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Lipopolysaccharide as a major virulence factor in the pathogenesis of *Burkholderia cepacia* syndrome.

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Abstract

In cystic fibrosis (CF), bacteria of the *Burkholderia cepacia* complex (Bcc) can induce a fulminant inflammation with pneumonitis and sepsis. Lipopolysaccharide (LPS) may be an important virulence factor associated with the increased morbidity and mortality seen in Bcc infection but little is known about the molecular pathogenesis of Bcc LPS.

In this study, the inflammatory response to highly purified LPS from different Bcc clinical isolates and the cellular signalling pathways employed was investigated.

It has been suggested that the large inflammatory response from BcLPS may be due to contaminants in the LPS preparation. Phenol-chloroform petroleum ether (PCP) purified BcLPS preparations were compared to more highly purified, enzyme treated preparations of BcLPS. There were no significant differences in the levels of IL-6 and TNF-α induced from monocytes (MM6) and in levels of IL-8 from epithelial cells (A549), which indicates that there were no contaminants present that could cause an inflammatory response from these cells.

The inflammatory response elicited by LPS from different Bcc species that were tested was seen to be varied. LPS from different clinical isolates of the same clonal ET12 strain of *Burkholderia cenocepacia* were found to induce a varied inflammatory response. Some isolate's LPS produced as much cytokine as prototypical *Escherichia coli* LPS and all Bcc isolates tested with the exception of environmental samples produced higher levels of inflammatory cytokine than LPS from the CF pathogen *Pseudomonas aeruginosa*. It was shown that passaging over time under laboratory conditions was not responsible for this variation. Bcc LPS samples were tested in their ability to prime MM6 cells for respiratory burst, samples that had previously produced high levels of cytokine from direct stimulation of MM6 cells were found to not be the most efficient primers of respiratory burst. It was found that the inflammatory response from Bcc LPS stimulated monocytes was separate and in some cases opposite to the ability to prime for respiratory burst.

Iinhibition experiments were used to investigate the Toll-like receptors and associated adaptor molecules and pathways utilized when monocytes were stimulated by Bcc LPS. The use of anti-CD14, anti-TLR4 and anti-TLR2 antibodies in Bcc LPS stimulated monocytes showed that all Bcc LPS samples tested signalled via CD14 and TLR4 and not via TLR2. Inhibition of MyD88 using an inhibitor peptide proved that all but one sample required MyD88 to signal. LPS from clinical isolate of *Burkholderia multivorans* was found to activate the inflammatory response via MyD88-independent pathways.

Using MAP kinase inhibitors to test for reduction of cytokine response from stimulated MM6 cells and direct analysis of activation through western blotting, LPS from all clinical Bcc isolates were seen to activate all three major MAPKs; p42/44, p38 and JNK. Degradation of the NF-κB inhibitory protein IκB-α was tested using anti-IκB-α antibodies and activation of the transcription factor NFκB was tested using an electrophoretic mobility shift assay (EMSA). IκB-α was degraded and NF-κB was activated in all the BcLPS samples tested.

This study suggests that LPS alone from clinical isolates of Bcc is major virulence factor in CF that it can cause a massive inflammatory response from cells, and that the LPS induced signalling cascade is via classical TLR4-mediated signalling pathways similar to highly inflammatory LPS purified from *E.coli*.

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Abbreviations

ASL	airway surface liquid
BALF	bronchoalveolar lavage fluid
Bcc	*Burkholderia cepacia* complex
BCESM	*Burkholderia cepacia* epidemic strain marker
BcLPS	*Burkholderia cepacia* complex LPS
BCSA	*Burkholderia cepacia* selective agar
BSA	bovine Serum Albumin
CF	cystic fibrosis
CFTR	cystic fibrosis trans-conductance regulator
CGD	chronic granulomatous disease
DPBS	Dulbecco's phosphate buffered saline
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ET-12	electrophoretic type 12
KDO	3-deoxy-D-*manno*-octulosonic acid
KO	D-*glycero*-D-*talo*-2-octulosonic acid
IL-8	interleukin 8
IL-6	interleukin 6
LDS	lithium dodecyl sulphate
LPB	LPS binding protein
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAPK	Mitogen activated protein kinases
MEP	mucoid exopolysaccharide
MM6	Monomac 6 cells (monocytic cell line)
PAMP	pathogen associated molecular pattern
PCP	Phenol-chloroform petroleum ether
PCR	polymerase chain reaction
PMB	Polymyxin B
PRR	pathogen recognition receptor
ROS	reactive oxygen species
RT	room temperature
TNF-α	tumour necrosis factor alpha
TLR	Toll-like receptor

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Introduction

1.1. Cystic fibrosis

1.1.1. Incidence and diagnosis

Cystic fibrosis (CF) remains the most common life shortening genetic disorder in the Caucasian population with an estimated survival age in the USA of 36.8 in 2006 according to the Cystic fibrosis foundation (http://www.cff.org/AboutCF). However survival ages do vary from country to country, the median estimated life expectancy of children with cystic fibrosis (CF) born in 1990 in the UK is 40 years which represents a doubling in the last 20 years (Jaffe & Bush 2001).

The incidence of CF is approximately 1 in 2500 live births with a carrier frequency of 1 in 25 people in European populations (Gazvani & Lewis-Jones 2004; Welsh *et al* 1995). This equates to approximately 1 in 10,000 people in the UK (450 babies born with CF annually).

Diagnosis of CF is usually straight forward with a child showing suggestive clinical symptoms or a family history, and a sweat test (collection of sweat and analysis of chloride concentration) confirms the diagnosis. Suggestive clinical features include recurrent chest infections, pancreatic insufficiency and a failure to thrive. Presently most CF patients are diagnosed in early childhood due to testing for the presence of the most common mutation (ΔF508) in babies in Northern Ireland, Wales and

Scotland from the heel-prick blood test. This screening procedure was extended to all babies in England from April 2007 (CF screening program (NHS), www.gosh.nhs.uk/newborn/cf/screening)

1.1.2. Genetic and molecular basis of CF

CF is an autosomal recessive disorder which is caused by mutations in a single gene on chromosome 7 that encodes for the Cystic fibrosis trans-conductance regulator (CFTR) (Riordan *et al* 1989).

CFTR is a cAMP-dependant chloride channel that influences the electrolyte and water balance of secretions from sweat glands, airways, pancreatic ducts, hepatobiliary ducts and intestinal glands. These organs are affected by the accumulation of viscous secretions with a progressive obstruction, scarring and destruction. Because of the effective management and treatment of most of the effects on these organs, especially in nutritional care, 84% of patients with CF die from respiratory disease rather than any other effect of the disease. (Liou *et al* 2001).

In the lung, the CFTR channel is found in surface airway epithelial cells and the cells of the submucosal glands. The CFTR mutation causes airway mucus to be poorly hydrated and together with changes in airway surface tension, hinders mucociliary clearance. This causes obstruction of small airways and promotes airway infection. Because of the respiratory systems' interaction with the outside world and constant re-exposure to infective bacteria the treatment of lung disease is very difficult. (Moran *et al* 1999)

CFTR is a member of the ATP-binding cassette transporter family of membrane proteins (Baldursson *et al* 2001). The CF gene is large (250 kb) and is comprised of 27 exons (Zielenski 1991), the gene is transcribed into a 6.5 kb messenger RNA that encodes a 1480 amino acid protein (Riordan *et al* 1989). Since the identification of the gene, over 800 disease associated mutations in the CF gene have been reported and a further 70 DNA sequence variants have been identified (Gazvani & Lewis-Jones 2004) with the most common being a three base pair deletion that codes for phenylalanine at position 508, Δ508 accounts for 70% of CF alleles in the white population (Gibson *et al* 2003).

The amino acids of CFTR form five domains: two membrane spanning domains each composed of six subunits, two nucleotide binding domains and a cytoplasmic regulatory domain. The membrane spanning domains seem to contribute to the formation of a chloride channel pore because mutation of specific residues in the first membrane spanning domain changes the anion selectivity of the channel (Welsh & Smith 1993). The nucleotide binding domain of CFTR is responsible for the binding and hydrolysis of ATP which provides energy necessary for channel function (Tsui 1995). The regulatory domain modulates the channel activity of CFTR (Rich *et al* 1993)

1.1.3. Pathological symptoms

CF manifests itself in a variety of organs and systems and leads to lung and sinus disease, gastrointestinal, liver and pancreatic disease, endocrine disease and growth problems, and infertility. In the past CF patients died from meconium ileus (failure to pass meconium), pancreatic insufficiency and hence nutritional deficiencies or lung infection as an infant or young child. The majority of CF patients presently die from pulmonary infection as adults.

Vulnerability to infection in CF occurs only in the airways and not at other sites such as the urinary tact or the skin, so there is no systemic immune defect in CF. However, excess inflammation occurs at several sites, for instance the presence of inflammatory bowel disease and pancreatitis are significantly higher amongst people with CF. (Lloyd-still 1994; Taylor CJ 2002)

As well as a pancreatic insufficiency and increased concentrations of chloride and sodium in sweat, CF causes chronic pulmonary disease which manifests itself in the airways and submucosal glands and then later the interstitium and alveolar spaces. (Gibson *et al*, 2003).

1.1.4. Abnormality in epithelial mucins

CF affects several organs of the body, the airways of the lung, the pancreas, small intestine, skin, and the vas deferens in males and cervical mucus in females. Defective chloride ion channel transport results in salty sweat and a build up of dehydrated and

concentrated secretions which block epithelial ducts at many sites, most notably in the pancreas and the lungs.

Symptoms are varied in CF and may or may not present antenatally, however the lungs of the foetus show no pathology prior to birth in CF. An accumulation of viscid mucous in the gut can lead to an intestinal obstruction in the newborn and consequently 10-15% of infants will present with meconium ileus. (Chaudry *et al* 2006).

Half a century ago postmortem studies on the lungs from infants that have died of meconium ileus have identified mucus plugging in terminal bronchioles, with emphysema secondary to obstruction of mucus filled bronchioles, but without signs of infection or inflammation (Zuelzer & Newton 1949). This suggested that mucus plugging was an early event (pre-infection) in the CF lung, in addition to this several other early observations indicated that abnormal airway surface liquid (ASL) volume regulation and failure to transport mucus plays a role in CF pathogenesis (Matthews *et al* 1963). There are currently two theories that explain why the CF lung is more prone to bacterial infection that the lungs of healthy individuals. The 'fluid' model states that hyper-absorption of fluid by the airway surface epithelium leads to a lower than normal airway-surface liquid (ASL) volume. The reduced volume and the defective fluid production by the submucosal glands leads to under-hydrated mucous and impaired mucociliary clearance. Impaired mucociliary clearance and thick airway secretions contribute to the establishment of an environment in the airway that promotes colonization of the lungs by bacteria (Matsui *et al* 1998). The 'salt' model, states that the salt content of airway fluid in CF is too high and prevents salt-sensitive

defensin molecules in the ASL from killing bacteria, leading to increased susceptibility to lung infections (Moskwa *et al.*, 2007; Smith *et al* 1996). Defensins are small (15-20 residues) cysteine-rich cationic proteins They are active against bacteria, fungi and enveloped viruses. Most defensins function by penetrating the microbe's cell membrane by way of electrical attraction, and once embedded, forming a pore in the membrane which allows efflux. An increase in salt concentration inhibits the activity of individual factors and attenuates synergy between defensins and this is the theory behind the 'salt' model.

1.1.5. Pancreatic insufficiency

After birth, obstruction of the pancreatic ducts due to concentrated secretions causes mal-absorption of nutrients due to the absence of pancreatic enzymes in the small intestine. This loss of pancreatic enzymes causes weight loss, fatigue and delayed growth if not managed properly. Over a longer time period, obstruction of the pancreatic ducts causes fibrosis of exocrine secretory tissue due to inflammation and tissue damage caused by proteolytic enzymes. This eventually impairs the ability of the pancreas to produce insulin which leads to diabetes mellitus. Development of pancreatic enzyme treatments, antibiotics and better diagnosis and management means that survival ages of CF patients are on the increase. Because of this successful management, the majority of patients suffering from CF now die due to pulmonary complications and not any other effect of the disease.

1.1.6. Underlying inflammatory response

At birth, the CF lung appears free from inflammation but typically within the first few months of life a heightened inflammatory state can be detected even in the absence of positive bacterial cultures (Nichols *et al* 2008). This has led to speculation that the inflammatory process in CF is autonomous and begins with a response to the misfolded CFTR protein within epithelial cells (DiMango *et al* 1998; Rao & Grigg 2006). However the majority of studies suggest that the inflammation is initiated in response to infection, which early in the disease can be transiently eradicated (Berger 2002; Bonfield *et al* 1999).

It is thought that when CF patients acquire an endobronchial infection a strong inflammatory response is elicited, this response is characterized by a marked influx of neutrophils into the lung, and elevations in inflammatory mediators such as TNF-α, IL-1β, IL-6, leukotriene B_4 (LTB_4), and IL-8 (Konstan *et al* 1993). Neutrophils are the predominant inflammatory cell in the lungs of patients with CF, even in individuals with mild pulmonary involvement (Konstan 1994).

