed with LPS and the proteins extracted. Figure 4.34 shows that abundant proteins are present in these extracts. While distinct banding patterns are difficult to make out, it is clear that a

large amount of proteins are migrating in the region expected to contain all CD1 isotypes (41-55 kDa).

However, immunoblots of these gels using anti-CD1 monoclonal antibodies continually failed to reveal the presence of CD1 (data not shown). For this reason, dot blots were performed to determine whether the antibody could detect CD1 in the context of nitrocellulose.

Figure 4.35a shows that MHC-II molecules present in the extract from these cells are readily detected while the isotype matched negative control antibody (anti-CD3) shows that non-specific staining is not occurring. However, Figure 4.35b shows that the presence of CD1 isotypes cannot be detected using this protocol. As detection of CD1 isotypes using these antibodies failed to occur in several similar experiments, planned experiments using radiolabelled LPS were abandoned.

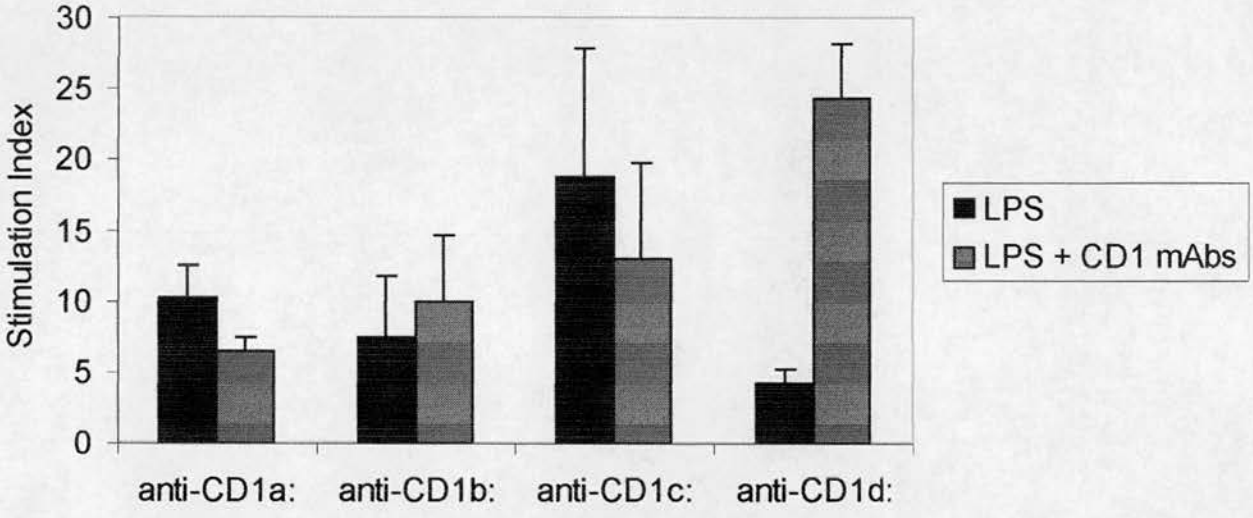


Figure 4.32 Effect of anti-CD1 mAbs on proliferation of PBMC exposed to *E.coli* R3 LPS
PBMC extracted from fresh venous blood of various donors were plated in either medium alone or medium supplemented with 10μg/ml *E.coli* R3 LPS or medium supplemented with 10μg/ml *E.coli* R3 LPS and 2.5μg/ml α-CD1a,b,d or 50μg/ml α-CD1c. Stimulation index was calculated as LPS treated cell proliferation divided by control cell proliferation. Mean stimulation indices for several individuals were combined for each antibody. α-CD1a represents mean stimulation indices from 1 donor; α-CD1b = 3 donors; α -CD1c = 4 donors; α -CD1d=1 donor.

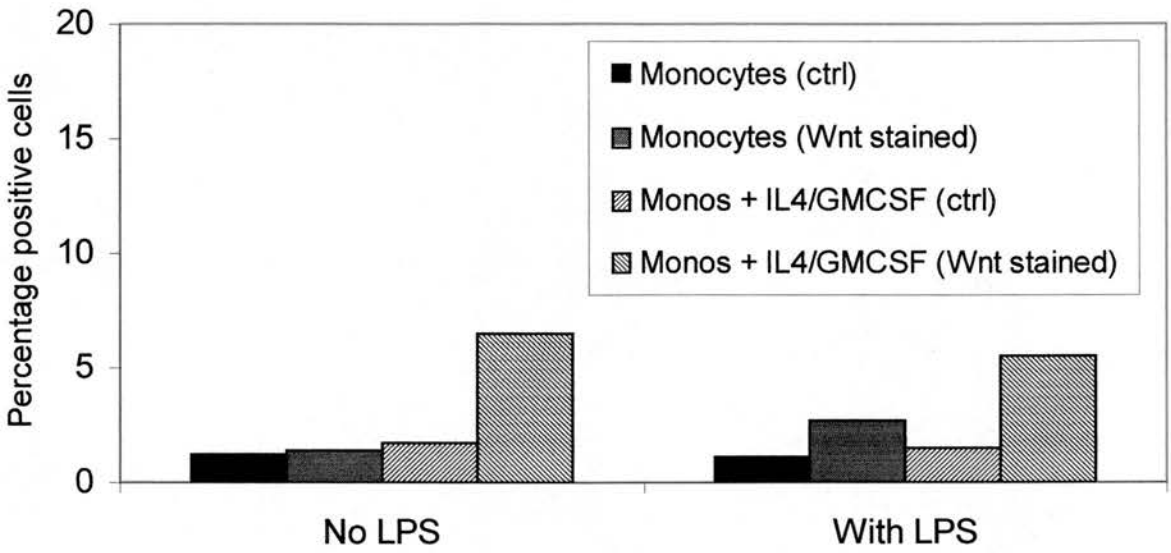


Figure 4.33 Expression of LPS core structures on cell surface of PBMC exposed to LPS and IL-4/GM-CSF

PBMC extracted from fresh human venous blood (donor 3) were plated in either medium alone (RPMI/10%HS) or medium supplemented with 200 IU/ml IL-4 and GM-CSF. Both cultures were supplemented with 10μg/ml *E.coli* R1 LPS. Cells were stained with either no primary antibody or the LPS core reactive monoclonal antibody Wnt-1 after 7 days incubation. Percentage of cells staining positively for LPS expression was assessed by flow cytometry.

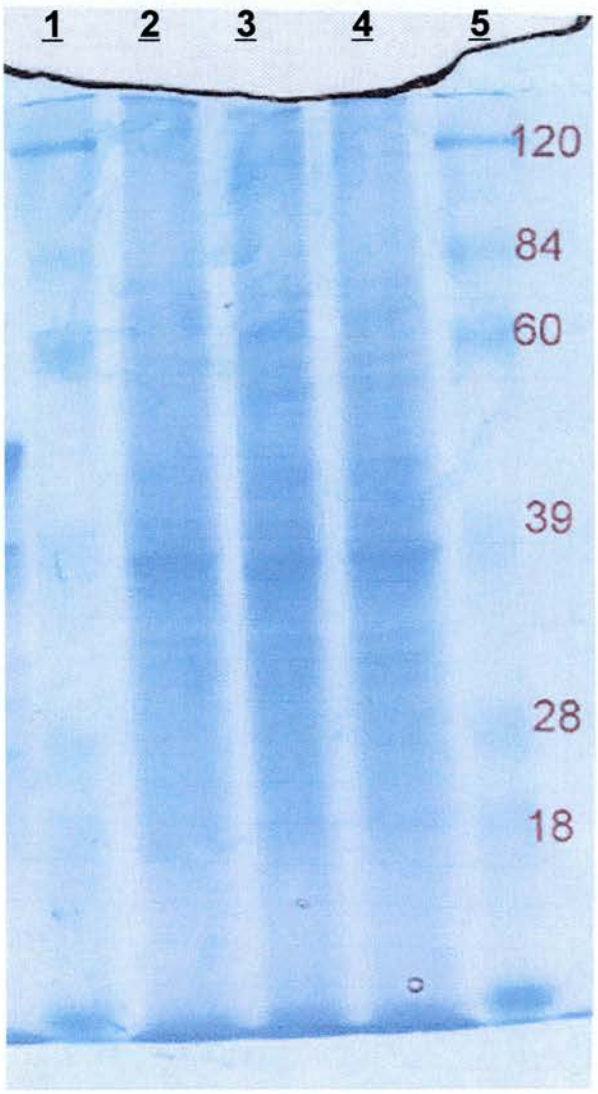
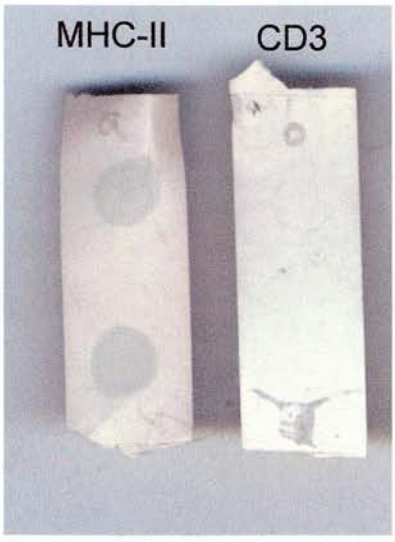


Figure 4.34 Coomassie blue stained gel of protein extract from IL-4/GM-CSF treated PBMC
Proteins were extracted from fresh human PBMC treated with 200IU/ml IL-4/GM-CSF for seven days, separated by PAGE electrophoresis and stained using Coomassie blue. Lanes 1+5, molecular weight markers (120kDa, 84kDa, 60kDa, 39kDa, 28kDa, 18kDa). Lanes 2-4, protein extracts from three separate experiments. CD1a,b,c or d if present should migrate at 41-55 kDa.

a)



b)

Figure 4.35 Dot blot of (a) MHC-II and CD3 and (b) CD1a,b,c,d proteins extracted from IL-4/GM-CSF treated monocytes

Proteins were extracted from fresh human monocytes treated with 200IU/ml IL-4/GM-CSF for seven days. Extracts were blotted in duplicate onto nitrocellulose and stained with (a) α-MHC-II or isotype matched control antibody (α-CD3) or (b) monoclonal antibodies specific for CD1a,b,c or d.

4.3 Discussion

4.3.1 Why look at T-cell responses to LPS?

While the response towards LPS of other immune cells such as neutrophils, monocytes and B-cells have been well characterised (reviewed in Martich et 1993, Rietschel et al 1994, Peavy et al 1970 respectively), much less is known of the role of T-cells in immune responses to LPS. For some time it was considered that human T-cells were incapable of proliferating in response to LPS (Peavy et al 1970, Greaves et al 1974, Ellner et al 1989, Fauci et al 1976), though this has now been shown not to be true (Mattern et al 1994). Since it is understood that T-cells have a strong role to play in both the orchestration and class switching of antibodies in any immune response, an understanding of the T-cell response to LPS was desired in order to further our understanding of how antibody responses to LPS are made. Information derived from such studies could provide insights that may be of use in the design of lipopolysaccharide-based vaccines.

4.3.2 Murine splenocyte responses to LPS

Several studies have demonstrated the ability of murine splenocytes to proliferate in response to LPS (Peavy et al 1970, Andersson et al 1977, Morrison et al 1981, Glode et al 1976). This has typically been considered to be mediated primarily by B-cells, with one estimate suggesting that 1 in 3 splenic B-cells are capable of proliferating in response to LPS (Andersson et al 1977). However, evidence also exists for the presence of LPS reactive T-cells in mice. In particular, murine T-cell proliferative responses have been reported to occur in response to both *in vitro* (Vogel et al 1983, Miller et al 1983) and *in vivo* (Tough et al 1997) LPS challenge. Of particular interest to the following study, however, is the observation by Vogel *et al* that a small subpopulation of T-cells purified from murine splenocytes (approximately 3%) were capable of

proliferating in response to LPS challenge. For this reason, early experiments investigated the response of murine splenocytes to LPS challenge.

Challenge of murine splenocytes with LPS resulted in dose dependent proliferation (Figure 4.1). Viable T-cells were clearly present within the splenocyte population as evidenced by the strong proliferative response to the T-cell mitogen ConA (Figure 4.1). The kinetics of this proliferation indicate strong responses at 4 days, with proliferation diminishing daily to background levels by day 7. Such kinetics are reminiscent of the proliferative responses of lymphocytes to other polyclonal mitogens (Peavy et al 1970, Greaves et al 1974, Fauci et al 1976).

Then in order to determine whether there was any effect of LPS core type on the strength of the proliferative response, *E.coli* R1, R2, R3 and R4 and *B.fragilis* LPSs were added to splenocytes at equal concentrations and the proliferation measured at three days. Figure 4.3 shows that the strongest responses are developed against *E.coli* R3 and R4 LPS, with responses to *E.coli* R1 and R2 LPS slightly reduced and responses to *B.fragilis* LPS much lower. The latter finding is consistent with the previously reported observation of the lower capacity of *B.fragilis* LPS to stimulate activation of monocytes (Delahooke et al 1996, Lindberg 1990, Erridge et al 2002).

In order to determine which cell type within the splenocyte population was proliferating in response to these antigens, purified cellular sub-populations were assessed for capacity to proliferate in response to LPS. Figure 4.4 shows that CD19+ve cells (B-cells) proliferate strongly in response to LPS, while the CD3+ve cells (T-cells) proliferate slightly and the CD1d+ve fraction do not proliferate at all. CD1d was chosen as one of the marker molecules as it has been implicated in the response of other immune cells to lipid antigens (Beckman et al 1994, Schofield et al 1999, Park et al 2000). However, the

proliferation seen in the CD3+ve cell fraction in this experiment most likely represents B-cell contamination of this fraction, as it was seen in a second experiment that depletion of CD19+ve cells from splenocytes was seen to result in complete abrogation of proliferation in response to LPS (Figure 4.5).

These experiments suggest that the murine splenocyte reactivity towards LPS is mediated entirely by CD19+ve B-cells. The splenic T-cell preparations reported by Vogel *et al* (1983) to be capable of responding to LPS may well have also been contaminated with B-cells. Their study employed an antibody affinity purification approach similar to the one used in this study. Further, they noted that FACS analysis of their preparations indicated a 98% purity of their T-cell preparations. No characterisation of the remaining 2% of cells was reported. In the light of the current study, it is likely that this impurity contained a contaminating B-cell population which imparted the proliferative response on preparations. For these reasons, subsequent attempts to identify the presence of LPS reactive T-cells in mice focused instead on cells extracted from murine lymph nodes.

4.3.3 Murine lymph node responses to LPS

In several experiments, cells from the lymph nodes of naïve mice did not show any proliferative response to either *E.coli* K12 LPS or the T-cell mitogen ConA (Figure 4.6a). For this reason, the cells from lymph nodes of pre-immunised mice were used in subsequent experiments.

Mice were initially immunised with either *E.coli* K12 LPS, heat killed *E.coli* O18K$^-$ bacteria or sheep red blood cells complexed with Freunds complete adjuvant. Lymph node cells were removed 8 days post immunisation and exposed to *E.coli* K12 LPS, heat-killed *E.coli* O18K$^-$ bacteria or sheep red blood cells *in vitro*. Figure 4.7 reveals that no proliferation occurs in response to these antigens at two, four or six days incubation *in vitro*.

It was thought that the 8 day delay between immunisation and harvesting of cells may not have been long enough to generate a significant T-cell response to these antigens, so lymph nodes from either naïve mice, mice immunised 10 days pre-harvesting or mice immunised 14 days before harvesting with heat-killed *E.coli* O18K$^-$ bacteria were assessed. Figure 4.8 shows that incubation of the cells with heat-killed *E.coli* O18K$^-$ bacteria resulted in no appreciable response to this antigen, though a strong response to ConA was seen at all time points, suggesting that the presence of viable T-cells is not lacking in these experiments. The response to ConA was seen to be strongest in day 14 animals, less in day 10 animals and least strong in naïve animals. This suggests that the immunisation protocol has increased either the number or the responsiveness of the T-cells in the lymph nodes of these animals.

As viable T-cells were not seen to be lacking in these assays, it was considered that perhaps the number of antigen presenting cells was limiting, so subsequent experiments attempted to supplement cultures with autologous splenocytes. In order to ensure that proliferation of the splenocytes did not occur and mask any T-cell response, these cells were transiently treated with the cell division inhibitor mitomycin C. Figure 4.9 shows that treatment of murine splenocytes with mitomycin C is capable of significantly blunting their capacity to proliferate in response to *E.coli* R4 LPS or ConA. Further, co-culture of untreated splenocytes with mitomycin-C treated cells is not seen to result in inhibition of the responses of untreated cells to LPS (Figure 4.9).

Figure 4.10 shows that addition of antibody purified naïve T-cells to mitomycin C treated splenocytes does not enhance the proliferative response to either *E.coli* R4 LPS or ConA. As the yield of T-cells from the magnetic bead purification system was relatively low, passage of murine splenocytes over nylon wool was

employed in the next series of experiments to provide larger numbers of purified T-cells.

Figure 4.11 shows that nylon wool-selected autologous T-cells do not proliferate in response to either LPS or ConA. Supplementation with mitomycin C-treated splenocytes does not rescue this phenotype. Further, addition of T-cells to mitomycin C-treated splenocytes is seen to completely remove the residual proliferative capacity of the mitomycin C-treated splenocytes. This is most likely explained by the fact that the concentration of mitomycin C-treated splenocytes in these wells are at half the concentration of unsupplemented wells, and may be below a critical cellular concentration required for measurable activity in this assay.

The same experiment was repeated using longer treatment of the splenocytes with mitomycin C. In this experiment, the T-cell fraction is seen to retain residual proliferative capacity in response to LPS, a finding which may reflect the possibility of B-cell contamination of these relatively crude preparations (Figure 4.12a). Mitomycin C-treated splenocytes do not respond to either stimulus, but are seen to remove the capacity of the T-cells to display their residual proliferation in response to LPS, suggesting that despite thorough washing, these cells retain some toxicity post treatment in this experiment.

In an attempt to reduce the potential B-cell contamination of the T-cell preparations, freshly purchased nylon wool was used in the next series of experiments. Figure 4.12b shows that the activity of the T-cell fraction in response to LPS is reduced, but since the response to ConA is also severely blunted, the viability of these cells is questionable. Supplementation with mitomycin C-treated antigen presenting cells does not rescue their ability to proliferate.

As consistent results from these experiments could not be obtained, the focus of the search for LPS reactive T-cells shifted towards the proliferative response of human peripheral blood mononuclear cells (PBMC). However, the success of previous workers in demonstrating the presence of LPS reactive T-cells in mice may perhaps be explained by the following observations. Positive responses of splenic T-cells to LPS (Vogel et al 1983, Miller et al 1983) may have occurred as a result of contamination of these preparations with responding B-cells (as described above). The demonstration of a murine T-cell line (CT6) capable of responding to LPS challenge *in vitro* reports that the cell line did not have this property when first isolated. This property was spontaneously acquired after repeated passages approximately two years post-cloning, and therefore most likely represents a long term tissue culture adaptation resulting in expression of TLR4. The report of *in vivo* LPS mediated T-cell proliferation most likely occurs as a result of indirect activation. Antigen presenting cells secrete a wide range of cytokines on exposure to LPS, several of which have been shown to induce proliferation of memory T-cells. In particular, IFN-α and β stimulate T-cell proliferation (Tough et al 1997), as do the cytokines IL-1β (Vogel et al 1983) and IL-15 (Grabstein et al 1994). Finally, since the *in vivo* proliferation of T-cells is absent in mice of the C3H/HeJ strain, and since functional TLR-4 is not required for presentation of antigens to T-cells, it is most likely that the response is occuring as a result of TLR4 mediated recognition of LPS, and not from antigen presentation of LPS to T-cells.

4.3.4 Human buffy coat PBMC responses to LPS

In order to attempt a large number of experiments, a readily available source of human PBMC was required. As buffy coats derived from the blood transfusion service had reliably provided viable monocytes for investigations of innate immunity in earlier experiments, these were chosen as the source of human cells in the first series of experiments looking for LPS-reactive human T-cells.

Figure 4.13 shows that while viable T-cells are clearly present in these preparations (there is strong proliferation in response to ConA), there is no proliferation in response to either LPS or heat-killed *E.coli* at either 3 days or 5 days incubation. PBMC from several different donors revealed the same lack of reactivity.

It was then considered that since *E.coli* K12 is a laboratory strain not widely encountered in the natural setting, perhaps lack of prior exposure to this antigen may be responsible for the lack of response seen in these individuals. For this reason, the next series of experiments investigated responses to LPS of *E.coli* R1, *E.coli* R2, *E.coli* R3, *E.coli* R4 and *B.fragilis* – all of which may be commonly found in the gut or are associated with infection (Gransden et al 1990, Patrick 1993). Figures 4.14 + 4.15 show the profile of response to these antigens, displayed by one such donor and which is broadly representative of all similar experiments. Proliferation in response to these antigens was seen to be no different from that of control cultures incubated for 3, 4 or 6 days. Challenge with the LPS of *Klebsiella aerogenes*, *K.friedlander*, *K.pneumoniae*, *Salmonella minnesota* or *S.typhimurium* in many similar experiments also revealed no significant proliferative response.

As the molecule CD1 has been shown capable of mediating T-cell recognition of other lipids (discussed later), it was considered that perhaps the presence of CD1+ve cells was required for T-cell proliferation in response to LPS. This was achieved in the next series of experiments by supplementing the culture medium with 200IU/ml of the cytokines IL-4 and GM-CSF, which together induce the expression of CD1a,b and c on human monocytes (Beckman et al 1994 and Figure 4.29).

Treatment of whole PBMC in this fashion did not result in the proliferation of cells in response to LPS at 4 days (Figure 4.16a) or at 6 days (Figure 4.16b).

Preincubating cells with the two cytokines for 3 days before LPS challenge also resulted in no proliferation (Figure 4.17), despite clear evidence of high levels of expression of the type I CD1 molecules (*ie* CD1a,b and c) on similarly treated cells (Figure 4.29).

Then, as concurrent experiments had revealed that use of foetal calf serum was capable of stimulating production of cytokines from unchallenged monocytes, foetal calf serum from three different manufacturers was tested (Gibco, Sigma and Imperial laboratories). None of these could remove the high (>1,000cpm) background proliferation observed in all experiments (Figure 4.19).

Suspecting that trace amounts of endotoxin may be present in the foetal calf serum that was activating both a cytokine and a proliferative response, further experiments were performed using buffy coat PBMC supplemented with pooled human serum. These experiments demonstrated much lower background counts, but did not reveal any responses to LPS higher than unstimulated cells.

4.3.5 Comparison with earlier studies

At this stage, a reference was found in the literature that demonstrated human T-cell proliferation in response to LPS. In contrast to previously held dogma, Mattern and colleagues had shown that a small proportion of human T-cells are capable of responding to LPS (Mattern et al 1994). Their explanation for the reason that this had not been seen before (Peavy et al 1970, Greaves et al 1974, Ellner et al 1989, Fauci et al 1976) was because other groups had measured proliferation at the more usual timepoint of 3-4 days, whereas the proliferation they measured was reached after seven days incubation.

This response seemed to be mediated by antigen presentation, as T-cell proliferation was absolutely dependent on contact with accessory monocytes

and the frequency of responding T-cells of between 1:400 and 1:1100 was in the range of antigen (not mitogen) specific T-cells.

Only 50% of the healthy volunteers they tested, however, responded with an appreciable proliferation in response to LPS – a finding they later related to the presence or absence of CD34+ blood stem cells (Mattern et al 1999).

As the same experimental conditions used by Mattern to demonstrate LPS reactive T-cells (seven days incubation and use of human serum) had already been tried several times in the present study, it was concluded that some property of the buffy coats may be hindering response to LPS. With hindsight, it is possible that the CD34+ blood stem cells that are required for this proliferation are absent from or unviable in buffy coat preparations, either as a result of the purification method of the cells at source, or the storage system employed following purification.

For this reason, subsequent experiments investigated the responses of PBMC freshly isolated from human venous blood.

4.3.6 Human PBMC responses to LPS

Figure 4.20 shows that freshly isolated PBMC are capable of mounting a strong proliferative response towards the LPS of *E.coli* R1, *E.coli* R2, *E.coli* R3, *E.coli* R4, *Klebsiella aerogenes* and *K.friedlander*. This figure also shows that a slight proliferative response can also be made by human PBMC to the lipoteichoic acid of the Gram-positive organism *S.aureus*, a finding which has not been documented before.

Figure 4.21b shows that human PBMC proliferation in response to LPS is minimal before six days incubation, reaching a maximum proliferative response between days 7 and 8, then tailing off with residual activity remaining after

almost two weeks incubation. The kinetics of this response are therefore starkly different to the response made by PBMC to the mitogen ConA, which demonstrates maximum proliferation by day 3, but tails off quickly to background levels by days 6-7 (Figure 4.21a). This provides further evidence for the fact that LPS acting as an antigen, not as a mitogen in these cells.

T-cell responses to LPS occurred in a dose dependent manner, with all concentrations above 0.01ng/ml inducing proliferation (Figure 4.23). This is of a similar order to the concentration of LPS required to initiate a TNF-α response from human monocytes (Table 3.4), and therefore does not preclude the possibility that both cell types may be employing the same type of receptor for recognition of LPS.

However, if this was the case and the monocyte receptor for LPS (TLR4) was responsible for the proliferation observed in the PBMC, it follows that the responses to LPS of different core types should all be largely similar, as only the presence of lipid A is recognised by TLR4 (Heine et al 1999, Medzhitov et al 1997). By contrast, Figures 4.24 a–f show that PBMC responses to LPS from *E.coli* R1, *E.coli* R2, *E.coli* R3, *E.coli* R4, *B.fragilis*, *S.minnesota*, *S.typhimurium* and LTA of *S.aureus* vary considerably between the six healthy volunteers tested. For example, while the PBMC of some donors respond to most core types of LPS (e.g. donor 3, Figure 4.24b), others respond to only a handful (e.g. donor 5, Figure 4.24d). These results suggest that an antigen specific reaction is occuring that is not dependent on the lipid A component of the LPS.

In order to verify Matterns finding that the cells proliferating in these assays are T-cells (Mattern et al 1994), PBMCs were taken from one volunteer (donor 4), exposed to *E.coli* R3 LPS for 7 days and flow cytometry performed on the markers CD3, CD14 and CD19 (markers for T-cells, monocytes and B-cells respectively). Figure 4.25 shows that a small expansion (~15%) of the CD3 and

CD19+ve cells is seen following LPS stimulation, while CD14+ve cells are seen to be slightly reduced in number after LPS stimulation. However, as the staining of the control culture is also increased following LPS incubation, the significance of this assay might be questioned. Repeat of Matterns transwell experiments involving plating of antibody purified monocytes and T-cells would be the best way to verify the fact that T-cells are responsible for the proliferation observed in PBMC exposed to LPS.

It was then determined whether the proliferative responses to different LPS core types varied from day to day. The same 7 healthy volunteers were assayed on either one or two further occasions. Figures 4.26 a-f show that while some donors exhibit a very similar profile from day to day (eg. donor 2, Figure 4.26a), others varied somewhat (eg. donor 5, fig 4.26d). The non-responder did not respond on any of the three days, once again as a result of a high background proliferation (Figure 4.26f). One possible explanation for the observation that responses to particular core types may rise and fall with time is that individuals may be constantly exposed to these antigens. Recent exposure to particular core types, either as a result of asymptomatic infection or translocation of specific LPS/bacteria from the gut may result in amplification of memory T-cells specific for these epitopes. Some dynamic interplay may also exist between the expansion of these cells and the establishment of gut-mediated tolerance which may ultimately result in down-regulation of responses to certain epitopes. Further work is required to establish the clinical relevance of T-cell reactivities towards individual core types.

Next, as immunological status has been shown to have a strong impact on the outcome of pre-surgical patients (Christou et al 1984, Christou et al 1989, Johnson et al 1979, Adami et al 1980, Christou et al 1983), an average of all of the stimulation indexes for each LPS tested was calculated to provide a more general measure of the day to day variation of overall LPS responsiveness

among the volunteers. Figure 4.27 shows that the overall responsiveness of individuals may either rise or fall with time, in some cases quite dramatically. Donor 1, for example, makes a clear transition from responder status to non-responder status. This observation prompted the hypothesis that should LPS-reactive T-cells turn out to play a protective role in either Gram-negative infection or endotoxaemia, then individuals may enter phases of LPS hyporeactivity during which they are at greater risk of complications from these pathologies. Should the mechanism be found for this particular immunosuppression, then therapeutic interventions may be devised to help reduce the likelihood of such episodes. This hypothesis is discussed in detail in the following chapter.

4.3.7 Human T-cell responses to liposomal LPS

As there is a role for T-cells in the regulation of antibody formation (Stavnezer 1996, Fujieda et al 1998), the capacity of the vaccine to induce a T-cell response was assessed. Figure 4.28 shows that the vaccine is capable of inducing T-cell proliferation at LPS concentrations as low as 10ng/ml. This concentration is approximately 100 fold lower than that seen capable of initiating a cytokine response in human monocytes (Table 3.3). This finding provides further evidence that T-cell recognition of LPS is not dependent on the same receptor utilised by monocytes to detect LPS – TLR4. Instead, presentation of internalised liposomal LPS by antigen presenting cells to T-cells is the more likely explanation for this enhanced sensitivity to liposomal LPS.

4.3.8 Role of CD1 in T-cell responses to LPS

In order to further investigate the possibilty that LPS or processed fragments of LPS were being presented to T-cells, the role of CD1 was investigated. The CD1 family of molecules share striking sequence and structural homology to the MHC class of molecules (Park et al 2000) and have been found to be present in every mammalian species so far examined (Calabi et al 1989). Humans, for example,

are seen to possess genes for five CD1 molecules (CD1a,b,c,d and e which is not expressed) and mice only CD1d (Cattoretti et al 1989, Balk et al 1991).

CD1 molecules are now regarded as having a key role in generating T-cell immunity against mycobacterial lipid antigens (Pamer et al 1999). Several studies have demonstrated CD1 mediated presentation of lipid antigens to T-cells including the mycobacterial lipids mycolic acid (Beckman et al 1994, Porcelli et al 1992), lipoarabinomannan (Sieling et al 1995), glucose monomycolate (Moody et al 1997), phosphoisoprenoids (Moody et al 2000), human self ceramides (Shamshiev et al 1999) and, curiously, the marine sponge lipid α-galactosylceramide (Natori et al 1994). Interestingly, all CD1-presented lipids have been shown to share a common general architecture. This can be broadly characterised as a di-acyl hydrophobic region attached to a strongly polar headgroup – the only exception so far documented being the monoacyl nature of the CD1c ligand glucose monomycolate (Park et al 2000).

4.3.9 Can LPS bind to CD1?

In order to answer the question of whether or not CD1-mediated presentation of LPS is possible, the question of whether or not LPS is capable of binding to CD1 must be answered first. Structurally speaking, the general architecture of LPS is similar to that displayed by the other CD1 ligands: namely a hydrophobic region attached to a highly polar headgroup. However, as a result of the substitution of the diglucosamine backbone with between 5 and 7 acyl chains, it is likely that unmodified LPS monomers will be too bulky to fit inside the di-acyl adapted groove of CD1. This conflict is further borne out by the observation that enterobacterial LPS monomers have been estimated to have a width of approximately 20-24A and a length of roughly 12-14A (Alexander et al 2001). This compares with the hydrophobic binding pocket of CD1 which is approximately 30A long by 10-15A wide (Zeng et al 1997). Thus, it appears that

while LPS molecules are not too long to fit inside the CD1 binding groove, they are too wide.

However, enzymatic degradation of LPS within cells may well be capable of trimming sufficient acyl and glycan groups to form a ligand of the appropriate structure and size for binding to CD1 (as discussed in section 1.4). Such enzymes have certainly been reported to be present in the intracellular compartments through which LPS trafficks (Prigozy et al 1997, Sugita et al 1996, Ernst et al 1998, Thiemblemont et al 1998, Detmers et al 1996).

Mattern *et al* dismissed the possibility of CD1-mediated T-cell recognition in their studies of LPS-dependent T-cell proliferation, citing a lack of expression of CD1 of either monocytes or CD34+ blood stem cells (Mattern et al 1999). However, as more recent studies have suggested that CD1 may be expressed constitutively at least at low levels on monocytes (Porcelli et al 1999), and CD1a,b and c on cytokine differentiated monocytes (Porcelli et al 1992, Kasinerk et al 1993), the possibility that CD1 presentation of LPS may be responsible for the T-cell proliferation seen in earlier experiments was investigated in the following study.

Expression of CD1 was not detected on resting monocytes (Figure 4.29), but all type I CD1 molecules were seen to be strongly expressed on IL-4/GM-CSF-treated monocytes (Figure 4.29). This induction could not be achieved using stimulation of PBMC with *E.coli* R3 LPS (Figure 4.30). This may be because the cytokines produced by PBMC in response to LPS are typically Th1 biased, whereas IL-4 and GM-CSF are more commonly associated with a Th2 response, which may be more likely to occur in response to a natural infection with bacteria.

Attempts to block the proliferation of PBMC in response to LPS using antibodies directed against CD1 isomers failed (Figure 4.31). Curiously, the monoclonal antibody specific for CD1d appears to actually increase the proliferative response of the PBMC to LPS. This antibody may exhibit cross binding activity with other activating molecules on these cells. The failure of α-CD1a,b,c mAbs to block proliferation may be due to internalisation of CD1-bound antibody due to cycling of membrane proteins over the relatively long seven day incubation, or the antibodies may target epitopes on the CD1 molecules that do not sterically hinder their interaction with either antigen or T-cells.

In order to determine whether or not LPS could bind to CD1, *E.coli* R1 LPS was added to human monocytes treated with cytokines to induce expression of CD1a,b and c. Presence of bound LPS on the surface of cells was probed for with a monoclonal antibody previously demonstrated to bind to the core structures of a wide range of enteric LPS, including *E.coli* R1 core structures (Di Padova et al 1993). This method did not reveal the presence of LPS on the surface of the cells after seven days incubation (Figure 4.33). The monoclonal antibody used also shows non-specific binding to cells that have been treated with IL-4 and GM-CSF. It remains possible, however, that the reason no core epitopes were detected on the surface of the cells by this antibody is that enzymic processing of internalised LPS may have resulted in alteration or removal of core epitopes that are required for antibody binding to unprocessed LPS molecules.

Protein extracts of IL-4/GM-CSF treated monocytes were then assessed for CD1 content. Figure 4.34 shows that abundant proteins are present in these extracts, particularly in the 41-55 kDa range expected to contain all CD1 isotypes, though repeated immunoblots of these gels using the same anti-CD1 monoclonals used in blocking experiments and flow cytometry could not reveal the presence of CD1.

Dot blots of the same protein extracts revealed the expected high expression of MHC-II and lack of expression of CD3, but no expression of CD1a,b,c or d could be seen in these assays (Figure 4.35). These antibodies clearly work in flow cytometry (Figure 4.29) but appear incapable of binding to CD1 in the context of nitrocellulose. This technical difficulty could not be overcome, and as a result co-immunoprecipitation experiments of LPS and CD1 could not be performed.

In summary, there is evidence that LPS or fragments of LPS are presented to T-cells. The fact that T-cell proliferation in response to LPS is dependent on cell contact with accessory cells (Mattern et al 1994), and that these cells are present only at frequencies typical of antigen specific T-cells (~1:1,000) support this hypothesis. The finding of this study that responses appear to be directed against core epitopes of LPS and not lipid A appear to rule out TLR4-mediated activation of the T-cells, as does the observation that T-cell responses to liposomal LPS occur at concentrations approximately 100-fold lower than those recognised by monocytes. However, the mechanisms by which presentation of LPS to T-cells is achieved remain unknown, and further work is required to determine whether or not LPS or breakdown products of LPS are capable of being presented by CD1.

CHAPTER 5

IMMUNE RESPONSES TO LIPOPOLYSACCHARIDE IN SEPSIS

5.1 Introduction

In order to understand the role played by the host immunological responses to LPS in outcome from disease, a prospective series of patients presenting with inflammatory disease associated with endotoxin exposure were recruited into the following study.

The capacity of patients' PBMC to proliferate in response to a panel of LPS was measured, as was the ability to proliferate in response to lipoteichoic acid of *S.aureus* and the T-cell mitogen ConA. Toll receptor-4 genotype was analysed and measurements of antibodies of the IgG and IgM subclass specific for the core structures of LPS were also made. Clinical parameters investigated included diagnosis, length of stay, whole blood neutrophil and lymphocyte counts, SIRS score, SOFA score, Glasgow score and presence or absence of sepsis on admission.

The following chapter investigates the possible relationships between these biological and clinical indicators and compares the responses of these patients with those of a control group of healthy volunteers.

5.2 Results

5.2.1 Patient and control recruitment

Over the course of 14 months, emergency surgical patients were enrolled for the following study if a clinical diagnosis was confirmed consistent with abdominal inflammation together with a documented source of infection. In total, 40 patients were enrolled with six patients agreeing to follow up blood sampling

on either one or two occasions. Five of the patients enrolled in the study had to be excluded from subsequent analysis on the grounds of more generalised inflammatory conditions. These diagnoses included 1 torted ovarian cyst, 1 hiatus hernia, 1 probable urinary tract infection, 1 case of pelvic inflammatory disease and 1 case that was undiagnosed.

Of the 35 remaining patients, a largely complete data set is only currently available for 27 patients. This cohort consists of 10 cases of appendicitis, 8 cases of pancreatitis, 5 cases of cholecystitis, 2 cases of diverticulitis and 2 cases of Crohn's disease. In the following analysis, data from the patients diagnosed with appendicitis, cholecystitis or diverticulitis have been pooled to form a further patient grouping, hereafter refered to as the A+C+D cohort. Data from these diagnoses in particular have been pooled since all of these cases involve inflammation of a lumenal organ and a likely resultant Gram-negative infection. Forty healthy controls were also enrolled.

5.2.2 Patient and control demographics

The 27 patients enrolled had a median age of 39 years (range 13-80 years), which was not significantly different from the median age of the control cohort (32 years, range 21-63). Only the 2 diverticulitis and 2 Crohn's patients showed significantly different mean age than the control population (diverticulitis patients were older at mean age 68, $p<0.05$; and Crohn's patients younger at mean age 16, $p<0.05$). The sex ratio of the whole patient group (17 males to 10 females) was not significantly different from the sex ratio of the control group (18 males to 22 females). These data are summarised in Table 5.1.

Table 5.1 Patient and control demographics

Patient Group:	n	Median Age (range):	Sex (M:F)	* *p*	** *p*
Controls:	40	32 (21-63)	18:22	-	-
All:	27	39 (13-80)	17:10	0.490	-
Appendicitis:	10	32 (18-52)	8:2	0.489	0.238
Cholecystitis:	5	53 (20-68)	1:4	0.626	0.795
Diverticulitis:	2	68 (63-73)	1:1	**0.021**	0.132
Crohn's:	2	16 (13-20)	1:1	**0.020**	0.093
Pancreatitis:	8	62 (19-80)	6:2	0.055	0.223
A+C+D:	17	39 (18-73)	10:7	0.675	0.791

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the median age of each group with the control population using the Mann-Whitney U-test. ** *p*-values represent comparison of the median age of each patient subgroup with that of the entire patient group using the Mann-Whitney U-test.

5.2.3 Patient clinical scores

On enrolment each patient was assessed for systemic inflammatory response syndrome (SIRS score), sepsis-related organ failure assessment (SOFA score) and if presenting with acute pancreatitis or Crohn's disease the modified Glasgow score or simple index of Crohn's activity respectively. As a result of incomplete filling in of forms, Glasgow scores are only available for 4 of the 8 pancreatitis patients and only one of the 2 Crohn's patients was assessed on the Crohn's index. Data relating to the concentrations of neutrophils and lymphocytes in patients whole blood were also recorded from the routine hospital blood analyses of 12 patients.

5.2.4 Patient SIRS scores

All patients were assessed on enrolment for systemic inflammatory response using the SIRS score of Bone *et al* (1992). Scores ranged from 0 to a maximum score of 4 (median score 1). 13 patients had a score of 2 or more and since all patients enrolled had a documented source of infection, are hence defined as suffering from sepsis. SIRS scores and frequency of diagnosis of sepsis for each patient group are summarised in Table 5.2. No individual patient subgroup had a SIRS score significantly higher or lower than the whole group median. The number of patients with a SIRS score of 2 or more was approximately half of the whole group number for each patient subgroup.

Table 5.2 Patient SIRS scores by subgroup

Patient group:	n	Median SIRS score (range):	* *p*	SIRS score > 1
All:	27	1 (0-4)	-	13
Appendicitis:	10	2 (0-4)	0.442	5
Cholecystitis:	5	2 (0-3)	0.876	3
Diverticulitis:	2	1 (0-2)	0.606	1
Crohn's:	2	0 (0-0)	-	0
Pancreatitis:	8	2 (0-3)	0.798	4
A+C+D:	17	2 (0-4)	0.763	9

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. SIRS > 1 column shows number of patients diagnosed with sepsis within each subgroup. * *p*-values represent comparison of the SIRS scores of each patient subgroup with the whole patient population using the Mann-Whitney U-test. Number of patients with SIRS scores greater than 1 represent subset diagnosed with sepsis.

5.2.5 Patient SOFA scores

All patients were assessed on enrolment for systemic organ failure using the SOFA score of Vincent *et al* (1996). Scores ranged from 0 to a maximum score of 5 (median score 1). The maximum possible score on the SOFA scale is 24. No individual subgroup of patients showed a significantly higher or lower organ failure score than the group average. SOFA scores of each patient subgroup are summarised in Table 5.3.

Table 5.3 Patient SOFA scores by subgroup

Patient group:	n	Median SOFA score (range):	* *p*
All:	27	1 (0-5)	-
Appendicitis:	10	1 (0-3)	0.811
Cholecystitis:	5	0 (0-0)	-
Diverticulitis:	2	2 (1-3)	0.366
Crohn's:	2	1 (0-1)	0.636
Pancreatitis:	8	2 (0-5)	0.336
A+C+D:	17	0 (0-3)	0.655

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the SOFA scores of each patient subgroup with the whole patient population using the Mann-Whitney U-test.

5.2.6 Patient length of stay

As mortality was low in this cohort (1 pancreatitis patient), length of stay was chosen as an objective measurement of patient outcome. In the whole patient group, length of stay ranged from 2 days to 31 days with a median stay of 4 days (Table 5.4). Only the group of appendicitis patients stayed a sigificantly

shorter time in hospital than the median time stayed by the entire patient cohort (2-5 days, median stay 3 days).

Table 5.4 Patient length of stay by subgroup

Patient group:	n	Median Stay (range):	* *p*
All:	27	4 (2-31)	-
Appendicitis:	10	3 (2-5)	**0.017**
Cholecystitis:	5	5 (4-13)	0.406
Diverticulitis:	2	11 (8-13)	0.245
Crohn's:	2	14 (12-15)	0.121
Pancreatitis:	8	4 (3-31)	0.409
A+C+D:	17	4 (2-13)	0.341

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the lengths of stay of each patient subgroup with the whole patient population using the Mann-Whitney U-test.

5.2.7 Glasgow scores of pancreatitis patients

Pancreatitis patients were assessed for severity of disease using the modified Glasgow score of Blamey *et al* (1984). Of the 8 pancreatitis patients enrolled, scores are available for 4 and were seen to have a range of 0-4 and a median of 1. The maximum possible score on the Glasgow scale is 8.

5.2.8 Crohn's index scores of Crohn's patients

Of the 2 Crohn's' patients enrolled in the study, only one was assessed for severity of disease using the simple index of Crohn's activity proposed by Harvey *et al* (1980). This patient showed a score of 7 (maximum possible 33) on this scale.

5.2.9 Patient whole blood neutrophil and lymphocyte counts

Neutrophil and lymphocyte count data are available for 12 of the 27 patients. Median cellular concentrations recorded for each patient subgroup are summarised in Table 5.5. Each patient subgroup is seen to have a substantially raised concentration of neutrophils when compared to that of the healthy population (normal range 2.0 - 7.5 cells/μl), and a slightly reduced concentration of lymphocytes (normal range 1.5 - 4.0 cells/μl). Comparison between patient subgroups revealed no individual subgroup to have significantly higher or lower neutrophil and lymphocyte counts than the group average.

Table 5.5 Neutrophil and lymphocytes counts of patient subgroups

Patient Group:	n	Neutrophils (range):	* *p*	Lymphocytes (range):	** *p*
All:	12	13.7 (6.1-17.4)	-	0.82 (0.4-2.3)	-
Appendicitis:	7	13.9 (9.8-17.4)	0.352	1.07 (0.4-2.3)	0.353
Cholecystitis:	0	-	-	-	-
Diverticulitis:	1	9.4	-	0.4	-
Crohn's:	1	6.1	-	0.7	-
Pancreatitis:	3	13.5 (9.3-14.7)	0.773	0.7 (0.6-1.0)	0.564
A+C+D:	8	13.9 (9.4-17.4)	0.563	1.0 (0.4-2.3)	0.671

All counts are represented in cells / μl. A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the neutrophil counts of each patient subgroup with the whole patient population using the Mann-Whitney U-test. ** *p*-values represent comparison of the lymphocyte counts of each patient subgroup with the whole patient population using the Mann-Whitney U-test.

5.2.10 Patient and control proliferative responses to LPS

PBMC from patients and controls were exposed to *E.coli* R1, *E.coli* R2, *E.coli* R3, *Bacteroides fragilis*, *Klebsiella pneumoniae* and *Salmonella typhimurium* LPS and the proliferation of the cells in response to these stimuli measured by thymidine incorportion. Additionally, cells of some patients and controls were incubated with lipoteichoic acid (LTA) of *Staphylococcus aureus* or the T-cell mitogen concanavalin-A (ConA). As the number of cells obtained from the blood of patients was not always sufficient to provide cultures for a complete set of experiments, the various stimuli were ranked in order of priority. Priority was given to *E.coli* R1 LPS, with the following being plated in order as availability of cells allowed: *E.coli* R2 LPS, *E.coli* R3 LPS, *S.aureus* LTA, *B.fragilis* LPS, *K.pneumoniae* LPS and *S.typhimurium* LPS. In the rare cases where sufficient cells were available for further cultures than these listed, a separate plate was set up to measure the capacity of the T-cells to respond to ConA after three days incubation.

Proliferative responses of patients cells to each stimuli were compared by calculation of the stimulation index (SI) for each antigen. This was calculated as the proliferative response (measured in counts per minute) of the challenged cell cultures divided by the background proliferative response (also measured in counts per minute) of unchallenged cell cultures.

5.2.11 Patient and control mean responses to all LPS

A generalised score was derived to represent the overall ability of each subject tested to mount a proliferative PBMC response to LPS. This index was calculated as the average of the stimulation indices for all the Gram-negative LPS tested for that individual. Table 5.6 summarises the mean responses to LPS of each patient and control subgroup tested. Using this measure, the median response of the whole patient group was 3.6, which was not

significantly less than the median response of 7.1 demonstrated by the cohort of 40 healthy controls (p=0.15). Responses of cholecystitis and diverticulitis patients to all LPS were higher than control responses (median SI 9.3 and 10.1 respectively), but this was not a significant difference. The most blunted responses were seen in the appendicitis, pancreatitis and Crohn's patient subgroups (scores of 2.2, 2.1 and 2.3 respectively), though no patient subgroup demonstrated an overall PBMC reactivity towards LPS significantly different from controls.

Patients and controls were then further subdivided into groups of high and low reactivity towards LPS. According to the convention of Mattern *et al* (1994), those demonstrating a stimulation index greater than 7 were classed as responders while those with a stimulation index of less than 7 were classed as non-responders. Using this test, 50% of healthy controls were classed as responsive to LPS in general (Table 5.6). Frequencies of responders within the various patient subgroups were then compared with the frequency of responders in the healthy control population using the Chi-squared test. The whole patient group shows a frequency of responders of 26% which, due to the small sample size, is not significantly different from controls (p=0.086). Similarly, despite the fact that only 10% of appendicitis patients were classed as overall responders, this frequency is not significantly different from controls (p=0.053).

Table 5.6 Patient and control mean responses to all LPS

Patient Group:	n	Median SI (range)	* *p*	>7	<7	** *p*
Controls	40	7.1 (0.1 - 46.1)	-	20	20	-
All	27	3.6 (0.4 - 66.4)	0.150	7	20	0.086
Appendicitis	10	2.2 (0.4 - 13.8)	0.085	1	9	0.053
Pancreatitis	8	2.1 (0.6 - 7.2)	0.054	2	6	0.364
Cholecystitis	5	9.3 (1.6 - 66.4)	0.448	3	2	0.958
Crohn's	2	2.3 (0.8 - 3.8)	0.226	0	2	0.512
Diverticulitis	2	10.1 (3.8 - 16.5)	0.658	1	1	0.469
A+C+D	17	3.7 (0.4 - 66.4)	0.469	5	12	0.254

A mean response to each of the LPS tested was derived for each individual patient to obtain a measure of overall responsiveness. Median SI represents the median of these scores for each of the patient groups examined. A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the mean responses to all LPS tested for each patient subgroup with the control population using the Mann-Whitney U-test. >7 or <7 indicates number of individuals within each subgroup with a stimulation index greater (or less) than 7 in this assay. ** *p*-values represent comparison of the frequencies of responders within each patient subgroup and the control population using the Chi-squared test.

5.2.12 Patient and control best response to any LPS

As it was seen that a number of patients classed as overall non-responders showed clear responses to particular individual types of LPS, a record was kept of the best response to any of the panel of LPS applied to the cells of each patient. The median best response to any LPS for the control group was 12.7 (range 0.2 - 87), while that of the whole patient group was 4.6 (range 0.7 - 171). No significant difference in median stimulation index or frequency of responders was observed between the various patient subgroups and the control cohort (Table 5.7).

Table 5.7 Patient and control best response to individual LPS types

Patient Group:	n	Median SI (range)	* *p*	>7	<7	** *p*
Controls	40	12.7 (0.2 - 87.0)	-	24	16	-
All	27	4.6 (0.7 - 171.0)	0.156	11	16	0.194
Appendicitis	10	4.6 (0.7 - 20.7)	0.152	3	7	0.178
Pancreatitis	8	4.0 (0.8 - 32.6)	0.158	3	5	0.435
Cholecystitis	5	9.7 (2.8 - 171.0)	0.600	3	2	0.628
Crohn's	2	2.7 (1.0 - 4.4)	0.165	0	2	0.347
Diverticulitis	2	17.9 (8.5 - 27.2)	0.637	2	0	0.696
A+C+D	17	6.7 (0.7 - 171.0)	0.536	8	9	0.542

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the best responses to all LPS tested for each patient subgroup with the control population using the Mann-Whitney U-test. >7 or <7 indicates number of individuals within each subgroup with a stimulation index greater (or less) than 7 in this assay. ** *p*-values represent comparison of the frequencies of responders within each patient subgroup and the control population using the Chi-squared test.

5.2.13 Patient and control responses to *E.coli* R1 LPS

27 patients and 40 controls were assessed for PBMC proliferation in response to *E.coli* R1 LPS. The median response for the control population was 5.6 (range 0.1 - 43.4), while the median response of the whole patient group was significantly lower at 1.8 (range 0.3 - 25.6, p=0.006). Every patient subgroup demonstrated a lower response to *E. coli* R1 LPS than healthy controls, though only the appendicitis, pancreatitis and A+C+D subgroups demonstrated significantly lower responses to *E.coli* R1 LPS than healthy controls (Table 5.8). The frequency of reponders in the whole patient group was also significantly lower than that of the control population (p=0.035).

Table 5.8 Patient and control PBMC response to *E.coli* R1 LPS

Patient Group:	n	Median SI (range)	* *p*	>7	<7	** *p*
Controls	40	5.6 (0.1 - 43.4)	-	15	25	-
All	27	1.8 (0.3 - 25.6)	**0.006**	3	24	**0.035**
Appendicitis	10	1.3 (0.4 - 6.5)	**0.028**	0	10	0.054
Pancreatitis	8	0.9 (0.4 - 18.3)	**0.028**	1	7	0.338
Cholecystitis	5	1.9 (0.3 - 25.6)	0.613	2	3	0.704
Crohn's	2	2.7 (1.0 - 4.4)	0.425	0	2	0.746
Diverticulitis	2	2.6 (2.2 - 3.1)	0.359	0	2	0.746
A+C+D	17	1.9 (0.3 - 25.6)	**0.035**	2	15	0.104

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the responses to *E.coli* R1 LPS of each patient subgroup with the control population using the Mann-Whitney U-test. >7 or <7 indicates number of individuals within each subgroup with a stimulation index greater (or less) than 7 in this assay. ** *p*-values represent comparison of the frequencies of responders within each patient subgroup and the control population using the Chi-squared test.

5.2.14 Patient and control responses to *E.coli* R2 LPS

26 patients and 39 controls were assessed for PBMC proliferation in response to *E.coli* R2 LPS. The median response for the control population was 7.6 (range 0.1 - 74.6), while the median response of the whole patient group was 2.7 (range 0.3 - 73.9). Responses of cholecystitis and diverticulitis patients to *E.coli* R2 LPS were higher than control responses (median SI 9.5 and 11.1 respectively), but this was not a significant difference. No significant differences were observed between patient subgroup and control responses to LPS when considering either median response or frequency of responders (Table 5.9).

Table 5.9 Patient and control PBMC response to *E.coli* R2 LPS

Patient Group:	n	Median SI (range)	* *p*	>7	<7	** *p*
Controls	39	7.6 (0.1 - 74.6)	-	20	19	-
All	26	2.7 (0.3 - 73.9)	0.258	8	18	0.167
Appendicitis	10	1.7 (0.3 - 20.0)	0.121	2	8	0.156
Pancreatitis	7	2.7 (0.6 - 18.2)	0.321	2	5	0.486
Cholecystitis	5	9.5 (2.8 - 73.9)	0.318	3	2	0.914
Crohn's	2	2.6 (1.0 - 4.3)	0.238	0	2	0.490
Diverticulitis	2	11.1 (2.0 - 20.2)	0.856	1	1	0.490
A+C+D	17	2.8 (0.3 - 73.9)	0.575	6	11	0.417

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the responses to *E.coli* R2 LPS of each patient subgroup with the control population using the Mann-Whitney U-test. >7 or <7 indicates number of individuals within each subgroup with a stimulation index greater (or less) than 7 in this assay. ** *p*-values represent comparison of the frequencies of responders within each patient subgroup and the control population using the Chi-squared test.

5.2.15 Patient and control responses to *E.coli* R3 LPS

25 patients and 39 controls were assessed for PBMC proliferation in response to *E.coli* R3 LPS. The median response for the control population was 7.6 (range 0.1 - 40.6), while the median response of the whole patient group was 3.6 (range 0.3 - 171.0). Responses of cholecystitis and diverticulitis patients to *E.coli* R3 LPS were higher than responses of healthy controls (median SI 11.4 and 17.9 respectively), but this was not a significant difference. No significant differences were observed between patient subgroup and control responses to LPS when considering either median response or frequency of responders, despite the very low response of the appendicitis patient subgroup to *E.coli* R3 LPS (median SI 1.6; Table 5.10).

Table 5.10 Patient and control mean PBMC response to *E.coli* R3 LPS

Patient Group:	n	Median SI (range)	* *p*	>7	<7	** *p*
Controls	39	7.6 (0.1 - 40.6)	-	20	19	-
All	25	3.6 (0.3 - 171.0)	0.259	9	16	0.347
Appendicitis	10	1.6 (0.3 - 20.7)	0.076	2	8	0.156
Pancreatitis	8	3.6 (0.6 - 29.4)	0.359	2	6	0.333
Cholecystitis	5	11.4 (3.4 - 171.0)	0.268	3	2	0.914
Crohn's	2	1.6 (0.5 - 2.6)	0.155	0	2	0.490
Diverticulitis	2	17.9 (8.5 - 27.2)	0.318	2	0	0.535
A+C+D	17	6.4 (0.3 - 171.0)	0.611	7	10	0.685

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the responses to *E.coli* R3 LPS of each patient subgroup with the control population using the Mann-Whitney U-test. >7 or <7 indicates number of individuals within each subgroup with a stimulation index greater (or less) than 7 in this assay. ** *p*-values represent comparison of the frequencies of responders within each patient subgroup and the control population using the Chi-squared test.

5.2.16 Patient and control responses to *B.fragilis* LPS

16 patients and 37 controls were assessed for PBMC proliferation in response to *B.fragilis* LPS. The median response for the control population was 5.0 (range 0.1 - 85.5), while the median response of the whole patient group was 2.9 (range 0.3 - 23.6). Responses of cholecystitis patients to *B.fragilis* LPS were higher than responses of healthy controls (median SI 22.1 vs 5.0), but this was not a significant difference. The lowest median SI was once again displayed by the appendicitis patient subgroup (2.2, not significant). No significant differences were observed between other patient subgroup and control responses to LPS when considering either median response or frequency of responders (Table 5.11).

Table 5.11 Patient and control PBMC response to *B.fragilis* LPS

Patient Group:	n	Median SI (range)	* *p*	>7	<7	** *p*
Controls	37	5.0 (0.1 - 85.5)	-	14	23	-
All	16	2.9 (0.3 - 23.6)	0.854	6	10	0.775
Appendicitis	7	2.2 (0.7 - 9.0)	0.847	2	5	0.969
Pancreatitis	5	2.9 (0.3 - 9.2)	0.484	1	4	0.776
Cholecystitis	3	22.1 (7.7 - 23.6)	0.057	3	0	0.137
Crohn's	0	-	-	-	-	-
Diverticulitis	1	1.9	-	0	1	0.782
A+C+D	11	2.9 (0.7 - 23.6)	0.492	5	6	0.918

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the responses to *B.fragilis* LPS of each patient subgroup with the control population using the Mann-Whitney U-test. >7 or <7 indicates number of individuals within each subgroup with a stimulation index greater (or less) than 7 in this assay. ** *p*-values represent comparison of the frequencies of responders within each patient subgroup and the control population using the Chi-squared test.

5.2.17 Patient and control responses to *K.pneumoniae* LPS

16 patients and 36 controls were assessed for PBMC proliferation in response to *K.pneumoniae* LPS. The median response for the control population was 5.0 (range 0.1 - 36.1), while the median response of the whole patient group was 2.2 (range 0.2 - 55.3). The appendicitis patient subgroup showed the lowest proliferative response to *K.pneumoniae* LPS (SI = 1.0), which was significantly lower than that seen in the healthy controls (p=0.048). Responses of cholecystitis patients to *K.pneumoniae* LPS were higher than responses of healthy controls (median SI 11.5 vs 5.0), but this was not a significant difference. No significant differences were observed between other patient subgroup and control responses to LPS when considering either median response or frequency of responders (Table 5.12).

Table 5.12 Patient and control PBMC response to *K.pneumoniae* LPS

Patient Group:	n	Median SI (range)	* *p*	>7	<7	** *p*
Controls	36	5.0 (0.1 - 36.1)	-	12	24	-
All	16	2.2 (0.2 - 55.3)	0.234	4	12	0.783
Appendicitis	7	1.0 (0.2 - 11.2)	**0.048**	1	6	0.579
Pancreatitis	5	1.4 (0.3 - 20.8)	0.449	1	4	0.930
Cholecystitis	3	11.5 (2.5 - 55.3)	0.268	2	1	0.596
Crohn's	0	-	-	-	-	-
Diverticulitis	1	4.4	-	0	1	0.704
A+C+D	11	2.5 (0.2 - 55.3)	0.309	3	8	0.994

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the responses to *K.pneumoniae* LPS of each patient subgroup with the control population using the Mann-Whitney U-test. >7 or <7 indicates number of individuals within each subgroup with a stimulation index greater (or less) than 7 in this assay. ** *p*-values represent comparison of the frequencies of responders within each patient subgroup and the control population using the Chi-squared test.

5.2.18 Patient and control responses to *S.typhimurium* LPS

16 patients and 36 controls were assessed for PBMC proliferation in response to *S.typhimurium* LPS. The median response for the control population was 6.0 (range 0.1 – 87.0), while the median response of the whole patient group was 3.8 (range 0.4 – 50.2). The lowest response to *S.typhimurium* LPS was observed in the appendicitis patient subgroup (SI = 1.5), but this was not significantly lower than the response of the healthy controls (p=0.122). Responses of cholecystitis patients to *S.typhimurium* LPS were higher than responses of healthy controls (median SI 19.8 vs 6.0), but this was not a significant difference. No significant differences were observed between other patient subgroup and control responses to LPS when considering either median response or frequency of responders (Table 5.13).

Table 5.13 Patient and control PBMC response to *S.typhimurium* LPS

Patient Group:	n	Median SI (range)	* *p*	>7	<7	** *p*
Controls	36	6.0 (0.1 - 87.0)	-	16	20	-
All	16	3.8 (0.4 - 50.2)	0.736	6	10	0.870
Appendicitis	7	1.5 (0.4 - 17.9)	0.122	1	6	0.284
Pancreatitis	5	9.0 (0.8 - 32.6)	0.661	3	2	0.861
Cholecystitis	3	19.8 (2.4 - 50.2)	0.225	2	1	0.889
Crohn's	0	-	-	-	-	-
Diverticulitis	1	2.6	-	0	1	0.890
A+C+D	11	2.6 (0.4 - 50.2)	0.474	3	8	0.506

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the responses to *S.typhimurium* LPS of each patient subgroup with the control population using the Mann-Whitney U-test. >7 or <7 indicates number of individuals within each subgroup with a stimulation index greater (or less) than 7 in this assay. ** *p*-values represent comparison of the frequencies of responders within each patient subgroup and the control population using the Chi-squared test.

5.2.19 Patient and control responses to *S.aureus* LTA

23 patients and 32 controls were assessed for PBMC proliferation in response to *S.aureus* LTA. The median response for the control population was 2.6 (range 0.1 - 20.6), while the median response of the whole patient group was 1.8 (range 0.2 - 47.1). The cholecystitis patient subgroup showed a significantly higher median response (21.6, range 5.0 - 47.1) to *S.aureus* LTA than the healthy controls ($p=0.0127$). Diverticulitis patients also expressed a proliferative response to *S.aureus* LTA higher than that of the healthy control cohort (SI=6.1), though this was not a significant difference. The appendicitis and pancreatitis patient subgroups shared the lowest responses to *S.aureus* LTA (SI=1.4), though neither was significantly different from the response of healthy controls. Other than the cholecystitis patient subgroup, no significant differences were observed between patient subgroup and control responses to LPS when considering either median response or frequency of responders (Table 5.14).

Table 5.14 Patient and control PBMC response to *S.aureus* LTA

Patient Group:	n	Median SI (range)	* *p*	>7	<7	** *p*
Controls	32	2.6 (0.1 - 20.6)	-	8	24	-
All	23	1.8 (0.2 - 47.1)	0.805	6	17	0.824
Appendicitis	10	1.4 (0.2 - 10.3)	0.238	1	9	0.570
Pancreatitis	8	1.4 (0.4 - 12.6)	0.237	0	8	0.277
Cholecystitis	5	21.6 (5.0 - 47.1)	**0.013**	3	2	0.286
Crohn's	0	-	-	0	0	-
Diverticulitis	2	6.1 (2.1 - 10.1)	0.558	1	1	0.961
A+C+D	17	2.1 (0.2 - 47.1)	0.648	5	12	0.994

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the responses to *S.aureus* LTA of each patient subgroup with the control population using the Mann-Whitney U-test. >7 or <7 indicates number of individuals within each subgroup with a stimulation index greater (or less) than 7 in this assay. ** *p*-values represent comparison of the frequencies of responders within each patient subgroup and the control population using the Chi-squared test.

5.2.20 Patient and control PBMC response to ConA

Only 2 pancreatitis patients and 12 healthy volunteers provided enough cells for measurement of proliferation in response to ConA (Table 5.15). The median response for the control population was 88.3 (range 7.5 - 151.3), while the median response of the patient group was 1.0 (range 0.8 - 1.3). In terms of both median response and frequency of responder these two patients show significantly lowered capacity to respond to ConA (p=0.036 and p=0.008 respectively).

Table 5.15 Patient and control PBMC response to Concanavalin-A

Patient Group:	n	Median SI (range)	* *p*	>7	<7	** *p*
Controls	12	88.3 (7.5-151.3)	-	12	0	-
Pancreatitis	2	1.0 (0.8-1.3)	**0.036**	0	2	**0.008**

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the mean proliferative responses to ConA for each patient subgroup with the control population using the Mann-Whitney U-test. >7 or <7 indicates number of individuals within each subgroup with a stimulation index greater (or less) than 7 in this assay. ** *p*-values represent comparison of the frequencies of responders within each patient subgroup and the control population using the Chi-squared test.

5.2.21 Post recovery patient PBMC responses to LPS

Six patients agreed to follow up blood sampling for measurement of PBMC responses to LPS after a period of between one and twelve weeks recovery. Table 5.16 summarises the changes in overall responsiveness to all LPS tested of these patients following recovery. Five of the six patients sampled showed an increased capacity to mount a proliferative response to LPS, with a post-recovery : pre-recovery response ratio (calculated as the post-recovery SI divided by the SI measured at admission) ranging from 1.7 to 37.2. One

cholecystitis patient who was a responder on admission (SI=9.3) showed a lower proliferative response to LPS post recovery (SI=1.9).

Table 5.16 Post recovery patient proliferative responses to LPS

Patient	Diagnosis	Age	Average SI (admission)	Average SI (recovery)	Response ratio (recovery / admission)
1	Appendicitis	26	0.7	2.1	3.0
2	Cholecystitis	68	1.6	59.5	37.2
3	Cholecystitis	20	9.3	1.9	-4.9
4	Diverticulitis	73	1.2	16.5	13.8
5	Pancreatitis	72	1.1	1.9	1.7
6	Pancreatitis	53	1.6	4.5	2.8

5.2.22 Patient and control endogenous core IgG antibody responses to LPS

Heparinised plasma was separated from all blood samples collected and stored at -20°C before measurement of antibodies specific for the core epitopes of enteric LPS (endogenous core antibodies - EndoCAb) as described in Materials and Methods sections 2.4.6 and 2.4.7.

27 patients and 40 healthy volunteers were assessed for plasma titre of endogenous core antibodies of the Immunoglobulin-G (IgG) isotype. The control population was seen to have a median antibody titre of 192 median units (MU)/ml (range 74 - 345) while the whole patient group showed a significantly lower IgG EndoCAb titre of 154 MU/ml (range 97 - 359, $p<0.05$). Table 5.17 shows that the diverticulitis patients demonstrated the highest IgG EndoCAb titre among the patient subgroups (198 MU/ml), but this was not significantly

different from either controls or the remainder of the patient group. Other patient subgroups showed quite similar median IgG EndoCAb titres, ranging from 146 MU/ml (appendicitis) to 158 MU/ml (Crohn's).

Table 5.17 Median endogenous core IgG antibodies of patients and controls

Patient group:	n	Median IgG (range)	* *p*
Controls	40	192.0 (74 - 345)	-
All	27	153.8 (97 - 359)	**0.0497**
Appendicitis	10	145.8 (101 - 223)	0.102
Pancreatitis	8	155.9 (97 - 243)	0.262
Cholecystitis	5	147.1 (107 - 359)	0.312
Crohn's	2	158.1 (130 - 166)	0.425
Diverticulitis	2	198.2 (146 - 251)	0.906
A+C+D	17	147.1 (101 - 359)	0.086

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the endogenous core IgG antibody titre for each patient subgroup with the titres of the control population using the Mann-Whitney U-test.

5.2.23 Patient and control endogenous core IgM antibody responses to LPS

27 patients and 40 healthy volunteers were assessed for plasma titre of endogenous core antibodies of the Immunoglobulin-M isotype (Table 5.18). The control population was seen to have a median antibody titre of 89 MU/ml (range 35 - 159) while the whole patient group showed a lower (but not statistically significant) IgM EndoCAb titre of 69 MU/ml (range 19 - 181). Every patient subgroup was seen to have lower median levels of IgM EndoCAb when

compared to controls, though this was not significant. In contrast to IgG EndoCAb titre, diverticulitis patients showed the lowest IgM EndoCAb titres of the patient subgroups (52 MU/ml).

Table 5.18 Median endogenous core IgM antibodies of patients and controls

Patient group:	n	Median IgM (range)	* *p*
Controls	40	89.1 (35 - 159)	-
All	27	69.2 (19 - 181)	0.117
Appendicitis	10	76.9 (22 - 181)	0.734
Pancreatitis	8	57.5 (47 - 164)	0.430
Cholecystitis	5	69.2 (19 - 90)	0.080
Crohn's	2	73.0 (56 - 90)	0.637
Diverticulitis	2	52.4 (23 - 82)	0.204
A+C+D	17	70.4 (19 - 181)	0.138

A+C+D subgroup represents data pooled from appendicitis, cholecystitis and diverticulitis patients. * *p*-values represent comparison of the endogenous core IgM antibody titre for each patient subgroup with the titres of the control population using the Mann-Whitney U-test.

5.2.24 Post recovery patient antibody responses to LPS

Five patients agreed to follow up blood sampling on either one or two occasions. Tables 5.19 and 5.20 summarises the changes in EndoCAb IgG and IgM antibody titres of these patients following recovery. It can be seen that antibodies to LPS of both the IgG and IgM isotype may increase or decrease following recovery, with no obvious trend apparent.

Table 5.19 Post recovery patient IgG antibody responses to LPS

Patient	Diagnosis	Age	t_1	t_2	t_3
Patient 1	Appendicitis	26	119	90	66
Patient 2	Cholecystitis	68	147	328	-
Patient 3	Cholecystitis	20	107	146	-
Patient 4	Diverticulitis	73	251	335	-
Patient 5	Pancreatitis	72	158	227	212

T1 = EndoCAb IgG titre at admission to hospital. T2 = First measurement of EndoCAb IgG titre following recovery from illness. T3 = Later measurement of EndoCAb IgG titre following recovery from illness. All titres are expressed in EndoCAb median units/ml.

Table 5.20 Post recovery patient IgM antibody responses to LPS

Patient	Diagnosis	Age	IgM-1	IgM-2	IgM-3
Patient 1	Appendicitis	26	60	33	38
Patient 2	Cholecystitis	68	19	61	-
Patient 3	Cholecystitis	20	76	15	-
Patient 4	Diverticulitis	73	82	93	-
Patient 5	Pancreatitis	72	58	43	40

IgM-1 = EndoCAb IgM titre at admission to hospital. IgM-2 = First measurement of EndoCAb IgM titre following recovery from illness. IgM-3 = Later measurement of EndoCAb IgM titre following recovery from illness. All titres are expressed in EndoCAb median units/ml.

5.2.25 Patient and control antibody responses to individual LPS core types

As materials allowed, titres of antibodies specific for the core epitopes of several enteric LPS were measured in the plasma of 17 patients and 6 healthy controls as described in Materials and Methods sections 2.4.6 and 2.4.7. Antibodies of the IgG isotype directed against the following LPS were measured: *E.coli* R1, *E.coli* R2, *E.coli* R3, *E.coli* R4, *B.fragilis* rough and smooth LPS, *K.aerogenes* and *S.typhimurium*. Optical densities were compared with that of the same standard high titre serum used to calibrate the EndoCAb IgG and IgM measurements, assuming it to have the same median units/ml for each antibody as demonstrated for EndoCAb IgG (392 MU/ml).

The antibody responses to each LPS were then sorted in descending order of magnitude according to the response profile of patient 1, leading to the following order of presentation in Figure 5.1: EndoCAb, *B.fragilis* rough, *E.coli* R1, *B.fragilis* smooth, *K.aerogenes*, *S.typhimurium*, *E.coli* R4, *E.coli* R2, *E.coli* R3. This allowed easier comparison of the antibody response profiles of each individual to the panel of LPS used.

It can be seen that the profile of response to each LPS is remarkably similar in all patients and controls, with only occasional deviations from the typical profile of response in response to particular core types. Of particular note, however, is the occasional high response to *S.typhimurium* LPS, as displayed by patients 4, 7 and 16 and healthy volunteer number 4. There was no significant difference between patient and control mean titres of any of the antibodies investigated.

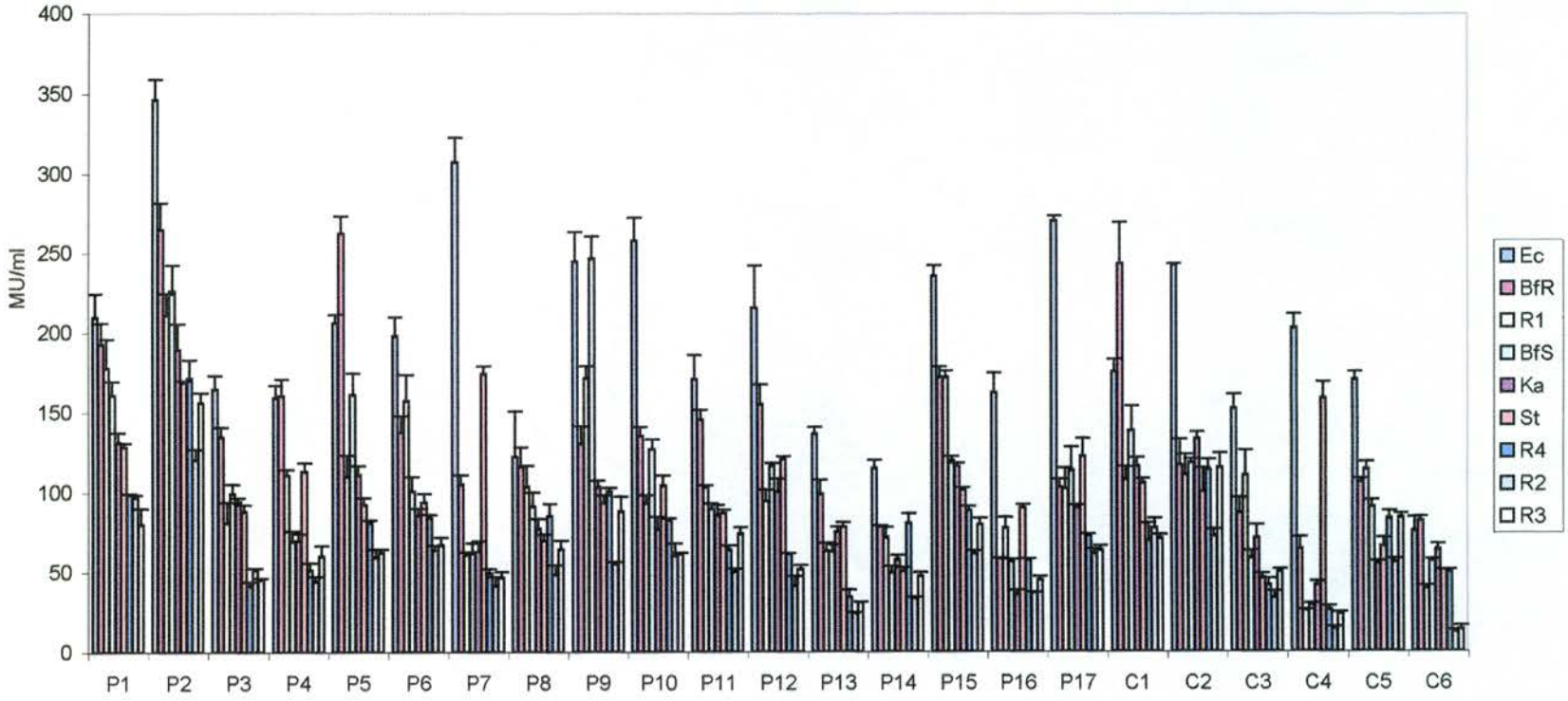


Figure 5.1: Patients and control IgG antibody responses to individual LPS

P1-P17, patients 1-17. C1-C6, healthy controls 1-6. Antibodies to Ec = EndoCAb core LPS, BfR = *B.fragilis* rough LPS, BfS = *B.fragilis* smooth LPS, Ka = *K.aerogenes* LPS, St = *Salmonella typhimurium*, R1,R2,R3,R4 = *E.coli* R1,R2,R3,R4 LPS. Error bars are sem of three independent measurements.

5.2.26 Frequencies of TLR4 mutations in patients and controls

Patient and control genomic DNA samples were screened for the presence of the mutations Asp299Gly and Thr399Ile in the Toll-like receptor 4 gene using the restriction enzyme digest method described in Materials and Methods section 2.5.8.

27 patients and 40 controls were screened for the presence of either mutation. Four individuals were found in each group to be heterozygous for both the Asp299Gly and the Thr399Ile mutation. No individual was seen to carry only one of the mutations and no individuals were found to be homozygous for either mutation. Analysis of the allele frequencies of the mutation in both groups (10% of controls vs 15% of patients) revealed that this difference was not significant. The four heterozygote patients included two appendicitis and two pancreatitis patients.

5.2.27 Effect of TLR4 mutations on proliferative responses to LPS

Proliferative responses to LPS were compared between TLR4 heterozygotes and wild types within the patient and control populations. Heterozygous patients exhibited slightly lower responses to LPS than wild-type patients (median SI 2.2 vs 3.7), though heterozygous controls exhibited slightly higher responses to LPS than wild-type controls (median SI 10.9 vs 7.1). A similar pattern was observed for best proliferative responses to LPS and median proliferative responses to LTA though none of these differences were statistically significant, most likely reflecting the very small sample size (n=4) of the heterozygote groups available for comparison (Table 5.21).

Table 5.21 Effect of TLR4 mutations on proliferative responses to LPS

Patients	TLR4 ++	TLR4 +-	* p
All LPS average	3.7	2.2	0.918
Best SI for any LPS	5.0	4.1	0.920
S.aureus LTA	1.8	1.3	0.441

Controls	TLR4 ++	TLR4 +-	** p
All LPS average	7.1	10.9	0.499
Best SI for any LPS	12.7	16.5	0.620
S.aureus LTA	2.7	4.6	0.576

TLR4++ = homozygous, wild-type cohort. TLR4+- = heterozygous cohort. *p* represents statistical comparison of the wild type and heterozygote population proliferative responses to LPS or LTA using the Mann-Whitney U-test.

5.2.28 Effect of Toll-receptor 4 mutations on antibody responses to LPS

EndoCAb antibody responses to LPS were then compared between TLR4 heterozygotes and wild types within the patient and control populations. Heterozygote IgG was lower than wild type IgG for the patient group (141 vs 157 MU/ml), though this trend was reversed in the control group (219 vs 186 MU/ml). Heterozygote IgM was higher than wild type IgM in both the patient (102 vs 62 MU/ml) and control groups (108 vs 87 MU/ml). None of these differences were statistically significant, most likely reflecting the very small sample size (n=4) of the heterozygote groups available for comparison (Table 5.22).

Table 5.22 Effect of TLR4 mutations on antibody responses to LPS

Patients:	TLR4 ++	TLR4 +-	* p
EndoCAb IgG	156.8	141.5	0.585
EndoCAb IgM	61.9	101.6	0.065
Controls	TLR4 ++	TLR4 +-	* p
EndoCAb IgG	186.4	219.0	0.379
EndoCAb IgM	86.6	108.2	0.380

TLR4++ = homozygous wild type cohort. TLR4+- = heterozygous cohort. *p* represents statistical comparison of the wild type and heterozygote population antibody responses to LPS using the Mann-Whitney U-test.

5.2.29 Statistical analysis of potential risk factors in sepsis

In order to assess which biological and clinical indicators had the strongest impact on patient outcome from abdominal inflammation, data from each assay was correlated against length of stay for each patient. Biological indicators investigated included proliferative responses to LPS and LTA, IgG and IgM antibody responses to LPS and Toll-receptor 4 genotype. Clinical indicators investigated included SIRS score, SOFA score and neutrophil and lymphocyte whole blood counts.

5.2.30 Correlation of patient PBMC responses to LPS with length of stay

Patient PBMC proliferative responses to the antigens *E.coli* R1 LPS, *E.coli* R2 LPS, *E.coli* R3 LPS, *B.fragilis* LPS, *S.typhimurium* LPS, *K.pneumoniae* LPS and *S.aureus* LTA were plotted against length of stay (Figures 5.2a-i). Linear regression analysis was applied to each plot to assess the coefficient of correlation (R^2).