Airway epithelial cells, macrophages, and neutrophils are all capable of producing cytokines. Several studies have found elevated concentrations of pro-inflammatory cytokines in the sputum and bronchoalveolar lavage fluid (BALF) of patients with CF (Richman-Eisenstat 1996).

The inflammatory response in patients with CF is excessive, and current management of pulmonary disease in CF is directed at the consequences of infection and inflammation (Ramsey 1996). Moreover, some infants with CF appear to have an

inflammatory response in the airways, even if infection is not detected (Khan *et al* 1995)

When compared to normal individuals the airway fluids of CF patients show an increased number of neutrophils, and increased levels of proinflammatory cytokines, TNF-α and IL-6, IL-8 and leukotriene B4, but decreased levels of anti-inflammatory IL-10. (Greally *et al* 1993; Bonfield *et al* 1995)

IL-8 is a powerful chemoattractant for neutrophils, its overproduction is the likely cause of excessive neutrophil infiltration in the CF lung. (Strieter 2002) Following this infiltration, live and dead neutrophils then release compounds such as oxidants, proteinases and DNA causing airway damage and inflammation. This combined with any bacterial infection and the reduced mucous clearance associated with CF leads to mucopurulent plugging bronchiectasis (Devaney *et al* 2003). TNF-α increases chemotaxis of neutrophils to the site of inflammation, adhesion of neutrophils to the endothelium and induces synthesis of chemoattractant neutrophils. IL-6 mediates the acute-phase reaction, matures B-lymphocytes and activates T-lymphocytes (Courtney *et al* 2003)

CF cell lines produce higher levels of pro-inflammatory cytokines than normal cell lines in response to *Pseudomonas aeruginosa* infection (DiMango *et al* 1995; Kube *et al* 2001). The absolute amounts of cytokines and chemokines produced are increased, and in *cftr* -/- mice they persist longer than in controls when challenged (Muir *et al* 2003). However it has been shown that cell origin and obviously cell species origin, and culture conditions can impact significantly on any observed responses in vitro, primary culture cells from the airway epithelium of CF patients have shown variable

production of IL-8 under different conditions when they are exposed to pro-inflammatory cytokines. (Aldallal *et al* 2002)

1.1.7. Microbiology

In individuals with CF, antenatally the lungs appear to be normal. Around the time of birth marked changes in fluid flux across the airway occur, the air-liquid interface at the epithelial surface is established and the pattern of ion transport is altered. When the lungs of neonates that have died of meconium ileus are examined, little histopathology and no inflammation can be seen. (Chmiel 2003)

In healthy individuals, mucus secreted by mucus gland and goblet cells of the large airways entraps particles that are then propelled by movement of cilia upwards. This mucociliary clearance provides an efficient system to clear pathogenic particles and is assisted by aerodynamic filtering and airway reflexes such as coughing and sneezing. In CF, after birth the airway is challenged repeatedly with various organisms which are normally dealt with by non-specific responses such as nitric oxide (NO) and antimicrobial peptides and proteins. In the airways of infants with CF these non-specific defences may be compromised, and the thick viscid mucus is not cleared as it is in healthy individuals.

In CF, chronic bacterial infections of the lungs are associated with a limited spectrum of pathogens including *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, *Stenotrophomonas*

maltophilia and *Alcaligenes xylosoxidans* (Bauernfeind *et al*, 1987; Gilligan 1991). *H.influenzae* and *S.aureus* predominate early in life, and until the age of antibiotics most infants would die early on from Staphylococcal infection. At the age of three 30% of patients are infected with *P.aeruginosa*, in young adults this rises to 80% (Bauernfeind *et al* 1987; Gilligan 1991).

Data collected in a 1998 multi-centre study of CF patients showed that *P. aeruginosa, S. aureus*, and *H. influenzae* could be cultured from the sputum or respiratory tract secretions of 61%, 47%, and 16% of tested CF patients, respectively (Lyczak *et al* 2002).

It is estimated that up to 50% of patients have fungus in their sputum, usually *Aspergillus fumigatus* (Nunley *et al* 1998)and it is of particular problem in lung transplant where it causes tissue invasive disease. CF patients are predisposed to this kind of fungal infection due to lung damage and long-term antibiotic therapy.

The role of viral infections in CF has not been firmly established. However several studies suggested that 40% of acute pulmonary exacerbations in cystic fibrosis are associated with respiratory viruses; these viruses also lead to pulmonary function abnormalities and disease progression. Armstrong *et al* showed that 52% of the infants with cystic fibrosis admitted to the hospital with respiratory symptoms were caused by respiratory viruses (Armstrong *et al* 1998). Thirty five percent of these hospitalised infants acquired *P aeruginosa* at follow up compared with 6% of those who were not hospitalised for respiratory symptoms. They concluded that respiratory viral infections

were associated with airway inflammatory changes, and admission to hospital was associated with early acquisition of *P aeruginosa*.

1.1.8. *Psuedomonas aeruginosa* infection in CF

Pseudomonas aeruginosa is Gram negative, aerobic bacillus with unipolar motility; it is an opportunistic pathogen in humans and does not normally cause a problem in immuno-competent individuals. An opportunistic pathogen, *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, and wounds and also causes sepsis. It colonises over 80% of CF adults (Kosorok *et al* 2001; Nixon *et al* 2001).

P. aeruginosa is intrinsically resistant to a range of antibiotics due to the concerted action of multi-drug efflux pumps with chromosomally encoded antibiotic resistance genes and the low permeability of the bacterial cellular envelope.

P.aeruginosa has the ability to develop resistance to multiple antibiotics either by horizontal gene transfer or by mutation in chromosomally encoded genes, some strains of *P.aeruginosa* have the ability to mutate rapidly in the lungs of patients with CF. These hypermutable strains demonstrate an increased ability to develop resistance to antibiotics (Oliver *et al* 2000).

During the initial infection of the airways of people with CF, *P.aeruginosa* continues its normal non-mucoid phenotype, but ultimately produces mucoid exopolysaccharide (MEP) or alginate. A steep oxygen concentration gradient exists between the airway

lumen and the interior of the mucus. The hypoxic environment created causes *P.aeruginosa* to produce even more MEP which contributes to the persistence of the bacteria in the airway by interfering with the host defences and delivery of antibiotics to the bacterial cell. (Pier *et al* 1993; Marshall & Caroll 1991; Worlitzch *et al* 2002) *P.aeruginosa* produces a number of toxins and virulence factors such as toxin A (Hollsing *et al*, 1987), elastase, alkaline proteinase (Sorensen *et al* 1983), alginate (Pedersen *et al*, 1992) and lipopolysaccharide (LPS).

Colonisation of the mucosal surface of the upper respiratory tract is the initial step leading to chronic lung infection and biofilm formation with microcolonies in the airways of the lung (Hoiby *et al* 1994). Chronic lung infection with mucoid *P.aeruginosa* is associated with a decline in pulmonary function and a much poorer prognosis in CF patients when compared to non-colonised patients (Pamukcu *et al* 1995). The LPS of *P.aeruginosa* is considered to be a major virulence factor (Pier 2007) and due to the relevance of *P.aeruginosa* infection in CF this organism has been used as comparison during this study.

1.1.9. Management of the CF lung

Management of the CF lung includes daily physiotherapy to drain the mucus from the airways. Topically nebulised amiloride is used in the lung to reduce the absorption of sodium and water and to increase mucociliary clearance (Kohler *et al* 1996)

Antibiotics are used to help with acute bacterial exacerbations in the lungs, these are delivered either intravenously, orally or in aerosol form. When lung infection

progresses past the point where other treatments are effective, bilateral lung transplantation is a clinical therapeutic option for selected patients that are suffering from advanced lung disease because of CF. (Egan *et al* 2002)

1.2.*Burkholderia cepacia* complex in CF

1.2.1. History

During the mid 1940's, vegetable growers in the USA became infected by an organism which had been previously known to cause onion bulbs to rot. Following this outbreak, three bacterial isolates were identified in the autumn of 1947 and a fourth in the autumn of 1948. These were given the latin name, "cepacia" meaning "of or like onion" by Walter H. Burkholder in his paper (Burkholder 1949).

The *B.cepacia* species have been isolated from a variety of natural habitats such as plant rhizosphere, soil and river water and from several urban environments such as playgrounds and athletic fields (Vermis *et al* 2003; Miller *et al* 2002; Fiore *et al* 2001). They have been found to have useful properties as plant pest antagonists, plant-growth-promoting (PGP) rhizobacteria and degradative agents of toxic substances (Chiarini *et al* 2006).

Since the 1950s the *B.cepacia* species have been reported as the cause of numerous catheter-associated urinary tract infections, wound infections, and intravenous catheter associated bacteraemia. In 1971, it was reported as the causative organism of "foot rot" in U.S. troops on swamp training exercises in northern Florida. (Taplin *et al* 1971)

Prior to the 1980s, reports of infections in humans caused by *B. cepacia* were infrequent and limited to small hospital outbreaks involving non-CF patients have and have been due to a contaminated common source, such as disinfectant preparations or intravenous solutions (Anderson *et al* 1991; Pegues *et al* 1993).

In the early 1980s, rising incidences in CF of B. cepacia complex infections were reported and in 1984, Isles and associates published their seminal paper describing *B. cepacia* as an emerging pathogen in CF (Isles *et al* 1984).

Since the 1980s, *B.cepacia* species have emerged as serious respiratory pathogens in CF patients (Govan *et al* 1996). It has been isolated from up to 40% of CF patients in some CF clinics and there have been several outbreaks of transmissible strains of *B.cepacia* species in CF centres in North America and the United Kingdom (Johnson *et al* 1994; Pitt *et al* 1996). Fifty years on from the Burkholder paper, this organism is now recognised as an important bacterial invader and coloniser of the lungs of patients with cystic fibrosis (CF).

1.2.2. Members of complex

The nomenclature for the cepacia group of organisms has changed over the years. They were formerly known as, amongst others, *Pseudomonas cepacia* or *Pseudomonas multivorans* (Stanier *et al* 1966) the species was moved to the new genus, Burkholderia, in 1992.

Since the creation of the Burkholderia genus 18 years ago, the number of species in it has risen to over 40 validly named species and candidate species (those which cannot be cultured outside the host but for which there is evidence to suggest that they are separate species) (http://www.bacterio.cict.fr/). These species are summarised along with their habitat in table 1.1.

In 1997 five sub-species were defined from within the *Burkholderia cepacia* species, known as 'genomovars' or 'genomic species'. The term 'genomovar' is used to denote species which is phylogenetically distinguishable from one another but which are not phenotypically distinguishable. (Vandamme *et al* 1997) Genomovars have specific different genotypes but are not distinguished by the usual biochemical tests that are carried out in a routine diagnostic microbiology laboratory; the genomovar becomes a species when a biochemical test is developed that allows it to be identified.

Presently, the formerly recognised genomovars of *B. cepacia* have been divided into species that include *B. cepacia* genomovar I, *B. multivorans* (genomovar II), *B. cenocepacia* (genomovar III), *B. stabilis* (*B.* cepacia genomovar IV), *B. vietnamiensis* (*B. cepacia* genomovar V), *B.dolosa* (genomovar VI), *B.ambifaria* (genomovar VII), *B anthina* (genomovar VIII) and *B. pyrrocinia* (genomovar IX).
At present ten Burkholderia species are combined into the *Burkholderia cepacia* complex (Bcc) group of bacteria (Table 1.2.), *B.cenocepacia* appears twice as there are 2 genetically different strains and these have not been designated with different names yet. *B.cenocepacia* and *B.multivorans* are the most clinically relevant species in the lungs of patients with CF but all members of Bcc have been isolated.