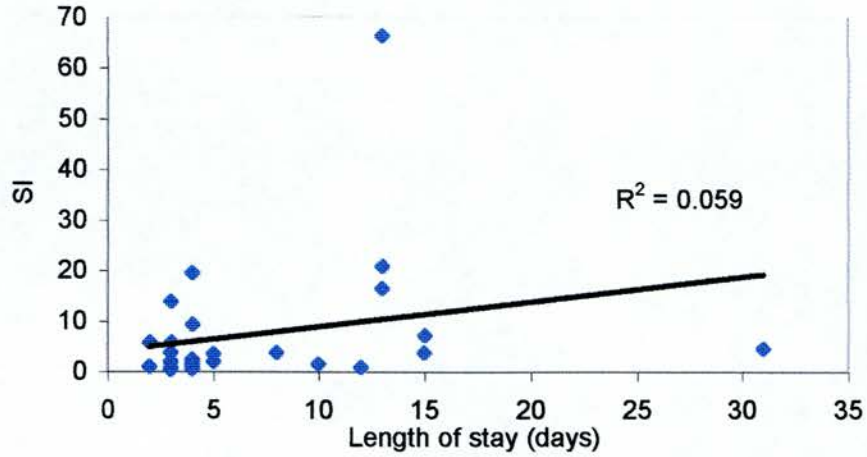


Figure 5.2a: Patient overall T-cell responses to LPS vs length of stay

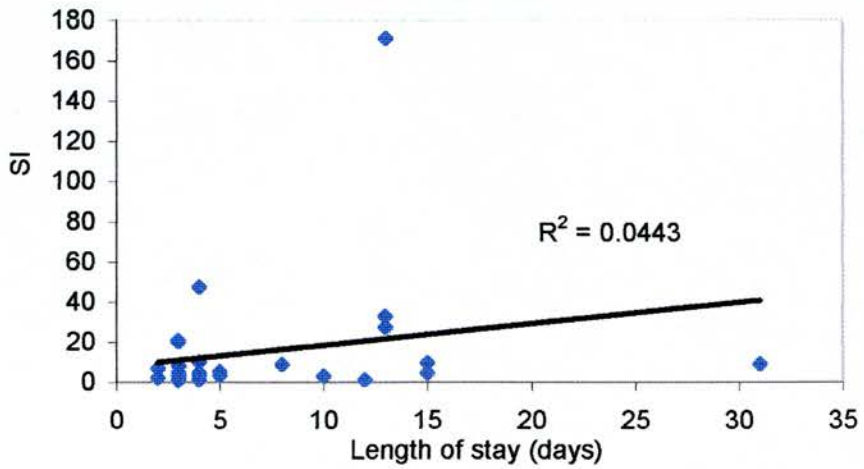


Figure 5.2b: Patient best stimulation index vs length of stay

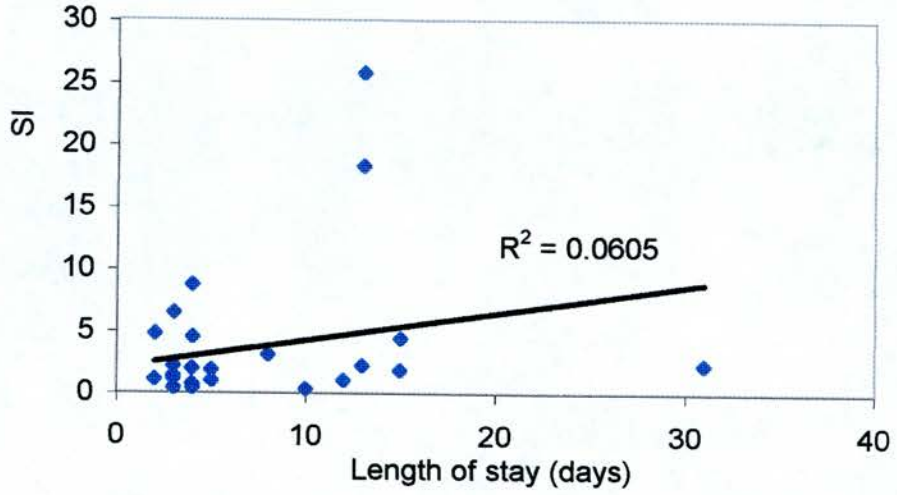


Figure 5.2c: Patient T-cell responses to *E.coli* R1 LPS vs length of stay

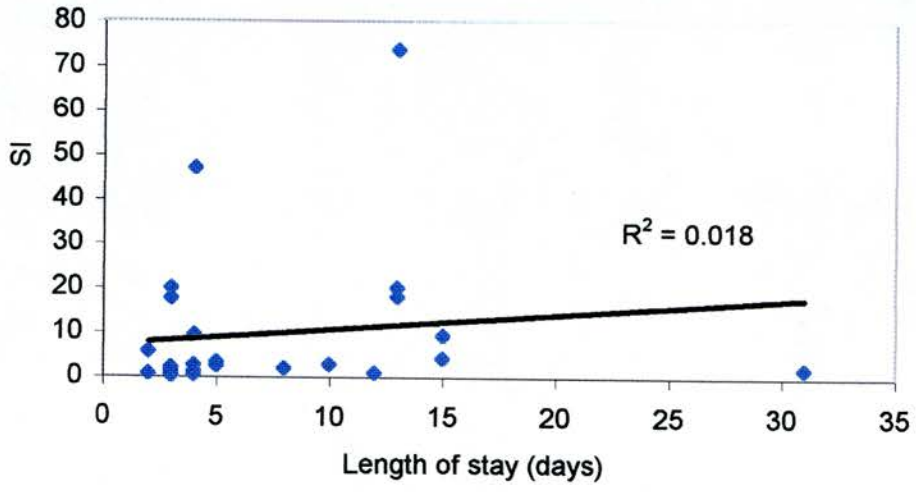


Figure 5.2d: Patient T-cell responses to *E.coli* R2 LPS vs length of stay

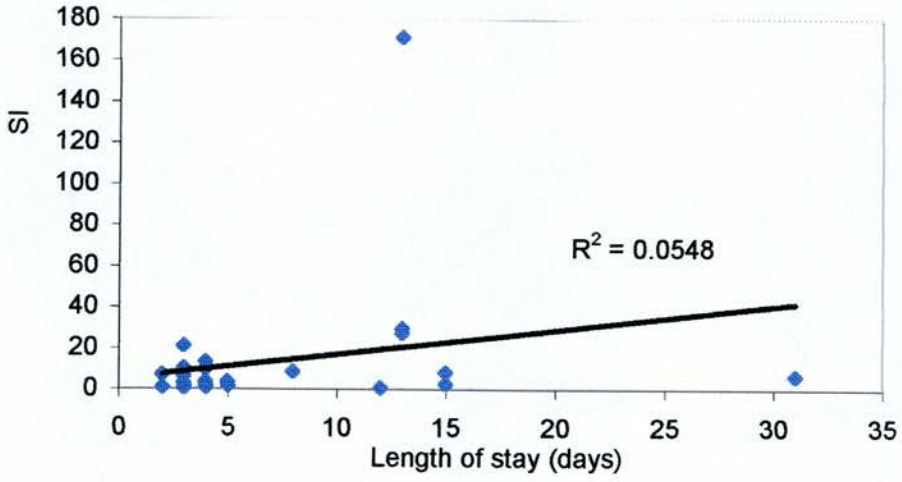


Figure 5.2e: Patient T-cell responses to *E.coli* R3 LPS vs length of stay

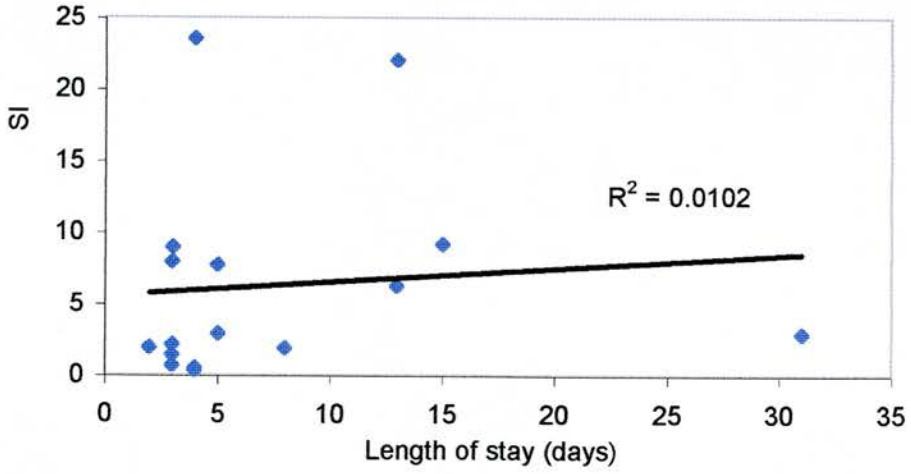


Figure 5.2f: Patient T-cell responses to *B.fragilis* LPS vs length of stay

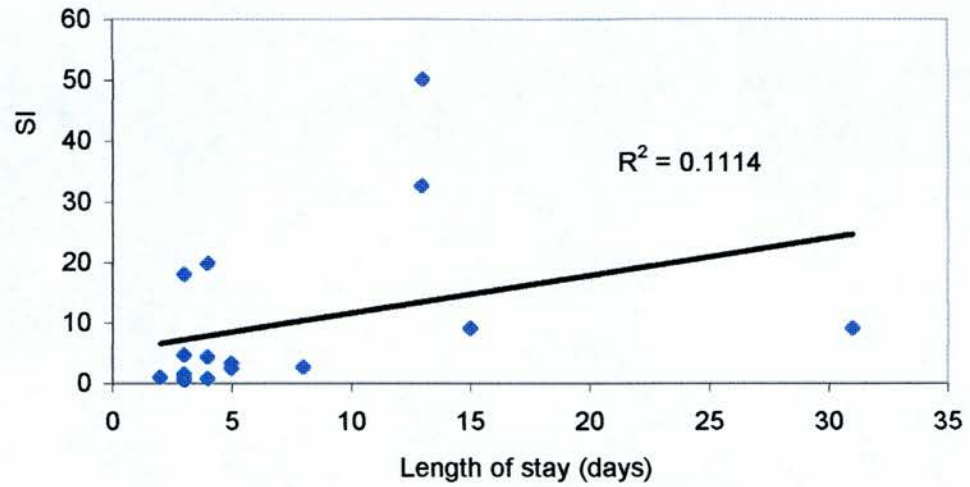


Figure 5.2g: Patient T-cell responses to *S.typhimurium* LPS vs length of stay

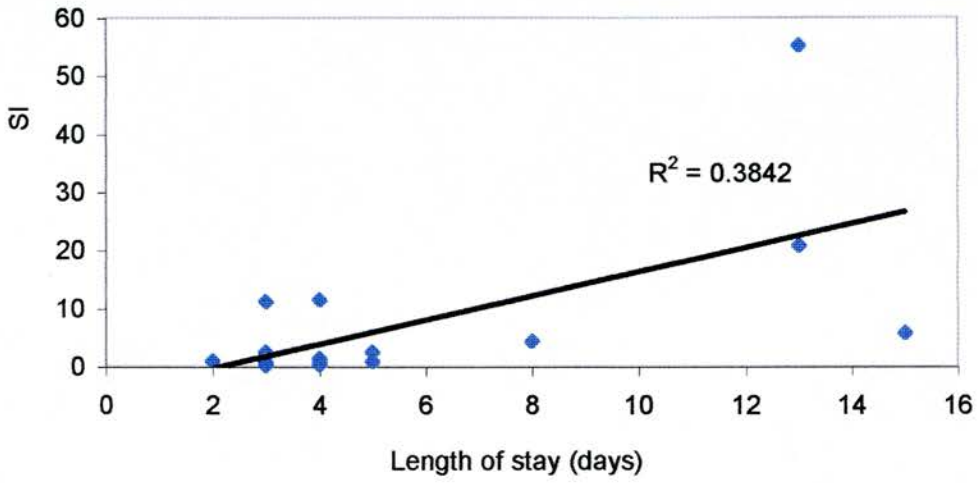


Figure 5.2h: Patient T-cell responses to *K.pneumoniae* LPS vs length of stay

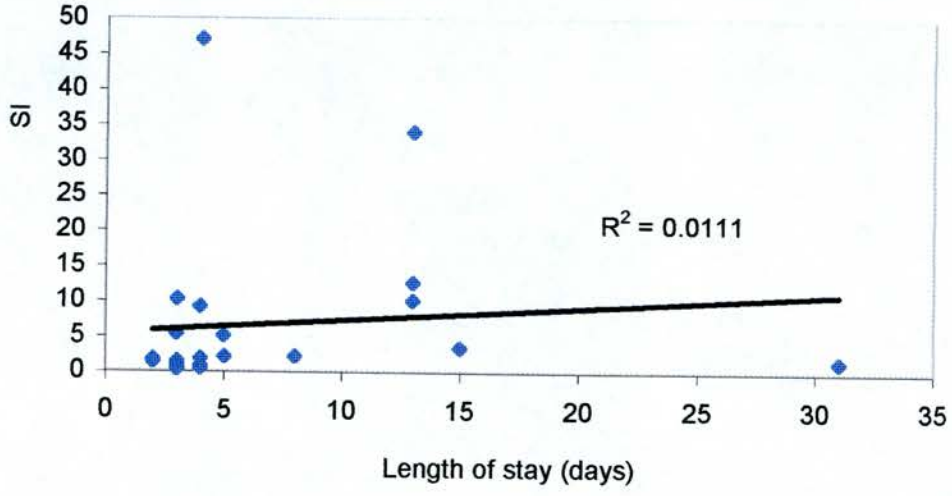


Figure 5.2i: Patient T-cell responses to *S.aureus* LTA vs length of stay

No statistically significant correlation was observed between patient length of stay and proliferative responses to any of the LPS or LTA tested except that of *K.pneumoniae*. Figure 5.2h shows that within this group of 16 patients, higher proliferative responses to *K.pneumoniae* LPS at admission tend to correlate with longer stay in hospital (R^2=0.384, p<0.01). This correlation is not nealy as clearly seen in the scatter plots of other LPS, which show a much wider scattering of points (R^2 for overall response to all LPS tested =0.06, best response to LPS =0.04, *E.coli* R1 LPS =0.06, *E.coli* R2 LPS =0.02, *E.coli* R3 LPS =0.05, *B.fragilis* LPS =0.01, *S.typhimurium* LPS =0.111, *S.aureus* LTA = 0.01). The strong correlation coefficient for *K.pneumoniae* LPS most likely reflects the fact that two patients who stayed a relatively long time in hospital (13 days) happened to demonstrate much higher proliferative responses to *K.pneumoniae* LPS than the other patients, and has therefore most likely occurred by chance.

Then, according to the protocol of Mattern *et al* (1994), patients were stratified according to responsiveness to each antigen. Those displaying a stimulation index greater than 7 were classed as responders while those displaying a stimulation index less than 7 were classed as non-responders. Table 5.23 summarises the median lengths of stay of the responding and non-responding patient populations with respect to each antigen. Non-responders in the context of each LPS stay a median of only 3.5 – 4 days in hospital. Median length of stay is longer and more variable among the groups classed as responders for each antigen (4.5 days to 13 days). This longer stay seen in the responding group is only a significant difference when considering the ability to respond to *S.typhimurium* LPS (median length of stay 13 days vs 3.5 days for non-responders, p=0.045).

Table 5.23 Effect of responder status on patient length of stay

Proliferative response	Responder median stay	Non-responder median stay	*p*
Mean response to all LPS	13	4	0.121
Best response to any LPS	8	4	0.139
E.coli R1 LPS	13	4	0.203
E.coli R2 LPS	8.5	4	0.291
E.coli R3 LPS	8	4	0.174
B.fragilis LPS	4.5	4	0.704
K.pneumoniae LPS	8.5	4	0.396
S.typhimurium LPS	13	3.5	**0.045**
S.aureus LTA	8.5	4	0.195

p represents statistical comparison of the responding and non-responding populations responses to LPS using the Mann-Whitney U-test.

5.2.31 Correlation of patient antibody responses to LPS with length of stay

Patient antibody responses to the LPS core epitopes were plotted against length of stay (Figures 5.3 a-b). Linear regression analysis was applied to each plot to assess the coefficient of correlation (R^2). The coefficient of correlation between patient length of stay and IgG anti-core LPS antibodies was (0.0013). The coefficient of correlation between patient length of stay and IgM anti-core LPS antibodies was (0.0358). Neither of these results represent a statistically significant trend.

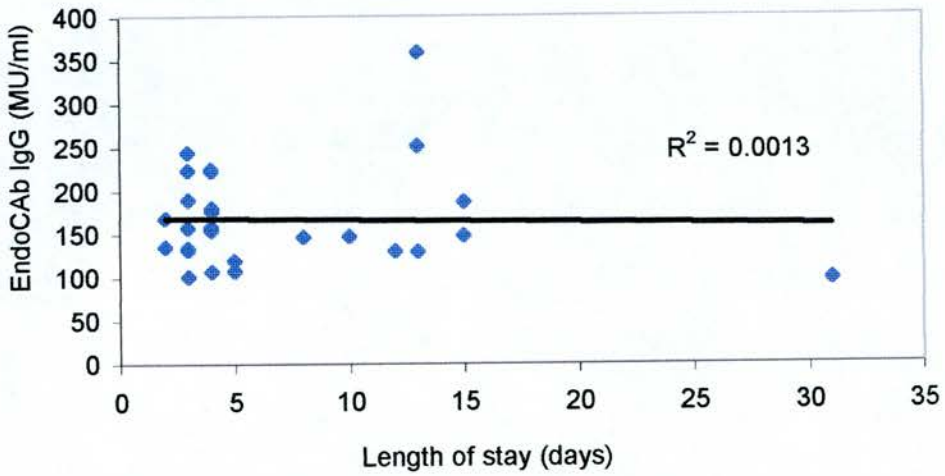


Figure 5.3a: Patient anti-core LPS IgG antibodies vs length of stay

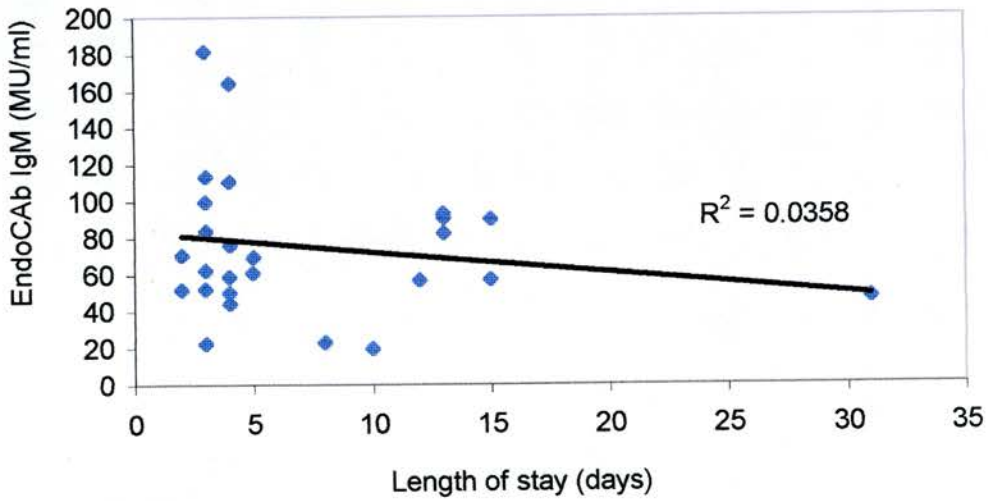


Figure 5.3b: Patient anti-core LPS IgM antibodies vs length of stay

5.2.32 Effect of Toll receptor mutation on length of stay and clinical scores

Patients were stratified according to Toll-like receptor 4 genotype into those harboring the Asp299Gly and Thr399Ile mutations (heterozygotes) and those without (wild types). Heterozygotes were younger than wild types (23years vs 48years) but this was not a significant difference (p=0.082). Heterozygotes stayed slightly longer in hospital (4.5days vs 4 days) and exhibited lower median SIRS scores (1 vs 2), lower median SOFA scores (0.5 vs 1) and a reduced incidence of sepsis (25% vs 52%), though none of these differences were of any statistical signicance, presumably reflecting the small sample number of heterozygotes available for study (n=4, Table 5.24).

Table 5.24 Effect of TLR4 mutations on patient clinical indicators and outcome

Clinical indicator:	TLR4 ++	TLR4 +-	*p*
Age	48.1	22.7	0.082
Length of stay (median)	4	4.5	0.838
SIRS (median)	2	1	0.865
SOFA (median)	1	0.5	0.633
Sepsis (frequency)	12/23	1/4	0.644

TLR4++ = homozygous wild type cohort. TLR4+- = heterozygous cohort. *p* represents statistical comparison of the wild type and heteroygote population antibody responses to LPS using the Mann-Whitney U-test or chi-squared test of equal variance if comparing frequencies.

5.2.33 Correlation of clinical indicators with length of stay

The patient clinical indicators SIRS score, SOFA score, whole blood neutrophil count and whole blood lymphocyte count were plotted against length of stay (Figures 5.4 a-d). Linear regression analysis was applied to each plot to assess

the coefficient of correlation (R^2). SIRS score showed no correlation at all (R^2=0.000) with length of stay. However, higher SOFA scores on admission were seen to correlate quite clearly with increased length of stay (R^2=0.156, $p<0.05$, Figure 5.4b). Lower lymphocyte counts were also seen to exert a statistically significant detrimental effect on length of stay (R^2=0.141, $p<0.05$, Figure 5.4d). Lower neutrophil counts also correlated with longer hospital stay, though this was not statistically significant (R^2=0.130, Figure 5.4c). Additionally, patients were stratified into those diagnosed with sepsis at admission (ie, those presenting with a SIRS score >=2) and those not. Both septic patients and non-septic patients stayed the same median length of time in hospital (4 days).

5.2.34 Correlation of clinical scores with PBMC and antibody responses to LPS

Since high scores in the SIRS and SOFA scales have been associated with increased morbidity and mortality, associations were sought between these scores and the proliferative and antibody responses to LPS. Figures 5.5 a-f show linear regression analyses of the two scores plotted against overall proliferative responiveness to LPS, anti-core LPS IgG antibodies and anti-core LPS IgM antibodies. No statistically significant correlation was observed when comparing SIRS scores with overall proliferative responses to LPS (R^2=0.023, Figure 5.5a), SOFA scores with overall proliferative responses to LPS (R^2=0.045, Figure 5.5b), SIRS scores with EndoCAb IgG (R^2=0.031, Figure 5.5c), SOFA scores with EndoCAb IgG (R^2=0.044, Figure 5.5d), SIRS scores with EndoCAb IgM (R^2=0.000, Figure 5.5e) or SOFA scores with EndoCAb IgM (R^2=0.029, Figure 5.5f).

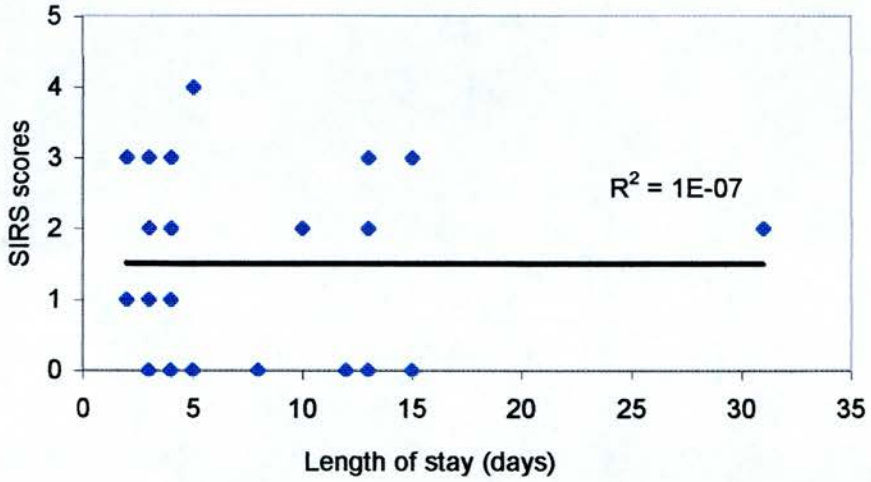


Figure 5.4a: Patient SIRS scores vs length of stay

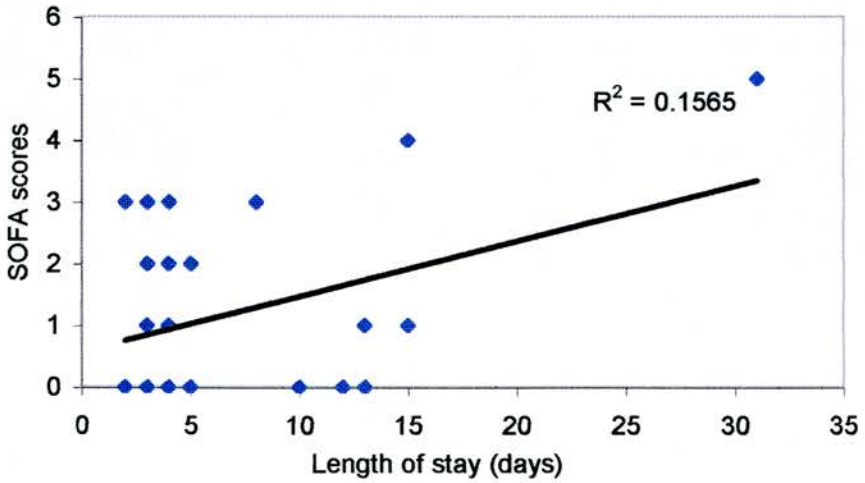


Figure 5.4b: Patient SOFA scores vs length of stay

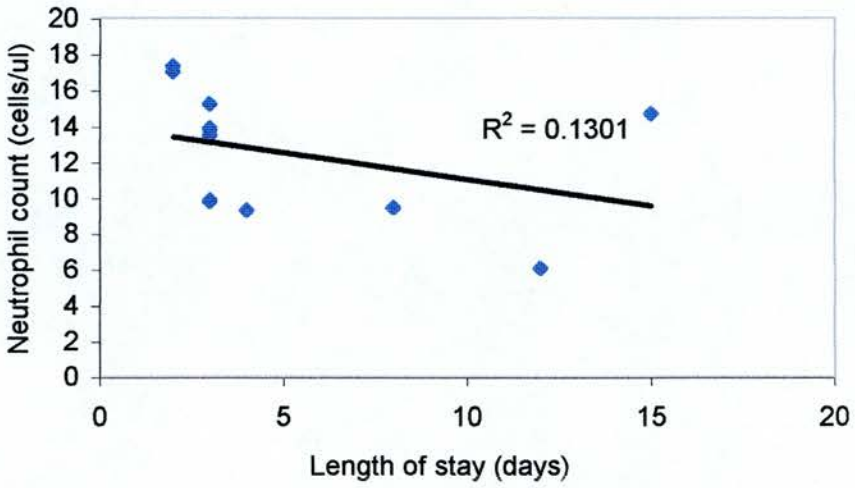


Figure 5.4c: Patient whole blood neutrophil counts vs length of stay

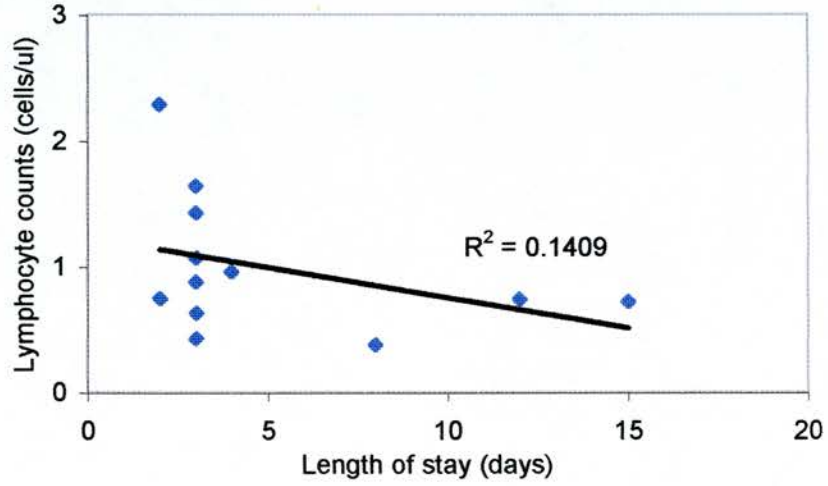


Figure 5.4d: Patient whole blood lymphocyte counts vs length of stay

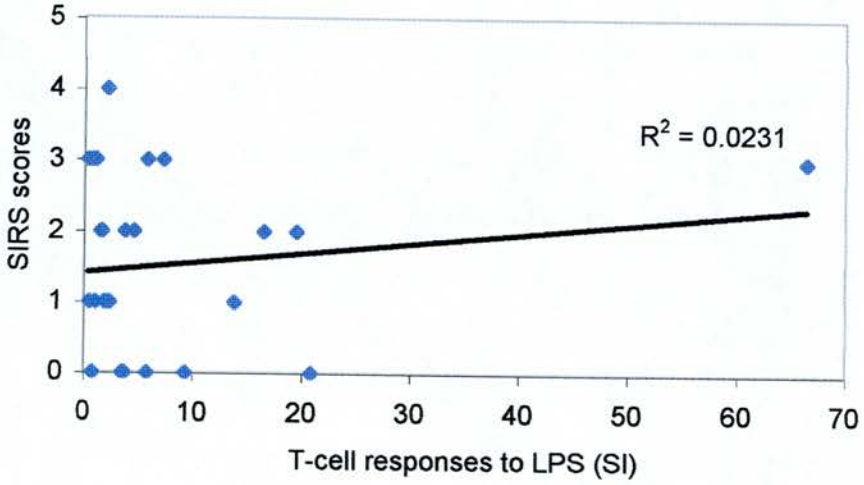


Figure 5.5a: Patient SIRS scores vs overall T-cell response to LPS

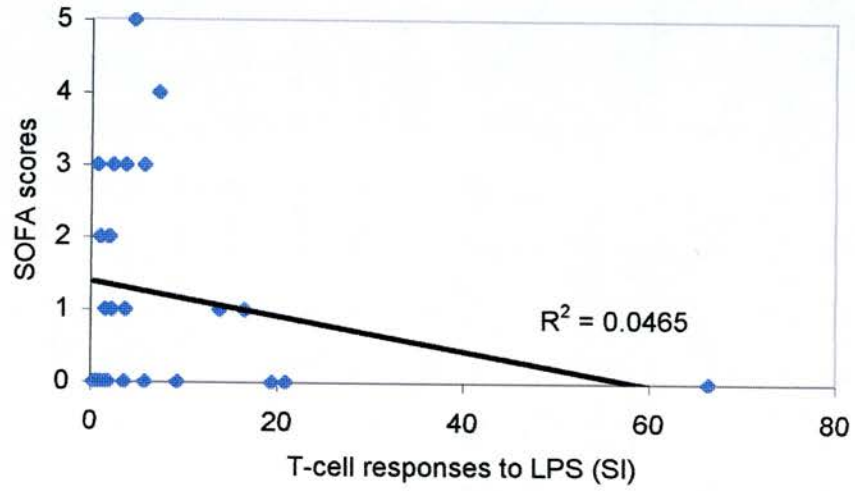


Figure 5.5b: Patient SOFA scores vs overall T-cell response to LPS

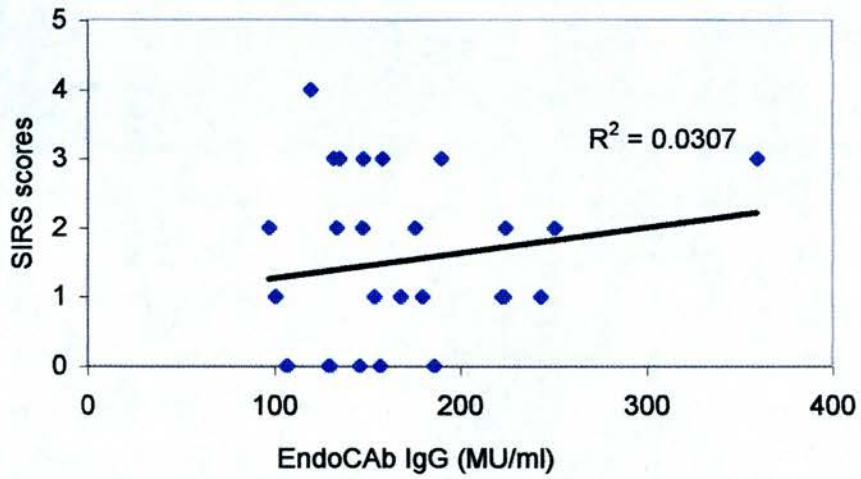


Figure 5.5c: Patient SIRS scores vs anti-core LPS IgG antibodies

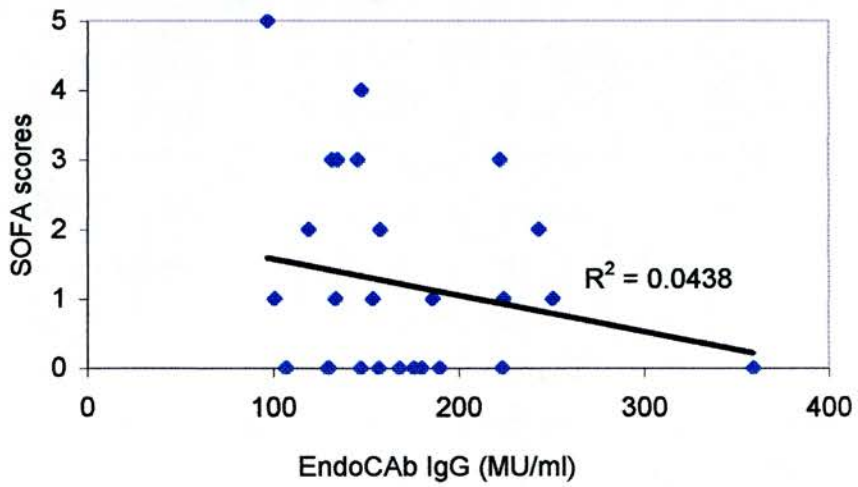


Figure 5.5d: Patient SOFA scores vs anti-core LPS IgG antibodies

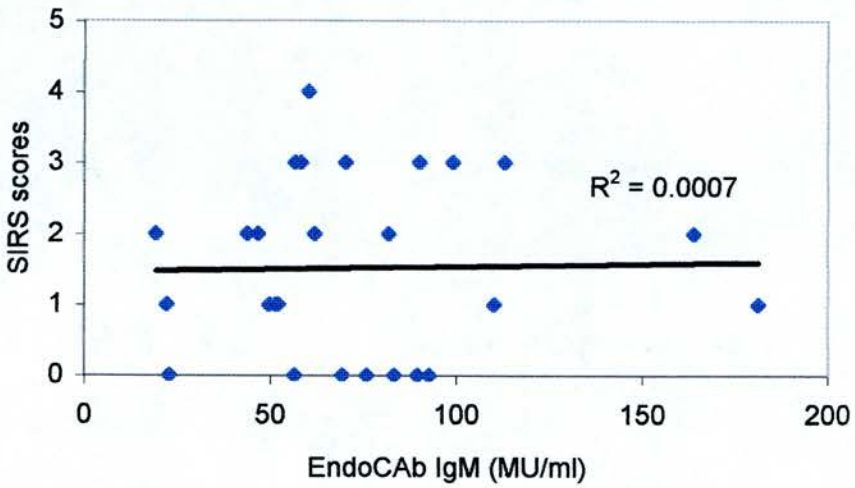


Figure 5.5e: Patient SIRS scores vs anti-core LPS IgM antibodies

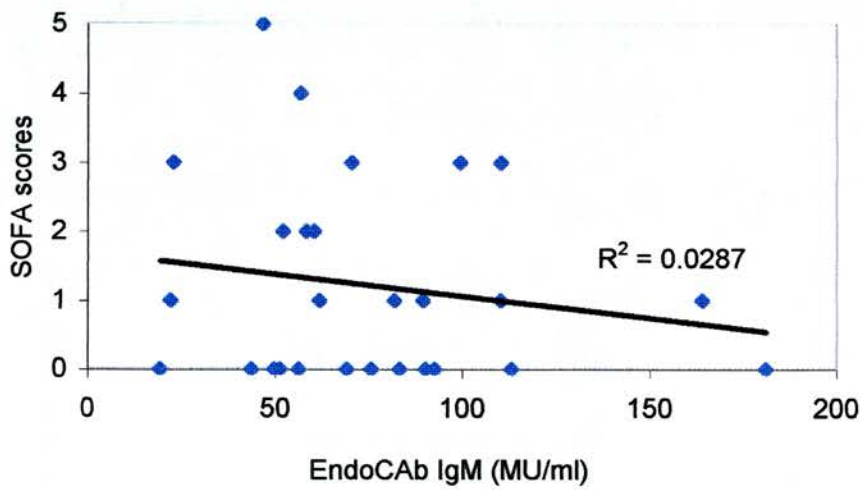


Figure 5.5f: Patient SOFA scores vs anti-core LPS IgM antibodies

5.2.35 Correlation of proliferative and antibody responses towards LPS

As the proposed liposomal LPS vaccine had been seen to induce a proliferative PBMC response, the question was asked whether proliferative responsiveness to LPS had any impact on titres of IgG or IgM anti-core LPS antibodies. Patient and healthy control overall proliferative responses to LPS were plotted against levels of antibodies to core LPS structures (Figures 5.6 a-d). Linear regression analysis was applied to each plot to assess the coefficient of correlation (R^2). No statistically significant correlation was observed between proliferative response to LPS and EndoCAb IgM in patients (R^2=0.000) or controls (R^2=0.111). Levels of EndoCAb IgG strongly correlated with overall PBMC responses to LPS in the patient group (R^2=0.319, $p<0.01$), but this trend was completely absent in the analysis of the control population (R^2=0.016).

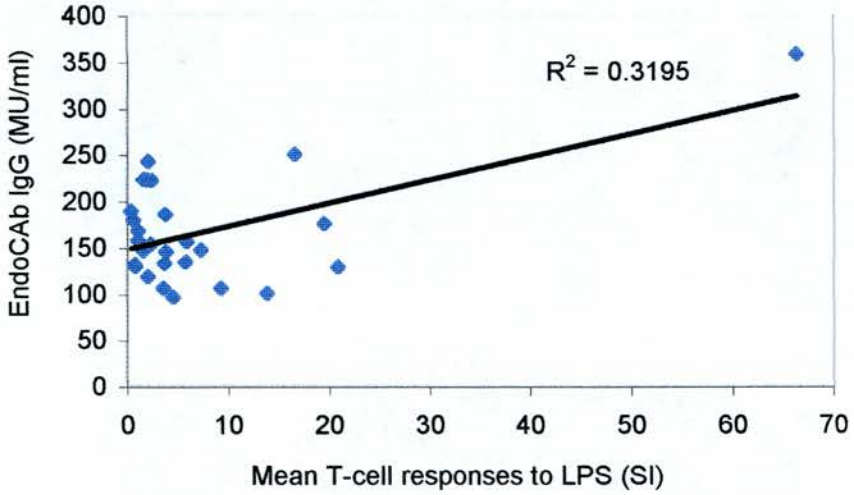


Figure 5.6a: Patient anti-core LPS IgG antibodies vs overall T-cell response to LPS

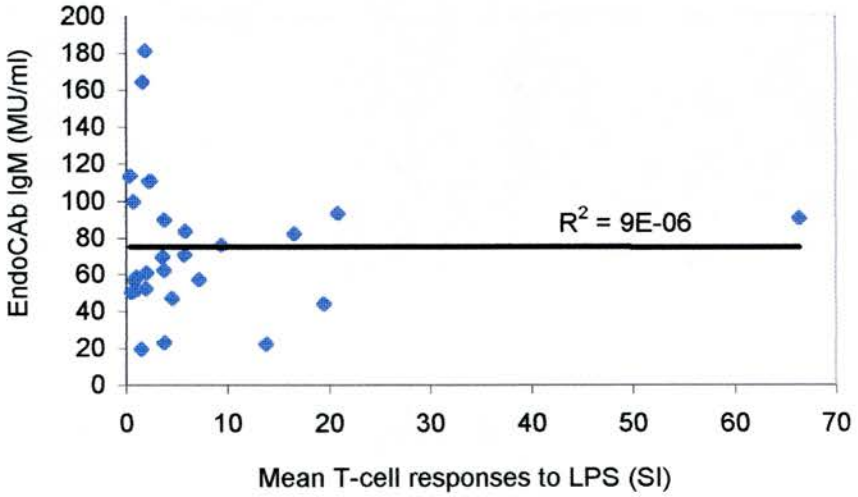


Figure 5.6b: Patient anti-core LPS IgM antibodies vs overall T-cell response to LPS

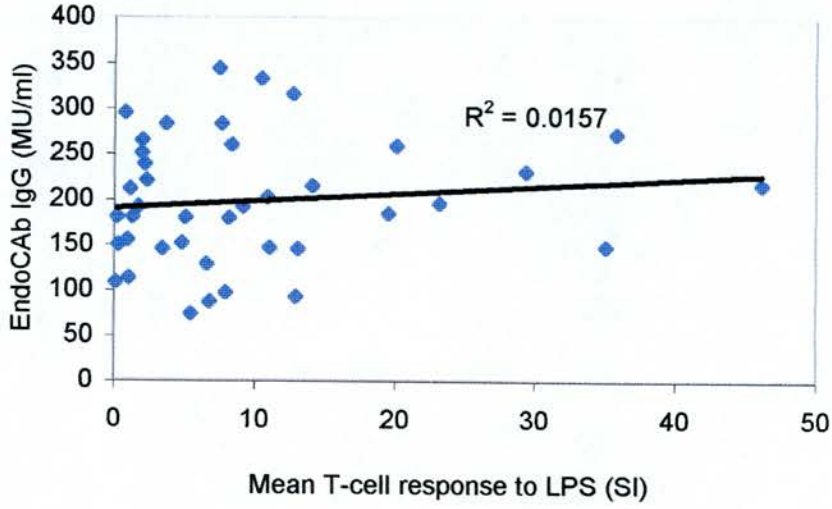


Figure 5.6c: Control anti-core LPS IgG antibodies vs overall T-cell response to LPS

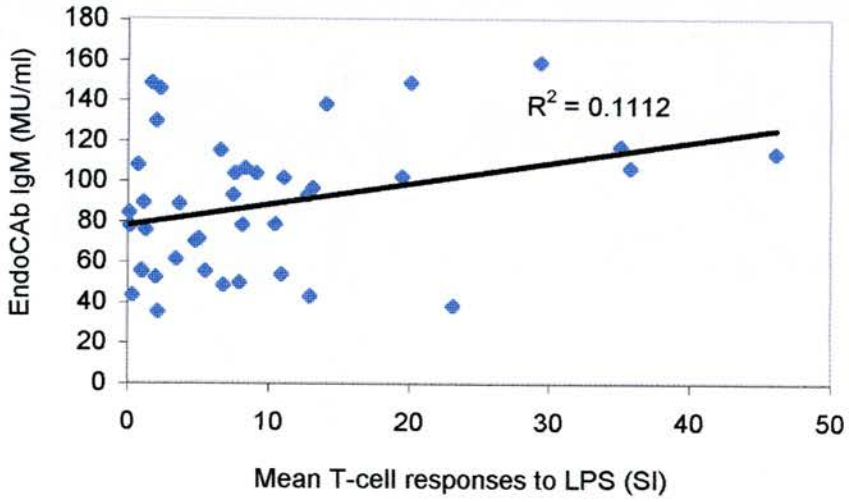


Figure 5.6d: Control anti-core LPS IgM antibodies vs overall T-cell response to LPS

5.3 Discussion

5.3.1 Why investigate this patient group?

The aim of the current study was to evaluate the role of PBMC proliferative and antibody responses to LPS in endotoxin-related disease. For this reason, patients admitted to hospital suffering from abdominal inflammation arising from an infective source were enrolled. These entry criteria led to the recruitment of diagnoses comprising appendicitis, cholecystitis, diverticulitis, Crohns' disease and pancreatitis – all of which are thought to involve either or both of Gram-negative infection and endotoxaemia (Prof. K. Fearon, personal communication). As each of these ailments are associated with different initiating pathologies, time courses and severities of disease, the following analysis of the results has been considered in terms of the different disease subgroups. Additionally, since the cases of appendicitis, cholecystitis and diverticulitis all relate to inflammation of a lumenal organ likely caused by Gram-negative infection, data from these patients have been collated into a further grouping (A+C+D) for analysis.

5.3.2 Patient and control demographic and clinical data

Healthy volunteers were chosen to match the age spread of the patient group as closely as possible. However, the slight difference of median age between the two groups (32 for controls vs 39 for patients) is unlikely to affect analysis of proliferative responses as there was seen to be no correlation between age and proliferative response to LPS (data not shown). This finding is surprising in itself as a clear trend was observed correlating increased age with reduced proliferative responsiveness to ConA in 12 healthy controls (data not shown). This may indicate that certain components of the human immune system are less affected by the human ageing process than others.

5.3.3 Patient clinical scores

The SIRS and SOFA scores of the various patient subgroups are seen to be not significantly different from the median scores exhibited by the whole group. This is almost certainly due to the low patient numbers present in the current study as there is a clear difference in the severity of disease between the different diagnoses. For example, while cholecystitis patients typically present with mild disease, pancreatitis patients often present with more severe symptoms (Prof. K. Fearon, personal communication).

A similar picture is seen when considering the lengths of stay of the various patient subgroups. Only the appendicitis cases are revealed to have significantly reduced hospital stay when compared with the mean stay for the whole group (Table 5.4), reflecting the acute nature of this condition. With greater patient numbers, it is almost certain that the other patient subgroups would show significantly longer or shorter lengths of stay in hospital.

5.3.4 Patient and control proliferative responses

When considering overall proliferative responsiveness to LPS, the entire patient group shows a median score approximately half that of the healthy controls. Further, while 50% of controls were considered responders, only 26% of patients were. These results are not significantly different however ($p=0.15$), once again as a result of the small sample number of the current study.

Of more interest, however, are the diffences in overall LPS responsiveness of the different patient subgroups. The appendicitis and pancreatitis patient subgroups in particular show a trend towards reduced responsiveness to LPS (Table 5.6). The cholecystitis group, on the other hand, shows a median overall responsiveness to LPS greater than that of the control group and therefore stands out from the rest of the patient group in this respect. This may reflect the

mildness of disease occuring in the cholecystitis patients, as their immune systems may not be as suppressed as the more severely ill patients such as the appendicitis and pancreatitis patients. This picture is largely similar when considering patient best responses to any LPS (Table 5.7).

Of patient responses to the individual LPS, the first thing to note is that overall responses of the whole patient group are reduced in response to every LPS tested when compared with the healthy controls (Tables 5.8-5.13). Interestingly, however, the strongest reduction in responses occurs with *E.coli* R1 LPS (5.6 vs 1.8 median SI, p=0.06) and *K.pneumoniae* LPS (6.0 vs 3.8). This may reflect a defecit of T-cells responsive to the core epitopes of these LPS in these patients, perhaps as a result of gut mediated tolerance towards particular organisms in these individuals. Further work will be required to investigate the mechanism responsible for this clonal deletion.

Of secondary interest is the fact that the appendicitis and pancreatitis patient subgroups are once again seen to suffer the greatest reductions in responsiveness to these LPS, while the cholecystitis group has a higher response than the control group to all LPS except *E.coli* R1, further suggesting a strong role for the R1 core type of *E.coli* LPS in the modulation of the immune response in these diseases (Tables 5.8-5.13).

Also of interest is the fact that the three components of the A+C+D cohort (appendicitis, cholecystitis and diverticulitis) show quite different responses to each LPS, despite the fact that they originate from a similar pathology. In general, appendicitis patients present the most blunted responses, while diverticulitis patients show less blunted responses and the responses of cholecystitis patients are either only mildly blunted or are enhanced when compared to the median control response (Tables 5.8-13). This suggests that

the progression or severity of the disease may have a significant impact on the pattern of resulting immunocompromise. In particular, the timecourse may have significance, as the length of time between onset of symptoms and admission to hospital is quite different between these subgroups. The course of appendicitis, for example, is typically very rapid, with patients being admitted to hospital sometimes within 24 hours of initial symptoms. Diverticulitis and cholecystitis, on the other hand, may progress more slowly with the inflammatory response proceeding at a similarly reduced rate. Thus, it may be that either the severity of the initial insult in the appendicitis patients is larger than that seen in the other groups, leading to stronger immunosuppression. Or it may be that the length of time between initial insult and admission to hospital allows some restoration of an earlier, more pronounced immunosuppression in the diverticulitis and cholecystitis patients. Further work will be required to answer this question.

Proliferative responses to the Gram-positive lipid lipoteichoic acid are, on the other hand, not nearly as markedly reduced in the patient group as those towards LPS (Table 5.14). The strong immune responses of the cholecystitis subgroup are once again highlighted by the significantly stronger response to *S.aureus* LTA than controls (median SI 21.6 vs 2.6). There are two possible explanations for this finding. The first is that an expanded memory T-cell pool reactive for LTA may exist in cholecystitis patients, implying that the course of their ailment has allowed them time to generate a significant cellular response to a Gram-positive infection. The second is that some feature of the pathology of cholecystitis produces an immunological priming effect that leads to a more generalised enhancement of T-cell capacity to respond to all antigens. Such an effect has been shown to be induced by the cytokine IFN-γ, itself secreted by LPS reactive T-cells (Mossman et al 1989, Morel et al 1998), though the mechanism responsible for the priming in this case remains to be determined.

Responses to the T-cell mitogen ConA show a much clearer difference between patients and controls. While only two ConA scores from within the 27 patient subgroup are available for comparison with controls at this time, 4 remaining patients with incomplete data sets show similarly blunted responses to ConA (data not shown), suggesting that these two results are statistically relevant. Indeed, comparison of these 2 ConA scores alone with controls demonstrates a highly significant ($p=0.036$) reduction in capacity to respond to ConA (control SI 88.3 vs patient SI 1.0). An explanation for the severity of the hyporesponsiveness of these patients to ConA may be found in the kinetics of proliferation in response to these antigens (Figure 4.21). ConA responses were measured at 3 days, while responses to LPS and LTA were measured at 7 days. Thus, it is possible that the agent responsible for the immunosuppression present in these patients continues to affect PBMC proliferative capacity over a 3 day incubation *in vitro*, while the effect is lost by the seven day timepoint at which LPS responses are measured. Further experiments, including for example challenge of cells in heterologous serum, would be required to determine what this factor is.

The findings of the present study are not the first observation of PBMC hyporeactivity in septic patients. Blunted responses of PBMC have been reported by previous authors examining similar patient groups. For example, PBMC of septic patients have been shown to exhibit significantly decreased IL-2 production in response to PHA stimulation (Wood et al 1984). Monocytes from similar patients have been shown to have a diminished capacity to release TNF-α, IL-1α, IL-1β, IL-6, IL-10 and IL-12 in response to LPS (Ertel et al 1997, Munoz et al 1991, Sekatrian et al 1994, Van Deuren et al 1994) and human volunteers injected with endotoxin show significantly reduced capacity to subsequently produce IL-1, TNF-α and IL-6 in response to various activating factors (Granowitz et al 1993). Severely blunted T-cell responses to activating

factors have been described in patients with post-operative sepsis due to intra-abdominal infection (Heidecke et al 1999). Similarly, animal models of intra-abdominal infection show temporary functional defects in B- and T-cells consistent with those seen in patients with peritonitis (Gough et al 1992, Martineau et al 2000).

Studies such as these and the present study may be interpreted in two different ways. On the one hand, it is possible that the immunosuppression observed is occuring as a result of the systemic response to infection. On the other hand, it is possible that immunological hyporesponsiveness present in the general population is a strong risk factor for the development of sepsis and is therefore present in a high percentage of sepsis patients before the onset of disease. Evidence to support both hypotheses exists.

In support of the former is the observation that caecal ligation and puncture of mice results in a systemic immunosuppression that eventually reverts to normal (Gough et al 1992). Similarly, rats inoculated intra-abdominally with bacteria show temporary functional defects in both B and T-cells (Martineau et al 2000). Immunosuppressive cytokines are seen to be released rapidly in response to both injury (Neidhardt et al 1997, O'Sullivan et al 1995) and minor surgery (Brune et al 1999, Hensler 1997) in humans and the long lived cytokine IL-6 released in response to infection in humans which has long been considered a pro-inflammatory cytokine is now realised to express anti-inflammatory properties (Xing et al 1998). Thus, the combined stresses of infection and inflammation in sepsis patients may result in the downregulation of the immune response observed.

In support of the latter hypothesis, however, are the many reports of a strong correlation existing between immunological hyporeactivity and poor outcome

following surgery. Patients displaying an immunologically hyporeactive state on admission before surgery or any episode of sepsis (so called 'walk-in anergic' patients) are seen to be at considerably more risk from poor outcome when compared to patients exhibiting normal immune function on admission (Christou et al 1984, Christou et al 1989, Johnson et al 1979, Adami et al 1980, Christou et al 1983). In particular, measurements of the delayed-type hypersensitivity (DTH) reaction in response to five common recall antigens have shown that anergic patients are much more likely to suffer an episode of sepsis or poor outcome (Christou et al 1984 & 1989). Low pre-operative proliferative responses of PBMC to PHA and ConA were also seen to correlate with post surgical episodes of sepsis and adverse outcome in thoracic esophagectomy patients (Takagi et al 2001).

This hypothesis is complemeted by the hypothesis of Munford and Pugin that the immunological hyporeactivity seen in sepsis patients may result as a natural reaction of the body to stress (Munford and Pugin 2001a and 2001b). They cite evidence that stress factors as diverse as major injury, strenuous exercise, cold exposure and even psychological stress are sufficient to induce stress hormones such as catecholamines which in turn have been shown capable of suppressing the activity of immune cells (Jones et al 1989), inhibiting production of TNF-α (Severn et al 1992) and promoting a state favouring production of the anti-inflammatory cytokine IL-10 (Suberville et al 1996, van der Poll et al 1996). While this response has obviously evolved to afford protection to the host in some way, (it may promote better focussing of immune resources to a localised point of infection), they point out that the associated systemic immunosuppression may also lead to secondary infection from viruses, bacteria or fungi.

The hypothesis that immunological status may rise and fall in health is also lent support by the findings from the current study, as summarised in Figure 4.27. This examination of the overall PBMC responsiveness to LPS of healthy volunteers clearly shows that this measure of immune status rises and falls with time. Indeed, it is seen that at least one individual (donor 1) makes a clear transition from responder status to non-responder status. Further, 5 of 6 patients assessed for post-recovery ability to demonstrate a proliferative response to LPS were seen to demonstrate improved immune function (Table 5.16), suggesting that the immunosuppression observed in this patient group may be quite transient.

Thus the question remains of whether the immunological hyporesponsiveness observed in the current study is initiated before or during an episode of sepsis. Unfortunately, this can only be answered from the testing of patients responsiveness to LPS at regular timepoints before, during and after an episode of sepsis. As this is obviously an impractical proposition, animal models of these diseases must be examined, though it should be pointed out that the responses of mice (which are often used to simulate sepsis related conditions – Spinas et al 1992, Gough et al 1992) to endotoxin are quite different from those of humans (Martich et al 1993, Alexander et al 2001), and caution should therefore be urged in the interpretation of such studies.

Nevertheless, regardless of when the immunosuppression occurs, the question remains of which mechanisms are responsible for its initation and perpetuation. On the one hand, it is possible that these T-cells have been rendered anergic via a mechanism that is retained cellularly. Alternatively, since autologous plasma has been used to supplement the medium in all cultures in the current study, it is possible that the cells are in fact capable of activation, but their responses are blunted due to the activity of immunomodulating factors present

in the plasma. Evidence certainly exists that stress hormones and certain cytokines can have immunosuppressive effects on diverse cell types *in vitro* (Randow et al 1995, Munford and Pugin 2001a and 2001b, Opal et al 1998). In particular, the observation that the responsiveness of neutrophils to endotoxin can be restored in septic patients to near normal levels if the patients plasma is treated extracorporeally provides strong support for this hypothesis (Tetta et al 2002). As this method of plasmafiltration has been shown to remove (by adsorption) multiple cytokines, activated complement components and lipid mediators from patient plasma (Tetta et al 2002), the study therefore lends support to the attractive hypothesis that alteration of cytokine concentrations in immunosuppressed patients may be able to restore immunological competence.

As for what possible role LPS-reactive T-cells have, the original hypothesis that they may help in assisting with antibody formation is not made any clearer by the present study. Levels of anti-core IgM antibodies do not correlate with PBMC responses to LPS in either the patient group (R^2=0.000) or in the control group (R^2=0.111). This might be expected as IgM responses are typically considered to be T-cell independent (Stavnezer 1996). However, while levels of EndoCAb IgG strongly correlated with overall proliferative responses to LPS in the patient group (R^2=0.319, $p<0.01$), this trend was completely absent in the control group (R^2=0.016). One possible explanation for this paradox may be that there is no correlation in health, but patients exposed to LPS suffer antibody depletion and concurrent T-cell immunosuppression, thereby resulting in the positive correlation seen.

Instead, the role of these T-cells may lie in their production of cytokine. It is clear that on ligation with their ligand, they do not release type II cytokines such as IL-4, IL-5 or IL-10 (Mattern et al 1994), but produce significant amounts of the type I cytokine IFN-γ (Mattern et al 1999). This cytokine is seen to be

capable of inhibiting the activation of Th2-cells and stimulating cell-mediated immunity (Mossman et al 1989, Morel et al 1998). Interestingly, many studies have observed this cytokine to be blunted during the phase of immunological hyporeactivity often seen in patients following an episode of sepsis (Cavaillon et al 2001, Oberholzer et al 2002, Berguer et al 1999, Naldini et al 1998, Brune et al 1999, O'Sullivan et al 1995). Thus, the role of these T-cells may simply be to address this imbalance by secreting this cytokine in sepsis, infection or endotoxaemia. A lack of LPS-reactive T-cells in patients may therefore predispose them to overzealous immunosuppression and resultant poor outcome.

5.3.5 Patient and control endogenous core antibody responses to LPS

Antibodies directed against the core epitopes of LPS have been shown to offer the best cross reactivity with a wide range of LPS (Poxton 1995, Barclay 1990, Chedid 1968), and possibly also cross protection against infection with heterologous strains of bacteria (Braude et al 1972, McCabe et al 1972, Cross et al 1988), as discussed in greater detail in the Introduction (section 1.3).

Levels of antibodies to the core structures of LPS have also been seen to correlate with outcome in studies of a number of patient groups. For example, anti-core antibodies were seen to be depleted during episodes of endotoxaemia, then restored in 2 of 3 patients studied with septic shock (Barclay et al 1989). Studies of anti-core antibodies in the sera of pre- and post-surgery patients have also revealed a correlation between high endogenous levels of anti-core LPS antibodies and protection from adverse post-operative outcome (Freeman et al 1985, Bennett-Guerrero et al 1997, Hamilton-Davies et al 1997, Bennett-Guerrero et al 2001). Further, non-survivors of intra-abdominal sepsis were seen to have depressed levels of IgG anti-endotoxin (Wakefield et al 1998), as were non-survivors of intensive care (Goldie et al 1995). Falling

titres of IgG anti-core LPS antibodies have also been reported to correlate with the development of multiple organ failure in acute pancreatitis (Windsor et al 1993).

While no correlation was observed between patient antibody titres and patient length of stay in the present study (Figure 5.3), IgG anti-core LPS antibodies are significantly lower in patients than in healthy controls (median titre 154 vs 192 MU, $p<0.05$, Table 5.17). A lower level of IgM anti-core LPS antibodies is also seen in the patient group when compared to controls, though this is not a significant difference (median titre 69 vs 89 MU, Table 5.18). The small differences in antibody levels observed between the different patient subgroups are not statistically significant in the present study, though a much larger study may provide useful insights.

In general though, the results of this study appear to agree with the previous studies listed above. However, caution must be urged in the interpretation of these data as at least two possible interpretations are possible from these findings.

The first is that LPS released during the disease processes in the patient group in this study is depleting LPS-specific antibody from the serum. However, mindful of the recent observations of the critical role of immunosuppression in these conditions, it is also possible that the depletion of anti-LPS antibodies are merely symptomatic of falling IgG titres in the more general sense. Thus, low levels of humoral immunity may also be a risk factor in the initiation or progression of these infections, thereby explaining the increased frequency of depleted titres in our study sample. Certainly, anti-LPS antibody levels can vary somewhat in healthy controls, varying from 74 to 345 MU in the forty controls tested here (Table 5.17), and an even wider range has been reported in studies

of larger numbers of healthy donors (Barclay 1990). Similarly, the study of Goldie *et al* (1995) point out that their measurements of both IgG and IgM EndoCAb levels are highly significantly correlated (IgG, $r=0.5$, $p<0.001$; IgM, $r=0.65$, $p<0.001$) to plasma levels of whole antibody as measured by the standard hospital method of laser nephelometry. Further experiments measuring the whole IgG and IgM content of the patients plasma samples of the current study will be required to shed some light on this matter.

5.3.6 Patient and control antibody responses to individual LPS core types

Looking at the order of the peak titres listed in Figure 5.1, it is tempting to speculate that an order of frequency exists for antibodies specific for individual LPS core types in the general population. In particular, it appears that the most common antibodies exist towards *B.fragilis* core, then *E.coli* R1 core, then *B.fragilis* O-polysaccharide, then *K.aerogenes* core LPS etc (as listed in legend at right). However, this conclusion cannot be made from this experiment alone. The reason for this is that the serum used to calibrate the experiment was a high EndoCAb titre serum from one individual, and all antibodies are measured relative to that particular individuals profile of response. Thus, only one main point may be extracted from consideration of the profiles presented in Figure 5.1 – antibodies present in the serum of each patient and control seem to show a similar profile of response between donors, with each individual showing similar proportions of antibody specific for each LPS. Only occasionally are individuals seen to deviate greatly from this pattern, for example, the strong antibody response to *S.typhimurium* LPS shown by control number 4 and patient number 7. These occasional 'spikes' may reflect recent challenge with an organism expressing similar LPS core structures to *S.typhimurium* LPS which has resulted in these specific high titres. Examination of T-cell responses to proteins specific to these organisms may be able to add further support to this hypothesis.

5.3.7 Toll-receptor 4 mutations in patients and controls

In the present study, 4 of the 27 patients studied were found to be heterozygous for the co-segregating mutations Asp299Gly and Thr399Ile in the TLR4 gene. This frequency is not significantly different from the frequency of the mutation found in 40 healthy controls, and indeed in the entire original group of 40 patients, the frequency is also 4 out of 40 patients.

Presence of the mutation had no significant effect on the length of stay of patient carriers (Table 5.24), nor on the strength of proliferative or antibody responses to LPS (Tables 5.21 & 5.22). Further, presence of the mutation did not adversely affect SIRS or SOFA clinical scores (Table 5.24).

These findings are consistent with the earlier finding of this study that monocytes from individuals heterozygous for both mutations show absolutely no impairment of response to LPS (Figure 3.12). The numbers of patients used in this study are obviously too low for any statistically significant findings to emerge, though they certainly do not add support to the recent study of the mutation in sepsis patients of Lorenz *et al* (2002a). That study of 91 septic shock patients compared the frequency of the Asp299Gly mutation with that of 73 healthy controls and found the mutation exclusively in the patient group (Lorenz et al 2002a). The group also noted a higher prevalence of Gram-negative infections occurred in the patients carrying the Asp299Gly mutation. However, the frequency of the mutation in the patient group was not compared with the frequency of the mutation in the wider population – data which was both created in and available to the same laboratory (Arbour et al 2000, Lorenz et al 2002b), raising some doubts as to the validity of their statistical tests. However, it remains possible that individuals homozygous for the mutation have altered responses to LPS that affect the progression of disease in these

pathologies. Only a much larger study will be able to determine whether these mutations confer any protective or adverse effect on sepsis patients.

CHAPTER 6

DISCUSSION

6.1 Is there a role for LPS in sepsis syndrome?

One of the most significant questions we can ask in the context of the current study is simply "Does LPS have a role to play in the pathogenesis of sepsis?" If the answer is yes, then investigations into the role of LPS in sepsis may ultimately lead to valuable new options for therapy. If not, our efforts are best directed at trying to understand and then influence the other pertinent aspects of sepsis syndrome. In order to answer this question, there are three main lines of evidence which we must look towards to make our assessment of the viability of this hypothesis.

The first of these is the observation that administration of LPS or even purified lipid A to humans is capable of inducing all of the clinical features characteristic of sepsis syndrome (Michie et al 1998), shock (Suffredini et al 1989) and even multiple organ failure (Taveira daSilva et al 1993, Martich et al 1993). Countering this, however, is the observation that sepsis and resultant episodes may be initiated from no infective source or other obvious source of LPS whatsoever. Cases of severe trauma, for example, are often seen to result in a systemic inflammatory response and clinical course very similar to that of sepsis syndrome, with no obvious sign of infection (Faist et al 1987, Abraham et al 1985, O'Sullivan et al 1995, Baker et al 1988). This may perhaps be explained by the observation that products of host tissue damage resulting from these injuries such as host heat shock protein 60 and fibronectin extra domain A have been shown to be capable of signalling via TLR4 and inducing expression of exactly the same genes upregulated on LPS recognition (Okamura et al 2001, Vabulas et al 2002). Thus, it is possible that a systemic inflammatory response could be initiated under certain circumstances in the absence of any bacterial products. However, the argument remains that LPS may still have a role to play in these cases of sepsis too. Several studies have demonstrated that the gut is

extremely sensitive to loss of splanchnic blood flow, and cite evidence that gut hypoperfusion can result in translocation of both bacteria and LPS into the systemic circulation of the host (Saadia et al 1990, Haglund et al 1975, Chiu et al 1970, Baker et al 1988, Bynum et al 1984, Haglund et al 1975). Thus it remains possible that the ill effects seen in the patient groups exhibiting no obvious infective source could yet be attributable to LPS, in these cases derived from gut commensals as a result of injury to the gut mucosal barrier.

The second line of evidence comes from the observation of the presence of LPS in the plasma of many patients with sepsis syndrome (Goldie et al 1995, Opal et al 1999, Casey et al 1993, Danner et al 1991, Barclay et al 1989, Dofferhoff et al 1992). However, the question remains, of course, of whether this plasma-borne LPS is responsible for the initiation of the septic state, or whether it is simply a marker for gut barrier break down caused by systemic inflammation brought on by some other (non-LPS mediated) pathology.

Finally, there is the evidence arising from the studies of antibodies specific for LPS in human plasma. Associations have been observed, for example, between low pre-operative serum anti-endotoxin antibody levels and adverse outcome following surgery (Bennett-Guerrero et al 1997, Freeman et al 1985, Gould et al 1989, Hamilton-Davies et al 1997, Nys et al 1993). Low or falling levels of anti-endotoxin have also been shown to correlate with adverse outcome in patients diagnosed with abdominal sepsis (Wakefield et al 1998) and pancreatitis (Windsor et al 1993). However, most of these studies have not correlated their EndoCAb findings with whole IgG levels, and it remains possible that low levels of antibodies specific for LPS merely reflect a more generalised depletion of all immunoglobulin in the patient. Indeed, one study has shown an almost absolute correlation ($p<0.001$) for levels of LPS-specific antibodies and whole IgG and IgM levels in septic patients (Goldie et al 1995). Thus it remains possible that

low titres of anti-LPS antibodies may simply reflect a more general compromise of the immune system.

These lines of evidence suggest that LPS may have a role to play both in the initiation and the pathology of sepsis and its related sequelae, though the hypothesis has yet to be proven. Only extensive sampling of patients blood before septic episodes or effective neutralisation of LPS in the plasma of pre-septic patients will be able to provide more substantial evidence for the role of LPS in these conditions.

6.2 Antibody treatments for sepsis - can they work?

The history of research into the potential of LPS-specific antibodies in the treatment of disease has something of a chequered past at best (discussed in detail in section 1.3). Certainly evidence exists that antibodies specific for the core structures of LPS become depleted in patients exposed to LPS, and associations have been made between low or falling levels of these antibodies and poor outcome in patient groups (Pollack et al 1983, Nys et al 1993, Freeman et al 1985, Gould et al 1989, Bennett-Guerrero et al 1997). Nevertheless, hard evidence for a beneficial effect of passive transfer of antibodies to either patients or animal models of sepsis is scarce.

Indeed, the most promising reports of such evidence came in the late 1970s with the efforts of McCabe and Ziegler, whose studies appeared to demonstrate that passive transfer of anti-LPS antibodies conferred a protective benefit on rats challenged with live bacteria (McCabe et al 1977, Ziegler et al 1979). However, these findings could not be repeated by later researchers (Baumgartner et al 1990, Baumgartner et al 1991a). Similarly, several human clinical trials of passive transfer of hyperimmune serum have provided initially encouraging results (Ziegler et al 1982, Baumgartner et al 1985, Teng et al

1985), but all have since been heavily criticised in terms of either the interpretation or general methodology of the study (Baumgartner 1994, Baumgartner et al 1987, Baumgartner et al 1991c, Wenzel 1992 – summarised in section 1.3). Further, two large clinical trials of supplementation of the plasma of septic patients with two different monoclonal antibodies purported to bind to LPS (E5 and HA1A) have shown no protective benefit whatsoever and have led to widespread criticism of the methods used in assessment of such antibodies for clinical use (Wenzel et al 1991, Various authors 1991, Baumgartner 1994, Baumgartner et al 1991c).

For the researcher considering the application of anti-LPS antibodies as a therapeutic agent in sepsis, the first question that must be answered is "What led to the failure of these trials?" In essence there are two main possibilities that may explain these failures.

The first is that antibodies specific for LPS have very little role in dealing with either endotoxaemia or disease. However, the study of Burd *et al* (1993) showed that an IgG monoclonal antibody specific for *E.coli* O111 LPS not only enhanced uptake of that LPS by macrophages but also inhibited TNF-α secretion in response to that LPS. The extension of their hypothesis, that IgG-bound LPS is internalised via Fc receptors thereby bypassing the signalling receptors, suggests that high levels of these antibodies in septic patients may serve to reduce the burden of pro-inflammatory cytokine produced in response to a certain amount of LPS. The question has not yet been answered, however, of how significant this effect should be in humans *in vivo*.

The second possibility is that antibodies specific for LPS may well provide benefit, but the binding specificity of the antibodies towards the LPS used in each study was not broad enough to deal with the bacteria or LPS the animals

or patients were exposed to. In support of this idea is the report of a monoclonal antibody (termed WN1-222.5), which is capable of binding to all smooth and most rough LPS from all *E.coli* and *Salmonella* strains so far investigated (Di Padova et al 1993). This antibody has been shown capable of inhibiting release of pro-inflammatory cytokines such as TNF-α and IL-6 from LPS-challenged murine peritoneal macrophages. Of most interest, however, is the demonstration that its administration neutralises the pyrogenic response to LPS in rabbits and protects D-galactosamine-treated mice from lethal challenge with both *E.coli* and *S.abortus-equi*. These data are certainly encouraging, and suggest that broadly cross-reactive antibodies may yet offer therapeutic value to patients at risk of exposure to endotoxin. However, WN1-222.5 has not been shown capable of binding to LPS of *Enterobacter*, *Klebsiella*, *Serratia*, *Proteus* or *Pseudomonas* spp. This suggests that for a monoclonal antibody-based approach to offer significant benefit to patients, it will have to be a cocktail of several monoclonals, each with a specificity as broad as that demonstrated by WN1-222.5. Acting together, they should be designed such that their activities overlap and are able to bind and neutralise the LPS of a much wider range of clinically relevant organisms.

6.3 Prospects of the vaccine

Attempts to boost human anti-LPS antibody titres have so far revolved around the prophylactic transfer of either hyperimmune plasma or monoclonal antibodies to patients (Ziegler et al 1982, McCutchan et al 1983, Baumgartner et al 1985, Teng et al 1985, Calandra et al 1988, Ziegler et al 1991). However, a vaccine-based approach may have several advantages over these therapies. These include low cost and the ability to generate high titre endogenous antibodies in recipients. Further, prophylactic vaccination may be more effective than passive immunisation in challenged patients, as it is clinically difficult to

detect the early signs of endotoxaemia in time to intervene effectively with a specific anti-endotoxin therapy (Cross et al 1994, Opal et al 1995).

The idea of using LPS O-chain or core epitopes as part of a vaccination strategy to induce anti-LPS antibodies has received a great deal of interest (Cryz et al 1987, Barclay et al 1986, Cryz et al 1991, Bhattercharjee et al 1996, McCabe et al 1972, McCabe et al 1977, Bhattacharjee et al 1999). However, in order to avoid problems associated with the inherent toxicity of lipid A, most of these studies have focused on the properties of protein-saccharide conjugates (Cryz et al 1987, Schiff et al 1993, Cryz et al 1991, Bhattacharjee et al 1994, Bhattacharjee et al 1996).

The present study shows that a liposomal vaccine containing complete core LPS of *Escherichia coli* K12, *Escherichia coli* R1, *Bacteroides fragilis* and *Pseudomonas aeruginosa* is capable of being added to human monocytes and inducing no inflammatory response (in terms of TNF-α release, at least) at concentrations up to 100,000 fold higher than the equivalent amount of native LPS. A previous study (Bennett-Guerrero et al 2000) has shown the vaccine capable of providing significant protection from lethality in galactosamine-sensitised mice exposed to lethal doses of *E.coli* O18 LPS and inducing broadly cross-reactive antibody responses in immunised rabbits. However, the concentration of liposomal LPS used to vaccinate rabbits in this study was 16μg/ml – a concentration which this study has shown results in considerable release of TNF-α from human monocytes (Figure 3.4). If minimum release of TNF-α is desired for the safety of vaccine administration, the highest concentration that can be applied to humans in its current form is around 0.1μg/ml (Figure 3.4).

However, while the vaccine has been shown to display low toxicity and an ability to generate a broadly cross reactive antibody response in rabbits, experiments involving animal models of sepsis need to be performed before any idea of how useful the preparation is in the prevention of disease. In particular, challenge of mice with clinically relevant bacteria should be performed, as should murine caecal ligation and puncture (the standard murine model of sepsis) to determine whether vaccinated animals fare better than non-vaccinated ones. Only if studies such as these demonstrate a positive effect should the vaccine continue to human trials in its current form.

6.4 Other potential therapies for sepsis.

The last fifty years has seen an increase in the incidence of sepsis syndrome while outcome has not improved (Wheeler et al 1999, Various authors 1990). Unsurprisingly this has led to some frustration among physicians, keen to apply new therapies to reverse this trend. To this end, more than a dozen clinical trials using antagonists of either the initial stimulators of the pro-inflammatory state or the pro-inflammatory mediators themselves have been attempted in order to try to improve outcome in this patient group (reviewed in sections 1.3 and 1.5). Unfortunately none of these have met with clear success.

These disappointing results suggest that a much more thorough understanding of the pathological processes underlying sepsis syndrome will be required. In particular, acknowledgements of a number of key facts must be borne in mind in these considerations. The first of these is that sepsis is an extremely heterogeneous disease. It may be initiated by a number of primary causes: physical trauma (Faist et al 1987), reperfusion injury (Bone et al 1997), infection (Martineau et al 2000), gut barrier failure (Saadia et al 1990) and possibly even immunocompromise resulting from a wide variety of potential stresses (Munford and Pugin 2001b). The nature and particularly the timecourse of these different

initiators of sepsis may well have some bearing on the underlying processes present in those patients (as evidenced by the quite different immunological profiles of the different patient subgroups examined in the present study).

The next fact that must be considered is that in very general terms, there are two main pathologies that lead to the mortality of septic patients. The first of these is the effect on the host of the systemic inflammation characteristic of sepsis – the problem being in particular the multiple organ failure catalysed by the presence of widespread cytokine-induced coagulation, vasodilation and aggressive proteolysis of endothelial surfaces (Oberholzer et al 1997). The second major cause of mortality in septic patients is the frequently observed phase of immunosuppression, which leaves some patients unable to defend against otherwise non-threatening nosocomial infections (Hensler et al 1997). Any approach to treat septic patients must, therefore, be capable of addressing both of these concerns.

Attempts to stem the damage resulting from systemic inflammation have so far revolved around blockade of the pro-inflammatory cytokines TNF-α (Fisher et al 1993), and IL-1β (Fisher et al 1994). Unfortunately, these large scale studies have failed to demonstrate any improvement in survival of treated patients despite the initial promise of animal studies (Tracey et al 1987, Ziegler et al 1987, Ohlsson et al 1990). It may therefore be the case that these cytokines are required by the host to halt the spread of and ultimately clear the infection underlying the septic episode. With this in mind, a better approach may be to target not the signalling molecules which have a multitude of roles to play in protecting the host from the underlying infection, but the particular mediators causing most organ damage. Protease inhibitors and anti-coagulants in particular may allow some protection of organ vasculature from the deleterious effects of systemic inflammation. For patients at risk of sepsis, such as trauma

patients and those undergoing major surgery, such medications may prove of some use in a prophylactic capacity.

However, it should be noted that such interventions will only be useful within a particular window of opportunity. Patients surviving this phase should be considered at risk of immunological hyporeactivity, and this abnormality should then be targeted for treatment. As discussed in section 1.5, by far the most often reported predictor of favourable outcome in septic patients is a strong immunological status prior to sepsis (Christou et al 1984, Heidecke et al 1999, Christou et al 1989, Johnson et al 1979, Adami et al 1980, Christou et al 1983). This suggests that augmentation of the patients immune system may well provide some benefit in these patients.

Theoretically this may be achieved in a number of ways. It has been suggested by Oberholzer and colleagues, for example, that the excessive lymphocyte apoptosis often observed during sepsis (Hotchkiss et al 1999a & 1999b) may represent a potential target for therapy of this condition (Oberholzer et al 2001). Not only would blockade of such apoptosis lead to higher numbers of circulating lymphocytes, it could also reduce the amount of anti-inflammatory cytokines such as IL-10 released by macrophages in response to phagocytosis of apoptotic bodies (Voll et al 1997).

The present study has shown that T-cell responses to LPS and ConA are blunted in patients suffering from abdominal inflammation arising from an infective source. Therefore it is possible that modulation of the function of T-cells may offer an attractive prospective target for the therapeutic treatment of patients at risk of, or suffering from, sepsis.

The role played by the LPS reactive T-cells examined in the present study has yet to be established, though some interesting points can be made out. The first is that on exposure to LPS these cells secrete the type I cytokine IFN-γ (Mattern et al 1994), a cytokine capable of inhibiting the activation of Th2-cells and stimulating cell-mediated immunity (Mossman et al 1989, Morel et al 1998). As previously discussed, the role of these T-cells may therefore be to balance the excessive production of type II cytokines in the phase of immunological hyporeactivity typically seen during or following sepsis. Interestingly, treatment of sepsis patients with native IFN-γ has been shown to restore not only HLA-DR expression on monocytes but also their ability to secrete IL-6 and TNF-α (Bone et al 1996). Further, administration of IFN-γ has also been shown to result in fortification of host cell-mediated immunity (Reed et al 1988). For this reason, restoration of the activity of these cells may benefit patients.

As for how an increase in the abundance or activity of such cells might be achieved, it is interesting to note that the accessory cell type required for presentation of LPS to these cells is the rare $CD34^+$ blood stem cell (Mattern et al 1998). T-cells from non-responding donors were shown capable of responding when supplemented with $CD34^+$ cells (Mattern et al 1998), so it may be possible that supplementation of patients blood with $CD34^+$ cell populations may provide the LPS-reactive T-cell response required. This may be achieved by prophylactic drug treatment, or, as the interaction between these cells and T-cells is not dependent on MHC-molecules, donor cells could be sourced from either cord blood (in which the $CD34^+$ population is particularly abundant) or from healthy responding donors.

Of further interest is the observation that the liposomal LPS vaccine described in this study is capable of inducing expansion of LPS reactive T-cells *in vitro* at concentrations lower than that required to stimulate release of pro-inflammatory

cytokines from human monocytes (sections 3.4 and 4.5). This suggests that a proliferative response can be generated to liposomal LPS at concentrations lower than the threshold required for detection by the innate immune response, and by extrapolation, TLR-4. This observation provides further weight to the hypothesis that the proliferation observed is not mediated via TLR-4 or other innate mechanisms, but may be due instead to presentation of LPS to T-cells, and resultant clonal expansion. Therefore, in addition to inducing high titres of LPS reactive antibodies, the vaccine may also provide a protective benefit in recipients from the resulting expansion of LPS-reactive T-cells.

6.5 Future work

Further investigation of the potential of the proposed liposomal LPS vaccine will require evaluation of its efficacy in animal models of sepsis. In particular, challenge of immunised mice with a wide range of clinically relevant bacteria should be performed, as should the effect of the vaccine on outcome in caecal ligation and puncture experiments. The low toxicity of the formulation demonstrated in this study and others (Bennett-Guerrero et al 2000) already lends support for its use in phase I trials in human volunteers.

The high frequency of the Asp299Gly and Thr399Ile alleles of the Toll-like receptor 4 gene in the European population also merits further investigation. The present study has shown that heterozygous expression of the mutant receptor has no effect on LPS signalling. TLR4 deficient cell lines (such as 293 cells) should therefore be transfected with constructs coding for each mutation and LPS signalling investigated. Additionally, challenge of mutant receptors with other TLR4 agonists, such as heat shock protein and respiratory syncytial virus F-protein should be investigated, as the high frequency of these mutations in the general population may yet indicate that some advantage exists in the recognition of these alternative TLR4 ligands.

The role of LPS reactive T-cells in the wider immune response to pathogens has yet to be determined. As this study has shown the murine model to be inappropriate for investigation of these cells, studies of human cellular preparations must continue. In particular, despite strong circumstantial evidence from this and other studies (Mattern et al 1994, 1998, 1999), cell surface molecule-mediated presentation of LPS to T-cells has not yet been confirmed. Co-immunoprecipitation of radiolabelled LPS with such a presentation molecule may yet be achieved should monoclonal antibodies be found capable of functioning in this assay.

Lastly, the role of immunological hyporeactivity in healthy patients and in septic patients requires investigation. In particular, as it has been shown in this study that the immunological status of healthy individuals varies considerably with time, the responses of cells from anergic and immunocompetent donors to various stimuli should be compared. The capacity of the serum from anergic patients to modulate the response of cells from immunocompetent individuals should be investigated, as this will determine whether such immunosuppression can be reversed by alteration of factors present in the serum. If this is the case, attempts should then be made to restore the function of anergic cells *in vitro*. These should begin with an investigation into the efficacy of blockade of type II and anti-inflammatory cytokines, followed by treatment with drugs blocking the immunosuppressive effects of stress hormones. Treatment of anergic cells with other cytokines should be perfomed, as should supplementation with $CD34^+$ cells, as the capacity of these cells to modulate the wider immune response has yet to be determined.

REFERENCES

Abraham E, Regan RF. The effects of hemorrhage and trauma on interleukin-2 production. *Arch. Surg.* **120**:1341-1344 (1985)

Adami GF, Terrizi A, Vita M. The assessment of skin tests in surgery. *Surgery in Italy* **10**:302-307 (1980)

Akashi S, Ogata H, Kirikae F, Kirikae T, Kawasaki K, Nishijima M, Shimazu R, Nagai Y, Fukudome K, Kimoto M, Miyake K. Regulatory roles for CD14 and phosphatidylinositol in the signalling via Toll-like receptor 4 - MD-2. *Biochem. Biophys. Res. Commun.* **268**:172-177 (2000)

Akashi S, Nagai Y, Ogata H, Oikawa M, Fukase K, Kusumoto S, Kawasaki K, Nishijima M, Hayashi S, Kimoto M, Miyake K. Human MD-2 confers on mouse Toll-like receptor 4 species specific lipopolysaccharide recognition. *Int. Immunol.* **13**:1595-1599 (2001)

Alexander C, Rietschel ET. Bacterial lipopolysaccharides and innate immunity *J. Endotoxin Res.* **7**(3):167-202 (2001)

American College of Chest Physicians. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis *Crit. Care. Med.* **20**:864-874 (1992)

Andersson J, Coutinho A, Melchers F. Frequencies of mitogen reactive B cells in the mouse. Distribution in different lymphoid organs from different inbred strains of mice at different ages. *J. Exp. Med.* **145**:1511 (1977)

Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, Frees K, Watt JL, Schwartz DA. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat. Genet.* **25**:187-191 (2000)

Bahrami S, Schlang G, Yao Y, Redl H. Significance of translocation/endotoxin in the developments of systemic sepsis following trauma and/or haemorrhage. In "From Genes to Therapy" pp197-208 Wilwy-Liss, Inc (1995)

Baker JW, Deitch EA, Li M, Berg RD, Specian RD. Hemorrhagic shock induces bacterial translocation from the gut *J. Trauma* **28**:896-906 (1988)

Balk SP, Bleicher P, Terhorst C. Isolation and expression of cDNA encoding the murine homologues of CD1. *J. Immunol.* **146**:768-774 (1991)

Balk SP, Burke S, Polischuk JE, Frantz ME, Yang L, Porcelli S, Colgan SP, Blumberg RS. β2-microglobulin independent MHC class 1b molecule expressed by human intestinal epithelium *Science* **265**:259-262 (1994)

Barclay GR, Yap PL, McClelland DB, Jones RJ, Roe EA, McCann MC, Micklem LR, James K. Characterisation of mouse monoclonal antibodies produced by immunisation with a single serotype component of a polyvalent Pseudomonas aeruginosa vaccine. *J. Med. Microbiol.* **21**:87-90 (1986)

Barclay GR, Scott BB. Serological relationships between Escherichia coli and Salmonella smooth and rough mutant lipopolysaccharides as revealed by enzyme linked immunosorbent assay for human immunoglobulin G anti-endotoxin antibodies. *Infect. Immun.* **55**:2706-2714 (1987)

Barclay GR, Scott BB, Wright IH, Rogers PN, Smith DGE, Poxton IR. Changes in anti-endotoxin IgG antibody and endotoxaemia in three cases of Gram negative septic shock. *Circ. Shock* **29**:93-106 (1989)

Barclay GR. Antibodies to endotoxin in health and disease. *Rev. Med. Micro.* **1**:133-142 (1990)

Bauer A, Rutenfranz I, Kirchner H. Processing requirements for T-cell activation by Mycoplasma arthritidis-derived macrophages *Eur. J. Immunol.* **18**:2109 (1988)

Baumgartner JD, Glauser MP, McCutchan JA, Ziegler EJ, van Melle G, Klauber MR, Vogt M, Muehlen E, Luethy R, Chiolero R. Prevention of Gram-negative shock and death in surgical patients by prophylactic antibody to endotoxin core glycolipid. *Lancet* **2**:59-63 (1985)

Baumgartner JD, Glauser MP. Controversies in the use of passive immunotherapy for bacterial infections in the critically ill patient. *Rev. Infect. Dis.* **9**:194-205 (1987)

Baumgartner JD, Heumann D, Gerain J, Weinbreck P, Grau GE, Glauser MP. Association between protective efficacy of anti-lipopolysaccharide (LPS) antibodies and suppression of LPS-induced tumor necrosis factor and interleukin 6. Comparison of O side chain specific antibodies with core LPS antibodies *J. Exp. Med.* **171**:889-896 (1990)

Baumgartner JD. Immunotherapy with antibodies to core LPS: a critical appraisal. *Infect. Dis. Clin. North Am.* **5**:915-927 (1991a)

Baumgartner JD, Heumann D, Calandra T, Glauser MP. Antibodies to lipopolysaccharides after immunization of humans with the rough mutant *Escherichia coli* J5. *J. Infect. Dis.* **163**:769-772 (1991b)

Baumgartner JD, Heumann D, Glauser MP. The HA-1A monoclonal antibody for Gram-negative sepsis. Letter to the editor. *N. Engl. J. Med.* **325**:281-282 (1991c)

Baumgartner JD, Glauser MP, McCutchan JA. Management of septic shock: new approaches. In: Remington JS, Swartz MN *eds.* Current clinical topics in infectious diseases, Vol 12. Cambridge, Blackwell scientific publications inc. 165-187 (1992)

Baumgartner JD. Anti-Endotoxin Antibodies as treatment for sepsis - lessons to be learnt. *Reviews in Med. Micro.* **5**:183-90 (1994)

Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB. Recognition of a lipid antigen by CD-1 restricted $\alpha\beta^+$ T-cells *Nature* **372**:691-694 (1994)

Beldjford K, Beldjford C, MacIntyre E, Even P, Sigaux F. Peripheral selection of Vδ1+ cells with restricted T-cell receptor delta gene junctional repertoire in the peripheral blood of healthy donors. *J. Exp. Med.* **178**:121-127 (1993)

Bendelac A, Rivera MN, Park SH, Roark JH. Mouse CD1 specific NK1 T-cells; Development, specificity and function. *Ann. Rev. Immunol.* **15**:535-562 (1997)

Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. In vivo identification of glycolipid antigen specific T-cells using fluorescent CD1d tetramers. *J. Exp. Med.* **191**:1895-1903 (2000)

Bennett-Guerrero E, Ayuso L, Hamilton-Davies C, White WD, Barclay GR, Smith PK, King SA, Muhlbaier LH, Newman MF, Mythen MG. Relationship of preoperative serum anti-endotoxin-core antibody levels and adverse outcomes following cardiac surgery *JAMA* **277**:646-50 (1997)

Bennett-Guerrero E, MacIntosh TJ, Barclay GR, Snyder DS, Gibbs RJ, Mythen MG, Poxton IR. Preparation and preclinical evaluation of a novel liposomal complete-core lipopolysaccharide vaccine *Infect. Immunity* **68**:6202-6208 (2000)

Bennett-Guerrero E, Panah MH, Barclay GR, Bodian CA, Winfree WJ, Andres LA, Reich DL, Mythen MG. Decreased endotoxin immunity is associated with greater mortality and/or prolonged hospitalisation after surgery. *Anesthesiology* **94**:992-998 (2001)

Berguer R, Bravo N, Bowyer M, Egan C, Knolmayer T, Ferrick D. Major surgery suppresses maximal production of helper T-cell type 1 cytokines without potentiating the release of helper T-cell type 2 cytokines *Arch. Surg.* **134**:540-544 (1999)

Bhattacharjee AK, Opal SM, Palardy JE, Drabick JJ, Collins H, Taylor R, Cotton A, Cross AS. Affinity purified E.coli J5 LPS-specific IgG protects neutropenic rats against Gram-negative sepsis. *J. Infect. Dis.* **170**:622-629 (1994)

Bhattacharjee AK, Opal SM, Taylor R, Naso R, Semenuk M, Zollinger WD, Moran EE, Young L, Hammack C, Sadoff JC, Cross AS. A non-covalent complex vaccine prepared with detoxified E.coli J5 LPS and Neisseria meningitidis group B outer membrane protein produces protective antibodies against gram-negative bacteria. *J. Infect. Dis.* **173**:1157-1162 (1996)

Bhattacharjee AK, Cross AS. Vaccines and antibodies in the prevention and treatment of sepsis *Infect. Dis. Clin. North Am.* **13**:355-369 (1999)

Blamey SL, Imrie CW, O'Neill J, Gilmour WH, Carter DC. Prognostic factors in acute pancreatitis. *Gut* **25**:1340-1346 (1984)

Blumberg RS, Terhorst C, Bleicher P, McDermott FV, Allan CH, Landau SB, Trier JS, Balk SP. Expression of a non-polymorphic MHC class I like molecule, CD1d, by human intestinal epithelial cells. *J. Immunol.* **147**:2518-2524 (1991)

Bone RC, Fisher C, Clemmer T, Slotman G, Metz C, Balk R. Sepsis syndrome: valid clinical entity. *Crit. Care Med.* **17**:389-93 (1989)

Bone RC. Sepsis, the sepsis syndrome, multi-organ failure: a plea for comparable definitions *Ann. Intern. Med.* **114**:332-334 (1991a)

Bone RC, Balk RA, Fein AM, Perl TM, Wenzel RP, Reines HD, Quenzer RW, Iberti TJ, Macintyre N, Schein RM. Results of a second double-blind, randomised, controlled trial of antiendotoxin antibody E5 in gram negative sepsis. Abstract 1170 in Program and Abstracts of the 31st Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington DC. American Society for Microbiology, p294 (1991b)

Bone RC. Let's agree on terminology: Definitions for sepsis *Crit. Care Med.* **19**:973-976 (1991)

Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM consensus conference committee. *Chest* **101**:1644-1655 (1992c)

Bone RC. Immunologic dissonance: a continuing evolution in our understanding of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS) *Ann. Intern. Med.* **125**:680-687 (1996)

Bone RC, Grodzin CJ, Balk RA. Sepsis: a new hypothesis for pathogenesis of the disease process *Chest* **121**:235-243 (1997)

Bounos G, Menard D, De Medicis E. Role of pancreatic proteases in the pathogenesis of ischemic enteropathy. *Gastroenterology* **73**:102-108 (1977)

Brade H, Brade L, Nano FE. Chemical and serological investigations on the genus-specific lipopolysaccharide epitope of Chlamydia. *PNAS* **84**:2508 (1987)

Brandtzaeg P, Bjerre A, Ovstebo R, Brusletto B, Joo GB, Kierulf P. Neisseria meningitidis lipopolysaccharides in human pathology *J. Endotoxin Res.* **7**(6):401-420 (2001)

Braude AI, Douglas H. Passive immunisation against the local Schwartzman reaction. *J. Immunol.* **108**:505-512 (1972)

Brune IK, Wilke W, Hensler T, Holzmann B, Siewert JR. Downregulation of T helper type 1 immune response and altered pro-inflammatory and anti-inflammatory T-cell cytokine balance following conventional but not laparoscopic surgery. *Am. J. Surg.* **177**:55-60 (1999)

Bulut Y, Faure E, Thomas L, Equils O, Arditi M. Co-operation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signalling molecules in Toll-like receptor 2 signalling. *J. Immunol.* **167**:987-994 (2001)

Burd RS, Battafarano RJ, Cody CS, Farber MS, Ratz CA, Dunn DL. Anti-endotoxin monoclonal antibodies inhibit secretion of tumor necrosis factor alpha by 2 distinct mechanisms. *Ann. Surg.* **218**:250-261 (1993)

Bynum TE, Gallavan RH, Jacobson ED. The pathophysiology of nonocclusive ischemia. In: Shepherd AP, Granger DN, eds, *Physiology of Intestinal Circulation*. New York Raven Press. 369-376 (1984)

Calabi F, Jarvis JM, Martin LH, Milstein C. Two classes of CD1 genes. *Eur. J. Immunol.* **19**:285-292 (1989)

Calandra T, Glauser MP, Schellekens J, Verhoef J. The Swiss – Dutch J5 immunoglobulin study group. Treatment of Gram-negative septic shock with human IgG antibody to Escherichia coli J5; a prospective, double blind, randomised study. *J. Infect. Dis.* **158**:312-319 (1988)

Calendra T, Baumgartner JD, Grau GE. Prognosis values of tumor necrosis factor/cachectin, interleukin-1, alpha-interferon and gamma interferon in the serum of patients with septic shock *J. Infect. Disease* **161**:982-987 (1990)