Name	Habitat	Reference
B.ambifaria	Humans (CF), soil, rhizosphere soil	Coenye *et al.*, 2001
B.andropogonsis	Plant material	Coenye *et al.*, 2001
B.anthina	Humans (CF), animals, soil, rhizosphere soil, river water	Vandamme *et al.*, 2002
B.caledonica	Soil, rhizosphere soil	Coenye *et al.*, 2001
B.caribensis	Soil, root nodules	Achouak *et al.*, 1999
B.caryophilli	Plant material	Vandamme *et al.*, 1997
B.cenocepacia	Human (CF and non-CF), animals, soil, rhizosphere soil, plant, water, industrial contaminant	Vandamme *et al.*, 2003
B.cepacia	Human (CF and non-CF), soil, rhizosphere soil, plant, water	Vandamme *et al.*, 1997
B.dolosa	Human (CF), root nodules, rhizosphere soil	Vermis *et al.*, 2004
B.fungoraum	Human (CF and non-CF), animals, soil, plant, water, industrial contaminant	Coenye *et al.*, 2001
B.gladioloi (incl. *B.cocovenenans, P.antimicrobica*)	Human (CF and non-CF), animals, plant material, rhizosphere soil	Palleroni, 1984
B.glathei	Plant material, rhizosphere soil	Zolg and Ottow, 1975
B.glumae	Plant material	Kurita and Tabei, 1967
B.graminis	Plant material, soil, rhizosphere soil	Viallard *et al.*, 1998
B.hospita	Soil	Goris *et al.*, 2003
B.koreensis	Soil	Kim *et al.*, 2006
B.kururiensis	Water, plant material	Zhang *et al.*, 2000
B.mallei	Horses, mules and donkeys	Palleroni, 1984
B.mimosarum	Root nodules	Chen *et al.*, 2006
B.oklahomensis	Human (non-CF), soil	Glass *et al.*, 2006
B.phenazinium	Soil, plant material	Bell and Turner, 1973
B.phenoliruptrix	Soil	Coenye *et al.*, 2005
B.phymatum	Root nodules	Vandamme *et al.*, 2002
B.phytofirmans	Soil, plant material	Sessitsch *et al.*, 2005
B.plantarii (incl. *B.vandii*)	Plant material	Azegami *et al.*, 1987
B.pseudomallei	Humans, animals, soil, water	Palleroni, 1984

B.pyrrocinia	Human (CF and non-CF), soil, rhizosphere soil, water	Vandamme *et al.*, 2002
B.sacchari	Soil	Bramer *et al.*, 2001
B.sordidicola	Fungus	Lim *et al.*, 2003
B.silvatlantica	Rhizosphere soil, plant material	Perin *et al.*, 2006
B.stabilis	Human (CF and non-CF), hospital equipment	Vandamme *et al.*, 2000
B.terrae	Soil	Yang *et al.*, 2006
B.terricola	Soil, rhizosphere soil, plant material	Goris *et al.*, 2003
B.thailandensis	Soil	Brett *et al.*, 1998
B.tropica	Plant material	Reis *et al.*, 2004
B.tuberum	Root nodules	Vandamme *et al.*, 2002
B.ubonensis	Human (non-CF), soil	Yabuuchi *et al.*, 2000
B.unamae	Plant material, rhizosphere soil	Caballero-Mellado *et al.*, 2004
B.vietnamiensis	Human (CF and non-CF), soil, rhizosphere soil, plant material, animal	Gillis *et al.*, 1995
B.xenovorans	Human (non-CF), soil, rhizosphere soil	Goris *et al.*, 2004
Candidatus *B.kirkii*	Plant material	Van Oevelen *et al.*, 2002
Candidatus *B.calva*	Plant material	Van Oevelen *et al.*, 2004
Candidatus *B.nigropunctata*	Plant material	Van Oevelen *et al.*, 2004

Table 1.1. : Bacteria from the Burkholderia species and where they are most commonly isolated. Adapted from Burkholderia: Molecular Microbiology and Genomics, Coeyne and Vandamme, 2007.

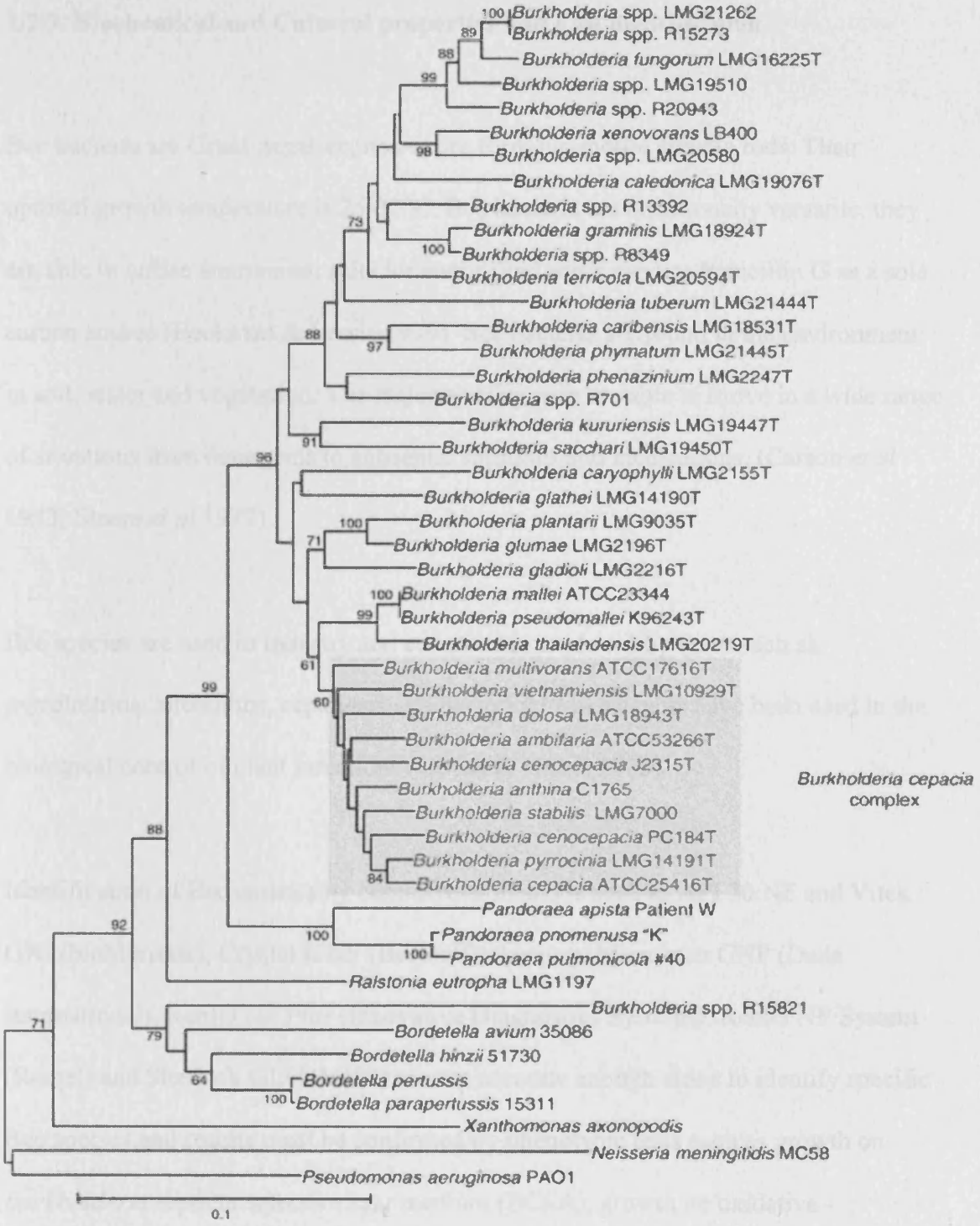

Table 1.2. : Phylogeny of the *Burkholderia* genus using *recA* gene sequences. Species name and strain designation are indicated (T denotes type strain). The tree was constructed using 760 nucleotides of the *recA* gene sequence and the neighbour-joining algorithm. Bootstrap values and genetic distance scale are shown. Bcc species (red text) are clustered in the shaded region of the tree. Reproduced with permission through RightsLink from Chiarini et al 2006

1.2.3. Biochemical and Cultural properties and Lab identification

Bcc bacteria are Gram-negative, non-spore forming, motile aerobic rods. Their optimal growth temperature is 25-30°C. Bcc bacteria are nutritionally versatile, they are able to utilise ammonium salts for energy and some can use Penicillin G as a sole carbon source (Beckman & Lessie 1979). Bcc bacteria are found in the environment in soil, water and vegetation. The majority of species are able to thrive in a wide range of situations from detergents to antiseptic solutions and medications. (Carson *et al* 1973; Steere *et al* 1977)

Bcc species are used in industry and compounds produced by them such as pyrrolnitrins, altercidins, cepalyans and bacteriocin-like agents have been used in the biological control of plant infections (Govan & Harris 1985).

Identification of Bcc isolates by commercial methods such as API 20 NE and Vitek GNI (bioMérieux), Crystal E/NF (Becton Dickinson), Microscan GNP (Dade International), RapID NF Plus (Innovative Diagnostics Systems), Remel NF System (Remel) and Sherlock GLC (MIDI) are not accurate enough alone to identify specific Bcc species and results must be confirmed by phenotypic tests such as growth on *Burkholderia cepacia* selective agar medium (BCSA), growth on oxidative - fermentative base, Polymixin B, bacitracim and lactose (OFPBL) agar, biochemical tests for lysine and ornithine decarboxylase, oxidase activity, sucrose and adonitol oxidation, hemolysis, pigment production and growth at 42°C. Following this a molecular method for identification can be chosen, most commonly *recA* analysis. The *recA* gene shows a sequence variability that allows Bcc to be differentiated from

closely related bacteria and sorted into species. The *recA*-based identification test involves restriction fragment length polymorphism analysis with the *Hae*III enzyme and species-specific PCR reactions in addition to sequencing of the entire *recA* gene (Chiarini *et al*, 2006).

Recently a system of identification using species-specific differences in fatty acid composition in cell lipidic structure and semi-automatic gas chromatography has been developed (Krejci & Kroppenstedtt 2006) but whether this is more accurate than methods already in use is still to be proven.

1.2.4. Pathogenicity

Bcc species members are recognised as opportunistic pathogens in humans. The majority of infections are found in patients suffering from CF but Bcc species can also colonise and cause infections in other patients, in particular those with chronic granulomatous disease (CGD) which is a rare immunodeficiency that renders patients' phagocytes unable to produce reactive oxygen species (ROS). Phagocytes require an enzyme to produce reactive oxygen species to destroy bacteria after they ingest the bacteria in a process called phagocytosis. This enzyme is termed "phagocyte NADPH oxidase" (*PHOX*). Defects in one of the four essential subunits of this enzyme can all cause CGD of varying severity, dependent on the defect (Bylund *et al* 2005). In rare cases Bcc infection has been reported in healthy individuals with no underlying lung disease. (Ledson *et al* 1998a)

Although they are not as common as some other pathogens in CF, organisms from the Bcc group have a major clinical impact. The clinical course that ensues after a patient has acquired a Bcc infection follows one of three patterns. First, there can be no discernable change in the clinical status and lung function. Second, there can be a chronic decline in lung function. A minority of patients show a rapid and uncontrollable clinical deterioration, which included necrotizing pneumonia and septicaemia that results in early death. The rapid decline in patient status has become known as 'cepacia syndrome' as the bacteraemia that is associated with this syndrome rarely occurs during infection with other CF pathogens (Isles *et al* 1984; Mahenthiralingam *et al* 2005).

The majority of patients who become infected with Bcc organisms follow the second pattern of infection with an accelerated decline in lung function compared to those infected with *P.aeruginosa*, so the majority of CF patients live with long term carriage of Bcc species. (McCloskey *et al* 2001)

The pathogenic mechanisms involved in severe Bcc infection are poorly understood, it has been shown that LPS from Bcc species elicits a much stronger inflammatory response from leukocytes compared with that of *P.aeruginosa* (Shaw *et al* 1995; Zughaier *et al* 1999a).

1.2.5. Distribution of Bcc in CF

Infection with Bcc bacteria can be highly transmissible in CF patients and epidemics have been seen to occur in a number of CF centres, in the UK, the USA and Canada. (Clode *et al* 2000)

Studies of the distribution of Bcc species in CF patients have revealed a highly disproportionate distribution, with most isolates belonging to either *B. multivorans* or *B. cenocepacia*; in contrast, *B. stabilis*, *B. ambifaria*, and *B. anthina* have rarely been isolated (Bevivino *et al* 2002; Speert *et al* 2002; Campana *et al* 2005), in fact over 85% of isolates identified from CF patients are found to be either *B.cenocepacia* or *B.multivorans* (Table 1.3.).

There has been compelling evidence shown by several studies that Bcc strains can spread between CF patients either by social contacts or via hospital admission. (Govan *et al* 1993; Pegues *et al* 1993) Epidemic strains predominate among patients in some CF clinics and may have an enhanced capacity for spread (LiPuma *et al* 1988, Mahenthiralingam *et al* 1997). The factors contributing to this are poorly understood, but putative transmissibility markers have been identified. Firstly there is the *B. cepacia* epidemic strain marker (BCESM), this is a 1.4-kb sequence containing an open reading frame (ORF) with homology to transcriptional regulatory genes. This has been found in several epidemic strains but is absent from strains for which there was no evidence of patient-to-patient spread (Mahenthiralingam *et al* 1997).

There is also the *cblA* gene that encodes the pilin subunit protein of cable pili that appears to facilitate infection by enhancing attachment to epithelial cells (Sajjan *et al* 1995). Cable pili are only found to be expressed by the *B. cepacia* ET-12 clonal lineage, a *B.cenocepacia* strain that predominates in Ontario, Canada, and is found in approximately one-third of all *B. cepacia*-colonized patients in the United Kingdom (Pitt *et al* 1996).

The strain ET-12 lineage, the so-called "epidemic" strain, has been a particularly virulent clone which has spread internationally from North America to Europe and subsequently within European countries. The ET-12 strain appears to have infected patients in Toronto in the late 1980's before spreading across Canada where it is the predominant *B.cenocepacia* lineage (Speert *et al* 2002), it was first introduced into the UK CF population in 1987 in Cardiff because of contact and transmission at a CF summer camp but it was not initially identified as the ET-12 strain (personal communication Henry Ryley), however it was first positively identified and reported to be in the UK CF population in 1989 (Govan *et al* 1993).