Callery MP, Kamei T, Mangino MJ, Flye MW. Organ interactions in sepsis. *Arch. Surg.* **126**:28-32 (1991)

Campbell WN, Hendrix E, Cryz S Jr, Cross AS. Immunogenicity of a 24-valent Klebsiella capsular polysaccharide vaccine and an eight-valent Pseudomonas O-polysaccharide conjugate vaccine administered to victims of acute trauma. *Clin. Infect. Dis.* **23**:179-181 (1996)

Canchis PW, Bhan AK, Landau SB, Yang L, Balk SP, Blumberg RS. Tissue distribution of the non-polymorphic major histocmpatibility complex I-like molecule CD1d. *Immunology* **80**:561-565 (1993)

Casey LC, Balk RA, Bone RC. Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. *Ann. Intern. Med.* **119**:771-778 (1993)

Castano AR, Tangri S, Miller JE, Holcombe HR, Jackson MR, Huse WD, Kronenberg M, Peterson PA. Peptide binding and presentation by mouse CD1. *Science* **269**:2232-226 (1997)

Cattoretti G, Berti E, Mancuso A, D'Amato L, Schiro' R, Soligo D, Delia D. CD1: a MHC class I related family of antigens with widespread distribution on resting and activated cells. In *Leukocyte Typing* III eds. McMichael A, Beverley PC. pp89-91 Oxford, UK: Oxford Univ. Press. (1987)

Cattoretti G, Berti E, Parravicini C, Buscaglia M, Cappio F, Caputo R, Cerri A, Crosti L, Delia D, Gaiera G, Polli N. Expression of CD1 molecules on dendritic cells: ontogeny, epitope analysis on normal and malignant cells and tissue distribution. *Leucocyte Typing IV*. Oxford: Oxford Univ. Press pp262-264 (1989)

Cavaillon JM, Adib-Conquy M, Cloez-Tayarani I, Fitting C. Immunodepression in sepsis and SIRS assessed by *ex vivo* cytokine production is not a generalised phenomenon: a review *J. Endotoxin Res.* **7**:85-93 (2001)

Chedid L, Parant M, Parant F, Boyer F. A proposed mechanism for natural immunity to enterobacterial pathogens *J. Immunol.* **100**:292-301 (1968)

Chiu CJ, McArdle AH, Brown R, Scott HJ, Gurd FN. Intestinal mucosal lesion in low-flow states. *Arch. Surg.* **101**:478-483 (1970)

Christou NV. Altered host defense. *Infect. Surg.* **2**:692-700 (1983)

Christou NV, Rode H, Larsen D, Loose L, Broadhead M, Meakins L. The walk in anergic patient: how best to assess the risk of sepsis following elective surgery *Ann. Surg.* **199**(4):438-444 (1984)

Christou NV, Tellado JR, Chartrand L, Giannas B, Kapadia B, Meakins J, Rode H, Gordon J. Estimating mortality risk in preoperative patients using immunologic, nutritional, and acute-phase response variables *Ann. Surg.* **210**:69-77 (1989)

Cipolle MD, Pasqualle NM, Cerra FB. Secondary organ dysfunction: from clinical perspectives to molecular mediators. *Crit. Care Clin.* **9**:261-298 (1993)

Cox AD, Taylor CJ, Anderson AJ, Perry MB, Wilkinson SG. Structures of the two polymers present in the lipopolysaccharide of burkholdaria (pseudomonas) cepacia serogroup O4 *Eur. J. Biochem.* **231**:784-789 (1995)

Cross AS, Opal SM. Therapeutic intervention in sepsis with antibody to endotoxin: is there a future? *J. Endotoxin. Res.* **1**:57-69 (1994)

Cross AS, Sadoff JC, Kaufman B, Zollinger W. Use of monoclonal antibody combinations in the prevention of bacteraemia caused by Escherichia coli O18:K1:H7. *Serodiag. Immunother.* **2**:127-136 (1988)

Cryz SJ, Furer E, Cross AS, Wegmann A, Germanier R, Sadoff JC. Safety and immunogenicity of a *Pseudomonas aeruginosa* O-polysaccharide-toxin A conjugate vaccine in humans. *J. Clin. Invest.* **80**:51-60 (1987)

Cryz SJ, Cross AS, Sadoff JC, Wegmann A, Que JU, Furer E. Immunogenicity of Escherichia coli O18 O-specific polysaccharide (O-PS)-toxin A and O-PS cholera toxin conjugate vaccine in humans. *J. Infect. Dis.* **163**:1040-1045 (1991)

da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. Transfer from CD14 to TLR-4 and MD-2. *J. Biol. Chem.* **276**:21129-21135 (2001)

Danner RL, Elin RJ, Hosseini JM, Wesley RA, Reilly JM, Parillo JE. Endotoxaemia in human septic shock. *Chest* **99**:169-175 (1991)

Deitch EA, Winterton J, Berg R. Thermal injury promotes bacterial translocation from the gastrointestinal tract in mice with impaired T-cell mediated immunity *Arch. Surg.* **121**:97-101 (1986)

DeJongh-Leuvenink J, Bouter AS, Marcelis JH, Schellekens J, Verhoef J. Cross reactivity of monoclonal antibodies against lipopolysaccarides of Gram-negative bacteria. *Eur. J. Clin. Microbiol.* **5**:148-151 (1986)

Delahooke D, Barclay G, Poxton I. TNF-α induction by an aqeous phenol-extracted lipopolysaccharide complex from *Bacteroides* species. *Infect. & Immunity* **63**:840-6 (1996)

DeMaria A, Johns MA, Berberich H, McCabe WR. Immunization with rough mutants of Salmonella minnesota: initial studies in human subjects. *J. Infect. Dis.* **158**:301-311 (1988)

Detmers PA, Thieblemont N, Vasselon T, Pironkova R, Miller DS, Wright SD. Potential role of membrane internalisation and vesicle fusion in adhesion of neutrophils in response to lipopolysaccharide and TNF *J. Immunol* **157**:5589-5596 (1996)

Di Padova F, Brade H, Barclay GR, Poxton IR, Liehl E, Schuetze E, Kocher HP, Ramsay G, Schreier MH, McLelland DBL, Rietschel ET. A broadly cross-protective monoclonal antibody binding to *Escherichia coli* and *Salmonella* lipopolysaccharides. *Infection and Immunity* **61**:3863-3872 (1993)

Dijkstra J, Ryan JL, Szoka FC. A procedure for the efficient incorporation of wild-type lipopolysaccharide into liposomes for use in immunological studies *J. Immunol. Methods* **114**:197-205 (1988a)

Dijkstra J, Larrick JW, Ryan JL, Szoka FC. Incorporation of LPS in liposomes diminishes its ability to induce tumoricidal activity and tumor necrosis factor secretion in murine macrophages *J. Leukocyte Biol.* **43**:436-444 (1988b)

Dijkstra J, Mellors JW, Ryan JL, Szoka FC. Modulation of the biological activity of bacterial endotoxin by incorporation into liposomes. *J. Immunol.* **138**:2663-2670 (1987)

Dijkstra J, Mellors JW, Ryan JL. Altered in vivo activity of liposome-incorporated lipopolysaccharide and lipid A. *Infect. Immun.* **57**:3357-3363 (1989)

Dofferhoff AS, Bom VJ, de Vries-Hospers HG, van Ingen J, vd Meer J, Hazenberg BP, Mulder PO, Weits J. Patterns of cyokines, plasma endotoxin, plasminogen activator inhibitor and acute phase proteins during the treatment of severe sepsis in humans. *Crit. Care Med.* **20**:185-192 (1992)

Drost AC, Burleson DG, Cioffi WG, Jordan BS, Mason AD, Pruitt BA. Plasma cytokines following thermal injury and their relationship with patient mortality, burn size, and time post burn. J. Trauma 35:335-339 (1993)

Ellner JJ, Spagnuolo PJ. Suppression of antigen and mitogen induced human T lymphocyte DNA by bacterial lipopolysaccharide: mediation by monocyte activation and production of prostaglandins. *J. Immunol.* **123**:2689 (1989)

Ernst WA, Maher J, Cho S, Niazi KR, Chatterjee D, Moody DB, Besra GS, Watanabe Y, Jensen PE, Porcelli SA, Kronenberg M, Modlin RL. Molecular interaction of CD1b with lipoglycan antigens. *Immunity* **8**:331-340 (1998)

Erridge C, Bennet-Guerrero E, Poxton IR. Structure and function of lipopolysaccharides *Microbes Infect.* **4**:837-851 (2002a)

Erridge C, Stewart J, Bennett-Guerrero E, McIntosh TJ, Poxton I. The biological activity of a liposomal complete core lipopolysaccharide vaccine. *J. Endotoxin Res.* **8**:39-46 (2002b)

Ertel W, Keel M, Neidhardt R, Steckholzer U, Kremer JP, Ungethuem U, Trentz O. Inhibition of the defense system stimulating interleukin-12 interferon-γ pathway during critical illness. *Blood* **89**:1612-1620 (1997)

Exley M, Garcia J, Balk SP, Porcelli S. Requirements for CD1d recognition by human invariant Va24+ CD4-CD8- T-cells. *J. Exp. Med.* **186**:109-120 (1997)

Fairhurst RM, Wang CX, Sieling PA, Modlin RL, Braun J. CD1 restricted T-cells and resistance to polysaccharide-encapsulated bacteria. *Immunol Today* **19**:257-259 (1998)

Faist E, Mewes A, Baker CC, Strasser T, Alkan SS, Rieber P, Heberer G. Prostaglandin E2 (PGE2)-dependent suppression of interleukin-2 (IL-2) production in patients with major trauma *J. Trauma.* **27**:837-848 (1987)

Fauci A, Pratt K. Activation of human B-lymphocytes. Direct plaque forming assay for the measurement of polyclonal activation and antigenic stimulation of human B-lymphocytes *J. Exp. Med.* **144**:674 (1976)

Fisher CJ Jr, Opal SM, Dhainaut JF, Stephens S, Zimmerman JL, Nightingale P, Harris SJ, Schein RM, Panacek EA, Vincent JL. Influence of an anti-tumor necrosis factor monoclonal antibody on cytokine levels in patients with sepsis. The CB0006 Sepsis Syndrome Study Group. *Crit. Care Med.* **21**:318-327 (1993)

Fisher CJ Jr, Dhainaut JF, Opal SM, Pribble JP, Balk RA, Slotman GJ, Iberti TJ, Rackow EC, Shapiro MJ, Greenman RL. Recombinant human interleukin-1 receptor antagonist in the treatment of patients with sepsis syndrome. Results from a randomized, double blind placebo controlled trial. Phase III rhIL-1ra Sepsis Syndrome Study Group. *JAMA* **271**:1836-1843 (1994)

Fitzgerald KA, Palsson-McDermot EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, Dunne A, Gray P, Harte MT, McMurray D, Smith D.E, Sims J.E, Bird T.A, O'Neill L.A.. Mal (MyD88-adaptor-like) is required for Toll like receptor 4 signal transduction. *Nature* **413**:78-83 (2001)

Flo TH, Halaas O, Lien E, Ryan L, Teti G, Golenbock DT, Sundan A, Espevik T. Human Toll-like receptor 2 mediates monocyte activation by *Listeria monocytogenes*, but not by group B streptococci or lipopolysaccharide. *J. Immunol.* **164**:2064-2069 (2000)

Freeman R, Gould FK. Prevention of fever and Gram negative infection after open heart surgery by antiendotoxin *Thorax* **40**:846-51 (1985)

Fujieda S, Sieling PA, Modlin RL, Saxon A. CD1-restricted T-cells influence IgG subclass and IgE production J. *Allergy & Clinical Immunology* **101**:545-551 (1998)

Galanos C, Luderitz O, Westphal O. Preparation and properties of antisera against the lipid-A component of bacterial lipopolysaccharides. *Eur. J. Biochem.* **24**:116-122 (1971a)

Galanos C, Rietschel ET, Luderitz O, Westphal O. Interaction of lipopolysaccharides with complement. *Eur. J. Biochem.* **19**:143-152 (1971b)

Galanos C, Luderitz O, Rietschel ET, Westphal O Brade H, Brade L, Freudenberg M, Schade U, Imoto M, Yoshimura H. Synthetic and natural E.coli free lipid A express identical endotoxic activities. *Eur. J. Biochem.* **148**:1-12 (1985)

Galanos C, Freudenberg MA. Mechanisms of endotoxic shock and endotoxin hypersensitivity *Immunobiology* **187**:346-356 (1993)

Gibb AP. The role of bacteria in sepsis syndrome *Reviews in Med. Micro.* **4**:59-64 (1993)

Glauser M, Zanetti G, Baumgartner JD, Cohen J. Septic shock: pathogenesis. *Lancet* **338**:732-736 (1991)

Glode LM, Rosenstreich DL. Genetic control of B-cell activation by bacterial lipopolysaccharide is mediated by multiple distinct genes or alleles. *J. Immunol.* **177**:2061-2067 (1976)

Godefroy DI, Hammond KJL, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. *Immunology Today* **21**:573-583 (2000)

Goldberg JB, Pier GB. Pseudomonas aeruginosa lipopolysaccharides and pathogenesis *Trends Microbiol.* **4**:490-494 (1996)

Goldie AS, Fearon KC, Ross JA, Barclay GR, Jackson RE, Grant IS, Ramsay G, Blyth AS, Howie JC. Natural cytokine antagonists and endogenous endotoxin core antibodies in sepsis syndrome. The Sepsis Intervention Group. *JAMA* **274**:172-177 (1995)

Gomez-Jimenez J, Salgado A, Mourelle M, Martin MC, Segura RM, Peracaula R, Moncada S. L-arginine:nitric oxide pathway in endotoxaemia and human septic shock. *Crit. Care Med.* **23**:253-258 (1995)

Gough D, Jordan A, Mannick JA, Rodrick ML. Impaired cell mediated immunity in experimental abdominal sepsis and the effect of interleukin 2. *Arch. Surg.* **127**:859-863 (1992)

Gould E.K, Harvey J.A, Dytrych J.K. Antibody to endotoxin is associated with decreased frequency of postoperative infection. *Am J Obstet Gynecol.* **160**:317-319 (1989)

Grabstein KH, Eisenman J, Shanebeck K, Rauch C, Srinivasan S, Fung V, Beers C, Richardson J, Schoenborn MA, Ahdieh M. Cloning of a novel T-cell growth factor that interacts with the β-chain of the IL-2 receptor *Science* **264**:965-968 (1994)

Granowitz EV, Porat R, Mier JW, Orencole SF, Kaplanski G, Lynch EA, Ye K, Vannier E, Wolff SM, Dinarello CA. Intravenous endotoxin suppresses the cytokine response of peripheral blood mononuclear cells of healthy humans *J. Immunol.* **151**:1637-1645 (1993)

Gransden WR, Eykyen SJ, Phillips I, Rowe B. Bacteraemia due to *Escherichia coli*: a study of 861 episodes. *Rev. Infect. Dis.* **6**:1008-1018 (1990)

Greaves M, Janossy G, Doenhoff M. Selective triggering of human T and B lymphocytes in vitro by polyclonal mitogens *J. Exp. Med.* **140**:1 (1974)

Haglund U, Hulten L, Ahren C, Lundgren O. Mucosal lesions in the human small intestine in shock. *Gut* **16**:979-984 (1975)

Hailman E, Lichenstein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M, Busse LA, Zukowski MM, Wright SD. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14 *J. Exp. Med.* **179**:269-277 (1994)

Hamilton-Davies C, Barclay G.R, Cardigan R.A, McDonald S.J, Purdy G, Machin S.J, Webb A.R. Relationship between preoperative endotoxin immune status, gut perfusion, and outcome from cardiac valve replacement surgery. *Chest* **112**:1189-1196 (1997)

Hammond SM, Claesson A, Jansson AM, Larsson LG, Pring BG, Town CM, Ekstrom B. A new class of synthetic anti-bacterials acting on lipopolysaccharide biosynthesis. *Nature* **327**:730-732 (1987)

Hammond KJ, Poulton LD, Palmisano LJ, Silveira PA, Godfrey DI, Baxter AG. α/β T-cell receptor (TCR) CD4-CD8- (NKT) thymocytes prevent insulin dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of IL-4 and/or IL-10 *J. Exp. Med.* **187**:1047-1056 (1998)

Hancock I, Poxton I. Bacterial cell surface techniques. Chichester, UK Wiley (1998)

Harvey RF and Bradshaw JM. A simple index of Crohn's disease activity. *Lancet* **1**:54 (1980)

Hassoun HT, Kone BC, Mercer DW, Moody FG, Weisbrodt NW, Moore FA. Post-injury multiple organ failure: the role of the gut *Shock* **15**:1-10 (2001)

Haziot A, Ferrero E, Kontgen F, Hijiya N, Yamamoto S, Silver J, Stewart CL, Goyert SM. Resistance to endotoxin shock and reduced dissemination of Gram-negative bacteria in CD14-deficient mice *Immunity* **4**:407-414 (1996)

Heidecke CD, Hensler T, Weighardt H, Zantl N, Wagner H, Siewert JR, Holzmann B. Selective defects of T lymphocyte function in patients with lethal intraabdominal infection *Am. J. Surg.* **178**:288-292 (1999)

Heine H, Kirschning CJ, Lien E, Monks BG, Rothe M, Golenbock DT. Cutting edge: Cells that carry a null allele for Toll-like receptor 2 are capable of responding to endotoxin *J Immunol* **162**:6971-6975 (1999)

Helander IM, Lindner B, Brade H, Altmann K, Lindberg AA, Rietschel ET, Zahringer U. Chemical structure of the lipopolysaccharide of *Haemophilus influenzae* strain I-69 Rd^-/b^+: description of a novel deep rough mutant. *Eur. J. Biochem.* **177**:483 (1988)

Hensler T, Hecker H, Heeg K, Heidecke CD, Bartels H, Barthlen W, Wagner H, Siewert JR, Holzmann B. Distinct mechanisms of immunosuppression as a consequence of major surgery. *Int. Immunol.* **65**:2283-2291 (1997)

Heumann D, Baumgartner JD, Jacot-Guillarmond H, Glauser MP. Antibodies to core lipopolysaccharide determinants: absence of cross reactivity with heterologous lipopolysaccharides *J. Infect. Diseases* **163**:762-768 (1991)

Heyderman RS, Fitzpatrick MM, Barclay GR. Haemolytic-ureamic syndrome. *Lancet* **343**:8904 (1994)

Hirschfield M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge:repurification of lipopolysaccharide eliminates signalling through both human and murine toll-like receptor 2. *J. Immunol.* **165**:618-622 (2000)

Hirschfield M, Weis JJ, Toschchakov V, Salkowski CA, Cody MJ, Ward DC, Quereshi N, Michalek SM, Vogel SN. Signalling by Toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages *Infect. Immun.* **69**:1477-1482 (2001)

Hitchcock PJ, Brown TM. Morphological heterogeneity among Slmonella lipopolysaccharide chemotypes in silver stained polyacrylamide gels. *J. Bacteriol.* **154**:269-277 (1983)

Horng T, Barton GM, Medzhitov R. TIRAP, an adaptor molecule in the Toll signalling pathway. *Nat. Immunol.* **2**:835-841 (2001)

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Toll like receptor 4 deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR-4 as the Ips gene product. *J. Immunol.* **162**:3749-3752 (1999)

Hotchkiss RS, Swanson PE, Knudson CM, Chang KC, Cobb JP, Osborne DF, Zollner KM, Buchman TG, Korsmeyer SJ, Karl IE. Overexpression of Bcl-2 in transgenic mice decreases apoptosis and improves survival in sepsis. *J. Immunol.* **162**:4148-4156 (1999a)

Hotchkiss RS, Tinsley KW, Swanson PE, Chang KC, Cobb JP, Buchman TG, Korsmeyer SJ, Karl IE. Prevention of lymphocyte cell death in sepsis improves survival in mice. *Proc. Natl. Acad. Sci. USA* **96**:14541-14546 (1999b)

Imoto M, Shiba T, Naoki H. Chemical structure of *E.coli* lipid A: a linkage site of acyl groups in the disaccharide backbone. *Tetrahedron Lett.* **24**:4017-4020 (1983)

J5 study group. Treatment of severe infectious purpura in children with human plasma from donors immunised with *Escherichia coli* J5: a prospective double blind study. *J. Infect. Dis.* **165**:695-701 (1992)

Jack RS, Fan X, Bernheiden M, Rune G, Ehlers M, Weber A, Kirsch G, Mentel R, Furll B, Freudenberg M, Schmitz G, Stelter F, Schutt C. Lipopolysaccharide-binding protein is required to combat a murine Gram-negative bacterial infection. *Nature* **389**:742-745 (1997)

Jansson PE, Lindberg AA, Lindberg B, Wollin R. Structural studies on the hexose region of the core in lipopolysaccharides from Enterobacteriaceae. *Eur. J. Biochem.* **115**:571-577 (1981)

Johnson WC, Ulrich F, Mequid MM, Lepak N, Bowe P, Harris P, Alberts LH, Nabseth DC. Role of delayed hypersensitivity in predicting postoperative morbidity and mortality *Am. J. Surg.* **137**:536-542 (1979)

Joiner KA, Schmetz MA, Goldman RC, Leive L, Frank MM. Mechanism of bacterial resistance to complement-mediated killing: inserted C5b-9 correlates with killing for Escherichia coli O111B4 varying in O-antigen capsule and O-polysaccharide coverage of lipid A core oligosaccharide. *Infect. Immun.* **45**:113-7 (1984)

Joiner KA, Grossman N, Schmetz M, Leive L. C3 binds preferentially to long-chain lipopolysaccharide during alternative pathway activation by Salmonella montevideo. *J. Immunol.* **136**:710-5 (1986)

Jones S, Romano F. Dose and time dependent changes in plasma catacholamines in response to endotoxin in conscious rats *Circ. Shock* **28**:59-68 (1989)

Joyce S, Woods AS, Yewdell JW, Bennik JR, De Silva AD, Boesteanu A, Balk SP, Cotter RJ, Brutkiewicz RR. Natural ligand of mouse CD1d: cellular glycosylphosphatidylinositol. *Science* **279**:1541-1544 (1998)

Kasinerk W, Baumruker T, Majdic O, Knapp W, Stockinger H. CD1 molecule expression on human monocytes induced by granulocyte-macrophage colony stimulating factor. *J. Immunol.* **150**:579-584 (1993)

Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M. CD1d restricted and TCR mediated activation of Va14 NKT cells by glycosylceramides. *Science* **278**:1626-1629 (1997)

Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M. Mouse Toll like receptor 4. MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol. *J. Biol. Chem.* **275**:2251-2254 (2000)

Kawasaki K, Gomi K, Nishijima M. Gln22 of mouse MD-2 is essential for lipopolysaccharide mimetic action of Taxol. *J. Immunol.* **166**:11-14 (2001)

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

Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, Willeit J, Schwartz DA. Toll-like receptor 4 polymorphisms and atherosclerosis *N. Engl. J. Med.* **347**:185-192 (2002)

Kirkland TN, Ziegler EJ. An immunoprotective monoclonal antibody to lipopolysaccharide. *J. Immunol.* **132**:2590-2592 (1984)

Knuefermann P, Nemoto S, Baumgarten G, Misra A, Sivasubramanian N, Carabello BA, Vallejo JG. Cardiac inflammation and innate immunity in septic shock: Is there a role for Toll-like receptors? *Chest* **121**:1329-1336 (2002)

Kohn FR, Kung AHC. Role of endotoxin in acute inflammation induced by gram negative bacteria: specific inhibition of lipopolysaccharide-mediated responses with an amino-terminal fragment of bactericidal/permeability-increasing protein. *Infect. Immun.* **63**:333-339 (1995)

Kosma P. Chlamydial lipopolysaccharide *Biochim. Biophys. Acta* **1455**:387-402 (1999)

Kox WJ, Bone RC, Krausch D, Docke WD, Kox SN, Wauer H, Egerer K, Querner S, Asadullah K, von Baehr R, Volk HD. Interferon gamma-1b in the treatment of compensatory anti-inflammatory response syndrome. A new approach: proof of principle. *Arch. Intern. Med.* **157**:389-393 (1997)

Kreger BE, Craven DE, McCabe WR. Gram negative bacteraemia. Re-evaluation of clinical features and treatment in 612 patients. *Am. J. Med.* **68**:344-355 (1980)

Kumada H, Haishima Y, Umemoto T, Tanamoto K. Structural study on the free lipid A isolated from lipopolysaccharide of *Porphyromonas gingivalis*. *J. Bacteriol.* **177**:2098-2106 (1995)

Kusumoto S. Chemical synthesis of lipid A. In: Bacterial Endotoxic Lipopolysaccharides, Vol I, Molecular Biochemistry and Cellular Biology *eds*. Morrison DC and Ryan JL. CRC press, Boca Raton, Florida, pp81-106 (1992)

Laemmli U. Cleavage of structural proteins during assembly of the head of bacteriohage T4. *Nature* **227**:680-685 (1970)

Levi M, ten Cate H, van der Poll T, van Deventer SJ. Pathogenesis of disseminated intravascular coagulation in sepsis *JAMA* **270**:975-979 (1993)

Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW, Carroll JD, Espevik T, Ingalls RR, Radolf JD, Golenbock DT. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J. Biol. Chem.* **274**:33419-33425 (1999)

Lien E, Means TK, Heine H, Yoshimura A, Kusumoto S, Fukase K, Fenton MJ, Oikawa M, Qureshi N, Monks B, Finberg RW, Ingalls RR, Golenbock DT. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J. Clin. Invest.* **105**:497-504 (2000)

Lindberg AA, Weintraub A, Zahringer U, Rietschel ET. Structure-activity relationships in lipopolysaccharides of *Bacteroides fragilis Rev. Infect. Dis.* **12**(Supp 2):S133-141 (1990)

Lorenz E, Frees KL, Schwartz DA. Determination of the TLR4 genotype using allele-specific PCR *Biotechniques* **31**:22-24 (2001)

Lorenz E, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock *Arch. Intern. Med.* **162**:1028-1032 (2002a)

Lorenz E, Scwartz DA, Martin PJ, Gooley T, Lin MT, Chien JW, Hansen JA, Clark JG. Association of TLR4 mutations and the risk for acute GVHD after HLA matched sibling hematopoietic stem cell transplantation *Biol. Blood Marrow Transplant* **7**:384-387 (2002b)

Lorenz E, Hallman M, Martilla R, Haataja R, Schwartz DA. Association between the Asp299Gly polymorphisms in the Toll-like receptor 4 and premature births in the Finnish population *Pediatr. Res.* **52**:373-6 (2002c)

Lorenz E, Patel DD, Hartung T, Schwartz DA. Toll-like receptor 4 (TLR4)-deficient murine macrophage cell line as an in vitro assay system to show TLR4-independent signaling of Bacteroides fragilis lipopolysaccharide *Infect. Immun.* **70**:4892-4896 (2002d)

Lynn WA. Anti-endotoxin therapeutic options for the treatment of sepsis *J. Antimicrob. Chem.* **41**(SA):S71-S80 (1998)

Mainous M, Tso P, Berg R, Deitch E. Studies of the route, magnitude and time course of bacterial translocation in a model of systemic inflammation. *Arch. Surg.* **126**:33-37 (1991)

Manthey C, Vogel S. Interactions of lipoplysaccharide with macrophages. In "*Macrophage-Pathogen Interactions*" Dekker NY (1994)

Martich GD, Boujoukos AJ, Suffredinin AF. Response of man to endotoxin *Immunobiology* **187**:403-416 (1993)

Martineau L, Shek PN. Changes in circulating lymphocyte subpopulations and mitogen-stimulated response in a rat infusion model of intra-abdominal infection *Crit. Care. Med.* **28**:2515-2521 (2000)

Mattern T, Thanhauser A, Reiling N, Toellner KM, Duchrow M, Kusumoto S, Rietschel E, Ernst M, Brade H, Flad HD, Ulmer A. Endotoxin and lipid A stimulate proliferation of human T-cells in the presence of autologous monocytes *J. Immunol.* **153**:2996-3004 (1994)

Mattern T, Flad HD, Brade L, Rietschel ET, Ulmer A. Stimulation of human T-lymphocytes by LPS is MHC unrestricted, but strongly dependant on B7 interactions *J. Immunol.* **160**:3412-3418 (1998)

Mattern T, Girroleit G, Flad HD, Rietschel ET, Ulmer A. CD34+ haematopoietic stem cells exert accessory function in lipopolysaccharide-induced T-cell stimulation and CD80 expression on monocytes *J. Exp. Med.* **189**:693-699 (1999)

Mayeux P. Pathology of lipopolysaccharide. *J. Toxicol. & Env. Health* **51**:415-35 (1997)

McCabe WR, Bruins SC, Craven DE, Johns M. Cross reactive antigens: their potential for immunisation-induced immunity to Gram-negative bacteria. *J. Infect. Dis.* **136S**:161-166 (1977)

McCabe WR, Greely A. Immunization with R mutants of S. minnesota. Protection against challenge with heterologous Gram-negative bacilli. J. Immunol. **108**:601-610 (1972a)

McCabe WR, Kreger BE, Johns M. Type-specific and cross reactive antibodies in Gram-negative bacteraemia. *New Eng. J. Med.* **287**:261-266 (1972b)

McCall CE, Grosso-Wilmoth LM, LaRue K, Guzman RN, Cousart SL. Tolerance to endotoxin induced expression of the interleukin-1 beta gene in blood neutrophils of humans with the sepsis syndrome *J. Clin. Invest.* **91**:853-861 (1993)

McCutchan JA, Wolf JL, Ziegler EJ, Braude AI. Ineffectiveness of single dose human anti-serum to core glycolipid (E.coli J5) for prophylaxis or bacteraemic, Gram-negative infection in patients with prolonged neutropenia. *Schweiz Med. Wschr.* **113S**:40-45 (1983)

Medzhitov R, Preston-Hurlburt P, Janeway CA Jnr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity *Nature* **388**:394-397 (1997)

Michie HR, Manogue KR, Spriggs DR, Revhaug A, O'Dwyer S, Dinarello CA, Cerami A, Wolff SM, Wilmore DW. Detection of circulating tumor necrosis factor after endotoxin administration *N. Engl. J. Med.* **318**:1481-1486 (1988)

Milner E, Rudbach JA, VonEschen KB. Cellular responses to bacterial lipopolysaccharide: T-cells recognise LPS determinants *Scand. J. Immunol.* **18**:21-26 (1983)

Minnikin DE. *in The Biology of the Mycobacteria; Physiology, Identification and Classification Vol 1 (*eds Rateledge C and Stanford J). 95-184 (Academic, London, 1982)

Mittelman A, Puccio C, Gafney E, Coombe N, Singh B, Wood D, Nadler P, Ahmed T, Arlin Z. A phase I pharmacokinetic study of recombinant human tumor necrosis factor administered by a 5-day continuous infusion. *Invest New Drugs* **10**:183-190 (1992)

Miyauchi T, Tomobe Y, Shiba R, Ishikawa T, Yanagisawa M, Kimura S, Sugishita Y, Ito I, Goto K, Masaki T. Involvement of endothelin in the regulation of human vascular tonus. *Circulation* **81**:1874-1880 (1990)

Moody DB, Reinhold BB, Guy MR, Beckman EM, Frederique DE, Furlong ST, Ye S, Reinhold VN, Sieling PA, Modlin RL, Besra GS, Porcelli SA. Structural requirements for glycolipid antigen recognition by CD-1b restricted T-cells *Science* **278**:283-286 (1997)

Moody DB, Ulrichs T, Muhlecker W, Young DC, Gurcha SS, Grant E, Rosat JP, Brenner MB, Costello CE, Besra GS, Porcelli SA. CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection *Nature* **404**:884-888 (2000)

Moran AP. Structure and conserved characteristics of campylobacter jejuni lipopolysaccharides *Rev. Infect. Dis.* **176**(Supp 2):S115-121 (1997)

Morel PA, Oriss TB. Crossregulation between Th1 and Th2 cells. *Crit. Rev. Immunol.* **18**:275-303 (1998)

Morrison DC, Rudbach JA. Endotoxin-cell-membrane interactions leading to transmembrane signalling. *Contemp. Top. Mol. Immunol.* **8**:187-195 (1981)

Morrison DC, Silverstein R, Luchi M, Shnyra A. Structure-function relationships of bacterial endotoxins *Infect. Dis. Clin. North Am.* **13**:313-340 (1999)

Mossman TR, Coffman RL. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties *Ann. Rev. Immunol.* **7**:145-173 (1989)

Munford RS, Pugin J. The crucial role of systemic responses in the innate (non-adaptive) host defense. *J. Endotoxin Res.* **7**:1-6 (2001a)

Munford RS, Pugin J. Normal responses to injury prevent systemic inflammation and can be immunosuppressive *Am. J. Respir. Crit. Care Med.* **163**:316-321 (2001b)

Munoz C, Carlet J, Fitting C, Misset B, Bleriot JP, Cavaillon JM. Dysregulation of in vitro cytokine production by monocytes during sepsis. *J. Clin. Invest.* **88**:1747-1754 (1991)

Mythen MG, Barclay GR, Purdy G, Hamiltondavies C, Mackie IJ, Webb AR, Machin SJ. The role of endotoxin immunity, neutrophil degranulation and contact activation on the pathogenesis of post-operative organ dysfunction. *Blood Coagulat. Fibrinol.* **4**:999-1005 (1993)

Naidenko OV, Maher JK, Ernst WA, Sakai T, Modlin RL, Kronenberg M. Binding and antigen presentation of ceramide-containing glycolipids by soluble mouse and human CD1d molecules *J. Exp. Med.* **190**:1069-1080 (1999)

Naldini A, Borreli E, Carraro F, Giomarelli P, Toscano M. IL-10 production in patients undergoing cardiopulmonary bypass: evidence of inhibition of Th1 type responses. *Cytokine* **11**:74-79 (1998)

Natori T, Morita M, Akimoto K, Koezuka Y. Agelasphins, novel anti-tumor and immunostimulatory cerebrosides from the sponge Agelas mauritanus. *Tetrahedron* **50**:2771-2784 (1994)

Neidhardt R, Keel M, Steckholzer U, Safret A, Ungethuem U, Trentz O, Ertel W. Relationship of IL-10 plasma levels to severity of injury and clinical outcome in injured patients *J. Trauma* **42**:863-870 (1997)

Nys M, Damas P, Joassin L, Lamy M. Sequential anti-core glycolipid immunoglobulin antibody activities with and without septic shock and their relation to outcome. *Ann. Surg.* **217**:300-306 (1993)

Oberholzer A, Oberholzer C, Moldawer LL. Sepsis syndromes: understanding the role of innate and acquired immunity *Shock* **16**:83-96 (2001)

Oberholzer A, Oberholzer C, Moldawer L. Interleukin-10: A complex role in the pathogenesis of sepsis syndromes and its potential as an anti-inflammatory drug. *Crit. Care Med.* **30**(S1):S58-S63 (2002)

Ohlsson K, Bjork P, Bergenfeldt M, Hageman R, Thompson RC. Interleukin 1 receptor antagonist reduces mortality from endotoxin shock. *Nature* **348**:550-552 (1990)

Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, Chow JC, Strauss JF 3rd. The extra domain A of fibronectin activates Toll-like receptor 4. *J. Biol. Chem.* **276**:10229-10233 (2001)

Opal SM. Lessons learned from clinical trials of sepsis *J. Endotoxin. Res.* **2**:1-6 (1995)

Opal SM, Wherry JC, Grint P. Interleukin-10: potential benefits and possible risks in clinical infectious diseases *Clin. Infect. Dis.* **27**:1497-1507 (1998)

Opal SM, Scannon PJ, Vincent JL, White M, Carroll SF, Palardy JE, Parejo NA, Pribble JP, Lemke JH. Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. *J Infect Dis* **180**:1584-9 (1999)

O'Sullivan ST, Lederer JA, Horgan AF, Chin DH, Mannick JA, Rodrick ML. Major injury leads to predominance of the T helper 2 lymphocyte phenotype and diminished interleukin-12 production associated with decreased resistance to infection *Ann. Surg.* **222**:482-492 (1995)

Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A. The repertoire for pattern recognition of pathogens by the innate immune system is defined by co-operation between toll-like receptors. *Proc. Natl. Acad. Sci. USA* **97**:13766-13771 (2000)

Pamer EG. The role of CD1-restricted T-cells in infectious diseases *Trends in Microbiology* **7**:13 (1999)

Park S, Bendelac A. CD1 restricted T-cell responses and microbial infection *Nature* **406**:788-792 (2000)

Patrick S. The virulence of Bacteroides fragilis. *Rev. Med. Micro.* **4**:40-49 (1993)

Peavy D, Adler W, Smith R. The mitogenic effects of endotoxin and staphylococcus enterotoxin B on mouse spleen cells and human peripheral lymphocytes *J. Immunol.* **105**:1453 (1970)

Petrov A, Semenov B, Vartanyan Y, Zakirov M, Torchilin V, Trubetskoy V, Koshkina N, L'Vov V, Verner I, Lopyrev I, Dmitriev B. Toxicity and immunogenicity of Neisseria meningitidis lipopolysaccharide incorporated into liposomes *Infect. Immunity.* **60**:3897-3903 (1992)

Pfeiffer R. Untersuchungen uber das Choleragift. *Z. Hygiene* **11**:393-412 (1892)

Plebani A, Proserpio AR, Guarneri D, Buscaglia M, Cattoretti G. B and T lymphocyte subsets in fetal and cord blood: age related modulation of CD1c expression. *Biol. Neonate* **63**:1-7 (1993)

Pollack M, Chia JKS, Koles NL, Miller M, Guelde G. Specificity and cross reactivity of monoclonal antibodies reactive with the core and lipid A regions of bacterial lipopolysaccharide *J. Infect. Diseases* **159**:168-184 (1989)

Pollack M, Huang AI, Prescott RK, Young LS, Hunter KW, Cruess DF, Tsai CM. Enhanced survival in Pseudomonas aeruginosa septicaemia associated with high levels of circulating antibody to Escherichia coli endotoxin. *J. Clin. Invest.* **72**:1874-1881 (1983)

Poltorak A, He X, Smirnova I, Liu MY, Huffel CV, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signalling in C3H/HEJ and C57Bl/10ScCr mice: mutations in Tlr4 gene. *Science* **282**:2085-2088 (1998)

Poltorak A, Ricciardi-Castagnoli P, Citterio S, Beutler B. Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation *PNAS* **97**(5):2163-2167 (2000)

Porcelli S, Brenner MB, Greenstein JL, Balk SP, Terhorst C, Bleicher PA. Recognition of cluster of differentiation I antigens by human CD4-CD8- cytolytic T lymphocytes *Nature* **341**:447-450 (1989)

Porcelli S, Morita CT, Brenner MB. CD1b restricts the response of human $CD4^{-}8^{-}$ T-lymphocytes to a microbial antigen *Nature* **360**:593-597 (1992)

Porcelli S. The CD1 family: a third lineage of antigen presenting molecules. *Adv. Immunol.* **59**:1-98 (1995)

Porcelli SA, Brenner MB. Antigen presentation: mixing oil and water. *Curr. Biol.* **7**:R508-R511 (1997)

Porcelli SA, Modlin RL. The CD1 system: Antigen presenting molecules for T-cell recognition of lipids and glycolipids *Ann. Rev. Immunol.* **17**:297-329 (1999)

Poxton I. Antibodies to lipopolysaccharide. *J. Immunol. Meth.* **186**:1-15 (1995)

Prigozy TI, Sieling PA, Clemens D, Stewart PL, Behar SM, Porcelli SA, Brenner MB, Modlin RL, Kronenberg M. The mannose receptor delivers lipoglycan antigens to endosomes for presentaion to T-cells by CD1b molecules *Immunity* **6**:187-197 (1997)

Pugin J, Schurer-Maly CC, Leturcq D, Moriarty A, Ulevitch RJ, Tobias PS. 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 (1993)

Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D. Endotoxin-tolerant mice have mutations in Toll like receptor 4 (Tlr4) *J. Exp. Med.* **189**:615-625 (1999)

Randow F, Syrbe U, Meisel C, Krausch D, Zuckermann H, Platzer C, Volk HD. Mechanism of endotoxin desensitisation: involvement of interleukin-10 and transforming growth factor beta. *J. Exp. Med.* **181**:1887-1892 (1995)

Read RC, Pullin J, Gregory S, Borrow R, Kaczmarski EB, Giovine FS, Dower SK, Cannings C, Wilson AG. A functional polymorphism of Toll-like receptor 4 is not associated with likelihood or severity of meningococcal disease *J. Infect. Dis.* **184**:640-642 (2001)

Redl H, Bahrami S, Schlag G, Traber DL. Clinical detection of LPS and animal models of endotoxaemia *Immunobiology* **187**:330-345 (1993)

Reed SG. In vivo administration of recombinant IFN-gamma induces macrophage activation and prevents acute disease, immunosuppression and death in experimental Trypanosoma cruzi infections. *J. Immunol.* **140**:4342-4347 (1988)

Rhodes RS, Depalma RG, Robinson AV. Intestinal barrier function in hemorrhagic shock. *J. Surg. Res.* **14**:305-312 (1973)

Rietschel E, Brade H. Bacterial Endotoxins. *Scientific American* August **267**:26-33 (1992)

Rietschel ET, Kirikae T, Schade FU, Ulmer AJ, Holst O, Brade H, Schmidt G, Mamat U, Grimmecke HD, Kusumoto S, Zahringer U. The chemical structure of bacterial endotoxin in relation to bioactivity *Immunobiology* **187**:169-190 (1993)

Rietschel ET, Kirikae T, Schade U, Mamat U, Scmidt G, Loppnow H, Ulmer AJ, Zahringer U, Seydel U, Di Padova F, Schreier M, Brade H. Bacterial endotoxin: molecular relationships of structure to activity and function *FASEB J* **8**:217-225 (1994)

Rietschel ET, Westphal O. Endotoxin: historical perspectives. In: Brade H, Opal SM, Vogel SN, Morrisson DC. (eds) *Endotoxin in health and disease*. New York, Dekker. 1-30 (1999)

Rinaldo JE, Gorry M, Strieter R, Cowan H, Abdolrasulnia R, Shepherd V. Effect of endotoxin induced cell injury on 70 kD heat shock proteins in bovine lung endothelial cells *Am. J. Respir. Cell.. Mol. Biol.* **3**:207-216 (1990)

Roark JH, Park SH, Jayawardena J, Kavita U, Shannon M, Bendelac A. CD1.1 expression by mouse antigen presenting cells and marginal zone B-cells. *J. Immunol.* **160**:3121-3127 (1998)

Rosen G, Sela MN, Naor R, Halabi A, Barak V, Shapira L. Activation of murine macrophages by lipoprotein and lipopolysaccharide of *Treponema denticola Infect. Immun.* **67**:1180-1186 (1999)

Saadia R, Schein M, MacFarlane C, Boffard KD. Gut barrier function and the surgeon *Br. J. Surgery* **77**:487-492 (1990)

Schaible UE, Hagens K, Fischer K, Collins HL, Kaufmann SH. Intersection of group I CD1 molecules and mycobacteria in different intracellular compartments of dendritic cells. *J. Immunol.* **164**:4843-4852 (2000)

Schiff DE, Wass CA, Cryz SJ, Cross AS, Kim KS. Estimation of protective levels of anti-O-specific lipopolysaccharide immunoglobulin G antibody against experimental Escherichia coli infection. *Infect. Immun.* **61**:975-980 (1993)

Schofield L, McConville M, Hansen D, Campbell AS, Fraser-Reid B, Grusby M, Tachado S. CD1d restricted IgG formation to GPI-anchored antigens mediated by NKT-cells *Science* **283**:225-229 (1999)

Scott BB, Barclay GR. Endotoxin polymyxin complexes in an improved enzyme linked immunosorbent assay for IgG antibodies in blood donor sera to Gram-negative endotoxin core glycolipids. *Vox. Sang.* **52**:272 (1987)

Sekatrian JC, Yee J, Christou NV. Reduced tumor necrosis factor-α production in lipopolysaccharide treated whole blood from patients in the intensive care unit *Arch. Surg.* **129**:187-192 (1994)

Severn A, Rapson NT, Hunter CA, Liew FW. Regulation of tumor necrosis factor production by adrenaline and by β-adrenergic agonists *J. Immunol.* **148**:3441-3445 (1992)

Seydel U, Labischinski H, Kastowsky M, Brandenburg K. Phase behaviour, supramolecular structure and molecular conformation of lipopolysaccharide *Immunobiology* **187**:191-211 (1993)

Shamshiev A, Donda A, Carena I, Mori L, Kappos L, DeLibero G. Self glycolipids as T-cell autoantigens *E. J. Immunol* **29**:1667-1675 (1999)

Sherry RM, Cue JI, Goddard JK, Parramore JB, DiPiro JT. Interleukin-10 is associated with the development of sepsis in trauma patients *J. Trauma* **40**:613-617 (1996)

Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med.* **189**:1777-1782 (1999)

Shumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ. Structure and function of lipopolysaccharide binding protein *Science* **249**:1429-1431 (1990)

Shwartz DA. The role of TLR4 in endotoxin responsiveness in humans *J. Endotoxin. Res.* **7**:389-393 (2001)

Sieling PA, Chatterjee D, Porcelli SA, Prigozy TI, Mazzaccaro RJ, Soriano T, Bloom BR, Brenner MB, Kronenberg M, Brennan PJ, Modlin RL. CD1 restricted T-cell recognition of microbial lipoglycan antigens *Science* **269**:227-230 (1995)

Sigurdsson GH, Christenson JT, el-Rakshy MB, Sadek S. Intestinal platelet trapping after traumatic and septic shock: am early sign of sepsis and multiorgan failure in critically ill patients? *JAMA* **20**:458-467 (1992)

Small TN, Knowles RW, Keever C, Kerman NA, Collins N, O'Reilly RJ, Dupont B, Flomenberg N. M241 (CD1) expression on B lymphocytes *J. Immunol.* **138**:2864-2868 (1987)

Smirnova I, Hamblin MT, McBride C, Beutler B, Di Rienzo A. Excess of rare amino acid polymorphisms in the Toll-like receptor 4 in humans *Genetics* **158**:1657-1664 (2001)

Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, Kawano T, Pelikan SB, Crowe NY, Godfrey DI. Differential tumor surveillance by natural killer (NK) and NKT cells. *J. Exp. Med.* **191**:661-668 (2000)

Spada FM, Grant EP, Peters PJ, Sugita M, Melian A, Leslie DS, Lee HK, van Donselaar E, Hanson DA, Krensky AM, Majdic O, Porcelli SA, Morita CT, Brenner MB. Self recognition of CD1 by γ/δ T-cells: implications for innate immunity. *J. Exp. Med.* **191**:937-948 (2000)

Spinas GA, Keller U, Brockhaus M. Release of soluble receptors for tumor necrosis factor (TNF) in relation to circulating TNF during experimental endotoxaemia. *J. Clin. Invest.* **90**:533-536 (1992)

Stavnezer J. Antibody class switching. *Adv. Immunol.* **61**:79-146 (1996)

Stenger S, Mazzaccaro RJ, Uyemura K, Cho S, Barnes PF, Rosat JP, Sette A, Brenner MB, Porcelli SA, Bloom BR, Modlin RL. Differential effects of cytolytic T-cell subsets on intracellular infection. *Science* **276**:1684-1687 (1997)

Stephens KE, Ishizaka A, Larrick JW, Raffin TA. Tumor necrosis factor causes increased pulmonary permeability and edema: comparison to septic acute lung injury *Am. Rev. Respir. Dis.* **138**:1364-1370 (1988)

Suberville S, Bellocq A, Fouqueray B, Philippe C, Lantz O, Perez J, Baud L. Regulation of IL-10 production by beta-adrenergic agonists *Eur. J. Immunol.* **26**:2601-2605 (1996)

Suffredini AF, Fromm RE, Parker MM. The cardiovascular response of normal humans to the administration of endotoxin *N. Engl. J Med.* **21**:280-5 (1989)

Sugita M, Jackman RM, van Donselaar E, Behar SM, Rogers RA, Peters PJ, Brenner MB, Porcelli SA. Cytoplasmic tail dependent localization of CD1b antigen presenting molecules to MIICs. *Science* **273**:349-352 (1996)

Sugita M, Grant E, Donselaar E, Hsu V, Rogers R, Peters P, Brenner M. Separate pathways for antigen presentation by CD1 molecules *Immunity* **11**:743-752 (1999)

Sumida T, Sakamoto A, Murata H, Makino Y, Takahashi H, Yoshida S, Nishioka K, Iwamoto I, Taniguchi M. Selective reduction of T-cells bearing invariant V alpha 24 J alpha Q antigen receptor in patients with systemic sclerosis. *J. Exp. Med.* **182**:1163-1168 (1995)

Tabeta K, Yamazaki K, Akashi S, Miyake K, Kumada H, Umemoto T, Yoshie H. Toll-like receptors confere responsiveness to lipopolysaccharide from Porphyromonas gingivalis in human gingival fibroblasts. *Infect. Immun.* **68**:3731-3735 (2000)

Takagi K, Yamamori H, Morishima Y, Toyoda Y, Nakajima N, Tashiro T. Preoperative immunosuppression: its relationship with high morbidity and mortality in patients receiving thoracic esophagectomy *Nutrition* **17**:13-17 (2001)

Takayama K, Quereshi N, Mascagni P. Complete structure of lipid A obtained from the lipopolysaccharides of the heptoseless mutant of *Salmonella typhimurium*. *J. Biol. Chem.* **258**:12801-12803 (1983)

Taveira daSilva AM, Kaulbach HC, Chuidian FS, Lambert DR, Suffredini AF, Danner RL. Shock and multiple organ dysfunction after self administration of Salmonella endotoxin *N. Engl. J. Med.* **328**:1457-1460 (1993)

Tellado JM, Giannias B, Kapadia B, Chartrand L, de Santis M, Christou NV. Anergic patients before elective surgery have enhanced non-specific host-defense capacity *Arch. Surg.* **125**:49-53 (1990)

Teng NNH, Kapian HS, Hebert JM. Protection against Gram-negative bacteraemia and endotoxaemia with human monoclonal IgM antibodies. *Proc. Natl. Acad. Sci. USA* **82**:1790-1794 (1985)

Tetta C, Bellomo R, Inguaggiato P, Wratten ML, Ronco C. Endotoxin and cytokine removal in sepsis *Ther. Apher.* **6**:109-115 (2002)

The Intravenous Immunoglobulin Collaborative Study Group. Prophylactic intravenous administration of standard immune globulin as compared with core-lipopolysaccharide immune globulin in patients at high risk of post-surgical infection. *N. Eng. J. Med.* **327**:234-240 (1992)

Thiemblemont N, Thieringer R, Wright S. Innate immune recognition of bacterial lipopolysaccharide: dependence on interactions with membrane lipids and endocytic movement. *Immunity* **8**:771-777 (1998)

Tough D, Sun S, Sprent J. T-cell stimulation in vivo by lipopolysaccharide (LPS) *J. Exp. Med.* **185**:2089-2094 (1997)

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications *Proc. Natl. Acad. Sci. USA* **76**:4350-4354 (1979)

Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia *Nature* **330**:662-664 (1987)

Tracey KJ, Lowry SF, Cerami A. Cachectin/TNF-alpha in septic shock and adult respiratory distress syndrome *Am. Rev. Respir. Dis.* **138**:1377-1379 (1988)

Tsai C, Frasch CE. A sensitive silver stain fro detecting LPS in polyacrylamide gels. *Analytical Biochemistry* **119**:115-119 (1982)

Uchida K, Mizushima S. A simple method for the isolation of lipopolysaccharides from Pseudomonas aeruginosa and some other bacterial strains *Agric. Biol. Chem.* **51**:3107-3114 (1987)

Underhill DM, Ozinsky A. Toll-like receptors: key mediators of microbe detection. *Curr. Opin. Immunol.* **14**:103-110 (2002)

Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *J. Biol. Chem.* **277**:15107-15112 (2002)

van der Poll T, Coyle SM, Barbosa K, Braxton CC, Lowry SF. Epinephrine inhibits tumor necrosis factor alpha and potentiates IL-10 production during human endotoxaemia *J. Clin. Invest.* **97**:713-719 (1996)

van Deuren M, van der Ven-Jongekrijg J, Demacker PN, Bartelink AK, van Dalen R, Sauerwein RW, Gallati H, Vannice JL, van der Meer JW. Differential expression of pro-inflammatory cytokines and their inhibitors during the course of meningococcal infections. *J. Infect. Dis.* **169**:157-161 (1994)

Various authors. Increase in national hospital discharge survey rates for septicaemia – united states, 1979-1987. *MMWR Morb Mortal Wkly Rep.* **39**:31-34 (1990)

Various authors. Letters to the editor: The HA-1A monoclonal antibody for gram-negative sepsis. *N. Engl. J. Med.* **325**:279-281 (1991)

Vasselon T, Hailman E, Thieringer R, Dietmers PA. Internalisation of monomeric lipopolysaccharide occurs after transfer out of cell surface CD14. *J. Exp. Med.* **190**:509-522 (1999)

Venezia ND, Minka S, Bruneteau M, Mayer H, Michel G. Lipopolysaccharides from Yersinia pestis *Eur. J. Biochem.* **151**:399-404 (1985)

Vincent JL, de Mendonca A, Cantraine F, Moreno R, Takala J, Suter PM, Sprung CL, Colardyn F, Blecher S. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the working group on sepsis-related problems of the European Society of Intensive Care Medicine. *Int. Care Med.* **22**:707-710 (1996)

Viriyakosul S, Tobias PS, Kitchens RL, Kirkland TL. MD-2 binds to lipopolysaccharide *J. Biol. Chem.* **276**:38044-38051 (2001)

Vogel S, Hilfiker M, Caulfield M. Endotoxin induced T-lymphocyte proliferation *J. Immunol.* **130**:1774-1779 (1983)

Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. *Nature* **390**:350-351 (1997)

Waage A, Halstensen A, Espevik T. Association between tumor necrosis factor in serum and fatal outcome in patients with meningococcal disease *Lancet* **338**:355-357 (1987)

Wakefield CH, Barclay GR, Fearon KCH, Goldie AS, Ross JA, Grant IS, Ramsay G, Howie JC. Pro-inflammatory mediator activity, endogenous antagonists and the systemic inflammatory response in intra-abdominal sepsis. *Br. J. Surg.* **85**:818-825 (1998)

Warren HS, Danner RL, Munford RS. Anti-endotoxin monoclonal antibodies. *N. Engl. J. Med.* **326**:1153-1157 (1992)

Weiss J, Elsbach P, Shu C, Castillo J, Grinna L, Horwitz A, Theofan G. Human bactericidal/permeability increasing protein and a recombinant NH_2-terminal fragment cause killing of serum resistant Gram-negative bacteria in whole blood and inhibit tumor necrosis factor release induced by the bacteria. *J. Clin. Invest.* **90**:1122-1130 (1992)

Wells CL, Rotstein OD, Pruett TL, Simmons RL. Intestinal bacteria translocate into experimental abdominal abscesses *Arch. Surg.* **121**:102-107 (1986)

Wenzel RP. The mortality of hospital acquired blood stream infections: need for a new vital statistic. *Int. J. Epidemiol* **17**:225-7 (1988)

Wenzel RP. Anti-endotoxin monoclonal antibodies – a second look. Editorial. *N. Engl. J. Med.* **326**:1151-1153 (1992)

Werts C, Tapping RI, Mathison JC, Chuang TH, Kravchenko V, Saint Girons I, Haake DA, Godowski PJ, Hayashi F, Ozinsky A, Underhill DM, Kirschning CJ, Wagner H, Aderem A, Tobias PS, Ulevitch RJ. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat. Immunol.* **2**:346-352 (2001)

Westphal O, Luderitz O, Bister F. Uber die Extraktion von Bacterien mit Phenol/Wasser. *Z. Naturforsch* **B7**:148-155 (1952)

Westphal O, Luderitz O. Chemische Erforschung von Lipopolysaccharidien Gram-negativer Bakterien. *Angew Chemie* **66**:407-417 (1954)

Westphal O, Westphal U, Sommer T. The history of pyrogen research. In: *Microbiology* ed. Schlessinger D. American Society for Microbiology, Washington DC, pp221-238 (1977)

Westphal O, Jann K, Himmelspach K. Chemistry and immunochemistry of bacterial lipopolysaccharides as cell wall antigens and endotoxins. *Prog. Allergy*. **33**:9-39 (1983)

Wheeler AP, Bernard GR. Treating patients with severe sepsis *N. Engl. J. Med.* **340**:207-214 (1999)

Windsor JA, Fearon KCH, Ross JA, Barclay GR, Smyth E, Poxton I, Garden OJ, Carter DC. Role of serum endotoxin and antiendotoxin core antibody levels in predicting the development of multiple organ failure in acute pancreatitis *Br. J. Surg.* **80**:1042-1046 (1993)

Wood JJ, Rodrick ML, O'Mahony JB, Palder SB, Saporoschetz I, D'Eon P, Mannick JA. Inadequate interleukin 2 production. A fundamental immunological deficiency in patients with major burns. *Ann. Surg.* **200**:311-320 (1984)

Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS-binding protein. *Science* **249**:1431-1433 (1990)

Wright SD. Multiple Receptors for Endotoxin. *Current Op. Immunol* **3**:83-90 (1991)

Wright SD. Toll, a new piece in the puzzle of innate immunity *J. Exp. Med.* **189**:605-609 (1999)

Wurfel MM, Monks BG, Ingalls RR, Dedrick RL, Delude R, Zhou D, Lamping N, Schumann RR, Thieringer R, Fenton MJ, Wright SD, Golenbock D. Targeted deletion of the 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 (1997)

Wurfel MM, Wright SD. Lipopolysaccharide (LPS) binding protein catalyses binding of LPS to lipoproteins *Prog. Clin. Biol. Res.* **392**:287-295 (1995)

Wurfel MW, Wright S. Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers. *J. Immunol* **158**:3925-3934 (1997)

Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, Achong MK. IL-6 is an anti-inflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J. Clin. Invest*. **101**:311-320 (1998)

Young LS, Gascon R, Alam S, Bermudez LE. Monoclonal antibodies for treatment of Gram-negative infections. *Rev. Infect. Dis.* **11S7**:1564-1571 (1989)

Zedler S, Bone RC, Baue AE, Donnersmarck G, Faist E. T-cell reactivity and its predictive role in immunosuppression after burns *Crit. Care Med.* **27**:66-72 (1999)

Zeng Z, Castano AR, Segelke BW, Stura EA, Peterson PA, Wilson IA. The crystal structure of mouse CD1: an MHC like fold with a large hydrophobic antigen binding groove. *Science* **277**:339-345 (1997)

Ziegler EJ, McCutchan JA, Braude AI. Treatment of Gram-negative bacteraemia with antiserum to core glycolipid. The experimental basis of immunity to endotoxin. *Europ. J. Cancer* **15**:71-76 (1979)

Ziegler EJ, McCutchan JA, Fierer J, Glauser MP, Sadoff JC, Douglas H, Braude AI. Treatment of Gram-negative bacteraemia and shock with human anti-serum to a mutant *Escherichia coli*. *N. Eng. J. Med*. **307**:1225-1230 (1982)

Ziegler EJ, Teng NNH, Douglas H, Wunderlich A, Berger HJ, Bolmer SD. Treatment of *Pseudomonas* bacteraemia in neutropenic rabbits with human monoclonal IgM antibody against *E.coli* lipid A *Clin. Res*. **35**:619A (Abstract) (1987)

Ziegler EJ, Fisher CJ Jr, Sprung CL, Straube RC, Sadoff JC, Foulke GE, Wortel CH, Fink MP, Dellinger RP, Teng NN. Treatment of gram-negative bacteraemia and septic shock with HA-1A human monoclonal antibody against endotoxin. A randomised double blind, placebo controlled trial. *N. Engl. J. Med*. **324**:429-46 (1991)

APPENDIX I

LOTHIAN RESEARCH ETHICS COMMITTEE
STANDARD CONSENT FORM

Title of proposed research: **Immune responses in abdominal complaints**

Name of Investigator: **Mr. Ian Currie**

Address: **Lister Laboratories, Royal Infirmary of Edinburgh.**

Further information is available from:
Mr. Rowan Parks, Senior Lecturer, Honorary Consultant Surgeon, Royal Infirmary of Edinburgh. Tel; 536 3817

List any drugs and procedures to be given in the study explaining their action:
No drugs will be given. One blood sample is required for healthy volunteers or clinic patients, two for inpatients.

- I agree to participate in this study
- I have read this consent form and the Information sheet and had the opportunity to ask questions about them
- I agree for notice to be sent to my General Practitioner about my participation in this study
- I agree to the provision of any clinically significant information to my General Practitioner
- I understand that I am under no obligation to take part in this study and that a decision not to participate will not alter the treatment that I would normally receive
- I understand that I have the right to withdraw from this study at any stage and that to do so will not affect my treatment
- I understand that this is non therapeutic research from which I cannot expect to derive any benefit.

Signature of Patient

Name of Patient

Signature of Investigator

Date

Four Copies to be made
Top copy to be retained by principal investigator
Second copy to be retained by patient/volunteer
Third copy to be sent to individual's General Practitioner
Fourth copy to be filed in case notes

Research article

The biological activity of a liposomal complete core lipopolysaccharide vaccine

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A vaccine that induces humoral immunity to lipopolysaccharide (LPS), while remaining non-pyrogenic should be beneficial, as high levels of antibodies against LPS are associated with a reduced risk of adverse outcome. However, pure LPS or bacteria expressing LPS are generally considered too toxic to be used as vaccines. Recently, a novel, immunogenic complete core lipopolysaccharide vaccine has been described, which has been designed to prevent endotoxin-related inflammatory reactions in surgical and high-risk hospitalized patients. *In vivo* studies have shown that while administration of the vaccine to rabbits results in no toxicity over 7 days, it does induce significantly enhanced antibody responses towards a broad range of clinically relevant Gram-negative LPSs. Here we show that encapsulation of the four complete core LPS types *Escherichia coli* K12, *Escherichia coli* R1, *Bacteroides fragilis* and *Pseudomonas aeruginosa* into liposomes greatly reduces the ability of a given amount of LPS to induce TNF-α production *in vitro* from human monocytes. In contrast to previous studies of liposomal LPS, we demonstrate a reduction in activity of approximately 100,000-fold; a reduction approximately 100–1,000-fold more than that previously described. The signalling by the liposomal LPS appears to be entirely dependent on serum factors, though this can be partially restored by soluble CD14 or, to a lesser extent, by lipopolysaccharide binding protein. Time-course experiments reveal that liposomal LPS signalling shows similar kinetics to pure LPS signalling. Therefore, as well as inducing specific antibody responses, liposomal LPS demonstrates characteristics suitable for use as a vaccine to be used in human beings.

INTRODUCTION

It has been estimated that endotoxin-mediated toxicity accounts for approximately 100,000 deaths per year in the US alone,[1] with a predisposition towards post-surgical patients. In surgical and high-risk hospitalized patients, it is thought that the gut barrier breaks down, leading to release of whole Gram-negative organisms, cell membranes or lipopolysaccharide (LPS: endotoxin) into the portal circulation.[2,3] Endotoxin-mediated toxicity is thought to occur as a result of the effect that LPS has on the cells of the immune system, and in particular, macrophages.[4] Endotoxin is capable of inducing macrophages to release a number of pro-inflammatory mediators, including TNF-α, IL-6, IL-8 and IL-1β. In greater than normal physiological concentrations, these mediators induce systemic pathological effects.[5–8]

Recent studies of anti-LPS antibodies in the sera of pre- and post-surgery patients have revealed a correlation between high endogenous levels of anti-core LPS antibodies and protection from adverse postoperative outcome.[9–12] Pre-surgical vaccination with LPS might benefit patients by increasing their anti-LPS antibody titre before surgery. However, even as little as several nanograms of LPS is enough, when administered to a human subject, to induce symptoms of systemic inflammation.[13] This toxicity must be reduced somehow if an LPS-based vaccine is to be

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viable, and for this reason the LPS used in the vaccine has been incorporated into liposomes.

However, while demonstrating lower toxicity than native LPS, the vaccine must also remain highly immunogenic. Therefore, in order to achieve the widest possible protective antibody response, the liposomal vaccine comprises a mixture of four complete core LPSs (*Escherichia coli* K12, *E. coli* R1, *Pseudomonas aeruginosa* PAC 608 and *Bacteroides fragilis*), since antibodies directed against these core epitopes cross-react with many LPS serotypes.[14] In addition, this liposomal cocktail has been shown to protect mice from challenge with a lethal dose of *E. coli* O18 LPS.[14]

The objective of this study was to investigate the toxicity of the vaccine in human cell systems. We compared the relative toxicity of the vaccine with the native unincorporated cocktail of LPSs mixed in equal ratio but not incorporated into liposomes. Measurement of TNF-α secretion from challenged human monocytes reveals the potential toxicity of the vaccine as applied to humans, as release of this cytokine is a major contributor to the symptoms of endotoxic shock.[6] The kinetics of release of these molecules was also investigated to determine whether the new preparation shows a delayed peak release of cytokines due to its encapsulation in the liposomes, or protracted cytokine release over time. Finally, the dependence of liposomal signalling on serum factors was investigated to determine whether the pathway utilized by macrophages to detect the liposomal LPS shared any similarities with the serum-dependent pathways used to recognize pure LPS.

Materials and Methods

LPS/liposome preparation

LPSs used in experiments were extracted from rough mutant *E. coli* K12 (NCTC 13116), *E. coli* R1 (NCTC 13114), *P. aeruginosa* PAC 608 (NCTC 13124), and *B. fragilis* (NCTC 9343). Bacteria were grown in nutrient broth (filter-sterilized, Gibco), overnight at 37°C. After harvesting and washing 3 times in PBS, they were heat killed (100°C, 10 min) and then frozen until LPS extraction. LPS was extracted from lyophilized bacteria by the phenol/chloroform/petroleum (PCP) method of Galanos *et al.* as described by Hancock and Poxton.[15] Purified LPS was checked for protein impurities by the Lowry assay (< 1% protein) and silver-stained SDS-PAGE (no visible bands), and for nucleic acid contamination by spectrophotometry (< 0.2%), then dissolved at 1 mg/ml in pyrogen-free water and stored at –20°C before use in assays. Multilamellar liposomes, consisting of dimyristoyl phosphatidyl choline, dimyristoyl phosphatidyl glycerol and cholesterol (4:1:4), containing 33 μg total LPS per ml (one-quarter of each LPS core type by weight, 10:1 lipid to LPS ratio w/w), were prepared according to the method of Dijkstra *et al.*,[16] with some modifications (as described by Bennett-Guerrero *et al.*[14]) at Duke University (Durham, NC, USA). For subsequent washes, liposomes were centrifuged for 10 min at 13,000 *g* in a bench centrifuge and resuspended in pyrogen-free PBS. LPS was recovered from liposomal preparations in some experiments by aqueous phenol extraction.[15]

Preparation of human monocytes

Human peripheral blood was diluted 1:3 in PBS then layered onto Histopaque 1077 (Sigma), centrifuged at 400 *g* for 30 min and the interface cells removed. Cells were then washed twice in PBS and resuspended in RPMI/10% human serum (HS; Sigma). Monocytes were counted in white cell diluting fluid (0.01% Gentian violet in 1% acetic acid). Cells were plated at 2 x 10^5 monocytes per well of a 96-well plate. After 1–1.5 h, the cells were washed with RPMI/10% HS to remove non-adherent cells, leaving a monolayer of monocytes.

Challenge with LPS preparations

LPS or liposome preparations were diluted to appropriate concentrations in RPMI/10% HS or RPMI without serum and 100 μl of each dilution was then added to monocytes in triplicate. In experiments using only one concentration of LPS, either 100 ng/ml LPS or 10 μg/ml liposomal LPS was chosen. Serum protein experiments used serum-free medium supplemented with 1 μg/ml purified human plasma lipopolysaccharide binding protein (HBT, Uden, The Netherlands) or 1 μg/ml recombinant human soluble CD14 (R&D). Samples of supernatant were collected and assayed for TNF-α content at specific time points (4 h for single time-point TNF-α experiments, various time-points for TNF-α release time-course experiments).

Measurement of TNF-α

Supernatant TNF-α concentrations were measured by bioassay as described by Delahooke *et al.*[17] Briefly, 10 μl of supernatant was added to L929 cells plated at 2 x 10^4 per well of a 96-well plate together with 90 μl DMEM/5% FCS/1 μg/ml actinomycin D. A standard curve was prepared using TNF-α (Peprotech) at 3.2-fold dilutions. After incubation at 37°C overnight, cell death was measured by staining with crystal violet for 2 min then releasing with 50 μl 20% (v/v) acetic acid. Values were read at 590 nm on an Anthos 2001 automated plate reader.

Statistics

Due to interexperimental variation in the maximum amount of TNF-α produced from each individual donor, the values for each experiment were normalized such that the maximum production of TNF-α in each experiment was taken to be 100. Data from 3–5 independent experiments were then combined and each datum point compared with control cell secretion by the Student's paired *t*-test. The end-point of dose response was taken as the first point that was not significantly different from background ($P > 0.05$).

Results

Relative biological activity of pure LPS and LPS-liposome preparations

The capacity for macrophage stimulation by PCP-purified LPS and LPS in liposomes was estimated by determining a dose-response curve for each preparation in terms of its ability to induce TNF-α from human peripheral blood monocytes. The end-point of dose response was taken as the first datum point that was not significantly different from background ($P > 0.05$).

The activities of *E. coli* R1 and *E. coli* K12 LPS show very similar levels of activity to the mixed (cocktail) LPS (Fig. 1). *B. fragilis* LPS appears to be approximately 10,000-fold less toxic than the *E. coli* and mixed LPSs, while *P. aeruginosa* LPS shows an ability to stimulate monocytes only at concentrations 10^5–10^6 times more than that obtained with *E. coli*-derived LPS.

The sensitivity of the monocytes from each individual donor to the different LPSs was very consistent for *E. coli*-derived and mixed LPS, but significant differences were observed between donors in their ability to recognise *B. fragilis* and *P. aeruginosa* LPS, as evidenced by

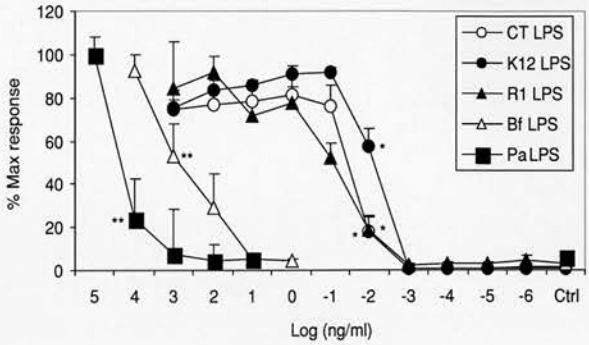

Fig. 1. Comparison of TNF-α production from monocytes exposed to free (unincorporated) *E. coli* K12, *E. coli* R1, *B. fragilis*, *P. aeruginosa* and the cocktail of these LPSs (CT). Results are normalized means ± SEM of five independent experiments. *Datum point indicating lowest concentration at which response is significantly different from control ($P < 0.05$). **For *B. fragilis* and *P. aeruginosa* LPS, the minimum concentration of LPS responded to was highly donor-dependent, so statistics were performed on the individual data sets from these donors and not the mean values presented here (see Table 1 and discussion).

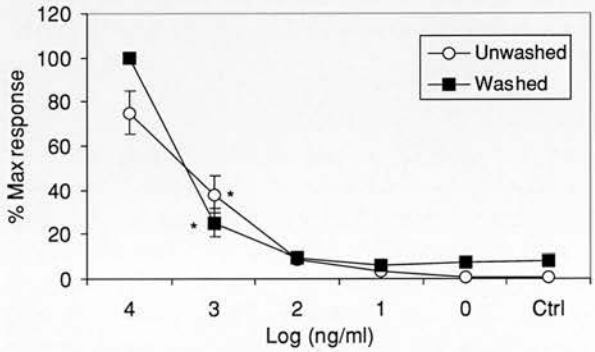

Fig. 2. Comparison of TNF-α production from monocytes stimulated with washed and unwashed liposomes containing cocktail LPS. Washed liposomes were centrifuged 5 times further than freshly prepared (unwashed) liposomes. Results are normalized means from four independent experiments ± SEM. *Indicates lowest concentration at which response is significantly different from control ($P < 0.05$).

Table 1. Comparative biological activities of LPS core types and liposomes

LPS type	Minimum concentration required to stimulate monocytes* (ng/ml)	Activity compared to liposomes (liposomes = 1)
E. coli R1	0.01	100,000
E. coli K12	0.01	100,000
B. fragilis	100–1000**	1–10
P. aeruginosa	1000–10,000**	0.1–1
Cocktail (CT) LPS	0.01	100,000
Liposomal CT LPS	1000	1
Washed liposomes	1000	1
Recovered LPS	0.1	10,000

*Datum point taken as lowest value significantly different ($P > 0.05$) from control, and hence concentration at which preparation does not stimulate cells. **Monocytes from certain individuals responded to different degrees with *B. fragilis* and *P. aeruginosa* LPS so range of sensitivity is given.

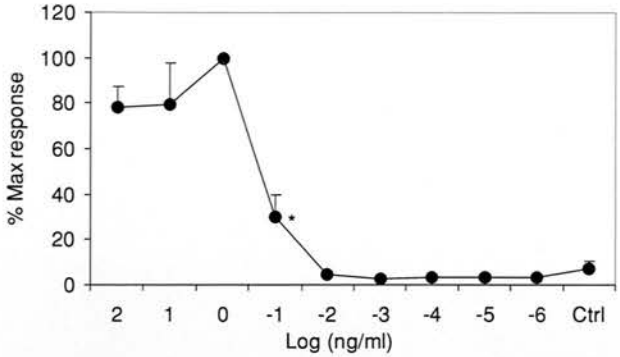

Fig. 3. TNF-α production from monocytes stimulated with LPS reclaimed from liposomes by phenol extraction. Results are normalized mean ± SEM of five independent experiments. *Indicates lowest concentration at which response is significantly different from control ($P < 0.05$).

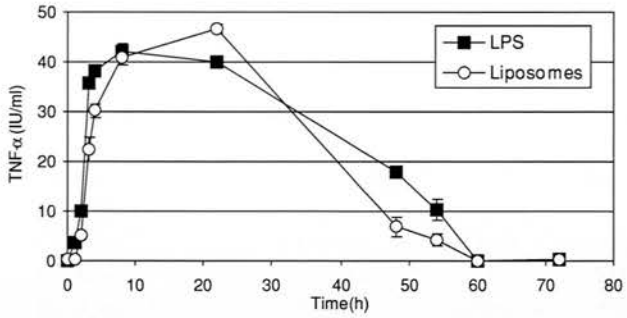

Fig. 4. Time course of TNF-α production by human monocytes in response to pure LPS and liposomal LPS. The amount of TNF-α produced in response to 100 ng/ml of pure LPS and 10 μg/ml of liposomal LPS was measured at various time points. Results show the mean of triplicate readings ± SEM from one experiment and are representative of three similar experiments.

the larger error bars in those series. The liposomal cocktail LPS proved to be ~10^5-fold less stimulatory than the equivalent mixture of unincorporated LPS (Figs 1 & 2), as summarised in Table 1. Liposomes containing no LPS do not induce TNF-α release from monocytes at any concentration.

Quantification of LPS incorporated into liposomes

To determine how much of the LPS added to the liposomal lipids became incorporated into the liposomes, a LAL assay was performed on the supernatant of centrifuged liposomes. This showed that 96–99.8% of the LPS was incorporated at this stage.[14] Challenge of monocytes with this liposomal supernatant and subsequent TNF-α measurement also revealed a biological activity in close accordance with what might be expected if this amount of LPS had been incorporated (data not shown).

However, to rule out the possibility that native LPS was co-pelleting with the liposomes in this assay, we performed a phenol extraction of the prepared liposomes and used the resultant re-purified LPS to challenge monocytes as described above. Figure 3 shows that the recovered LPS is approximately 10,000-fold more toxic than the liposomes (Fig. 2) – slightly less than might be expected if the recovery of the LPS from the liposomes were 100% efficient. Then, to determine whether subsequent washing steps could reduce the activity of the liposomes yet further, liposomes (already washed 5 times during manufacture) were washed a further 5 times. The activity of the liposomes following these additional washes remained largely unchanged (Fig. 2). Finally, in order to determine whether free LPS could co-precipitate with the liposomes in these washing steps, centrifugation of stock LPS was performed according to the same protocol. No pellet was observed and supernatant retained biological activity suggesting that centrifugation under these conditions is not sufficient to pellet soluble LPS.

Kinetics of cytokine release and dependency on serum factors

When human peripheral blood monocytes were incubated with 100 ng/ml pure LPS or 10 μg/ml LPS complexed in liposomes the release of TNF-α showed similar kinetic profiles (Fig. 4). TNF-α production was seen to peak at 8 h (LPS) and 24 h (liposomes) before decreasing substantially in both cases by 48 h and becoming undetectable by 60 h.

Removal of serum from the medium reveals the dependency of the signalling on substances within serum. Figure 5 shows the relative TNF-α produced from human monocytes exposed to either 100 ng/ml of pure LPS or 10 μg/ml

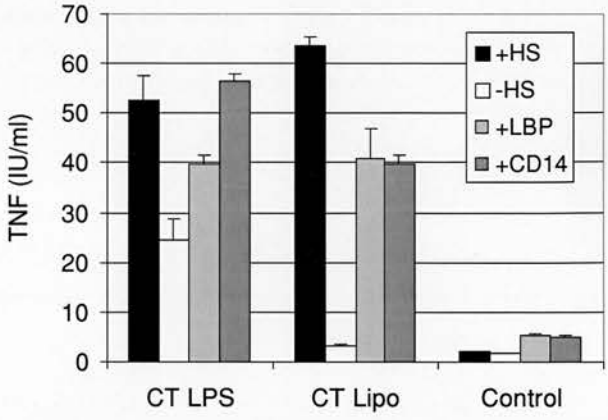

Fig. 5. Dependence of LPS signalling on serum factors. Human peripheral blood monocytes were challenged with 100 ng/ml LPS or 10 μg/ml liposomal LPS with human serum, without serum and without serum but supplemented with 1 μg/ml human LBP or soluble CD14. Results are representative of three similar experiments, error bars are SEM. CT LPS = 100 ng/ml cocktail LPS. CT Lipo = liposomes containing 10 μg/ml cocktail LPS.

liposomal LPS. On removal of serum, approximately one-half of the serum response remained following challenge of cells with native LPS preparations. This could be partially rescued by addition of purified LBP or to a greater extent by soluble CD14. The response towards liposomal LPS is completely abolished on removal of serum, yet this can also be recovered to approximately two-thirds of maximum activity by restoration of either LBP or soluble CD14, with each of these proteins having a similarly restorative effect in this case.

DISCUSSION

Evidence is now accumulating to support the hypothesis that low endogenous levels of anti-LPS antibody is a significant risk factor in the outcome of high-risk hospitalized patients. For example, a study of 86 cardiac surgery patients revealed that those with detectable anti-LPS antibodies before surgery had a lower incidence of wound infection (3% versus 29%) and fever (30% versus 64%).[9] A later study revealed that patients with low anti-LPS IgM levels had a higher incidence of post-surgery complications, while those with high anti-LPS IgM levels had a much lower incidence of post-surgical complications.[10] In light of these observations, several groups have attempted to provide passive protection from endotoxin-mediated complications by administration of monoclonal anti-LPS antibodies to patients prior to surgery, but these have met with limited success for a variety of reasons (for a review see Baumgartner[18]). In general, however, these problems were mainly concerned with the poor specificity for LPS or low cross reactivity between different LPS core types of the monoclonal antibodies used in these studies. An alternative approach is active immunisation with a cocktail of LPS preparations. In order to provide the widest possible protective antibody response, a liposomal vaccine comprising a mixture of four complete core LPSs (*E. coli* K12, *E. coli* R1, *P. aeruginosa* PAC 608, and *B. fragilis*) was developed. Antibodies directed against these core epitopes cross-react with LPS from many potential pathogenic organisms.[14] It should be noted that the antibodies produced by the vaccine are against core epitopes rather than specific O-antigens. The use of this cocktail of complete LPS core molecules has resulted in an immunogenic vaccine that produces a broadly cross reactive response to a range of LPSs from a large panel of Gram-negative bacteria. It has been shown to protect mice from lethal challenge with *E. coli* O18 LPS.[14]

In addition to immunogenicity, however, any proposed LPS-based vaccine must first be shown to be significantly less toxic than native LPS. Administration of pure LPS alone as a vaccine to hospitalized patients is an impractical proposal, as even very small amounts of pure LPS can elicit toxic reactions.[13] Pure LPS is also unlikely to produce an IgG T-dependent antibody response. For this reason, we have compared the relative toxicity of the cocktail LPS liposomal preparation with that of the individual LPS components in soluble form by looking at the TNF-α secretion of challenged human monocytes.

In order to do this, the concentration of the LPS within the liposomes had to be determined accurately. This was achieved in principle by measuring the concentration of LPS in a stock solution by LAL assay before incorporating the whole amount into liposomes.[14] Subsequently, the liposomes were centrifuged and the remaining supernatant and the liposomes themselves re-checked by LAL assay to ensure that negligible LPS remained in the supernatant. In practice, 96–99.8% of the LPS was incorporated, and the final LAL assay of the washed liposomes revealed an activity of less than 0.1% of the native LPS.[14]

This small amount of endotoxin remaining in the supernatant surrounding the liposomes was also confirmed by measuring the TNF-α release from monocytes challenged with the supernatant. This, therefore, prompted an experiment to determine whether the activity of the liposome preparations could be lowered yet further through further centrifugation and washing steps to remove this extra-liposomal endotoxin. Figure 2 shows that, in fact, the freshly manufactured liposomes (already washed 5 times during manufacture) have a biological activity largely similar to that of liposomes washed a further 5 times. In addition, it was noted that centrifugation of native, soluble LPS according to the same protocol was unable to produce a pellet. These findings suggest that the endotoxic activity existing in the supernatant outside the liposomes cannot be separated from them in this way, possibly indicating that an equilibrium exists between the liposomal and soluble compartments such that a small amount of endotoxin always remains in the medium.

However, to rule out the possibility that the low activity of our liposomes was purely due to extremely poor incorporation of LPS during manufacture, we performed a phenol extraction of the prepared liposomes and used the resultant re-purified LPS to challenge human monocytes. Figure 3 shows that, in terms of TNF-α production, the recovered LPS is approximately 10,000-fold more toxic than the original liposomes (Fig. 2). This is not quite the 100,000-fold difference that might be expected if the initial incorporation and subsequent extraction of the LPS had returned a 100% efficiency, and given the LAL assay data are more likely to be a reflection of the poor yield of the simple phenol extraction method used rather than the incorporation protocol.

We then compared the biological activity of the liposomal cocktail LPS with that of the individual component LPSs. Table 1 summarises the results of our comparisons of

the TNF-α release from human monocytes challenged with free and liposomal LPS. *E. coli* R1, *E. coli* K12 and 'cocktail' LPS showed very similar biological activities in this assay, all being easily detected by the monocytes at concentrations down to around 0.01 ng/ml (Fig. 1). Interestingly, however, while each of the five individual donors' monocytes responded similarly to the *E. coli*-derived LPSs, a much larger diversity was noted in their sensitivity to *B. fragilis* and *P. aeruginosa* LPS. Responses to *B. fragilis* LPS occurred from 100–1000 ng/ml and *P. aeruginosa* from 1000–10,000 ng/ml. This may indicate a heterogeneity in the sensitivity or expression of the receptor molecules for these LPSs in these individuals that is not seen in the largely TLR-4-dependent signalling process used by the monocytes to detect *E. coli* type LPSs. Further experiments will be required to determine how this heterogeneity in sensitivity to *P. aeruginosa* and *B. fragilis* LPS arises and whether or not differential TLR usage is involved.

When comparing the activity of the liposomal LPS with that of the equivalent free cocktail LPS, a reduction in activity of at least 100,000-fold was observed (Table 1). This reduction in biological activity is approximately 100–1000-fold more than that seen previously by other workers. For example, studies of *Salmonella minnesota* rough (Re) and wild-type LPS showed that incorporation of LPS into liposomes according to the method of Dijkstra *et al.*[16] results in reduction of IL-1β secretion from RAW 264.7 macrophages by 100–1000-fold,[19] and TNF-α release of 100–1000-fold.[20] A reduction in activity as measured by LAL assay has been observed to be in the order of 100–1000-fold,[16] and actinomycin D sensitized mice have been shown to have a 10-fold reduction in lethality when challenged with liposomal lipid A compared with native lipid A.[21] Finally, encapsulation of *Neisseria meningitidis* LPS within liposomes has been shown to reduce the pyrogenicity of LPS in rabbits by up to 1000-fold.[22] Why our liposomal preparations demonstrate lower biological activities than those seen in earlier studies is unclear, though perhaps some explanations may lie in the fact that the techniques used to prepare them are slightly different. A modified version of the method described by Dijkstra *et al.*[16] was employed.[14] Thus, our liposomes might contain more internal lamellae or are larger than those previously described, or perhaps the distribution of the LPS within the liposomes is different.

In order to understand how this reduction in activity is conferred, the recognition pathways utilised by the macrophages to recognise native LPS must be considered. It is thought that proteins present in serum (such as LBP and soluble CD14) are able to catalyze transfer of LPS from micelles or the outer membranes of bacteria to the cell surface of macrophages.[23,24] Here they appear to be inserted into the plasma membrane for recognition by Toll-like receptors (TLRs). It is not clear where this final Toll-mediated recognition occurs, but evidence exists that TLR-4[25,26] is involved in recognizing the presence of LPS and mediates subsequent internalization and transport of the LPS molecules to the Golgi.[24] Here, a poorly defined signalling cascade leads to LPS recognition and the stimulation of the macrophage, through NF-κB and AP-1 transcription factors, to release molecules such as TNF-α, IL-6, IL-8, and IL-1β.[26] However, alternative pathways exist for the uptake of LPS that do not elicit secretory responses. Both CD18 and scavenger receptor are capable of binding LPS and, following either pinocytosis of LPS or engulfment of whole bacteria, the LPS is degraded in lysosomes without inducing strong cytokine release.[27] These pathways are utilized by spleen and liver cells, for example, to detoxify LPS from the blood.

Bearing these pathways in mind, several possible explanations for the reduced toxicity of the liposomal LPS arise. First, it is possible that the multilamellar liposomes contain only a very small proportion of their LPS in their outermost lipid leaflets, and hence only a small proportion of the LPS is accessible to macrophage receptor molecules to induce signalling. In this model, the majority of the LPS would be contained within the central layers of the liposomes where it is shielded from interaction with proteins such as LBP or macrophage CD14. Alternatively, direct phagocytosis of the liposomes could direct them through less stimulatory degradative pathways, and there is already evidence to suggest that this is the case, as following endocytosis liposomes appear to be quickly trafficked towards secondary lysosomes.[20] In this model, it may simply be that the comparatively large size of the liposomes predisposes them towards rapid engulfment by the macrophages and hence non-stimulatory degradation of LPS, while the LPS micelles may remain too small to be dealt with efficiently in this manner. It may be also the case that the small size of the LPS micelles (~0.01 μm)[27] compared with the liposomes simply offers a much increased surface area for interaction with serum lipid transfer proteins in a fashion similar to the effect particle size has on reaction kinetics. Finally, since we were unable in our experiments to remove all endotoxic activity from the liposomal supernatant, the possibility remains that the larger part of the liposomal toxicity comes from this compartment. In this view, the preparation can then be seen as simply being a low concentration solution of LPS with the liposomes playing little or no role in monocyte activation.