There is now a high incidence of *B.cenocepacia* ET-12 in the UK with one study showing that 56% of isolates were ET-12 (McDowell *et al* 2004).
The ET-12 lineage appears to be disproportionately associated with cepacia syndrome-related deaths (Ledson *et al* 2002), and patients infected with this strain are at especially high risk of poor outcome after lung transplantation (De Soyza *et al.*, 2001; 2004).

Species (former genomovar designation)	Approximate %[a]
B. cepacia (I)	3
B. multivorans (II)	40
B. cenocepacia (III)	45
B. stabilis (IV)	<1
B. vietnamiensis (V)	6
B. dolosa (VI)	4
B. ambifaria (VII)	1
B. anthina (VIII)	<1
B. pyrrocinia (IX)	<1
Indeterminate	1

[a]Approximate % of infection among >1200 *Burkholderia cepacia* complex – infected cystic fibrosis patients in the United States

Table 1.3. : Distribution of *Burkholderia cepacia* complex species in US cystic fibrosis patients. Adapted from: LiPuma: Curr Opin Pulm Med, Volume 11(6).November 2005.528-533

1.2.6. Clinical management of CF patients colonised with Bcc species

Patients with CF and a chronic Bcc infection are managed with similar principals as those with a chronic *P.aeruginosa* infection, that is, with careful attention to airway clearance, nutrition, bronchodilator therapy and mucolytic therapy. However the treatment of acute episodes in patients with Bcc infection is more of a problem as Bcc organisms are nearly always multi-resistant to antibiotics. Antibiotic combinations have been shown to have a reasonable effect on acute exacerbations, especially those containing Menopenem. Patients with stable Bcc infections do not seem to show any more frequent exacerbations compared to those with *P.aeruginosa* infections alone (Moore & Elborn 2003).

Transmission of Bcc species is an ongoing problem for CF clinics, control measures have included segregating patients colonised with Bcc species from those who are not colonised. This policy has proved to be successful in minimising cross-infection of Bcc species between patients but has not affected initial acquisition (Thomassen 1986; Govan *et al* 1993; Whiteford *et al* 1995; Muhdi *et al* 1996; Cazzola *et al* 1996) It has been suggested that in the UK, only patients colonised with transmissibility marker positive *B.cenocepacia* strains should be strictly segregated from those patients infected with transmissibility marker negative *B.cenocepacia* strains and other Bcc species (Ledson *et al* 1998b; Clode *et al* 2000). However data from the USA suggests that cblA gene and the other transmissibility marker BCESM may not be sufficient markers to indicate transmissibility of *B.cenocepacia*. (LiPuma *et al* 2001; Sajjan *et al* 2002)

The current UK, USA and Canadian advice to CF patients and clinics is to discourage Bcc positive patients from attending CF meetings and conferences because of potential transmission, however individual clinics make the decision as to whether to segregate or not. Often segregating patients brings about a whole new set of problems as it isolates certain patients. (Moore & Elborn 2003)

Some patients with CF may transiently acquire infections with a known transmissible strain of Bcc species. The CF trust currently recommend a period of at least one year between negative cultures, during which at least 3 different sputum cultures have been taken, before a patient can be declared as having eradicated a Burkholderia species (Jones & Webb 2003).

1.2.7. Virulence factors in Bcc

Bcc species have many cellular and extracellular products that are considered to be putative virulence factors such as adhesions, extracellular polysaccharide, lipopolysaccharide, proteases, lipases, haemolysin and siderophores.
Bcc isolates from CF patients have been found to produce significantly more catalase, ornithine decarboxylase, valine, aminopeptidase, C14 lipase, alginase and protease than non-CF isolates and also produced complete haemolysis on bovine red blood cells. (Gessner & Mortensen 1990)

Intracellular invasion of Bcc species is an important factor to take into account when discussing virulence factors, several investigators have shown that Bcc species can survive in A549 human epithelial cells and macrophages (Saini *et al* 1999; Martin &

Mohr 2000; Keig *et al* 2001). Bcc species have also been localized by immunohistology in patients with "cepacia syndrome" in the exudates within bronchial lumens and between airway epithelial cells (Sajjan *et al* 2001). These CF patients were infected by the transmissible clonal ET-12. The isolates of this clone carry the *cblA* gene, which encodes the major subunit for surface cable pili (as mentioned previously), which have been shown to mediate binding to respiratory mucins (Sajjan & Forstner 1992), and therefore may facilitate invasion. Invasion of cells means that eradication of the bacterium by conventional antimicrobial therapy is more difficult.

The roles of Bcc virulence factors in CF pulmonary disease are under current investigation; they are incredibly complex and are slowly being elucidated. An understanding of these virulence factors may provide a route to a more effective treatment and a better prognosis.

1.3. LPS

1.3.1. Innate immunity

The innate immune system is phylogenetically conserved and is present in almost all multicellular organisms (Hoffman 1999). Activation of innate immunity constitutes the first line of defence for the host against infection and generates signals to the adaptive immune system that indicates the presence of infection by microbes. The innate or 'non-specific' immune system consists of soluble elements including the alternative and mannan binding lectin pathways of the complement system, acute phase proteins and cytokines. It also consists of cellular elements such as monocytes, macrophages, neutrophils, dendritic cells and natural killer cells.

Some elements of the innate immune system are pre-formed, such as natural antibodies and the alternative complement system, while others are mobilised following cellular activation via receptors called pathogen recognition receptors (PRRs) which recognise conserved molecular patterns that are found in micro-organisms. These patterns are known as pathogen associated molecular patterns (PAMPs) examples of which are LPS, peptidoglycan, flagellin and bacterial DNA. (Beutler 2000) Cell stimulation through these receptors results in the up-regulation of anti-microbial killing mechanisms and pro-inflammatory mediator release. (Takeda *et al* 2003)

Innate immune responses must be tightly regulated as unbalanced inflammatory and immune reactions can result in either uncontrolled microbial growth or overwhelming

inflammatory responses which result in tissue injury, vascular collapse and multi-organ failure (Bochud & Calandra 2003).

1.3.2. LPS

LPS is a major component of the outer membrane of Gram-negative bacteria (Figure 1.1.), contributing greatly to the structural integrity of the bacteria, and protecting the membrane from bile salts, digestive enzymes and antibiotics, it also enables the bacterium to evade innate immune responses such as complement, lysozyme and cationic proteins (de Maagd *et al* 1987; Nikaido *et al* 1979). It is a prerequisite for bacterial viability.

Lipopolysaccharides are complex amphiphilic molecules with an mw of about 10kDa, which vary widely in chemical composition both between and among bacterial species. LPS is located on the outer face of the bacterial cell membrane, where it mediates contact with the environment. LPS is essential to the function of the outer membrane, and as a structural component of the cell. It is not toxic when incorporated into the bacterial cell wall but after release, (due to cell division or death) its toxic moiety, lipid A, is exposed to immune cells and induces a strong inflammatory response.

LPS comprises three parts an outer O-polysaccharide chain, a core polysaccharide and a lipid A moiety (Figure 1.2.). The Lipid A component of LPS is the main determinant of pro-inflammatory activity (Khan *et al* 1998) and the secondary

acylation status of the Lipid A is starting to be recognized as important as well (Vorachek-Warren *et al* 2002). All bacterial species carry a unique LPS and some of the variations reside in the lipid A moiety in the acylation pattern, length of fatty acid residues, presence of 4-amino deoxy-L-arabinose and/or phosphoethanolamine linked to the phospho groups on the glucosamine sugars and the number of fatty acids present (Dixon 2005).

Immunogenicity is associated with the polysaccharide components of LPS, the composition of the O-side chain varies between different bacterial species and strains. O-side chains are easily recognised by the host; however the chain can be modified by the bacteria in order to avoid detection by the host immune system. LPS with complete O antigen are considered as called smooth type LPS, whereas bacteria with LPS that lack the O antigen are considered to be rough type LPS due to their appearance when growing on a plate.

LPS in large doses plays a role in the pathogenesis of a variety of different clinical conditions, primarily sepsis but also liver disease, inflammatory bowel disease, adult respiratory distress syndrome and toxic shock syndrome (Morrison & Ryan 1987).

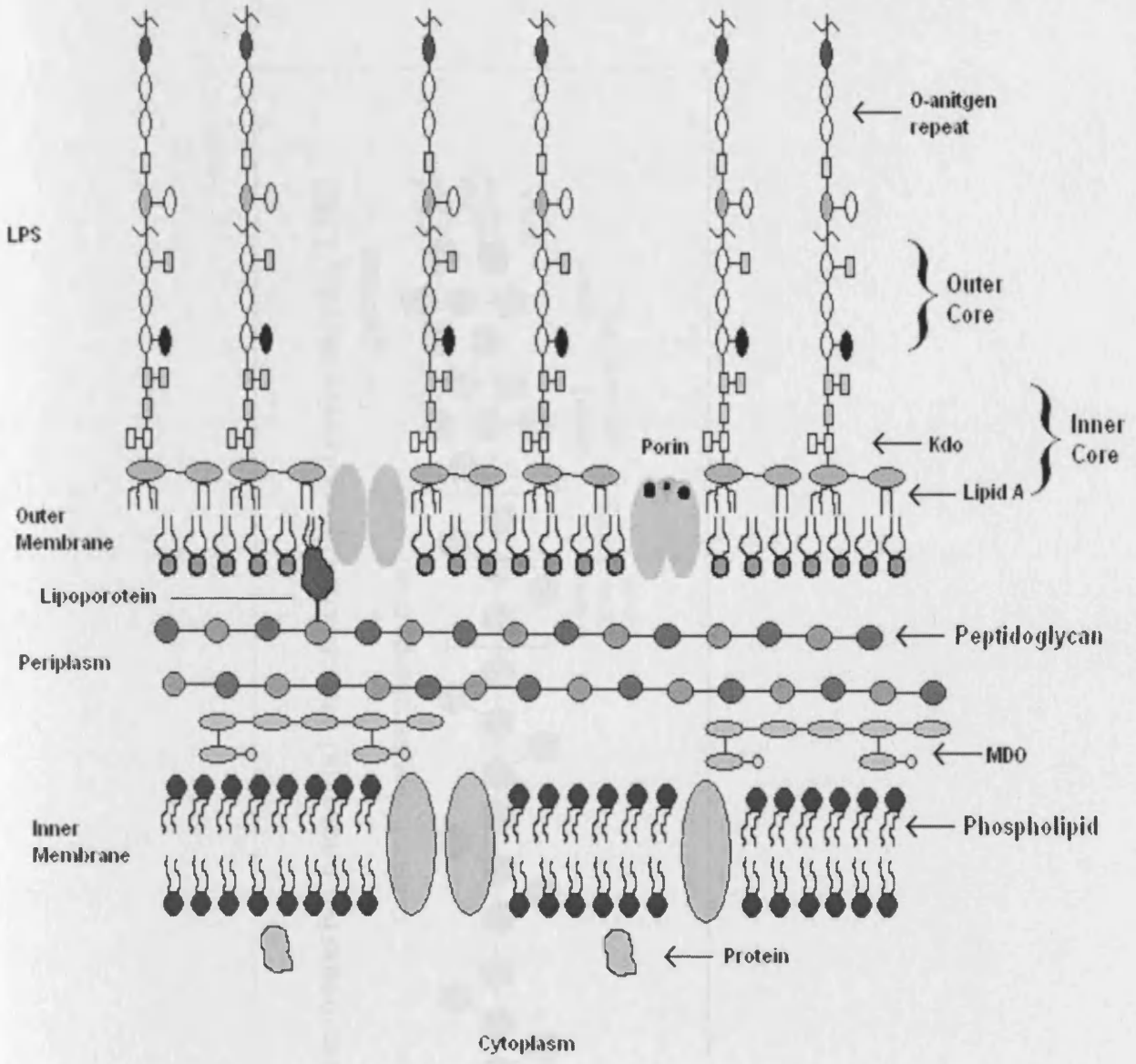

Figure 1.1. : Cross section of a Gram negative bacterial cell membrane. MDO-membrane derived oligosaccharides. KDO- 3-Deoxy-D-manno-octulosonic Acid, LPS- lipopolysaccharide.

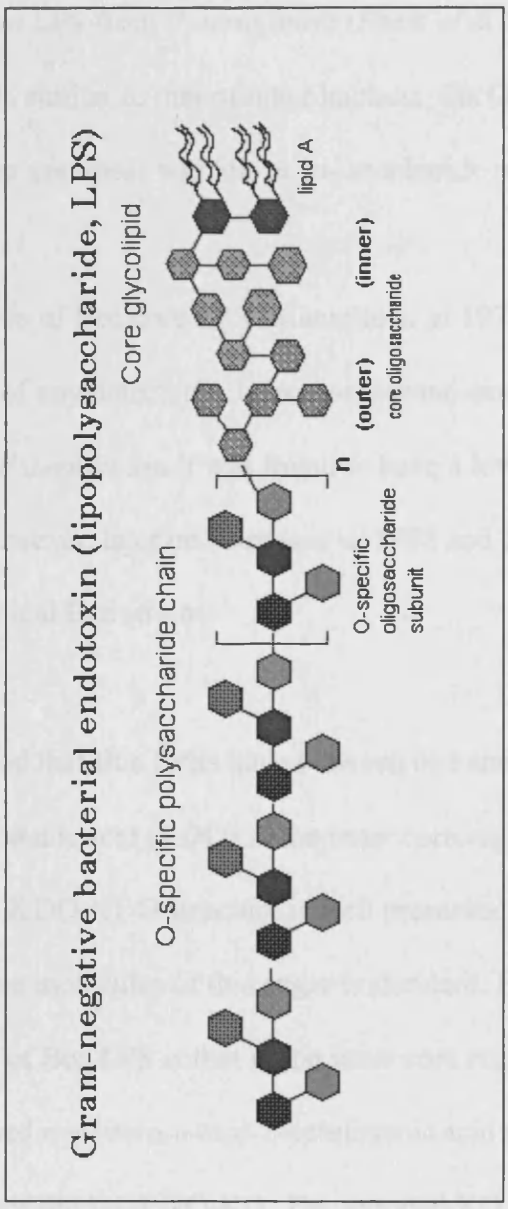

Figure 1.2. : Schematic diagram of Gram negative bacterial lipopolysaccharide (copywrite free image).

1.3.3. Bcc LPS

LPS from Bcc has been shown to elicit a nine-fold higher release of cytokines from leukocytes compared to LPS from *P.aeruginosa* (Shaw *et al* 1995). The basic structure of Bcc LPS is similar to that of other bacteria; the O antigen consists of un-branched polymers that are linear with di- or tri- saccharide repeating units.

Initial chemical analysis of Bcc core LPS (Maniello *et al* 1979; Anwar *et al* 1983) indicated the absence of any detectable 3-deoxy-D-*manno*-octulosonic acid (KDO), and in comparison to *P.aeruginosa* it was found to have a lower phosphorous content and more heptose. However, later on (Straus *et al* 1988 and 1990) isolation of KDO was reported from clinical Bcc strains.

It has now been verified that Bcc LPSs have between one and three molecules of 3-deoxy-D-*manno*-octulosonic acid (KDO) in the inner core region attached to the lipid A moiety. The lipid A KDO-KDO structure is well preserved among many bacterial species and one to three molecules of this sugar is standard. However the distinguishing feature of Bcc LPS is that in the inner core region, there is a unique sugar component named D-*glycero*-D-*talo*-2-octulosonic acid (KO), which has been found in the structure of lipid A-KDO-KO. The terminal KO residue of the disaccharide (KO/KDO) has been found to be non-stoichiometrically substituted at position 8 (C-8) with 4-amino-4-deoxy-β-**L**-arabinopyranose (Ara4N) (Issiki *et al* 2003).

LPSs containing Ara4N have been obtained from polymyxin-resistant strains and mutants of enterobacterial species, such as *E. coli*, *Salmonella enterica*, and *Proteus mirabilis*, and it has been found that substitutions of Ara4N occur in the ester-linked phosphate group in the lipid A region and/or in the KDO in the inner core region (Boll *et al* 1994). It has been suggested that this may explain Bcc species resistance to polymyxin. The presence of the Ara4N residue and its positive charge reduces the electronegativity of LPS and decreases binding of polymyxin, causing LPS to be resistant to its antagonistic action (Vinion-Dubiel 2003).

1.3.4. LPS mode of action and general clinical outcome

LPS is a potent initiator of non-specific immune responses. Monocytes orchestrate the innate immune response to LPS by producing a variety of inflammatory cytokines that include TNF-α, IL-6. The over reaction to the presence of LPS can lead to sepsis, septic shock or systemic inflammatory response syndrome. (Guhu & Mackman, 2001) Sepsis occurs as a result of a systemic inflammatory response to a severe bacterial infection (Pinsky 2004). A cellular response to bacterial products under controlled conditions is necessary to protect the host from infection. When sepsis occurs then there is an over-activation of the immune response which leads to the production of pro-inflammatory cytokines. It is the receptors of the innate immune system present on cells which are activated by bacterial products such as LPS, which are the first step in the initiation of sepsis.

1.4. Cellular pathways in response to LPS

1.4.1. LPS binding protein and CD14

Response to LPS is controlled by a range of different receptors and proteins and accessory molecules along a pathway that allows the host to initiate the production of pro-inflammatory molecules and respond to the bacteria. LPS is well recognized in the body and provokes a robust response from a variety of cells. LPS and LPS containing particles (such as intact bacteria) form complexes with a plasma protein called LPS binding protein (LBP). In humans LBP is present in plasma at 3-10 μg/ml but levels rise dramatically after an acute phase response to LPS. (Schumann *et al* 1990)

LBP deficient mice showed that LBP was not required *in vivo* for the clearance of LPS from the circulation, but was essential for the rapid induction of an inflammatory response by small amounts of LPS or Gram-negative bacteria. (Jack *et al* 1997)
The role of LBP in the activation of monocytes and macrophages by LPS has been demonstrated, immuno-depletion of LBP from whole blood lowers the sensitivity of monocytes to LPS by at least two orders of magnitude (Schuann *et al* 1990). Studies with LBP deficient mice have shown that LBP is not required *in vivo* for the clearance of LPS from the circulation, but is essential for rapid induction of an inflammatory response by small amounts of LPS or whole bacteria (Jack *et al* 1997)

CD14 is an accessory molecule that is expressed by monocytes, macrophages and neutrophils, it accepts the LPS molecule from LBP or can bind it directly. CD14 is a

myeloid marker antigen (Goyert *et al* 1986). The mature protein is expressed on the surface of myeloid cells via a glycosylphophatidylinositol (GPI) tail which anchors the protein to the membrane without a transmembrane segment, this membrane bound CD14 is known as mCD14 (Haziot *et al* 1988; Simmons *et al* 1989).

CD14 is also found free in plasma at 2-6 μg/ml (Bazil *et al* 1991) and is referred to as soluble CD14 (sCD14). sCD14 mediates activation of CD14 negative cells such as endothelial cells and some epithelial cells when LPS is present. (Pugin *et al* 1993).

Over-expression of human CD14 in transgenic mice causes a hyper-sensitivity to LPS and increases their susceptibility to endotoxin shock (Fererro *et al* 1993) .CD14 deficient mice are hypo-responsive to LPS and are 10 times less sensitive to LPS than wild type mice (Haziot *et al* 1995). CD14 also promotes responses to other bacterial cell wall components including lipoteichoic acid from Gram positive bacteria and lipoarabinomannan, however much larger doses of these bacterial components are needed to activate cells compared to LPS (Means *et al* 1999).

1.4.2. Toll-like receptors

Toll-like receptors (TLRs) were named after the fruit-fly receptor Toll which was first discovered because it has an important role in early fly development and was later recognised as contributing to innate immunity in adult flies.

The first event in an innate immune response in mammals is the activation of macrophages. Their role is to engulf invading pathogens and to secrete cytokines that activate other parts of the immune response. In *Drosophila*, Toll recognizes pathogens and provokes an innate immune response through activation of the Rel transcription factors Dorsal, Dif and Relish which are homologous to NF-κB in mammals. (Imler *et al* 2000) Shortly after the discovery of the role of Drosophila Toll in the host defence against fungal infection, a mammalian homologue of Drosophila Toll was discovered. (Medzhitov *et al* 1997)

Thirteen TLRs have been identified in mammals so far. In humans 11 members of the TLR family (TLR1-TLR11) have been identified all of which comprise a sub-family within the larger super-family of interleukin (IL) receptors (Takeda *et al* 2003).

TLRs are characterised by an amino-terminal extracellular domain composed of repeated motifs high in leucine and known as leucine rich repeats (LRRs), followed by a single transmembrane domain and a globular cytoplasmic domain called the Toll/interleukin 1 receptor (TIR) domain (Akira *et al* 2003). The LRR regions in the extracellular domain of TLRs have been implicated in the recognition of pathogens (Guhu & Mackman 2001).

TLRs are amongst a subset of a group of molecules called pattern recognition receptors (PRRs) and their main role is to mobilize the host's defences through several mechanisms including opsonization of foreign antigens, induction of host complement, coagulation, phagocytic and pro-inflammatory signalling cascades. The molecular patterns recognized by these receptors are called pathogen-associated

are essential to the integrity, function or replication of microbes and are unique to single celled organisms.

1.4.3. TLR2

Initial studies showed that TLR2 mediates LPS signalling, this conclusion was based on transfection experiments utilizing the 293 cell line and over expression of TLR2 (Kirschning *et al* 1998; Yang *et al* 1998). However gene targeting experiments showed that TLR2 deficiency does not disturb any responses to LPS (Takeuchi *et al* 1999) and it was found that TLR2 deficient Chinese hamster-derived cells can respond to LPS (Heine *et al* 1999). It has now been confirmed that TLR2 recognises a variety of microbial components including lipoproteins, peptidoglycan and lipoteichoic acid. TLR2 also recognises several atypical forms of LPS from *Leptospira interrogans* and *Porphyromonas gingivalis* (Hirschfeld *et al* 2001; Werts *et al* 2001).

1.4.4. TLR4

TLR4 has been identified as an essential signal transducer for LPS, it has been found to recognise the lipid A core of the LPS molecule (Han & Ulevitch 2005). C3H/HeJ and C57BL/10ScCr mice contain a mutation in the *Lps* gene that makes them hypo-responsive to LPS. Studies on these mice determined that the defective LPS signalling

seen is due to mutations in TLR4 (Poltorak *et al* 1998; Qureshi *et al* 1999) which indicated that this was the signalling receptor for LPS. This was further proven when macrophages from mice with a targeted inactivation of he TLR4 gene did not respond to LPS (Hoshino *et al* 1999). TLR4 also functions as a signal transducer for ligands other than LPS, for example, gram positive bacteria derived lipoteichoic acids and the plant derived anti-cancer agent taxol (Ding *et al* 1990; Takeuchi *et al* 1999). Both TLR4 and TLR2 (along with TLRs 1 and 6) have been found to function on the cell surface within large receptor complexes, or 'activation clusters' on lipid rafts or microdomains (Soong *et al* 2004).

1.4.5. MD-2

Additional proteins are required for LPS activation of cells via TLR4. It has been shown that expression of the extracellular protein, MD-2, enhanced TLR4-dependant activation of NF-κB in transiently transfected human embryonic kidney (293) cells. (Shimazu *et al* 1999) MD-2 is required for LPS activation of the mitogen-activated protein kinase (MAPK) pathways P44/42, JNK and P38 (Yang *et al* 2000).

TLR4-MD2 complex was identified on the surface of mouse peritoneal macrophages which indicates that a TLR4-MD2 complex functions as the LPS signalling receptor on the surface of monocytes (Akashi *et al* 2000)

1.4.6. MyD88 (dependant and independent pathway)

MyD88 is a cytoplasmic adaptor protein that consists of an N-terminal death domain and a C-terminal TIR domain. MyD88 is recruited to the TIR domain of activated TLRs and mediates a signalling cascade that results in the activation of various Mitogen activated protein (MAP) kinases and activation and nuclear translocation of NF-κB. Studies on MyD88 deficient mice showed that they lack the ability to respond to LPS (Kawai *et al* 1999)

MyD88 is used by all TLRs (except TLR3) to activate NF-κB MyD88-adaptor-like (Mal) protein is necessary to recruit MyD88 to TLR2 and TLR4. TLRs can utilise MyD88 dependant and independent pathways (Broad *et al* 2006).

Upon stimulation, MyD88 recruits a death domain-containing serine/threonine kinase, the IL-1R-associated kinase (IRAK). IRAK is activated by phosphorylation and then associated with TRAF6, leading to activation of two distinct signalling pathways. These two TLR-associated signaling cascades, each has a distinct adapter. A preliminary response is dependent upon a MyD88 adapter and a delayed response makes use of TRIF, or TIR-domain-containing adapter-inducing interferon-β. TLR4 signals through four adaptor proteins which operate in functional pairs, MyD88 with Mal (TIRAP) and TRIF with TRAM. (Palsson-McDermott *et al* 2004)

The TRIF cascade is a MyD88-independent signaling pathway associated with TLR3 and TLR4. A second adapter protein identified as TRAM acts as an essential bridge between TRIF and TLR4, acting upstream of the TRIF protein. These TIR domain-

containing adapters are differentially recruited to TLRs upon stimulation by pathogenic patterns.The TRIF cascade results in the activation of IRF-3, which then activates interferons α and ß, which are essentially for an inflammatory immune response. This pathway also stimulates NF-κβ, which upregulates costimulatory molecules CD40, CD80, and CD86 that in turn activate T-cell production and immune response (Boraschi *et al* 2006).