The possibility that the liposomes might release the LPS slowly over time leading to stronger and/or longer lasting monocyte activation was investigated, as this outcome could negate some of the benefits of the vaccine. Such a delayed peak in TNF-α release could occur by fusion of the internal liposomal lamellae with TLR-4 bearing intracellular vesicles or the plasma membrane.

However, this seems unlikely given the data presented in Figure 4, as both free and liposomal LPS were revealed to show a similar kinetic profile. TNF-α production was seen to peak at 8 h (LPS) and 24 h (liposomes) before decreasing substantially in both cases by 48 h and becoming undetectable by 60 h. This profile of monocyte activation suggests that the observed reduction in biological activity of the liposomal LPS is not due simply to a delayed response of the macrophages to the LPS, but rather to a lack of availability of the LPS to the macrophage recognition pathways.

To clarify further the role of serum proteins on liposomal LPS recognition, experiments were performed in the absence of serum, since activation of macrophages under these circumstances could indicate direct liposomal fusion with the macrophage plasma membrane or with subsequent internal vesicles linked to the LPS signalling pathways. On removal of serum, approximately one-half of the serum response remained following challenge of cells with native LPS preparations (Fig. 5). This observation (that high concentrations of pure LPS do not require serum factors for monocyte activation) has been made before.[28] This activity could be restored partially, however, by addition of purified LBP or entirely by addition of an equivalent amount of soluble CD14. By contrast, the response towards liposomal LPS is seen to be completely abolished on removal of serum, yet this too can be restored to approximately two-thirds of maximum activity by supplementation of the medium with either LBP or soluble CD14. However, in this case, both proteins seem to have a similar restorative capacity. These findings suggest that the biological activity of the liposomes is occurring via the traditional LPS shuttle proteins and receptors used to detect low concentrations of soluble LPS. We are thereby unable to rule out the possibility that most or all of the liposomal activity seen comes from the small amount of endotoxin present in the supernatant surrounding the liposomes.

Interestingly, while the biological activity of the LPS has been clearly diminished by incorporation into liposomes, the immunogenicity of the vaccine has not. This can perhaps be best explained by suggesting that the liposomal LPS is processed in the cell via pathways that are much more likely to result in antibody formation than the pathways used to deal with native LPS. At present, however, these mechanisms remain largely unclear.

Conclusions

We have provided data in support of the potential benefits of this new liposomal LPS by showing it to have greatly reduced macrophage stimulatory potential *in vitro*. The immunogenicity of this new liposomal preparation has been shown capable of inducing production of broadly cross-reactive and protective anti-LPS antibodies in small animals[14] and we await clinical trials in pre-surgery patients.

Acknowledgements

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References

1. Anon. Increase in national hospital discharge survey rates for septicaemia – United States, 1979–1987. *Morb Mortal Wkly Rep* 1990; **39**: 31–34.
2. Saadia R, Schein M, MacFarlane C, Boffard KD. Gut barrier function and the surgeon *Br J Surg* 1990; **77**: 487–492.
3. Mainous M, Tso P, Berg R, Deitch E. Studies of the route, magnitude and time course of bacterial translocation in a model of systemic inflammation. *Arch Surg* 1991; **126**: 33–37.
4. Manthey C, Vogel S. Interactions of lipopolysaccharide with macrophages. In: Zwilling BS, Eisenstein TK eds. *Macrophage–Pathogen Interactions*. New York: Dekker, 1994; 63–78.
5. Manthey C, Vogel S. The role of cytokines in host responses to endotoxin. *Rev Med Microbiol* 1992; **3**: 72–79.
6. Calendra T, Baumgartner JD, Grau GE. Prognosis values of tumor necrosis factor/cachectin, interleukin-1, alpha-interferon and gamma interferon in the serum of patients with septic shock. *J Infect Dis* 1990; **161**: 982–987.
7. Bennett-Guerrero E. Systemic inflammation. In: Kaplan JA, Reich DL, Konstadt SN. (eds) *Cardiac Anesthesia*, 4th edn. Philadelphia, PA: Saunders, 1998; 297-318.
8. Taveira da Silva AM, Kaulbach HC, Chuidian FS, Lambert DR, Suffrendini AF, Danner RL. Shock and multiple-organ dysfunction after self-administration of *Salmonella* endotoxin. *N Engl J Med* 1993; **328**: 1475–1460.
9. Freeman R, Gould FK. Prevention of fever and Gram-negative infection after open heart surgery by anti-endotoxin *Thorax* 1985; **40**: 846–851.
10. Bennett-Guerrero E, Ayuso L, Hamilton-Davies C *et al.* Relationship of pre-operative serum anti-endotoxin-core antibody levels and adverse outcomes following cardiac surgery *JAMA* 1997; **277**: 646–650.
11. Hamilton-Davies C, Barclay GR, Cardigan RA, Purdy G, Machin SJ, Webb AR. Relationship between pre-operative endotoxin immune status, gut perfusion and outcome from cardiac valve replacement surgery. *Chest* 1997; **112**: 1189–1196.
12. Bennett-Guerrero E, Panah MH, Barclay GR *et al.* Decreased endotoxin immunity is associated with greater mortality and/or prolonged hospitalisation after surgery. *Anesthesiology* 2001; **94**: 992–998.
13. Suffredini AF, Fromm RE, Parker MM. The cardiovascular response of normal humans to the administration of endotoxin. *N Engl J Med* 1989; **321**: 280–285.
14. Bennett-Guerrero E, McIntosh TJ, Barclay GR *et al.* Preparation and preclinical evaluation of a novel liposomal complete core lipopolysaccharide vaccine. *Infect Immun* 2000; **68**: 6202–6208.
15. Hancock IC, Poxton IR. *Bacterial Cell Surface Techniques*. Chichester: Wiley, 1988.
16. Dijkstra J, Ryan JL, Szoka FC. A procedure for the efficient incorporation of wild-type lipopolysaccharide into liposomes for

use in immunological studies. *J Immunol Methods* 1988; **114**: 197–205.
17. Delahooke D, Barclay G, Poxton IR. TNF-α induction by an aqueous phenol-extracted lipopolysaccharide complex from *Bacteroides* species. *Infect Immun* 1996; **63**: 840–846.
18. Baumgartner JD. Anti-endotoxin antibodies as treatment for sepsis – lessons to be learnt. *Rev Med Microbiol* 1994; **5**: 183–190.
19. Dijkstra J, Mellors JW, Ryan JL, Szoka FC. Modulation of the biological activity of bacterial endotoxin by incorporation into liposomes. *J Immunol* 1987; **138**: 2663–2670.
20. Dijkstra J, Larrick JW, Ryan JL, Szoka FC. Incorporation of LPS in liposomes diminishes its ability to induce tumoricidal activity and tumor necrosis factor secretion in murine macrophages. *J Leukocyte Biol* 1988; **43**: 436–444.
21. Dijkstra J, Mellors JW, Ryan JL. Altered *in vivo* activity of liposome-incorporated lipopolysaccharide and lipid A. *Infect Immun* 1989; **57**: 3357–3363.
22. Petrov AB, Semenov BF, Vartanyan YP *et al.* Toxicity and immunogenicity of *Neisseria meningitidis* lipopolysaccharide incorporated into liposomes. *Infect Immun* 1992; **60**: 3897–3903.
23. Wurfel MW, Wright S. Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers. *J Immunol* 1997; **158**: 3925–3934.
24. Thiemblemont N, Thieringer R, Wright S. Innate immune recognition of bacterial lipopolysaccharide: dependence on interactions with membrane lipids and endocytic movement. *Immunity* 1998; **8**: 771–777.
25. Brightbill HD, Libraty DH, Krutzik SR *et al.* Host defence mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science* 1999; **285**: 732–736.
26. Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 1997; **388**: 394–397.
27. Wright S. Multiple receptors for endotoxin. *Curr Opin Immunol* 1991; **3**: 83–90.
28. Shumann RR, Leong SR, Flaggs GW *et al.* Structure and function of lipopolysaccharide binding protein. *Science* 1990; **249**: 1429–1431.

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Review

Structure and function of lipopolysaccharides

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Abstract

The lipopolysaccharides of Gram-negative bacteria have a profound effect on the mammalian immune system and are of great significance in the pathophysiology of many disease processes. Consideration is given in this review to the relationship between structure and function of these lipopolysaccharides.

Keywords: Lipopolysaccharides; Molecular structure; Endotoxins

1. Introduction

Lipopolysaccharide (LPS) is vital to both the structural and functional integrity of the Gram-negative bacterial outer membrane. Ubiquitously expressed by all Gram-negative bacteria, and containing several well-conserved domains, LPS also serves as one of the primary targets of the innate arm of the mammalian immune system. Recognition of the presence of LPS by cells such as monocytes and macrophages has evolved over the millennia to provide the mammalian host with a rapid recognition of and reaction towards Gram-negative infection. This rapid, innate response against LPS typically involves the release of a range of pro-inflammatory mediators, such as TNF-α, IL-6 and IL1β, which in local sites of infection and in moderate levels benefit the host greatly by promoting inflammation and otherwise priming the immune system to eliminate the invading organisms.

However, in conditions where the body is exposed to LPS excessively or systemically (as when LPS enters the blood stream), a systemic inflammatory reaction can occur, leading to multiple organ failure, shock and potentially death. Studies demonstrate that the majority of endotoxin exposure in these patients does not originate from an infection per se [1], but instead arises from translocation of endotoxin from the gastrointestinal tract [2]. The LPS of Gram-negative bacteria also shows quite a diversity of causative or modulating effects in a number of other disease processes. As much of the pathophysiological potential of these organisms derives directly from the exact structure of the LPS they express, this review will focus on the relationship between the structure and function of a number of clinically significant LPSs.

2. General structure of LPS

In the early part of the last century, it was becoming clear that endotoxin consisted of lipid and carbohydrate, and hence the term 'lipopolysaccharide' came into usage. The first main attempts at elucidation of the structure of the molecule came in the 1950s with the efforts of Luderitz and Westphal, whose acid treatment of LPS preparations yielded the first carbohydrate-free portions of LPS (termed lipid A). By the 1960s, it was becoming clearer that it was this lipid portion of the molecule that contained the endotoxic activity, as the so-called 'deep-rough' mutants (lacking the major part of the oligosaccharide portion of the molecule) were found to retain strong biological activity. Other experiments, such as mild alkali removal of fatty acids and protein binding experiments, pointed towards the same conclusion. However, it was only in the 1980s when free lipid A was finally prepared synthetically by Tetsuo Shiba et al. in Japan [3] that it was proven to be the endotoxic centre of the molecule.

Today, with procedures such as NMR, HPLC and epitope studies, the structures of the LPSs of many Gram-negative organisms have been characterised, and they all conform, in

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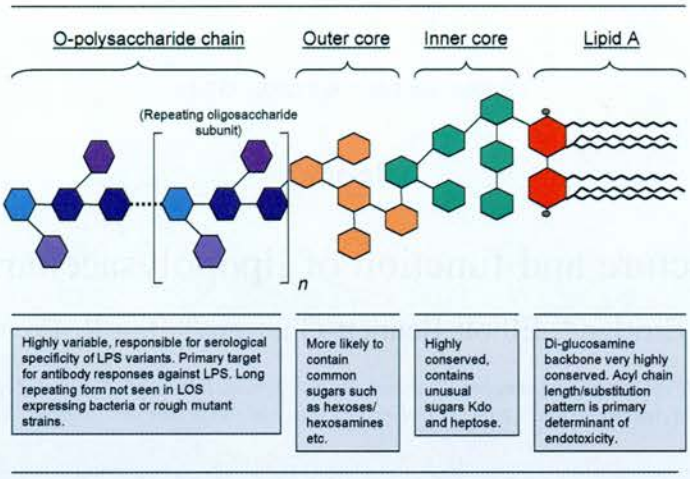


Fig. 1. General structure of Gram-negative LPS.

the main, to a common general architecture (Fig. 1). Modifications of this basic structure account for the endotoxins of all Gram-negative organisms so far investigated and can be formally classified as containing three separate regions. Lipid A is the highly hydrophobic and endotoxically active part of the molecule. Covalently attached to this is the core section of the molecule which can itself be formally divided into inner and outer core. The inner core is proximal to the lipid A and contains a high proportion of unusual sugars such as 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-manno heptose (Hep). The outer core extends further from the bacterial surface and is more likely to consist of more common sugars such as hexoses and hexosamines. Onto this is attached, in most cases, a polymer of repeating saccharide subunits called the *O*-polysaccharide, or *O*-chain, also typically composed of common hexoses. This *O*-chain is not ubiquitous, however, as it is seen to be truncated or lacking in a number of Gram-negative strains. Bacteria, which colonise mucosal surfaces, for example, often express LPS with a truncated non-repeating *O*-chain known as lipo-oligosaccharide (LOS). In addition, certain strains carry mutations in the otherwise well-conserved '*rfb*' locus (which contains a selection of genes involved in *O*-chain synthesis and attachment) and are termed 'rough mutants' to differentiate them from the wild-type 'smooth' strains which express *O*-chain-bearing LPS.

2.1. *O-polysaccharide*

The repeating units of the *O*-polysaccharide region consist between one and eight glycosyl residues and differ between strains by means of the sugars, sequence, chemical linkage, substitution, and ring forms utilised. As can be expected, this leads to an almost limitless diversity of *O*-chain structure and is verified in nature with the observation of hundreds of serotypes for particular Gram-negative species. In addition, the number of subunits used to complete the chain varies between 0 and ~50 and a single organism will produce a wide range of these lengths as a result of incomplete synthesis of the chain. This gives rise to the classical ladder pattern of molecular weights seen when smooth LPS is visualised by SDS-PAGE.

The *O*-polysaccharide is also the outermost part of the LPS molecule expressed on bacteria and is therefore the major antigen targeted by host antibody responses. These responses can be highly *O*-chain specific, and for this reason the *O*-chain is often also referred to as the *O*-antigen. As a result of this, serology of *O*-antigens has become a useful tool in typing strains of organisms and LPS.

The *O*-polysaccharide region is also recognised by the innate arm of the immune system, playing a role in both the activation and inhibition of complement activation [4]. For many organisms (e.g. *Salmonella*), the *O*-chain is essential for survival in host serum as it prevents penetration of the complement membrane attack complex [5].

2.2. *Core polysaccharide*

While the *O*-polysaccharide region of bacterial endotoxins is extremely variable, the oligosaccharide structures in the core part of the molecule are much more limited, with some regions being highly conserved between different strains and species. As an illustration, while there are now over 160 identified *O*-serotypes for *Escherichia coli*, only five unique core structures have so far been determined (R1–R4 and K12) [6]. Fig. 2 shows some examples of the range of core structures seen in a selection of wild-type and 'rough' strain Gram-negative bacteria.

The outer core typically consists of common hexose sugars such as glucose, galactose, *N*-acetyl galactosamine and *N*-acetyl glucosamine (hence alternatively referred to as the hexose region) and is generally more variable than the inner core.

The inner core is characterised by more unusual sugars, particularly Kdo and heptose. The former of these is seen in almost every LPS looked at to date, being α-bound to the carbohydrate backbone of lipid A in every case. The only

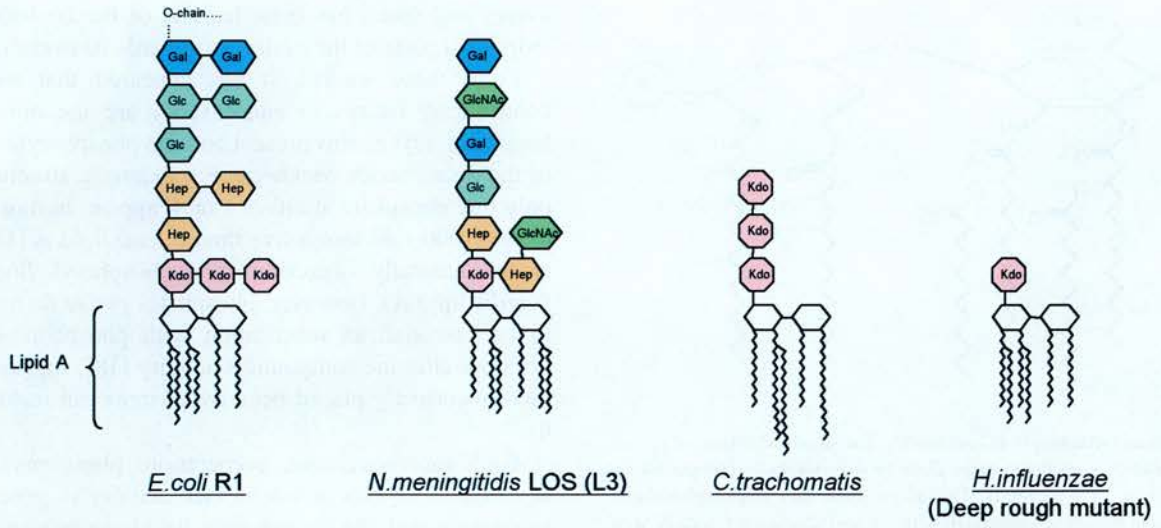


Fig. 2. Core structures of selected Gram-negative pathogens. Examples of core types from selected Gram-negative pathogens showing a variety of viable core structures. The *E. coli* core shown is that of rough mutant R1; the structure given for *N. meningitidis* relates to the entire LOS of the organism; all isolates of *C. trachomatis* so far investigated have shown only the Kdo triplet depicted; and the deep rough mutant of *H. influenzae* (I-69 Rd^{-}/b^{+}) represents the smallest core structure yet seen for any viable Gram-negative bacteria. NB: linkages of sugars shown in this scheme are for diagrammatic purposes only.

exceptions yet seen to this rule are *Acinetobacter* and *Burkholderia cepacia* LPS which employ the alternative 2-keto-D-glycero-D-talo-octonic acid (Ko) in its place. The bond between the lipid A and this first Kdo residue is normally very acid labile with moderate pH (<4.4) easily hydrolysing the bond to release the core (and with it the *O*-antigen) from the lipid A.

Interestingly, while the *O*-chain and the majority of the core can be dispensed with in some viable mutants, this Kdo residue is always absolutely required for bacterial viability. Evidence for this is seen in the fact that the smallest saccharide component seen in the LPS of any organism is that of a deep rough mutant of *Haemophilus influenzae* [7] which has only one Kdo residue attached to its lipid A, and of the naturally occurring bacteria, the smallest core yet seen is that of *Chlamydia* spp., which consists of only a triplet of Kdo units [8] (Fig. 2). This primary Kdo residue always has a negatively charged substituent on it (such as phosphate, typically at position 4) and it appears that this is essential for viability too.

The fact therefore that Kdo synthesis is absolutely essential for Gram-negative bacteria viability presents rational drug designers with a tantalising opportunity. Drugs targeting the Kdo synthesis enzymes would show not only a wide specificity towards Gram-negative organisms but would also represent an entirely new class of antibiotics [9].

A second unusual sugar seen in the inner core is heptose, typically in the L-glycero-D-manno configuration, but also occasionally as D-glycero-D-manno heptose. *Yersinia* strains, for example, use this isomer exclusively [10].

Both inner and outer core sugar residues can be substituted with charged groups like phosphate, pyrophosphate, 2-aminoethylphosphate and 2-aminoethylpyrophosphate. In the inner core, it is thought that these substituents maintain a close association with the Ca^{2+} and Mg^{2+} ions that are required for membrane structure and function [11].

Since the inner core is reasonably well conserved (the structure is identical, for example, for all strains of *E. coli* and *Salmonella*), this provides the possibility of an epitope capable of inducing an antibody of broad enough specificity to cross-react antigenically with the LPS of many different strains of Gram-negative LPS. This thought has already been applied in the consideration of a new vaccine to elicit such widely cross-reactive antibodies in human sera [12] and in the production of a monoclonal antibody capable of binding to a wide range of *E. coli* and *Salmonella* LPSs [13].

Finally, it should be pointed out that while the endotoxically active part of the molecule is lipid A, the nature and number of attached saccharide residues and substituents do have considerable impact on modulating this activity. The mechanisms for this will be discussed later.

2.3. General structure of lipid A

In 1985, it was shown that synthetic lipid A exhibits identical biological properties to *E. coli* lipid A. [14], thus proving that lipid A is the part of the molecule responsible for its endotoxic activity. Structurally, lipid A is typically composed of a β-D-GlcN-(1-6)-α-D-GlcN disaccharide carrying two phosphoryl groups (at positions 1 and 4'). Both phosphates can be further substituted with groups such as phosphate, ethanolamine, ethanolamine phosphate, ethanolamine diphosphate, GlcN, 4-amino-4-deoxy-L-arabinopyranose and D-arabino-furanose.

To this structure are attached up to four acyl chains by ester or amide linkage. These chains can then in turn be substituted by further fatty acids to provide LPS molecules

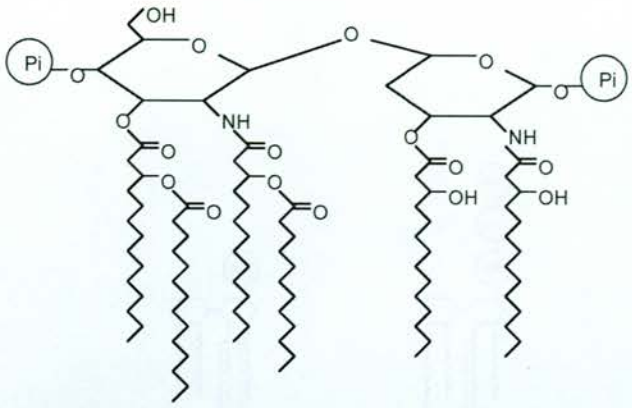


Fig. 3. Chemical structure of *E. coli* lipid A. The structural format of *E. coli* lipid A is widely considered to be close to that optimally recognised by human cellular LPS receptors. Deviations from this bisphosphorylated diglucosamine backbone substituted with six acyl chains are typically seen to result in molecules of lower biological activity.

with up to seven acyl substituents, which vary quite considerably between species in nature, number, length, order and saturation. These can be attached to the lipid A either symmetrically (3 + 3, e.g. *Neisseria meningitidis*) or asymmetrically (4 + 2, e.g. *E. coli*). Unsaturated fatty acids are rarely seen in lipid A, but have been reported in *Rhodobacter sphaeroides* and *R. capsulatus* LPS and in enterobacteriaceae grown at low temperature. It is possible though not yet proven that this helps the bacteria regulate membrane fluidity.

The polysaccharide portion of the molecule is almost invariably linked (see above) to the lipid A by means of a linking Kdo residue at position 6' (Fig. 3). This portion of the molecule therefore constitutes the most highly conserved region seen between LPS molecules: a phosphorylated diglucosamine backbone attached to at least one Kdo (or Ko) residue. (*Campylobacter jejuni* LPS is the notable exception to this rule, in which GlcN II is replaced by the related molecule 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N) [15].)

Some examples of lipid As from selected human pathogens and chemically synthesised derivatives are depicted in Fig. 4. These are the chemically elucidated complete structures of lipid A, though it should be borne in mind that like *O*-chain, extracted lipid A often possesses a considerable proportion of partial or incomplete structures as a result of incomplete biosynthesis.

3. Lipid A structure vs. function

In order to understand how the structure of lipid A affects its endotoxic activity, much use has been made of chemically synthesised and modified lipid As. In the main, these lipid A partial structures were prepared to contain the typical β-(1-6)-linked D-GlcN disaccharide while differing in patterns of acylation and phosphorylation. To date, a wide range of synthetic structures have been tested in biological assays and much has been learned of the contribution of individual parts of the molecule towards its overall toxicity.

From these studies, it has appeared that the major contributing factors to endotoxicity are the number and lengths of acyl chains present and the phosphorylation state of the disaccharide backbone. For example, structures with only one phosphate at either 1 or 4' appear, in most assays, to be ~1000-fold less active than *E. coli* lipid A [16,17] (as is the naturally occurring monophosphoryl *Bacteroides fragilis* lipid A). However, phosphates per se do not appear to be essential, as substitution with phosphono-oxyethyl does not alter the compound's activity [18], suggesting that merely correctly placed negative charges can restore activity.

GlcN monosaccharide preparations phosphorylated and acylated in various positions lack activity in general [19], suggesting that the disaccharide backbone is also required for optimum recognition by humoral/cellular receptors. Lipid A with GlcN3N replacing GlcN (as seen naturally in *C. jejuni*) have similar activity to those expressing GlcN.

Naturally, much work has concentrated on the role of the nature, number and lengths of acyl chains attached to the lipid A. For example, structures similar to *E. coli* lipid A with two phosphates but seven or five fatty acids are less active by a factor of approximately 100 [6].

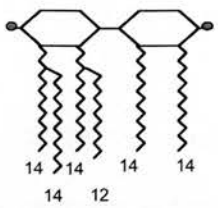
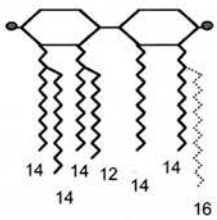
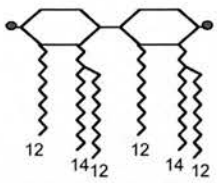
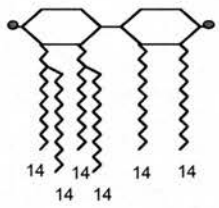
From these studies and many others, it appears that it is the diphosphorylated *E. coli*-like hexa-acyl lipid A containing two β(1-6)-linked D-glucosamine (GlcN or GlcN3N) residues which is optimally recognised by mammalian receptors to express the full spectrum of endotoxic activities. In almost all structures looked at, deviations from this pattern reduce the activity of the molecule.

These predictive rules for toxicity tend to hold true when looking at naturally occurring LPSs, as evidenced by the structures recently obtained for endotoxins of lower activity (cf. lipid As of *B. fragilis*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis* and *R. sphaeroides* depicted in Fig. 4).

The excellent work of Rietschel et al. [11] has looked in particular at the role of individual subunits within this structure in conferring overall toxicity, and is summarised in Fig. 5.

Interestingly, it appears that this optimum structure for cellular activation is not the same as the optimum structure for cell binding. Experiments have revealed that the optimum structure for binding to cells follows a much less strict template, being merely a bisphosphorylated disaccharide, together with some fatty acids (minimum 2) and in no particular arrangement. Structures that bind very well to cells do not necessarily induce strong monokine release, further suggesting that it is the number, nature and distribution of fatty acids, i.e. the acylation pattern of the lipid A which determines the endotoxic activity of these compounds.

In addition to providing a number of structures with reduced biological activity, these studies have also produced

E.coli lipid A 	*S.minnesota* lipid A 	*N.meningitidis* lipid A 	*H.influenzae* lipid A 
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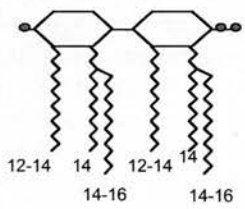
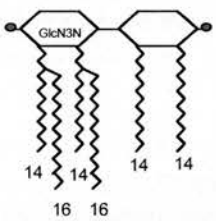
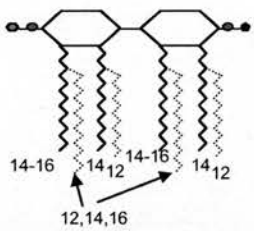
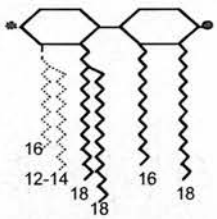
K.pneumoniae lipid A 	*C.jejuni* lipid A 	*Y.pestis* lipid A 	*H.pylori* lipid A 
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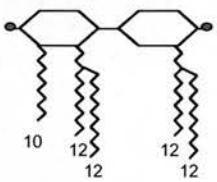
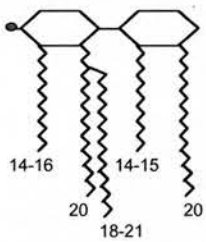
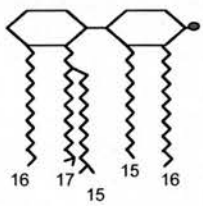
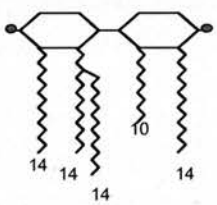
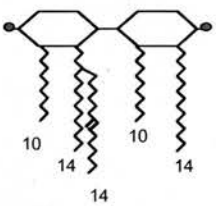
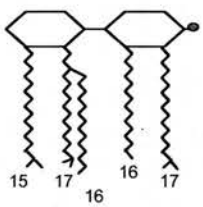
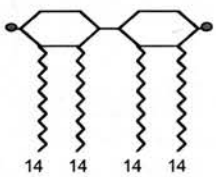
P.aeruginosa lipid A 	*C.trachomatis* lipid A 	*B.fragilis* lipid A 	*B.pertussis* lipid A 
Endotoxic Activity: +	Endotoxic Activity: +	Endotoxic Activity: +	Endotoxic Activity: + (?)
R.sphaeroides lipid A 	*P. gingivalis* lipid A 	Compound 406 (Ia) 	Lipid X 
Endotoxic Activity: - **(LPS antagonist)**	Endotoxic Activity: + (TLR-2 agonist)	Endotoxic Activity: - **(LPS antagonist)**	Endotoxic Activity: - (Very weak antagonist)

Fig. 4. Selected Gram-negative lipid A and derivative structures. Structures shown are complete forms. Commonly seen partial substitutions (as a result of incomplete biosynthesis) are indicated by dashed lines. NB. The endotoxic activities given for each compound are included as a qualitative guide only and represent only a subjective appraisal of the results from the many disparate (and occasionally conflicting) in vitro studies—and therefore cannot be assumed to reflect the overall in vivo endotoxicity of any structure shown. For LPS where no such data are available (indicated with a question mark), the activity shown is an estimate based on the structure of that molecule.

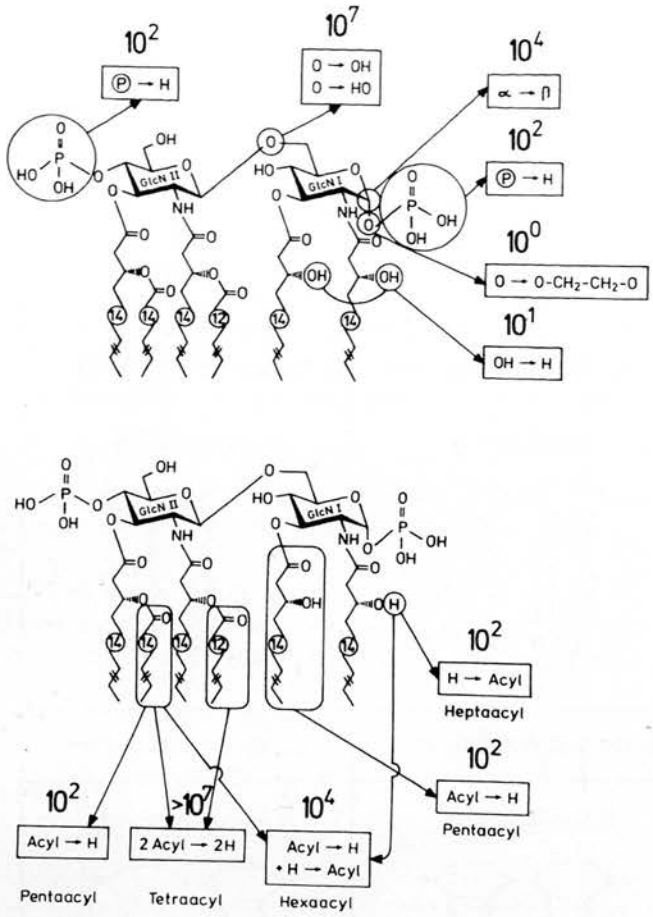


Fig. 5. Effect of structural modifications on lipid A bioactivity. Figures shown represent factors by which modified structures are reduced in bioactivity when compared to complete *E. coli* lipid A. Top: modifications to hydrophilic section. Bottom: modifications to hydrophobic section. Reproduced with permission from Rietschel et al. [11].

some compounds with pronounced inhibitory effects. One of the most interesting of these is compound 406 (also called precursor Ia, as it is a biological precursor to natural lipid A) which is tetra-acyl (Fig. 4). This structure is essentially the same as compound 506 (i.e. synthetic *E. coli* lipid A) but with the two acyl-substituted fatty acids removed. In a number of biological assays, this analogue not only completely lacks endotoxic activity, but is also able to inhibit normal lipid A signalling. Moreover, compound 406 is not antagonistic for other stimuli, such as lectins, Gram-positive bacteria, lipoproteins or cytokines. Of perhaps yet more interest is the fact that this compound, while exhibiting no endotoxicity in human assays, does show moderate activity in murine monocytes. The implications of this observation will be discussed later.

Additionally, it may be of note that on biodegradation of *E. coli* lipid A by the enzyme 3-acyloxyacyl-hydrolase within macrophages, the product yielded is essentially identical to compound 406 [20], suggesting the possibility that such a pathway may be utilised by the cells to detoxify LPS and thus counteract subsequent lipid A recognition.

Finally, it should be pointed out that natural antagonists to *E. coli* lipid A signalling also exist. LPS or lipid A from *R. capsulatus* and *R. sphaeroides* have also been shown to have antagonistic properties, as has LPS isolated from *B. fragilis* [21,22].

4. Physical conformation and endotoxicity of LPS

LPS molecules like other amphipathic molecules will aggregate into larger supra-molecular structures above a particular critical concentration. The three-dimensional configuration of this structure depends to a large extent on the shape of the individual contributing molecules and the environment in which it forms. Much work has recently been performed on how the structure of these aggregates affects the endotoxicity of LPS preparations. From these studies, some striking observations have been made as to the relationship between these structures, the molecular archi tecture of individual LPS molecules and resultant endotoxic activity.

Early studies looked initially at the phase transition temperature of lipid A and LPS preparations [23]. The acyl chains of LPS can assume two main phase states: liquid crystalline (α) or gel state (β), and above or below a particular temperature (T_c), this phase state can reverse. T_c depends to a large extent on the length and degree of saturation of the acyl chains and on the conformation and charge density of the hydrophilic head group region. Of note, while bacteria are able to alter the T_c of their plasma membrane quite widely to adapt to the current growth temperature, the LPS T_c cannot be altered by nearly as much (perhaps lending a role to the partial structures and unsaturated acyl chains occasionally seen in naturally occurring LPS).

Seydel et al. have noted a striking correlation between T_c and the biological activity of LPS preparations. In addition to many other examples, they cite that for instance: (i) the induction of leukotrine C4 (LTC4) release from mouse peritoneal macrophages after stimulation with *Salmonella minnesota* LPS is highest with ReLPS (lowest T_c), lowest for lipid A (highest T_c) and decreases with longer oligosaccharide (T_c increasing); and (ii) murine spleen cell proliferation is high for rough mutant LPS (Rd is best), with responses to lipid A and smooth LPS significantly lower (reviewed in [17]). From this point of view, it appears that low T_c gives rise to high endotoxic activity.

However, other studies have taken an alternative approach, looking instead at the physical superstructure of LPS aggregates. It was initially observed that structural presentation of the LPS to cell receptors must play a role, following the work of Takayama et al. [24], who managed to prepare solutions of LPS monomers by using concentrations below the critical micelle concentration. They found that the activity of a given lipid is significantly increased when monomers are applied to the cells.

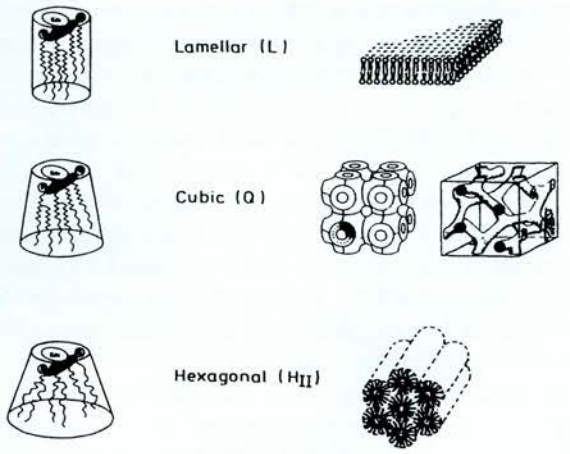


Fig. 6. Shape of LPS molecules and resultant supra-molecular structures. Three commonly seen conformations of LPS. The ratio between the upper (hydrophilic) and lower (hydrophobic) surface areas has been proposed to be the primary determinant of supra-molecular structure (see text). Reproduced with permission from Seydel et al. [23].

In general, biologically active lipid A adopts (at physiological conditions of water content, pH, temperature and Mg^{2+} concentration) exclusively non-lammelar structures [25]. These are of either cubic or hexagonal conformation (see Fig. 6). In contrast, biologically inactive lipid As adopt lammelar structures (e.g. *R. capsulatus*).

These researchers then suggest that the superstructure itself is purely a function of molecular shape. Effectively, LPS can be viewed as having two easily measured surface areas: that of the hydrophilic domain and the hydrophobic domain. The ratio between these two parameters essentially defines the shape of the molecule to be, for example, conical or cylindrical, and it is these parameters that dispose the LPS towards a particular type of superstructure, and hence endotoxicity (see Fig. 6). Thus, from comparison of many lipid A and LPS structures, they conclude that endotoxically active lipid A is conical, whereas inactive lipid A is cylindrical.

Of course it is quite possible that T_c and superstructure are highly related, though the question of which structural parameter of LPS (i.e. individual molecular shape or aggregate conformation) is being recognised by cellular receptors has yet to be determined.

However, a word of caution should be inserted at this stage. The assays used to determine the endotoxicity of these lipid A preparations have in the main been in vitro using highly chemically defined products. In the natural setting, the LPS presented to cells would almost certainly not be in this context. Instead, the LPS will be present in both large and small aggregates, complexed with bacterial proteins and other contaminants and would be heterogeneous in composition. In addition, plasma proteins will be at least partly complexed with the LPS and will also have a strong influence on the in vivo endotoxicity of any given preparation. Thus, while much can be said of the endotoxic potential of any given LPS by analysis of its phosphate and acyl composition, it does not necessarily follow that this will be reflected in the natural setting.

5. Receptors for LPS

While it had been recognised for some time that the molecule CD14 was required by cells to confer sensitive responses to LPS, it was seen that it could not function as the sole receptor molecule for LPS. Being a GPI anchored protein, CD14 lacks a transmembrane domain with which to facilitate intracellular signalling. In addition to this, it was noted that CD14-deficient human cells could still respond to LPS even in serum-free conditions lacking soluble CD14 [26].

This mystery remained unsolved until 1999, when the *lps* gene responsible for the LPS hyporesponsive phenotype of C3H/HeJ mouse was at last discovered and shown to code for a mutated version of the protein TLR-4 (Toll-like receptor, so called because of its homology with the *Drosophila* Toll protein) [27,28]. Examination of the TLR-4 knockout mouse that same year revealed a phenotype similar to that of the C3H/HeJ mouse, and thus the function of TLR-4 as an essential component of the LPS receptor was confirmed [29].

However, it was soon realised that this was not yet the complete picture. Transfection of some TLR-4-deficient cell lines with TLR-4 was not in itself sufficient to render them sensitive to LPS [27,29]. Thus, at least one further molecule was seen to be required to complete the LPS receptor complex. This was discovered shortly afterwards to be the protein MD-2 when it was seen that co-transfection of these cells with MD-2 restored LPS sensitivity [30]. An extracellular protein lacking a transmembrane domain, MD-2 remains attached to the cell surface via its interaction with TLR-4.

However, this picture of the TLR-4/MD-2 complex being the sole signalling receptor for LPS went through a period of some controversy. Many groups were able to show responses to their LPS preparations that were independent of TLR-4. Moreover, it appeared that another Toll-like receptor, TLR-2, was critical in these responses. Only stringent purification protocols capable of providing LPS extracts free from protein contaminants were finally able to reveal that the majority of LPSs signal solely through TLR4, while the TLR-2 signalling previously seen appeared to be due to lipoprotein contamination of those preparations [31].

Nevertheless, two very interesting exceptions to this rule have recently been described. The LPSs from *Porphyromonas gingivalis* and *Leptospira interrogans* have both been shown to be recognised not by TLR-4, but by TLR-2 [32,33]. Interestingly, the five branched acyl chains and mono-phosphorylated lipid A of *P. gingivalis* differs substantially from the typical hexa-acyl diphosporylated *E. coli* lipid-A-like template recognised by TLR-4. While the structure of *L. interrogans* lipid A has yet to be determined,

it is tempting to speculate that it may share structural features with *P. gingivalis* lipid A. Further, it is interesting to note that LPS extracted from *B. fragilis* has already been shown capable of activating TLR-4 mutant mouse (C3H/HeJ) cells and shares a lipid A of striking resemblance (see Fig. 4) to that of *P. gingivalis* lipid A [22]. Determining the chemical structures of lipid A from other organisms with LPS capable of stimulating TLR-4-deficient cells (such as that of *Treponema denticola* [34]) may well shed some light on the matter of which domains or particular three- dimensional arrangements of LPS are differentiated by TLR-2 and TLR-4.

5.1. Mechanisms for recognition of LPS by TLR-4/MD-2

Much evidence has recently emerged to shed some light on the mechanisms by which the TLR-4/MD-2 complex might recognise LPS. Firstly, it appears that the main role of LPS binding protein in the serum is to deliver LPS to either soluble or membrane-bound CD14 [35]. Then, as it is now becoming clearer that CD14 does not play an essential role in the TLR-4/MD-2 receptor complex (see above), it seems that the likely role of CD14 is to catalyse insertion of LPS molecules into either the plasma membrane (a function of CD14 demonstrated by Wurfel and Wright [36]) or directly into the receptor complex. In support of this hypothesis, Ulevitch and co-workers have used photo-activated cross-linking to reveal LPS in close proximity with both TLR-4 and MD-2, but only when in the presence of CD14 [37]. In addition, it has also been shown that LPS is transferred out of mCD14 before it is internalised [38].

As for which of TLR-4 and MD-2 participates in the discrimination of active lipid As, evidence is now emerging to suggest a role for both of these proteins in this function. For example, it has been shown that expression of murine TLR-4 in human cells renders them sensitive to the partial structure lipid IVa, whereas expression of human TLR-4 in unresponsive C3H/HeJ mouse cells does not [39,40]. On the other hand, Akashi et al. [41] have shown that while transfection of Ba/F3 cells with murine TLR-4 and MD-2 allows them to respond to the partial structure lipid IVa, replacement of the murine MD-2 with the human equivalent removes this ability. In addition, this chimaeric complex was seen to confer an antagonistic effect on lipid IVa, which remains an agonist in cells expressing the purely murine complex [41]. MD-2 has also been shown to have a direct effect on the discrimination of another TLR-4 ligand, the anti-mitotic compound Taxol. Signalling in response to Taxol has been shown to be ablated following the mutation of a single glycine residue in MD-2, whereas LPS signalling remains unaffected [42]. Further, distinct differences have been observed in the abilities of human and mouse MD-2 molecules to recognise Taxol [43].

Nevertheless, it seems clear that dimerisation of TLR-4 appears to be the key to activation of the complex, as chimaeric constructs of TLR-4/CD4 have been shown to dimerise at the cytosolic domains and induce cellular activation [44]. However, the question of how LPS is able to induce dimerisation of TLR-4 remains as yet unclear. Depicted in Fig. 7 are three models which suggest how particular types of LPS may result in dimerisation of TLR4 monomers and ultimately, cellular activation.

In the first (Fig. 7a), LPS is recognised within the complex of MD-2 and TLR-4. MD-2 has been shown to have a high affinity for LPS (Kd = 65 nM, similar to that of CD14 [45]) and may function to hold the LPS in place for TLR-4-mediated pattern recognition. Alternatively, the incorporation of the LPS into either molecule may induce a conformational change which then promotes dimerisation of TLR-4. In this model, LPS molecules are delivered directly to the complex by CD14.

Alternatively, since it has been shown that CD14 rapidly catalyses the insertion of LPS into the plasma membrane of host cells [36], it may be possible that this is where TLR-4 and MD-2 encounter the LPS (Fig. 7b). In this scenario, the shape of the lipid A is recognised by the transmembrane domains of TLR-4, while the (exposed) charged head group may be recognised by MD-2.

Finally, it is possible that disruptions to the physical properties of the host cell membrane occurring as a result of the incorporation of the unusual lipid A are recognised by TLR-4/MD-2, as first suggested by Wright [46]. Evidence

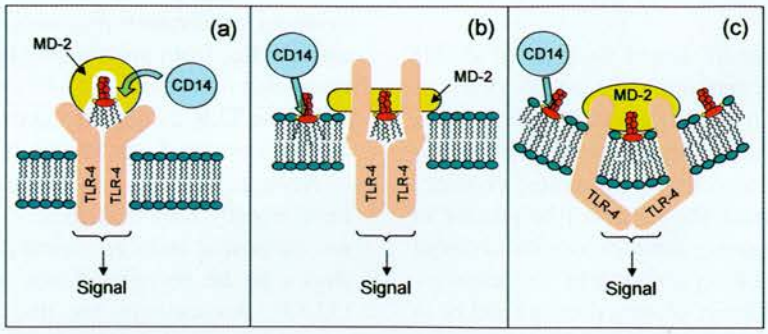


Fig. 7. Possible mechanisms for LPS recognition by the TLR-4/MD-2 complex. (a) CD14 delivers LPS molecules directly to the TLR-4/MD-2 complex inducing dimerisation; (b) CD14 catalyses insertion of LPS into the plasma membrane, where the hydrophobic domain interacts with the transmembrane domain of TLR-4 and the head group with MD-2; (c) LPS inserted into the membrane by CD14 causes changes in the membrane architecture sensed by the TLR-4/MD-2 complex.

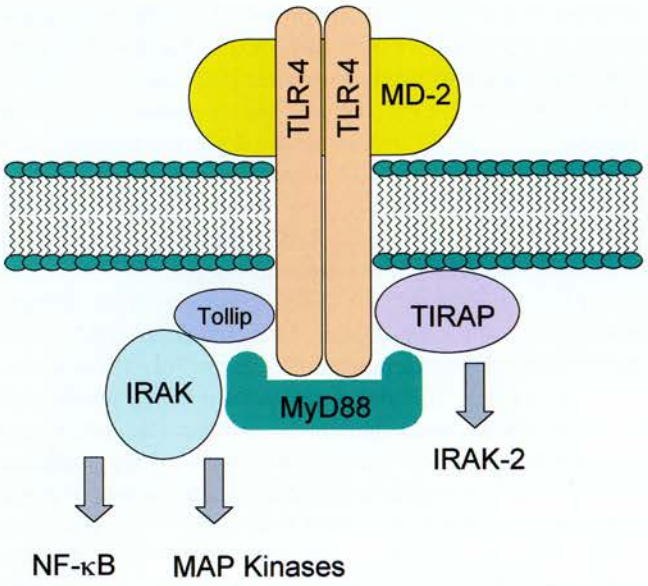


Fig. 8. Proposed signalling complex of TLR-4/MD-2. Dimerisation of TLR-4 in response to ligand occurs with the assistance of extracellular MD-2 to form a signalling complex containing MyD88 (Myeloid differentiation factor 88), TIRAP (Toll/interleukin-1-receptor-domain-containing adaptor protein), TOLLIP (Toll interacting protein) and IRAKs (IL-1 receptor-associated kinases).

for this includes the striking correlation between lipid A T_c and toxicity (discussed earlier) and the fact that the membrane active agent chlorpromazine is able to render cells usually insensitive to lipid IVa, sensitive [47]. The effects of lipid A antagonists can also be explained in this model by functioning to counteract the physical changes in the membrane caused by lipid A. In this model, local membrane architecture is altered in such a way as to dimerise the TLR subunits with the assistance of MD-2 resulting in signalling (Fig. 7c).

5.2. *Intracellular signalling of the TLR-4/MD-2 complex*

The TLR-4 signalling pathway shows striking similarities to that employed by the IL-1 receptor. In both pathways, the adaptor protein MyD88 (myeloid differentiation factor 88) is thought to be recruited to activated (i.e. dimerised) IL-1R or TLR-4 cytosolic domains where it catalyses the activation of serine/threonine kinases of the IL-1 receptor-associated kinase (IRAK) family. These then act through TRAF-6 (TNF-receptor associated factor 6) to promote both MAP kinase cascades and the NF-κB-inducing cascade (reviewed in [48]).

Recently, a protein has also been identified that interacts both with the cytoplasmic tail of the IL1 receptor and with IRAKs. Termed Tollip, this protein has also been shown to co-precipitate with TLR-2 and TLR-4 [49], and is also likely to play a role in recruiting IRAKs to the dimerised Toll receptors (see Fig. 8).

Evidence is accumulating, however, to suggest that LPS signalling may proceed along alternative parallel pathways. For example, certain cellular responses towards LPS do not require MyD88, and while the activation of NF-κB in response to LPS is merely delayed in MyD88-deficient cells, it is completely abolished in TLR-4-deficient cells [50]. Another recently discovered protein TIRAP (or MAL) has been shown to interact with both TLR-4 and IRAK-2 and on over-expression also activates NF-κB [51,52]. It is therefore possible that this adaptor protein is responsible for at least one MyD88-independent signalling pathway [51,52], though its full function remains to be determined.

6. Comparison of Gram-negative LPS structures

While the endotoxicity of lipid A is certainly of great significance in many disease processes, it is not the only aspect of naturally occurring LPSs to affect the pathophysiology of Gram-negative infections. The saccharide domains are also of great significance, and much variation occurs as to the general role of LPS in infections. Most notably, the *O*-polysaccharide portion of the molecule is often seen to confer serum resistance on organisms [53]. It may act as a cellular adhesion molecule, for example, *Actinobacillus pleuropneumoniae* which utilises its LPS as a major adhesin for lung tissue [54]. Finally, it may act either directly or indirectly as a virulence factor in many disease processes.

Further consideration is now given to the roles played by particular LPS types in the pathology of different Gram-negative infections and to the relationship between structure and function of these LPSs.

6.1. E. coli *LPS*

E. coli is the most common cause of urinary tract infections and is also responsible for many different gastrointestinal diseases including travellers' diarrhoea and infantile diarrhoea. However, the LPS of *E. coli* alone can be the causative agent of a number of diseases. Being a major gut commensal, the human gut therefore contains many grams of *E. coli* LPS. Under normal conditions, limited diffusion of this into the portal circulation is dealt with by Kupffer cells in the liver via non-stimulatory degradative pathways. However, on release of larger amounts of LPS into the blood stream (as can occur following surgery or other causes of hypo-volaemic shock) the resulting systemic inflammatory response syndrome can lead to multiple organ failure, shock and potentially death. In this respect, *E. coli* LPS is often regarded as the most potent initiator of monokines.

The high toxicity of *E. coli* LPS can be explained to a large extent by its structure. The hexa-acyl diphosphorylated lipid A with acyl chains of length C12–C14 is widely believed to comprise or at least be very close to the structure optimally configured to provide maximal activation of TLR-4, and hence activation of monocytes. In vitro studies of many different LPS types have shown that in the main,

deviations from this structural format lead to sometimes quite dramatic reductions in the endotoxic potential of a molecule (cf. Figs. 4 and 5).

6.2. S. minnesota *LPS*

Salmonella spp. are a leading cause of gastroenteritis. The LPS from *Salmonella* is typically regarded as having almost as much endotoxic activity as *E. coli* LPS. This could be expected of *S. typhimurium* LPS, which shares an identical lipid A structure with that of *E. coli*, but might be considered unusual of *S. minnesota* LPS, as hepta-acyl LPS has been shown to have reduced activity when compared to *E. coli* LPS. However, while the hepta-acyl structure given for *S. minnesota* lipid A in Fig. 4 is the most complete structure seen, it is not the most numerous. Studies of *S. minnesota* Re595 LPS and lipid A have revealed that only 15% of structures are complete (i.e. hepta-acyl) with the most abundant structure being a hexa-acyl analogue of the *E. coli* arrangement [55]. This therefore provides the most likely explanation for the high endotoxicity seen in naturally occurring *S. minnesota* LPS.

6.3. Klebsiella pneumoniae *LPS*

K. pneumoniae is an opportunistic pathogen causing bacteraemia, pneumonia and urinary tract infections in humans. Often associated with nosocomial infections, it is second only to *E. coli* as the culprit behind Gram-negative sepsis syndrome and is a major cause of mortality in hospital-acquired infections. Several *O*-antigens have now been described with approximately 80% of *K. pneumoniae* clinical isolates belonging to serotypes O1, O2, O3 and O5 [56]. Properties of *K. pneumoniae* LPS include the unusual observation of Kdo existing in the outer core and the existence of homo-polysaccharides in some *O*-chains. It has also been observed that *K. pneumoniae* LPS itself is a significant virulence factor in these infections. For example, injection of purified LPS significantly enhances *K. pneumoniae* virulence in a mouse model of infection [57], though it is not yet clear how this occurs. Characteristic lung damage can also be seen in *K. pneumoniae* infections and is caused by the release of a toxic complex from the bacterial cell surface during growth of strains of *K. pneumoniae* serotypes O1:K1 and O1:K2. LPS is the critical component of this complex.

In terms of endotoxicity, *K. pneumoniae* LPS is considered to be strongly active—an observation in accordance with its bisphosphorylated lipid A substituted with six acyl chains of 12–16 carbon length, thereby making it close in structure to the optimally recognised template.

6.4. P. aeruginosa *LPS*

P. aeruginosa is an opportunistic pathogen found ubiquitously in the environment and is capable of infecting patients whose health is compromised. At least 20 serogroups of *O*-chain have been identified and at least four core structures have now been determined [58]. Unusual features of *P. aeruginosa* LPS include the presence of several unusual diacetamido uronic acids of gluco-, manno-, and gulo-configurations in the *O*-polysaccharide region [59] and the substitution of some of these sugars with amino acids. Unusually, about 80% of the LPS expressed on the surface of *P. aeruginosa* is of the rough variety [60,61]. *P. aeruginosa* LPS also contains an additional polysaccharide antigen (sometimes called the A band LPS) which is common to many serogroups. Composed primarily of D-rhamnose monosaccharides, it is not clear if this polymer attaches to the same core as the *O*-chain or to a different lipid A. Either way, the *O*-polysaccharide region is required for virulence as R-mutant *P. aeruginosa* tested in animal models of acute infection are virtually avirulent [62].

However, *P. aeruginosa* infection in the cystic fibrosis patient reveals an interesting exception to this rule. Strains causing chronic infections are seen to lose the *O*-chain and become serum sensitive [63]. The reason for this is unclear. In healthy patients, functional CFTR is capable of binding to the *P. aeruginosa* LPS core region [64], thus mediating ingestion and removal of the bacteria. It has been proposed that this CFTR-mediated uptake is a key component of host innate defence against *P. aeruginosa* [65], as it appears to be the only bacteria recognised by these receptors. Cystic fibrosis patients expressing the defective CFTR dF508, on the other hand, only poorly ingest these bacteria and are therefore prone to chronic infection.

In structural terms, *P. aeruginosa* lipid A exhibits significantly less toxicity than *E. coli* and *Salmonella* lipid A, presumably as a result of the penta-acyl format which at C10–C12 is significantly shorter than the optimum *E. coli*-like template (Fig. 4).

6.5. B. cepacia *LPS*

B. cepacia was first identified as a phytopathogen causing soft rot in onions. Now, it is more widely recognised as a significant nosocomial pathogen and an agent of recalcitrant respiratory infections in cystic fibrosis patients in particular. *B. cepacia* LPS has been looked at as a possible virulence factor [66], but the effect on pathophysiology and colonisation remains unclear.

Nevertheless, *B. cepacia* LPS has some unusual structural features which may affect its pathogenicity. The most striking of these is the replacement of the otherwise very highly conserved lipid attached Kdo with the related 2-keto-D-glycero-D-talo-octonic acid (Ko). Of note, the bond of Ko to lipid A is not nearly as acid labile as Kdo.

B. cepacia *O*-chains are all linear with simple disaccharide or trisaccharide repeating units often containing unusual sugar residues (e.g. 2-amino-2-deoxy-L-glucose, D-fucose, L-glycero-D-manno-heptose) and in several serotypes, the LPS is seen to contain two antigenically distinct

polymers. *B. cepacia* LPS has a low anionic character as a result of the limited phosphorylation and the presence of 4-amino-4-deoxy-L-arabinose in the core polysaccharide [66]. This low anionic character may account for the high resistance to (especially cationic) antibiotics of *B. cepacia*. Antibody responses to *B. cepacia* are typically strong but do not clear the infection [67]. In most environments, *B. cepacia* LPS is of the smooth form, though in a similar fashion to *P. aeruginosa* isolates from cystic fibrosis patients, can be either R- or S-form.

The fine structure of *B. cepacia* lipid A has not yet been determined, though as it is seen to be strongly endotoxic in vitro, it is likely that it does not share the penta-acyl format of *P. aeruginosa* LPS, most likely adopting instead an *E. coli*-like hexa-acyl format.

6.6. Helicobacter pylori *LPS*

H. pylori is able to cause chronic infection of the human gastric mucosa and is the primary cause of active chronic gastritis. Structurally, *H. pylori* LPS has been well characterised. While R-LPS is tetra-acylated, S-LPS is seen to contain some hexa-acylated LPS. In addition, while R-form LPS is only mono-phosphorylated (at position 1 and partially substituted by ethanolamine), a second phosphate is partially substituted at position 4'. The acyl chains are longer than those seen in *E. coli* lipid A, averaging about 16–18 carbons long. Thus, it might be predicted that as a result of this under-phosphorylation and under-acylation *H. pylori* lipid A should display reduced endotoxic activity. This is indeed found to be the case: pyrogenicity and mitogenicity is 1000-fold less than that observed for *S. typhimurium* LPS, lethal toxicity in mice is 500-fold lower and LAL assay reveals an activity 100–500-fold less [68].

Of more interest, however, is the fact that *H. pylori* *O*-antigens can share Lewis x and y blood group antigens found in the gastric mucosa. Monteiro et al. [44] have shown that *H. pylori* can express both type 1 and type 2 Lewis antigens and it has even been shown that *H. pylori* derived from infected individuals express Lewis antigens consistent with the erythrocyte phenotype of the host [69, 70]. It has therefore been postulated that these structures are present to minimise the risk of host attack on these organisms [71].

6.7. B. fragilis *LPS*

Bacteroides spp. constitute the most numerous commensal bacteria in the human gut, outnumbering *E. coli* by at least 100 to 1. *B. fragilis* is one of the most commonly encountered anaerobic species in clinical specimens, appearing to be more virulent than its close relatives. Traditionally, the endotoxic activity of *B. fragilis* LPS (when extracted from whole bacteria by the petroleum/chloroform/phenol method for R-form LPS) is considered to be low when compared to *E. coli* and *Salmonella* LPS: pyrogenicity, the ability to promote local Shwartzman reaction and IL-1β induction from monocytes are all reduced 100–1000-fold [72]. This weak endotoxicity has been proposed to be due to the mono-phosphorylated (on the C1 of the reducing amino sugar) lipid A, the relatively long (C15–C17) acyl chains and the fact that there is an average of only five fatty acids per molecule with some of these showing unusual branching patterns.

However, when the LPS was extracted by the aqueous phenol method (for S-form LPS), the resultant product was much more highly endotoxic, being equivalent to that of enterobacterial LPS in terms of *Limulus* activity and TNF induction from mononuclear cells. It also appeared to produce a fine ladder pattern on silver-stained SDS-PAGE gels, reminiscent of smooth LPS [73,74].

6.8. C. jejuni *LPS*

C. jejuni is an increasingly recognised cause of intestinal infection. Human campylobacter infections are characterised by intestinal mucosal invasion, haemorrhagic inflammation and crypt abscess formation. *C. jejuni* lipid A is similar to that of related species, but has replaced one (or both) of the glucosamine residues of the lipid A backbone with the related sugar 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N). Other than this, *C. jejuni* lipid A is antigenically similar to enterobacterial lipid A, and has comparable endotoxic activity in biological test systems [15].

Inner core oligosaccharides of *C. jejuni* strains share a common unique tetrasaccharide (Glc–Hep–Hep–Kdo) and also possess a trisaccharide that occurs in the inner core of other bacterial species. The inner and outer core may be substituted with sialic acid [15] in the form of ganglioside mimicry. This is a common feature shared by a number of but not all *C. jejuni* serotypes and may play a significant role in the induction of autoantibodies in Guillain-Barré syndrome following *C. jejuni* infection [75].

As with other LPSs, there is some heterogeneity in naturally occurring lipid As. For example, at position 2' of the lipid A backbone, 16:0 on 14:0 (3-OH) is only partially (20%) substituted and non-stochiometric substitution of phosphate groups occurs (pos. 1: ethanolamine or ethanolamine phosphate; pos. 4': ethanolamine phosphate).

6.9. Yersinia pestis *LPS*

Less work has been performed on *Y. pestis* LPS, though the following general points can be made. Rough and smooth forms have been observed. Lipid A consists of the typical (1-6) β-linked D-glucosamine disaccharide carrying two phosphate groups—further substituted with either phosphoethanolamine, a 4-amino-4-deoxy-L-arabinosyl residue or the rarely seen D-arabinofuranose [10]. The exact locations of the acyl chains remain to be determined, though a proposed structure [10] is given in Fig. 4. The chain lengths (C12–C16) and presence of two lipid A bound phosphates

suggest that if the lipid A of this species turns out to be predominantly hexa-acylated, it is likely to show strong endotoxic activity.

6.10. N. gonorrhoeae *and* N. meningitidis *(LOS)*

Not all Gram-negative LPSs have a long repeating *O*-chain structure. Some bacteria express only core saccharide joined to a short non-repeating polysaccharide unit. Such LPSs have been termed 'lipo-oligosaccharide' (LOS) to differentiate them. Both *N. gonorrhoeae* and *N. meningitidis* express LOS—the latter of which is of particular interest as most of the shock and complications involved in meningococcal meningitis are due to *N. meningitidis* endotoxin, which causes extensive vascular damage.

The LOS of *N. meningitidis* and *N. gonorrhoeae* is non-repeating and less than 10 residues in length (see Fig. 2). Both are able to mimic carbohydrates present in the glycosphingolipids of humans by sialylating their LOS by means of host-derived enzymes [76]. For example, neuraminic acid (Neu5Nac) is often expressed at the terminal glycosyl residue. It has been hypothesised that this allows *Neisseria* to evade host immune responses by mim icking Neu5Nac-carrying host antigens [25]. This may be achieved in a number of ways. Firstly, it has been seen that the presence of sialic acid in membranes is able to down-regulate the alternative complement pathway component C3 convertase, though it should be pointed out that strains lacking this sialylation are not necessarily rendered sensitive to serum killing. Sialylation also inhibits phagocytosis of cells by human neutrophils. Some reports suggest that sialylation decreases adherence to human cells, while others have pointed out that there is specific binding of gonococcal LPS to ciliated epithelial cells, suggesting that a human lectin may exist to recognise specific carbohydrate epitopes on LOS. LOS receptors on human monocytes have also been suggested. Other suggested roles for sialylated LOS include intracellular survival and altered regulation of host cells. Lastly, *Neisseria* LOS can also be modified in vivo by host substances/secretions, thereby allowing it to be differentially expressed in different environments. (For literature relating to all of these points, see [76].)

In terms of endotoxicity, *Neisseria* LOS is generally agreed to be very strongly active, as might be predicted from the C12–C14 hexa-acyl bisphosphorylated lipid A structure which closely resembles that of *E. coli* lipid A.

6.11. H. influenzae *(LOS)*

H. influenzae is an exclusively human pathogen that routinely colonises the upper respiratory tract [77]. The capsular type B of the bacterium was the most frequent agent of meningitis in children under 4 years of age and was responsible for many hundreds of thousands of childhood deaths around the world. However, this has been greatly reduced since the introduction of the Hib conjugate capsular vaccine. Like *N. meningitidis*, *H. influenzae* also expresses an LOS. This is a critical component of the non-capsulated strains which are a common cause of otitis media, sinusitis and lower respiratory tract infections.

However, despite the lack of a long *O*-polysaccharide, strong serological variations are still observed [77]. *H. influenzae* can change its LOS epitopes via phase (or antigenic) variation by altering expression of the so-called 'contingency genes' [78].

In each type of *H. influenzae* LOS chemically identified so far, there is a common inner core consisting of three heptoses linked via a single Kdo to the lipid A (providing a promising target for vaccine development). The remainder of the core then tends to be composed of neutral hexoses outwards. A complete core seems to be required for both commensal and invasive behaviour. For example, the deep rough mutant I69 of a type b strain (lacking much of the core, Fig. 2) was found to be non-virulent in the infant rat model of infection [79], and a minimum of five sugars attached to lipid A are required for efficient intra-vascular survival. Again like *N. meningitidis*, sialylation is often seen on terminal sugar residues. This appears to be endogenous, but is enhanced when NANA is supplied in the growth medium.

Structurally speaking, the hexa-acyl, C12–C14 format of *H. influenzae* lipid A suggests a strongly toxic molecule and in vitro systems prove this to be the case. Indeed, the lipid A region is seen to be associated with damage during disease.

6.12. Bordetella pertussis *(LOS)*

B. pertussis, the agent of whooping cough, also produces an LOS which has been directly implicated as being the mediator behind whooping cough syndrome [80].

The lipid A of *B. pertussis* LOS is covalently linked to a single Kdo residue and then to a branched chain oligosaccharide, thereby making it unlike most other strains of the genus, which produce a longer *O*-antigen [81]. Also, unlike most other enteric bacteria, *Bordetella* LOS contains several charged sugars, and can be further substituted with other charged sugars such as *N*-acetyl-*N*-methylfucosamine (FucNAcMe), 2,3-dideoxy-2,3-di-*N*-acetylmannosaminuronic acid (2,3-di-NacManA) and *N*-acetylglucosamine (GlcNAc). It has been noted that the distal tri- and pentasaccharide epitopes provide potentially good targets for vaccine design [81].

In vitro challenge data are not available for the LOS of *B. pertussis*, though it might be suspected to exhibit a low activity as there are only five acyl chains, and while most are of *E. coli*-like length (C14), one is relatively short at C10.

6.13. Chlamydia *LPS*

Chlamydiae are obligate intracellular parasites responsible for a variety of clinical manifestations. For example, strains of *C. trachomatis* affect between 300 and 500 million people in developing countries, causing trachoma which can lead to blindness. In industrialised countries, *C. trachomatis* is the most prevalent cause of sexually transmitted uro-genital infections. *C. pneumoniae* is the causative agent of ~5–10% of pneumoniae and bronchitis cases and has even been implicated in coronary heart disease. (For literature see [82].)

Chlamydiae express LPS of a unique structure. While species expressing LOS typically attach between 10 and 20 sugars to their lipid A, chlamydial LPS makes do with only a Kdo tri-saccharide joined α-Kdo-2-8-α-Kdo-2-4-α-Kdo.

Chlamydial LPS is of low toxicity, and much of this can be attributed to its unusual structural features. Long-chain fatty acids (C14–C21) in a penta-acyl arrangement and mono-phosphorylation of the diglucosamine backbone differ greatly from the optimum structure of *E. coli*.

7. Summary

LPS is one of the most potent activators of the mammalian immune system, and is essential for the viability of all Gram-negative bacteria. A wide variety of LPS types are expressed in nature, but all share some common principles: a phosphorylated diglucosamine backbone substituted with several acyl chains and at least one Kdo residue.

Differences between species and strains exist in all parts of the molecule, but most variation occurs in the *O*-polysaccharide, followed by the core region and finally the lipid A component. The endotoxicity of LPS molecules is determined primarily by the number, nature and arrangement of acyl chains and phosphate groups on the lipid A part of the molecule, with molecules displaying an acylation pattern similar to that of *E. coli* being the ones optimally recognised by human monocytes.

References

[1] S.M. Opal, P.J. Scannon, J.L. Vincent, M. White, S.F. Carroll, J.E. Palardy, N.A. Parejo, J.P. Pribble, J.H. Lemke, Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock, J. Infect. Dis. 180 (1999) 1584–1589.

[2] A.E. Martinez-Pellus, P. Merino, M. Bru, R. Conejero, G. Seller, C. Munoz, T. Fuentes, G. Gonzalez, B. Alvarez, Can selective digestive decontamination avoid the endotoxemia and cytokine activation promoted by cardiopulmonary bypass? Crit. Care. Med. 21 (1993) 1684–1691.

[3] K. Tanamoto, U. Zahringer, G.R. Mckenzie, C. Galanos, E.T. Rietschel, O. Luderitz, S. Kusumoto, T. Shiba, Biological activities of synthetic lipid A analogues: pyrogenicity, lethal toxicity, anti-complement activity and induction of gelation of limulus amoebocyte lysate, Infect. Immun. 44 (1984) 421–426.

[4] K.A. Joiner, N. Grossman, M. Schmetz, L. Leive, C3 binds preferentially to long-chain lipopolysaccharide during alternative pathway activation by *Salmonella montevideo*, J. Immunol. 136 (1986) 710–715.

[5] K.A. Joiner, M.A. Schmetz, R.C. Goldman, L. Leive, M.M. Frank, Mechanism of bacterial resistance to complement-mediated killing: inserted C5b-9 correlates with killing for *Escherichia coli* O111B4 varying in *O*-antigen capsule and *O*-polysaccharide coverage of lipid A core oligosaccharide, Infect. Immun. 45 (1984) 113–117.

[6] E.T. Rietschel, T. Kirikae, F.U. Schade, A.J. Ulmer, O. Holst, H. Brade, G. Schmidt, U. Mamat, H.D. Grimmecke, S. Kusumoto, U. Zahringer, The chemical structure of bacterial endotoxin in relation to bioactivity, Immunobiology 187 (1993) 169–190.

[7] I.M. Helander, B. Lindner, H. Brade, K. Altmann, A.A. Lindberg, E.T. Rietschel, U. Zahringer, Chemical structure of the lipopolysaccharide of *Haemophilus influenzae* strain I-69 Rd^{-}/b^{+}: description of a novel deep rough mutant, Eur. J. Biochem. 177 (1988) 483–492.

[8] H. Brade, L. Brade, F.E. Nano, Chemical and serological investigations on the genus-specific lipopolysaccharide epitope of Chlamydia, PNAS 84 (1987) 2508–2512.

[9] S.M. Hammond, A. Claesson, A.M. Jansson, L.G. Larsson, B.G. Pring, C.M. Town, B. Ekstrom, A new class of synthetic anti-bacterials acting on lipopolysaccharide biosynthesis, Nature 327 (1987) 730–732.

[10] N.D. Venezia, S. Minka, M. Bruneteau, H. Mayer, G. Michel, Lipopolysaccharides from *Yersinia pestis*, Eur. J. Biochem. 151 (1985) 399–404.

[11] E.T. Rietschel, T. Kirikae, U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A.J. Ulmer, U. Zahringer, U. Seydel, F. Di Padova, M. Schreier, H. Brade, Bacterial endotoxin: molecular relationships of structure to activity and function, FASEB J. 8 (1994) 217–225.

[12] E. Bennett-Guerrero, T.J. McIntosh, G.R. Barclay, D.S. Snyder, R.J. Gibbs, M.G. Mythen, I.R. Poxton, Preparation and preclinical evaluation of a novel liposomal complete-core lipopolysaccharide vaccine, Infect. Immun. 68 (2000) 6202–6208.

[13] F. Di Padova, H. Brade, G.R. Barclay, I.R. Poxton, E. Liehl, E. Schuetze, H.P. Kocher, G. Ramsay, M.H. Schreier, D.B.L. McLelland, E.T. Rietschel, A broadly cross-protective monoclonal antibody binding to *Escherichia coli* and *Salmonella* lipopolysaccharides, Infect. Immun. 61 (1993) 3863–3872.

[14] C. Galanos, O. Luderitz, E.T. Rietschel, O. Westphal, H. Brade, L. Brade, M. Freudenberg, U. Schade, M. Imoto, H. Yoshimura, Synthetic and natural *E. coli* free lipid A express identical endotoxic activities, Eur. J. Biochem. 148 (1985) 1–5.

[15] A.P. Moran, Structure and conserved characteristics of *Campylobacter jejuni* lipopolysaccharides, Rev. Infect. Dis. 176 (Suppl. 2) (1997) S115–121.

[16] H. Takada, S. Kotani, in: D.C. Morrison, J.L. Ryan (Eds.), Bacterial Endotoxic Lipopolysaccharides. Vol. I. Molecular Biochemistry and Cellular Biology, CRC Press, Boca Raton, USA, 1992, pp. 107–131.

[17] E.T. Rietschel, H. Brade, L. Brade, K. Brandenburg, F.U. Schade, U. Seydel, U. Zahringer, C. Galanos, O. Luderitz, O. Westphal, H. Labischinski, S. Kusumoto, T. Shiba, Lipid A, the endotoxic centre of bacterial lipopolysaccharides: relation of chemical structure to biological activity, Prog. Clin. Biol. Res. 231 (1987) 25–34.

[18] A.J. Ulmer, H. Heine, W. Feist, S. Kusumoto, T. Kusama, H. Brade, F.U. Schade, E.T. Rietschel, H.D. Flad, Biological activity of synthetic phosphono-oxyethyl analogues of lipid A and lipid A partial structures, Infect. Immun. 60 (1992) 3309–3314.

[19] I. Aschauer, A. Grob, J. Hildebrandt, E. Schutze, P. Stutz, Highly purified lipid X is devoid of immunostimulatory activity. Isolation and characterisation of immunostimulating contaminants in a batch of synthetic lipid X, J. Biol. Chem. 265 (1990) 9159–9164.

[20] A.L. Erwin, R.S. Munford, in: D.C. Morrison, J.L. Ryan (Eds.), Bacterial Endotoxic Lipopolysaccharides. Vol. I. Molecular Biochemistry and Cellular Biology, CRC Press, Boca Raton, USA, 1992, pp. 405–430.

[21] D.K. Magnuson, A. Weintraub, H.T. Pohlmann, R.V. Maier, Human endothelial cell adhesiveness for neutrophils, induced by *E. coli* lipopolysaccharide in vitro, is inhibited by *Bacteroides fragilis* lipopolysaccharide, J. Immunol. 143 (1989) 3025–3030.

[22] D. Delahooke, G. Barclay, I. Poxton, TNF-α induction by an aqueous phenol-extracted lipopolysaccharide complex from *Bacteroides* species, Infect. Immun. 63 (1996) 840–846.

[23] U. Seydel, H. Labischinski, M. Kastowsky, K. Brandenburg, Phase behaviour, supramolecular structure and molecular conformation of lipopolysaccharide, Immunobiology 187 (1993) 191–211.

[24] Z.Z. Din, P. Mukerjee, M. Kastowsky, K. Takayama, Effect on solubility and ionic state of lipopolysaccharide obtained from the deep rough mutant of *E. coli*, Biochemistry 32 (1993) 4579–4586.

[25] A. Nowotny, J.J. Spitzer, E.J. Ziegler (Eds.), Cellular and Molecular Aspects of Endotoxin Reactions, Excerpta Medica, Amsterdam, 1990.

[26] S. Akashi, H. Ogata, F. Kirikae, K. Kawasaki, M. Nishijima, R. Shimazu, Y. Nagai, K. Fukudome, M. Kimoto, K. Miyake, Regulatory roles for CD14 and phosphatidylinositol in the signalling via Toll-like receptor 4 – MD-2, Biochem. Biophys. Res. Commun. 268 (2000) 172–177.

[27] S.T. Qureshi, L. Lariviere, G. Leveque, S. Clermont, K.J. Moore, P. Gros, D. Malo, Endotoxin-tolerant mice have mutations in Toll like receptor 4 (, Tlr4), J. Exp. Med. 189 (1999) 615–625.

[28] A. Poltorak, H. Xialong, I. Smirnova, M.Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, B. Beutler, Defective LPS signalling in C3H/HEJ and C57Bl/10ScCr mice: mutations in Tlr4 gene, Science 282 (1998) 2085–2088.

[29] K. Hoshino, O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, S. Akira, Toll like receptor 4 deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR-4 as the lps gene product, J. Immunol. 162 (1999) 3749–3752.

[30] R. Shimazu, S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, M. Kimoto, MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4, J. Exp. Med. 189 (1999) 1777–1782.

[31] M. Hirschfield, Y. Ma, J.H. Weis, S.N. Vogel, J.J. Weis, Cutting edge: repurification of lipopolysaccharide eliminates signalling through both human and murine toll-like receptor 2, J. Immunol. 165 (2000) 618–622.

[32] M. Hirschfield, J.J. Weis, V. Toschchakov, C.A. Salkowski, M.J. Cody, D.C. Ward, N. Quereshi, S.M. Michalek, S.N. Vogel, Signalling by Toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages, Infect. Immun. 69 (2001) 1477–1482.

[33] C. Werts, R.I. Tapping, J.C. Mathison, T.H. Chuang, V. Kravchenko, I. Saint Girons, D.A. Haake, P.J. Godowski, F. Hayashi, A. Ozinsky, D.M. Underhill, C.J. Kirschning, H. Wagner, A. Aderem, P.S. Tobias, R.J. Ulevitch, Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism, Nat. Immunol. 2 (2001) 346–352.

[34] G. Rosen, M.N. Sela, R. Naor, A. Halabi, V. Barak, L. Shapira, Activation of murine macrophages by lipoprotein and lipopolysaccharide of *Treponema denticola*, Infect. Immun. 67 (1999) 1180–1186.

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

[36] M.W. Wurfel, S. Wright, Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers, J. Immunol 158 (1997) 3925–3934.

[37] J. da Silva Correia, K. Soldau, U. Christen, P.S. Tobias, R.J. Ulevitch, Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. Transfer from CD14 to TLR-4 and MD-2, J. Biol. Chem. 276 (2001) 21129–21135.

[38] T. Vasselon, E. Hailman, R. Thieringer, P.A. Dietmers, Internalisation of monomeric lipopolysaccharide occurs after transfer out of cell surface CD14, J. Exp. Med. 190 (1999) 509–522.

[39] E. Lien, T.K. Means, H. Heine, A. Yoshimura, S. Kusumoto, K. Fukase, M.J. Fenton, M. Oikawa, N. Qureshi, B. Monks, R.W. Finberg, R.R. Ingalls, D.T. Golenbock, Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide, J. Clin. Invest. 105 (2000) 497–504.

[40] A. Poltorak, P. Ricciardi-Castagnoli, S. Citterio, B. Beutler, Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation, PNAS 97 (2000) 2163–2167.

[41] S. Akashi, Y. Nagai, H. Ogata, M. Oikawa, K. Fukase, S. Kusumoto, K. Kawasaki, M. Nishijima, S. Hayashi, M. Kimoto, K. Miyake, Human MD-2 confers on mouse Toll-like receptor 4 species specific lipopolysaccharide recognition, Int. Immunol. 13 (2001) 1595–1599.

[42] K. Kawasaki, K. Gomi, M. Nishijima, Gln22 of mouse MD-2 is essential for lipopolysaccharide mimetic action of Taxol, J. Immunol. 166 (2001) 11–14.

[43] K. Kawasaki, S. Akashi, R. Shimazu, T. Yoshida, K. Miyake, M. Nishijima, Mouse Toll like receptor 4. MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol, J. Biol. Chem. 275 (2000) 2251–2254.

[44] R. Medzhitov, P. Preston-Hurlburt, C.A. Janeway Jr, A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity, Nature 388 (1997) 394–397.

[45] S. Viriyakosul, P.S. Tobias, R.L. Kitchens, T.L. Kirkland, MD-2 binds to lipopolysaccharide, J. Biol. Chem. 276 (2001) 38044–38051.

[46] S. Wright, Toll, a new piece in the puzzle of innate immunity, J. Exp. Med. 189 (1999) 605–609.

[47] N. Thiemblemont, R. Thieringer, S. Wright, Innate immune recognition of bacterial lipopolysaccharide: dependence on interactions with membrane lipids and endocytic movement, Immunity 8 (1998) 771–777.

[48] D.M. Underhill, A. Ozinsky, Toll-like receptors: key mediators of microbe detection, Curr. Opin. Immunol. 14 (2002) 103–110.

[49] Y. Bulut, E. Faure, L. Thomas, O. Equils, M. Arditi, Co-operation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and *Borrelia burgdorferi* outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signalling molecules in Toll-like receptor 2 signalling, J. Immunol. 167 (2001) 987–994.

[50] T. Kawai, O. Adachi, T. Ogawa, K. Takeda, S. Akira, Unresponsiveness of MyD88-deficient mice to endotoxin, Immunity 11 (1999) 115–122.

[51] K.A. Fitzgerald, E.M. Palsson-McDermot, A.G. Bowie, C.A. Jefferies, A.S. Mansell, G. Brady, A. Dunne, P. Gray, M.T. Harte, D. McMurray, D.E. Smith, J.E. Sims, T.A. Bird, L.A. O'Neill, Mal (MyD88-adaptor-like) is required for Toll like receptor 4 signal transduction, Nature 413 (2001) 78–83.

[52] T. Horng, G.M. Barton, R. Medzhitov, TIRAP, an adaptor molecule in the Toll signalling pathway, Nat. Immunol. 2 (2001) 835–841.

[53] D.J. McGee, R.F. Rest, Enhancing effect of cholera toxin on interleukin-6 secretion by IEC-6 intestinal epithelial cells: mode of action and augmenting effect of inflammatory cytokines, Infect. Immun. 64 (1996) 4630–4637.

[54] S.E. Paradis, D. Dubreuil, S. Rioux, M. Gottschalk, M. Jacques, High-molecular-mass lipopolysaccharides are involved in *Actinobacillus pleuropneumoniae* adherence to porcine respiratory tract cells, Infect. Immun. 62 (1994) 3311–3319.

[55] S. Chan, V.N. Reinhold, Detailed structural characterisation of lipid A: electrospray ionisation coupled with tandem mass spectrometry, Anal. Biochem. 218 (1994) 63–73.

[56] D.S. Hansen, F. Mestre, S. Alberti, S. Hernandez-Alles, D. Alvarez, A. Domenech-Sanchez, J. Gil, S. Merino, J.M. Tomas, V.J. Benedi, *Klebsiella pneumoniae* lipopolysaccharide *O*-typing: revision of prototype strains and *O*-group distribution among clinical isolates from different sources and countries, J. Clin. Microbiol. 37 (1999) 56–62.

[57] P. Domenico, D.L. Diedrich, D.C. Straus, Extracellular polysaccharide production by *Klebsiella pneumoniae* and its relationship to virulence, Can. J. Microbiol. 31 (1985) 472–478.

[58] J.B. Goldberg, G.B. Pier, *Pseudomonas aeruginosa* lipopolysaccharides and pathogenesis, Trends Microbiol. 4 (1996) 490–494.

[59] P.V. Salimath, R.N. Tharanathan, J. Weckesser, H. Mayer, The structure of the polysaccharide moiety of *Rhodopseudomonas sphaeroides* ATCC 17023 lipopolysaccharide, Eur. J. Biochem. 144 (1984) 227–232.

[60] S.G. Wilkinson, L. Galbraith, Studies of lipopolysaccharides from *Pseudomonas aerugionsa*, Eur. J. Biochem. 52 (1975) 331–343.

[61] R.P. Darveau, R.E.W. Hancock, Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains, J. Bacteriol. 155 (1983) 831–838.

[62] S. Cryz, T.L. Pitt, E. Furer, R. Germanier, Role of lipopolysaccharide in virulence of *Pseudomonas aeruginosa*, Infect. Immun. 44 (1984) 508–513.

[63] R.E. Hancock, L.M. Mutharia, L. Chan, R.P. Darveau, D.P. Speert, G.B. Pier, *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains, Infect. Immun. 42 (1983) 170–177.

[64] G.B. Pier, M. Grout, T.S. Zaidi, J.C. Olsen, L.G. Johnson, J.R. Yankaskas, J.B. Goldberg, Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections, Science 271 (1996) 64–67.

[65] S.J. Cryz Jr, E. Furer, A.S. Cross, A. Wegmann, R. Germanier, J.C. Sadoff, Safety and immunogenicity of a *Pseudomonas aeruginosa O*-polysaccharide toxin A conjugate vaccine in humans, J. Clin. Invest. 80 (1987) 51–60.

[66] A.D. Cox, C.J. Taylor, A.J. Anderson, M.B. Perry, S.G. Wilkinson, Structures of the two polymers present in the lipopolysaccharide of *Burkholdaria* (*Pseudomonas*) *cepacia* serogoup O4, Eur. J. Biochem. 231 (1995) 784–789.

[67] E. Evans, I.R. Poxton, J.W. Govan, Lipopolysaccharide chemotypes of burkholdaria cepacia, J. Med. Microbiol. 48 (1999) 825–832.

[68] A.P. Moran, B. Lindner, E.J. Walsh, Structural characterisation of the lipid A component of *Helicobacter pylori* rough and smooth form lipopolysaccharides, J. Bacteriol. 179 (1997) 6453–6463.

[69] M.A. Monteiro, K.H.N. Chan, D.A. Rasko, D.E. Taylor, P.Y. Zheng, B.J. Appelmelk, H. Wirth, M. Yang, M.J. Blaser, S.O. Hynes, A.P. Moran, M.B. Perry, Simultaneous expression of type 1 and type 2 Lewis blood group antigens by *Helicobacter pylori* lipopolysaccharides: molecular mimicry between *H. pylori* lipopolysaccharides and human gastric epithelial cell surface glycoforms, J. Biol. Chem. 273 (1998) 11533–11543.

[70] A.P. Moran, E.J. Walsh, In vitro production of endotoxin by the gastroduodenal pathogen *Helicobacter pylori* – relevance to serotyping, Ir. J. Med. Sci. 162 (1993) 384–387.

[71] A.P. Moran, J.J. McGovern, B. Lindner, E.J. Walsh, Preliminary structural analysis of *Helicobacter pylori* lipid A, Gut 37 (Suppl. 1) (1995) A16–A18.

[72] A.A. Lindberg, A. Weintraub, U. Zahringer, E.T. Rietschel, Structure–activity relationships in lipopolysaccharides of *Bacteroides fragilis*, Rev. Infect. Dis. 12 (1990) S133–141.

[73] I.R. Poxton, D.M. Edmond, Biological activity of bacteroides lipopolysaccharide – reappraisal, Clin. Infect. Dis. 20 (1995) S149–153.

[74] D. Delahooke, G. Barclay, I. Poxton, A re-appraisal of the biological activity of bacteroides LPS, Med. Microbiol. 42 (1995) 102–112.

[75] A.P. Moran, M.M. Prendergast, B.J. Appelmelk, Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease, FEMS Immunol. Med. Microbiol. 16 (1996) 105–115.

[76] R.E. Mandrell, M.A. Apicella, Lipo-oligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host modifications of LOS, Immunobiology 187 (1993) 382–402.

[77] D.W. Hood, J.C. Richards, E.R. Moxon, Haemophilus influenzae lipopolysaccharide, Biochem. Soc. Trans. 27 (1999) 493–498.

[78] E.R. Moxon, P.B. Rainey, M.A. Nowak, R.E. Lenski, Adaptive evolution of highly mutable loci in pathogenic bacteria, Curr. Biol. 4 (1994) 24–33.

[79] A. Zwahlen, L.G. Rubin, E.R. Moxon, Contribution of lipopolysaccharide to pathogenicity of *Haemophilus influenzae*: comparative virulence of genetically-related strains in rats, Microb. Pathogen 1 (1986) 465–473.

[80] T.A. Flak, W.E. Goldman, Signalling and cellular specificity of airway nitric oxide production in pertussis, Cell. Microbiol. 1 (1999) 51–60.

[81] M. Caroff, J.R. Brisson, A. Martin, D. Karibian, Structure of the *Bordetella pertussis* 1414 endotoxin, FEB Lett. 477 (2000) 8–14.

[82] P. Kosma, Chlamydial lipopolysaccharide, Biochim. Biophys. Acta 1455 (1999) 387–402.