With very few exceptions all forms of LPS signal through TLR4 (Poltorak *et al* 1998) and through a MyD88-dependant pathway involving the adaptors MyD88 and Mal, or through a MyD88 independent pathway involving the adaptors TRIF and TRAM (Yamamoto *et al* 2003)

1.4.7. MAP Kinase and NF-κB activation

Mitogen activated protein kinases (MAPK) control many cellular events from complex programs such as embryogenesis, cell differentiation, cell proliferation and cell death, to short term changes required for homeostasis and acute hormonal responses (Chen *et al* 2001). MAPKs constitute more than a dozen proteins belonging to three families, these are extracellular signal-regulated kinases (ERKs), p38 MAPKs and c-Jun N-terminal kinase/stress activated protein kinases (JNK/SAPKs) (Rao 2001).

To date, LPS from enterobacteraceae has been shown to be a potent activator of three MAP kinase pathways, ERK, JNK, and p38 in macrophages. (Guhu & Mackman 2000; Lim *et al* 2002).

The transcription factor, nuclear factor (NF)-κB was originally identified as a regulator of the expression of the kappa light-chain gene in immune B lymphocytes. Later NF-κB was observed to be ubiquitously expressed. NF-κB regulates the expression of pro-inflammatory cytokines, chemokines, enzymes that generate mediators of inflammation, immune receptors, and adhesion molecules, all of which play a key part in the initiation of inflammatory reactions. Inducers of NF-κB activation include proinflammatory cytokines, growth factors, microbial infections oxidative stress and LPS.

IκB is a protein which inhibits NF-κB by complexing with and trapping it in the cytoplasm. However, the unphosphorylated form which is re-synthesized after cell stimulation is able to bind NF-κB, allowing its transport to the nucleus and protecting it to further IκB-dependent inactivation. This allows NF-κB to transcribe genes that produce chemokines and cytokines that start the inflammatory response.

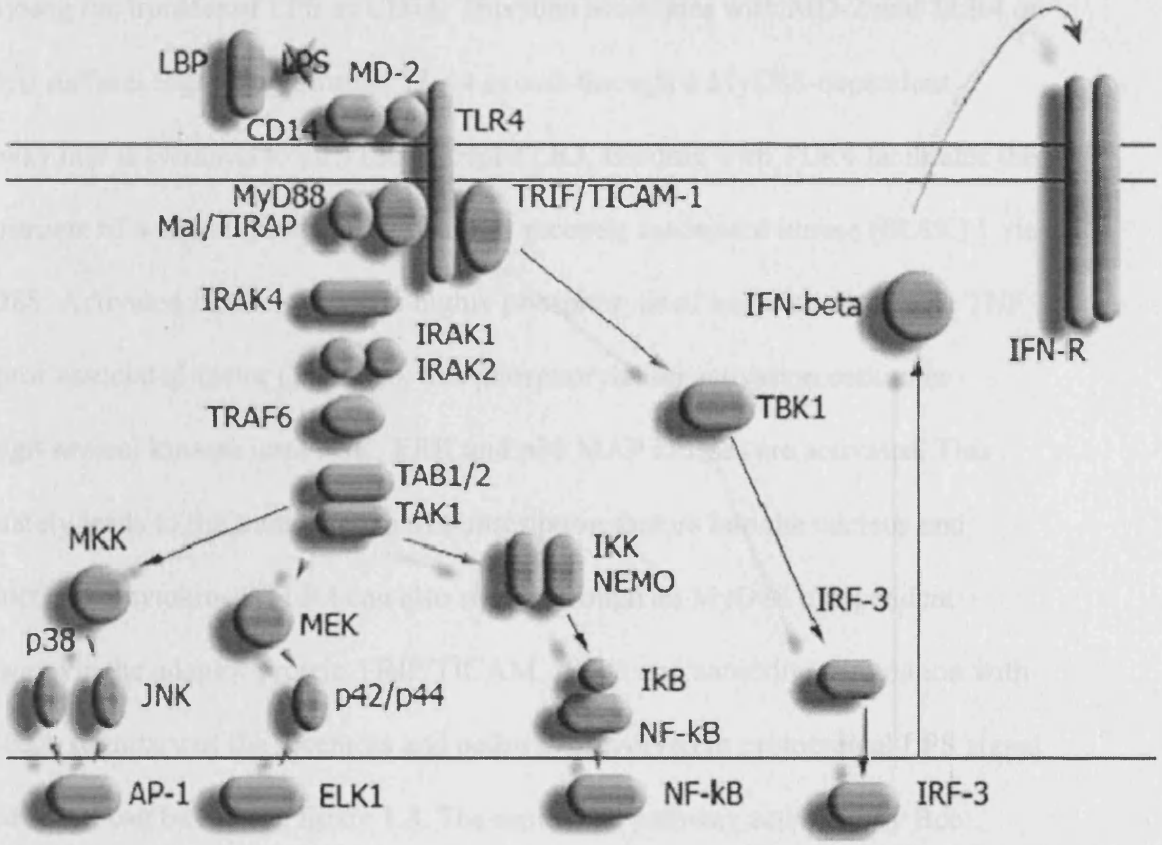

Figure 1.3. : TLR4 signalling pathway. Taken, with permission through Rightslink from Broad *et al* 2006.

1.4.8. Summary of LPS signalling pathway

In summary, the signalling pathway activated by prototypical LPS involves LBP catalysing the transfer of LPS to CD14. This then associates with MD-2 and TLR4 on the cell surface. Signalling through TLR4 occurs through a MyD88-dependant pathway that is common to all TLRs except TLR3. Binding with TLR4 facilitates the recruitment of a serine/threonine kinase IL-1 receptor associated kinase (IRAK) 1 via MyD88. Activated IRAK1 becomes highly phosphorylated and associates with TNF receptor associated factor (TRAF) 6, this phosphorylation activation continues through several kinases until JNK, ERK and p38 MAP kinases are activated. This ultimately leads to the translocation of transcription factors into the nucleus and production of cytokines. TLR4 can also signal through an MyD88 independent pathway via the adaptor protein TRIF/TICAM, IRF-3 and autocrine stimulation with IFN-ß. A summary of the receptors and pathways involved in prototypical LPS signal transduction can be seen in figure 1.3. The molecular pathway activated by Bcc species LPS has not been fully investigated until now.

Aims of this study

Some cystic fibrosis patients colonised with Bcc bacteria suffer a rapid deterioration in lung function with a small percentage suffering a fatal necrotising fulminant pneumonia referred to as 'cepacia syndrome'. The overall aim of this study is to investigate Bcc LPS as a virulence factor in the inflammatory response seen in CF lung disease. Briefly the aims are:

- To investigate the inflammatory response, as measured by cytokine production (TNF-α, IL-6, IL-8) in human cell lines (MM6, A549) when stimulated with highly purified LPS from different clinical and environmental Bcc isolates, and to compare this response to LPS from *P.aeruginosa* and highly potent LPS from *E.coli*.
- To investigate the intrinsic resistance of Bcc species to the antibiotic Polymyxin B (PMB). This will be done by testing the ability of PMB to bind BcLPS and inhibit cytokine production in stimulated human monocytes (MM6). This is of relevance as Polymyxin has a similar mode of action to host defensins.
- To investigate the ability of highly purified LPS from Bcc isolates to prime human monocytes (MM6) for respiratory burst and compare that ability to the inflammatory responses seen during direct stimulation with BcLPS. Over-production of reactive oxygen species (ROS) causes host tissue damage.
- To investigate the receptors that are bound and the signalling pathways that are activated by BcLPS from clinical CF isolates as compared to the pathways stimulated by *E.coli* LPS.

Materials and Methods

2.1. Cell Lines:

2.1.1. MM6 Cells: The human acute monocytic leukaemia (FAB M5)-derived cell line Mono Mac 6 (Ziegler-Heitbrock 1988), was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

For culture MM6 cells were maintained in RPMI 1640, supplemented with 10% heat-inactivated foetal bovine serum, 5ml 200mM L-glutamine, 1% v/v non-essential amino acids, 100U/ml penicillin, 100ug/ml streptomycin, and 1mM sodium pyruvate. Cells were cultured in a humidified incubator at 37°C with 5% CO_2

2.1.2. A549 Cells: Human pulmonary epithelial cells derived from a human lung carcinoma (Giard et al 1973). A549 cells were maintained in RPMI 1640, supplemented with 10% heat inactivated foetal bovine serum, 5ml 200mM L-glutamine and 100U/ml penicillin, 100ug/ml streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO_2.

Culture medium RPMI-1640 and the supplements L-glutamine, penicillin, streptomycin, sodium pyruvate and non-essential amino acids were obtained from

Cambrex, UK. Foetal bovine serum was obtained from Biosera, UK. Bovine insulin was obtained from Sigma, UK.

All media and supplements were tested for endotoxin contamination by Limulus Amoebocyte Lysate (LAL) assay in our lab and were found to contain less than 50 pg/ml endotoxin in all cases.

2.2. Bacterial Strains:

Table 2.2.1. Cardiff B.cepacia complex strains used in this study.

B.cepacia Strain	Species	Former Genomovar	Source
NCTC 10743	*B.cepacia*	I	Onion
NCTC 10661	*B.cepacia*	I	Environmental
Cardiff A1	*B.multivorans*	II	CF-Clinical
Cardiff A2	*B.multivorans*	II	CF-Clinical
Cardiff P2	*B.cenocepacia* ET12 epidemic	III	CF-Clinical (ET12 lineage)
Cardiff P5	*B.cenocepacia* ET12 epidemic	III	CF-Clinical (ET12 lineage)
Cardiff P1	*B.cenocepacia* ET12 epidemic	III	CF-Clinical (ET12 lineage)
Cardiff P3	*B.cenocepacia* ET12 epidemic	III	CF-Clinical (ET12 lineage)
Cardiff P4	*B.cenocepacia* ET12 epidemic	III	CF-Clinical (ET12 lineage)

Table 2.2.2. Other B.cepacia complex strains used in this study.

Edinburgh	*B.cenocepacia* ET12 epidemic	III	CF-Clinical
Liverpool	*B.cenocepacia* ET12 epidemic	III	CF-Clinical
London	*B.cenocepacia* ET12 epidemic	III	CF-Clinical
Manchester	*B.cenocepacia* ET12 epidemic	III	CF-Clinical
New Zealand	*B.cenocepacia* ET12 epidemic	III	CF-Clinical

Table 2.2.3. All other bacterial LPS isolates that were used in this study.

Bacterial Strain	Source
E.coli O111:B4	Sigma
P.aeruginosa PA01	Sigma
S.minnesota wild-type (smooth)	Sigma
S.minnesota R7 (rough)	Sigma

2.3.LPS characterisation methods

2.3.1. LPS extraction by phenol-water method

(based on the method of Galanos *et al* 1969 as adapted by Qureshi *et al* 1982)

Source of bacteria

Each bacterial isolate was stored at -80°Cin the Microbank®system that involves porous beads in cryopreservative (Pro Lab Diagnostics, Nestor, Wirral). A bead is used to inoculate a Columbia agar plate which is then incubated for 24 hours at 37°C. The pure culture was then transferred to 1 litre of tryptone soya broth (Oxoid Ltd, Basingstoke, UK) in a 3l flask and incubated at 37°C for 48 hours on a rotary shaker. The bacteria were harvested by centrifugation at 3770 xg for 30 min using a MSE Mistral 3000 centrifuge, washed and centrifuged again at 12189 xg for 15 min. The pellet was frozen, lyophilised and stored at -20°C in an air-tight UC until used.

Method

1g of the dried bacteria were suspended in 15ml of phenol extraction reagent (90% Phenol- 20ml, Chloroform- 50ml, Petroleum ether- 80ml) and homogenised for a minimum of 10 min using a tissue grinder with PTFE pestle (Kartell) cooled in an ice bath. The homogenised mixture was then transferred into a 15ml polypropylene (organic solvent resistant) tube and centrifuged for 30 min at 3770 xg in a Mistral 3000 centrifuge at 4°C. The resulting supernatant was removed and filtered through a

Whitman No 1 filter paper into a 250ml round bottom flask. Using a rotary evaporator, the chloroform and petroleum spirit were removed by evaporation for 15 min. The remaining solution was transferred to another 15 polypropylene tube and 14ml of diethyl ether/acetone (diethyl ether- 100ml, acetone- 500ml) reagent was added. After standing for a minimum of 1 hour at RT, the precipitated rich LPS fraction was collected by centrifugation at 3770 xg for 30 min in a Mistral 3000 centrifuge. The supernatant was discarded and the pellet was washed twice more with 14ml diethyl ether/acetone reagent followed by centrifugation. The LPS rich pellet was frozen and lyophilised for 30 min. It was then suspended in 3ml sterile pyrogen free water by heating in a 37°C water bath and by suction and displacement of the solution using a 5ml syringe with a 23 gauge needle. Vigorous vortexing also helped to disperse the pellet. The LPS suspension was centrifuged at 541745 xg for 1 hour in TLA-100.4 rotor using the Beckman Optima centrifuge. The pellet was then dispensed in 500 µl pyrogen free water and transferred into a glass bijoux, frozen and lyophilised. The dried LPS pellet was then referred to as BcLPS-1. It was either weighed and made up with pyrogen free water as a stock solution at a concentration of 1mg/ml and aliquoted and saved at -20°C, or further purified.

2.3.2. Enzymatic treatment of BccLPS-1

Method

For further purification of the LPS preparations, BcLPS-1 (suspended at 1mg/ml in pyrogen free water) was treated with 1μg of DNase/ml (from bovine pancreas; Sigma Chemical Co) and 1μg RNase/ml (from bovine pancreas; BDH, Poole, UK). The mixture was incubated at 30°C for 90 minutes at pH 7.5, followed by the addition of 1μg/ml of trypsin (from bovine pancreas, Sigma Chemical Co) which was added for a further incubation at 30°C for 90 minutes. Finally 1μg of Proteinase K/ml (from fungi; Sigma Chemical Co) was added and the mixture incubated at 30°C for 90 minutes. Following these enzymatic treatments the solution was dialysed against distilled water overnight and lyophilized.

A solution of pyrogen free water containing 0.2% triethylamine and 0.5% Sodium deoxycholate was prepared and 1ml of this solution was added to every 5 mg of dried LPS. An equal volume of 9:1 phenol:water solution was added and the-samples were then vortexed intermittently over 5 min. The phases were allowed to separate by gravity for 5 min. The solution was then put on ice for 10 min, followed by centrifugation for 2 min at 14,000 g. The phenol phase was then removed and submitted to re-extraction with 1 ml water containing 0.2% triethylamine and 0.5% Sodium deoxycholate and the first and second aqueous phases were pooled. The pooled aqueous phases were then subjected to re-extraction with an equal volume of 9:1 phenol:water solution and the phenol phase was discarded.

The aqueous phases were adjusted to make the solution 75% ethanol and 30 mM Sodium acetate and the LPS was allowed to precipitate at -20°C for 1 hour. The LPS precipitate was then collected by centrifugation for 10 min at 14,000 g and then the pellet was washed with cold ethanol and spun down again. The resulting pellet was then allowed to air-dry. This preparation was then referred to as BcLPS-2. It was either weighed and made up with pyrogen free water as a stock solution at a concentration of 1mg/ml and aliquoted and saved at -20°C.

2.4. Direct characterisation of *B.cepacia* complex LPS using whole organisms by silver stain.

2.4.1. B.cepacia complex preparation for analysis

Bacteria were inoculated on a Columbia agar plate and incubated overnight at 37°C. The bacteria were harvested from the whole plate and were washed with 10 ml sterile PBS and re-suspended again in 10 ml DPBS. The optical density of the suspension was then adjusted to 1.0 at 550 nm. 1.4 ml of the suspension was transferred into an Eppendorf tube and centrifuged at 13,000 rpm for 5 min in a micro-centrifuge. The pellet was re-suspended in 50 µl lysis buffer and the mixture was boiled for 10 min in a water bath. 10 µl of Proteinase K digestion buffer was then added to the solution, this was then mixed and incubated at 60°C for 1 hour. The resulting lysate was then analysed by electrophoresis.

2.4.2. Electrophoresis of LPS fraction on 14% SDS-PAGE

(This method is based on Laemmli,1970).

2.4.3. Preparation of SDS polyacrylamide discontinuous gel

18x16 cm BioRad Electrophoresis cassettes with 1.5 mm spacers were cleaned and fitted onto the base unit and were checked for leakage using water. 30 ml of resolving gel and 20 ml of stacking gel were required for the preparation of one gel.

Firstly the resolving gel was prepared and poured immediately into the cassette using a 20 ml syringe avoiding production of air bubbles, the solution was then overlaid with 5 ml distilled water and left to polymerise for approximately 30 minutes. The distilled water was then removed. A stacking gel was then prepared and poured onto the resolving gel in the cassette again using a syringe, and the comb was inserted slowly to avoid trapping any air bubbles. The gel was then left to polymerise for a minimum of 1 hour. The gel was then stored overnight at 4°C before use. (resolving gel buffer: 3 M Tris-HCl pH 8.8, stacking gel buffer: 0.5 M Tris-HCl pH 6.8)

2.4.4. SDS-PAGE

The prepared gel was allowed to stand at RT for 1 hour before being loaded. The comb was removed from the top of the gel cassette and the spaces were filled with 1:4 stacking buffer (0.5 M Tris-HCl pH 6.8). Fine pipette tips were used to load 20 µl sample per well. The cassette was then attached to the upper reservoir and the lower tank filled with 3 litres reservoir buffer (stock reservoir buffer: 0.25 M Tris, 1.92 M glycine, 1% SDS, pH 8.3) and the upper reservoir and cassette was placed on top and filled with 1 litre of the same reservoir buffer. The unit was closed and the electrodes were fitted to a transformer with the voltage set to 100 V. When the samples had completely entered the stacking gel (about 30 min) the voltage was increased to 250 V. When the bromophenol blue marker was approximately 10 mm from the bottom of the gel the run was ended and the cassette was removed from the buffer and carefully

opened using a plastic spatula. The stacking gel was then removed from the top of the gel making it ready for silver staining.

2.4.5. Silver staining of LPS

Method based on Hitchcock and Brown (1983). (Solutions listed in appendix)

The staining steps took place in glass staining trays with continuous gentle mixing on a Rotastat orbital shaker. The gel was firstly immersed overnight in 250 ml of the fixing reagent with gentle shaking. The gel was then transferred into 250 ml oxidising reagent for 5 min followed by 8x30 min washes with 250 ml de-ionised water. The gel was then placed in 250 ml of staining solution for 10 min followed by 4 washes of de-ionised water for 30 min. During this washing stage, 250 ml of developing reagent was warmed to above 25°C and added to the washed gel with gentle shaking. The development of colour was stopped when the banding was strong by adding 250 ml of stopping solution for 20 min. The gel was then washed briefly and then visualised using a UVP scanner and camera.

2.5. Investigation of the biological activity of B.cepacia LPS

2.5.1.Neutralization of LPS induced cytokine production by polymyxin

Polymyxin B sulphate: 7730 unit/mg (Sigma-Aldrich, UK). Stock solutions of Polymyxin B sulphate were prepared at different concentrations, 50 mg/ml, 5 mg/ml and 500 μg/ml in pyrogen free water. The stock solutions were diluted 10 fold prior to use in the neutralisation experiments.

Mono Mac 6 cells (Human monocytes)

Mono Mac 6 cells were grown in the conditions and media stated (2.1.1.). Cell viability (>95%) was checked using the trypan blue exclusion method and the cell count was then adjusted to $1x10^6$ cell/ml.

LPS: LPS stock solutions (1 mg/ml) were diluted using sterile pyrogen free water and added to MM6 cells ($1x10^6$ cell/ml) to make a final concentration of 100 ng/ml and 500 ng/ml. Stock LPS solutions were stored at -20°C until needed in pyrogen free glassware.

Method

MM6 cells were suspended at $1x10^6$ and dispensed into 24 well plates in 1 ml aliquots. Stock LPS solutions were thawed and vigorously vortexed for 5 min. The LPS solution was added to each 1 ml well to make a final concentration of either 100

ng/ml or 500 ng/ml. Then the Polymyxin solution was added to each well to make final concentrations of 5 μg/ml and 50 μg/ml, each experiment was setup in triplicate. Controls containing Polymyxin alone, LPS only and unstimulated control cells were also included. The cells were then incubated at 37°C, 5% CO_2 in a humidified incubator overnight. Positive controls of cells stimulated with LPS alone were included and negative controls with Polymyxin only and cells with no stimulus were included. The cells were then centrifuged at 2500 rpm for 5 min and the supernatants were collected. The supernatants were placed in sterile Eppendorf tubes and then either stored at -80°C or immediately tested for levels of TNF-α by ELISA.

2.5.2. TNF-α ELISA

A 96 well microtitre plate (Nunc) was coated with 100 μl/well of the anti-human TNF-α monoclonal antibody (diluted 1/125, 80 μl from a stock of 500 μg/ml was diluted in 10 ml PBS to coat 1 plate). The plate was covered with an adhesive seal and left to incubate at RT overnight. The plate was then washed 4 times using 300 μl/well of 0.05% Tween 20 in PBS and blocked with 300 μl/well PBS containing 1% BSA and 5% sucrose for 1 hour at RT then washed 4 times. A starting standard (human recombinant TBF-α) containing 1000 pg/ml was diluted from a stock standard containing 10 μg/ml. Subsequent standard points were prepared by doubling dilutions down to 15.6 pg/ml.

A 100 μl of sample or standard (diluted in 0.1% BSA, 0.05% Tween 20 in Tris-buffered saline, pH 7.3) was added per well, mixed by gentle taping of the plate frame

for 1 min, samples were always added in duplicate. The plate was then covered with an adhesive seal and left to incubate for 2 hours at RT.

A detection antibody (biotin conjugated goat polyclonal secondary antibody) was diluted 1/250 (50 μg/ml stock diluted to 200 ng/ml, 40 μl of stock diluted in 10 ml diluent). The plate was washed 4 times and 100 μl of the diluted biotinylated TNF-α was added to each well. The plate was then covered again and left for 2 hours at RT. The plate was then washed 4 times and 100 μl of Streptavidin horseradish peroxidise (HRP) (1/20,000 of a 1.25 mg/ml solution) was added per well, sealed and incubated at RT for 20 min. The plate was washed 4 times and 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) micowell peroxidase substrate solution was added per well and incubated for 20-30 min in the dark at RT. The reaction was halted by the addition of 50 μl 0.5 M H_2SO_4 per well and mixing by gentle tapping. The optical density of each well was determined using a microtitre plate reader set to 450 nm and 540 nm for wavelength correction.

The results were calculated by averaging the sample duplicate's optical density and subtracting the zero standard reading from it. The resulting optical densities from the standards were plotted against the actual concentrations of the standards and linearised by using a log-log best fit curve. The TNF-α concentrations in the samples that were tested were calculated from the best fit curve.

2.5.3. IL-6 ELISA

The IL-6 ELISA method was similar to the TNF-α ELISA except the IL-6 capture antibody was diluted 1/125 to give a final concentration of 4 μg/ml from a stock solution of 500 μg/ml.

A starting standard containing 1200 pg/ml was prepared from a stock standard containing 10 μg/ml. The subsequent standards points were prepared by doubling dilutions to 18.75 pg/ml. Biotinylated antibody was diluted 1/2000 (50 μg/ml stock diluted to 25 ng/ml, 5 μl of stock diluted in 10 ml diluent), all the other steps were performed identically to the TNF-α method.

2.5.4. IL-8 ELISA

The IL-8 method was similar to the TNF-α ELISA except the IL-8 capture antibody was diluted 1/125 to give a final concentration of 4 μg/ml from a stock solution of 500 μg/ml.

A starting standard containing 2000 pg/ml was prepared from a stock standard containing 10 μg/ml. The subsequent standards points were prepared by doubling dilutions to 31.25 pg/ml. Biotinylated antibody was diluted 1/2500 (50 μg/ml stock diluted to 20 ng/ml, 4 μl of stock diluted in 10 ml diluent), all the other steps were performed identically to the TNF-α method.

2.5.5. Comparison of BcLPS-1 andBcLPS-2 purified LPS samples

MM6 cells were grown in conditions the conditions and media stated earlier (2.1.1.). Cell viability (>95%) was checked using the trypan blue exclusion method and the cell count was then adjusted to $1x10^6$ cell/ml. The cells were then dispensed in 1 ml aliquots into 24 well culture plates. *B.cepacia* LPS that had either been purified to BcLPS1 or BcLPS2 level were added to each well in duplicate to make a final concentration of 1 μg/ml, control wells containing 1μg/ml *E.coli* LPS and non-stimulated wells were included on each plate. The plate was then mixed by gentle tapping and incubated at 37°C, 5% CO_2 for 16 hours. The cells were harvested and spun down in a centrifuge at 2500 rpm and the supernatants were collected and either stored at -80°C until testing or tested immediately for level TNF-α or IL-6 by ELISA.

2.5.6. Comparison of LPS from different ET12 strains

MM6 cells were grown in the conditions and media stated earlier (2.1.1.). Cell viability (>95%) was checked using the trypan blue exclusion method and the cell count was then adjusted to $1x10^6$ cell/ml. The cells were then dispensed in 1 ml aliquots into 24 well culture plates. *B.cepacia* LPS extracted from various ET12 epidemic strains were added to each well in duplicate to make a final concentration of 1 μg/ml, control wells containing 1μg/ml *E.coli* LPS and non-stimulated wells were included on each plate. The plate was then mixed by gentle tapping and incubated at 37°C, 5% CO_2 for 16 hours. The cells were harvested and spun down in a centrifuge

at 2500 rpm and the supernatants collected and either stored at -80°C until testing or tested immediately for TNF-α or IL-6 by ELISA.

2.5.7. Effect of B.cepacia LPS on the production of IL-8 in A549 cells

A549 cells were maintained in the conditions and media stated earlier (2.1.1.). Cells were cultured in a humidified incubator at 37°C with 5% CO_2. When the cells were confluent they were trypsinised for ~5 min and centrifuged at 2500rpm for 5 min. The cells were then re-suspended in medium and dispensed in 1 ml aliquots into 24 well culture plates at a concentration of $1x10^5$ cells/ml. The cells were then maintained at 37°C, 5% CO_2 in a humidified incubator until confluent, the media was refreshed everyday. When the cells were confluent they were washed in sterile PBS and then maintained for 2 days in the same media but without foetal bovine serum. To duplicate test wells, 1% human serum was added with 1 μg of LPS either from the *B.cepacia* isolates or *E.coli* to duplicate test wells. Controls were set up containing LPS alone for each sample, human serum alone and a negative control containing neither. The cells were incubated for 16 hours overnight and the supernatants collected and either tested immediately for IL-8 concentration by ELISA or stored until use at -80°C.

2.6. Respiratory burst measured by enhanced chemiluminescence

2.6.1. B.cepacia LPS effect on MM6 cells respiratory burst

Priming:

MM6 cells were grown in the conditions and media stated earlier (2.1.1.). The cells were tested for viability (>95%), counted, washed and suspended in medium in 5 ml flasks at a concentration of $1x10^6$ cells/ml. The cell were then stimulated with 100 ng *B.cepacia* LPS or *E.coli* LPS and incubated at 37°C, 5% CO_2 for 24 hours. A negative control without LPS was always included.

The primed cells were then spun down at 2500 rpm and washed twice in DPBS in plastic centrifuge tubes, the cells were re-suspended in standard buffer at a concentration of $1.5x10^6$ cells/ml.

Respiratory burst activation:

100 µl of 3.5 mM of luminol was added to each 1 ml of cell suspension.
150 µl aliquots of the cell solution were then dispensed in quadruplicate into a white 96 well plate (FluoroNunc-PolySorp, Gibco, UK). The plate was then allowed to equilibrate in a luminometer for 5 min. Finally 50 µl of opsonised zymosan (500 µg/ml) was added per well and the plate returned to the luminometer which was set to take a reading every 5 min for a total of 40 min (or until the reaction had finished). Chemiluminescence was measured in relative light units (RLU) which is a measurement of the number of photons generated by the reaction each time.

2.7. Investigations into the signalling pathway triggered by B.cepacia LPS

2.7.1. Cell stimulation with B.cepacia LPS and protein extraction

MM6 cells were grown up in conditions and media stated earlier (2.1.1.). Cell viability (>95%) was checked using the trypan blue exclusion method and the cell count was then adjusted to $1x10^6$ cell/ml and dispensed in 10 ml aliquots into T25 tissue culture flasks.

LPS stock solutions (1 mg/ml) were allowed to thaw and vigorously vortexed for at least 5 min, they were then diluted using sterile pyrogen free water and added to each flask to obtain a final concentration of 1 μg/ml LPS. A flask of non-stimulated cells was always included as a negative control. The cells were then incubated at 37°C for 1 hour and then transferred to 10 ml centrifuge tubes, centrifuged at 2500 xg and washed in ice-cold DPBS. The cells were then transferred to 1 ml Eppendorf tubes and washed again in DPBS. The supernatant was entirely removed and 300 μl of ice-cold RIPA buffer working solution was added to lyse the cells, the cells were then left for 30 min on ice. After this time the cell lysates were then subjected to sonication (on ice) for 3x10 seconds for each sample. The samples were then centrifuged at 2500 xg for 5 min at 4°C to remove cell debris. 40 μl volumes of the cell fractions were then transferred into separate tubes and either used straight away of stored at -80°C until needed.

2.7.2.Coomassie blue G dye binding method for protein estimation

After LPS stimulation and cell fractionation, samples were tested for protein concentration to ensure equal amounts of protein could be loaded onto the gel for electrophoresis. Bovine serum albumin (BSA) standards were added in triplicate to a 96 well plate. The standards ranged from 200 μg/μl up to 1200 μg/μl and included a blank sample. The cell lysate samples were added to wells in triplicate. Coomassie Blue G working solution was made up from a stock solution on the day of use and 200 μl of this was added to each test well, this was left for a few minutes for the colour to develop and then the optical density was read in a microtitre plate reader set to 620 nm. The protein concentration was determined for each sample using a standard curve derived from the BSA standards.

2.7.3. Western blotting

Electrophoresis using Novex system and NuPAGE gels.

Test samples were mixed with calculated volumes of LDS, water and reducing buffer to ensure equal amounts of protein in each sample. The samples were then vortexed and placed in a 70°C water bath for 10 min to fully denature proteins. The samples were then removed and kept on ice until loading. A NuPAGE pre-cast gel (Bis-Tris-HCl buffered 10%) was inserted into a mini-cell unit and sealed in. The inner chamber was filled with ~200 ml of reducing buffer ensuring the wells were covered. 10 μl volumes of the test samples were then loaded into the wells using a fine pipette

tip. For each run a MagicMark (Invitrogen) protein ladder, SEE Blue visual marker and positive and negative control samples were included. The outer chamber was then filled with ~600 ml running buffer, the gel was then electrophoresed at 200V for 45 min or until the SEE Blue marker was within 10 mm of the bottom of the gel.

2.7.4. Transfer to nitrocellulose membrane

Firstly NuPAGE transfer buffer was made up and blotting pads and filter paper were immersed in it for an hour, the nitrocellulose membrane was cut to size and also immersed in the transfer buffer for 10 min. The gel was then removed from the Mini-cell, and the plastic plate was removed carefully using a spatula, the gel forming the wells at the top was removed. The soaked nitrocellulose membrane was laid over the gel and then the gel was sandwiched in-between 2 soaked filter papers. 2 soaked blotting pads were placed on top and below the gel and filter paper and the entire 'sandwich' was rolled with a glass pipette to remove any trapped air bubbles, it was then assembled in the blotting module. The blotting module was placed in a Mini-cell unit a small amount of transfer buffer was poured into the blotting module to ensure the membrane and gel were completely covered and the rest of the transfer buffer was poured into the outer chamber. The unit was run for 75 min at 30 V constant current at RT.

2.7.5. Chemiluminescent detection using anti-rabbit Western Breeze kit (Invitrogen).

All steps in this procedure were carried out at RT. The nitrocellulose membrane was removed from the Mini-cell transfer module and placed in a dish, the membrane was then rinsed with 20 ml ultra-pure water for 5 min on a rotary shaker, this wash was repeated once. The membrane was then blocked with 10 ml of prepared blocking solution for 30 min, after this time the blocking solution was decanted and the membrane was washed in 20 ml water twice for 5 min on a rotary shaker. The membrane was then incubated with 10 ml of prepared primary antibody solution which contained 10 µl primary antibody (see below) in 10 ml blocking solution, this was left for 1 hour.

The antibody solution was decanted and the membrane washed with 20 ml of prepared antibody wash for 5 min on a rotary shaker, this was repeated 3 times. The membrane was then incubated with 10 ml secondary antibody solution for 30 min on a rotary shaker and then washed with 20 ml antibody wash 3 times as before. The membrane was finally washed with 20 ml water for 2 min twice and then placed on a sheet of transparent plastic and 2.5 ml of chemiluminescent substrate was applied for 5 min during this time the membrane was not allowed to dry out. The excess substrate was removed by blotting with filter paper and another piece of transparent plastic was placed over the top of the membrane and any air bubbles were removed using a glass pipette. The membrane was then exposed to photographic film in the darkroom for 5 min or longer if needed.

2.7.6. Detection of phosphorylated p38 MAP Kinase in LPS treated MM6 cells.

MM6 cells were stimulated with various *B.cepacia* LPS as described above, non-stimulated controls and an *E.coli* LPS was included with each incubation. Phosphorylated p38 MAPK was detected using the Novex system and Western Breeze detection as described above. P38 control cell extracts for phosphorylated and non-phosphorylated MAP kinases (Cell Signaling Technology) were included in each run. Detection antibodies for phosphorylated p38 MAP Kinase were included in the primary antibody solution at 1:500 dilution (20 µl in 10 ml). The detection antibody for phosphorylated p38 only binds when both threonine 180 and tyrosine 182 (Thr180/Tyr182) are phosphorylated.

2.7.7. Detection of phosphorylated and total P42/44 MAP Kinases in LPS treated MM6 cells

MM6 cells were stimulated with various *B.cepacia* LPS as described above, non-stimulated controls and an *E.coli* LPS was included with each incubation. The total and the phosphorylated p44/42 MAP kinase was detected using the Novex system and Western Breeze detection as described above. P44/42 control cell extracts for phosphorylated and non-phosphorylated MAP kinases (Cell Signaling Technology) were included in each run. Detection antibodies for phosphorylated p44/42 MAPK and total p44/42 MAPK were included in the primary antibody solution at a dilution of 1:1000 (10 µl in 10 ml). The detection antibody for phosphorylated p44/42 only

binds when both threonine 202 and tyrosine 204 (Thr202/Tyr204) of p44 are phosphorylated.

2.7.8. Detection activated IκB in LPS treated MM6 cells

MM6 cells were stimulated with various *B.cepacia* LPS as described above, non-stimulated controls and an *E.coli* LPS was included with each incubation. The IκB-α detection antibody (Santa Cruz Biotechnology, INC.) was included in the primary antibody solution at a dilution of 1:1000 (10 µl in 10 ml), the antibody only bound to IκB when proteolysis and phosphorylation had not occurred.

2.7.9.Investigation of the role of CD14 in LPS induced TNF-α production

MM6 cells were grown in the conditions and media previously stated (2.1.1.). Cell viability (>95%) was checked using the trypan blue exclusion method and the cell count was then adjusted to $1x10^6$ cell/ml and dispensed in 250 µl aliquots into 96 well culture plates. The cells were then pre-incubated with 5 µg of monoclonal anti-CD14 antibody (R&D systems). Test LPS was then added to each well at the concentration of 25 ng/ 250 µl. All experiments were conducted in duplicate each time, and a control with LPS alone and a non-stimulated control were included. The cells were incubated at 37°C, 5% CO_2 for 16 hours, harvested, centrifuged at 2500 rpm for 5 min and the supernatants collected. The supernatants were then either stored at -80°C until use or tested immediately for TNF-α concentration by ELISA.

2.7.10. Investigation of the role of TLR4 in LPS induced TNF-α production

MM6 cells were grown in the conditions and media previously stated (2.1.1.). Cell viability (>95%) was checked using the trypan blue exclusion method and the cell count was then adjusted to $1x10^6$ cell/ml and dispensed in 250 µl aliquots into 96 well culture plates. The cells were then pre-incubated with 5 µg of HTA125 monoclonal anti-TLR4 antibody (eBioscience) or with an IGg2a isotype control antibody (eBioscience). Test LPS was then added to each well at the concentration of 100 ng/ml. All experiments were conducted in duplicate each time, and a control with LPS alone and a non-stimulated control were included. The cells were incubated at 37°C, 5% CO_2 for 16 hours harvested, centrifuged at 2500 rpm for 5 min and the

supernatants collected. The supernatants were then either stored at -80°C until use or tested immediately for TNF-α concentration by ELISA.

2.7.11. Investigation of the role of TLR2 in LPS induced TNF-α production

MM6 cells were grown in the conditions and media previously stated (2.1.1.). Cells were incubated at 37°C with 5% CO_2. Cell viability (>95%) was checked using the trypan blue exclusion method and the cell count was then adjusted to $1x10^6$ cell/ml and dispensed in 250 µl aliquots into 96 well culture plates. The cells were pre-incubated with 5 µg of monoclonal anti-TLR2 antibody (Abcam) and test LPS was added to each well at the concentration of 100 ng/ml. All experiments were conducted in duplicate each time, and a control with LPS alone and a non-stimulated control were included. The cells were incubated at 37°C, 5% CO_2 for 16 hours, then harvested, centrifuged at 2500 rpm for 5 min and the supernatants collected. The supernatants were then either stored at -80°C until use or tested immediately for TNF-α concentration by ELISA.

2.7.12. MyD88 inhibition

MM6 cells were grown in the conditions and media previously stated. Cells were incubated at 37°C with 5% CO_2. Cell viability (>95%) was checked using the trypan blue exclusion method and the cell count was then adjusted to $1x10^6$ cell/ml and dispensed in 250 µl aliquots into 96 well culture plates. The cells were then pre-incubated with 100 µM MyD88 homodimerization inhibitory peptide or a control peptide (Imgenex, USA) for 24 hours. Test LPS was then added to each well at the concentration of 25 ng/ 250 µl. All experiments were conducted in duplicate each time, and a control with LPS alone and a non-stimulated control were included. The cells were incubated at 37°C, 5% CO_2 for 6 hours and then harvested, centrifuged at 2500 rpm for 5 min and the supernatants collected. The supernatants were then either stored at -80°C until use or tested immediately for TNF-α concentration by ELISA.

2.7.13. MAPK inhibition

MM6 cells were grown in the conditions and media previously stated (2.1.1.). Cell viability (>95%) was checked using the trypan blue exclusion method. Cells were dispensed in 1 ml aliquots at a concentration of $1x10^6$ into 24 well culture plates.

The cells were then pre-incubated with 10 µl of either PD98059 (P42/44 inhibitor), SB203580 (P38 inhibitor) or SP600125 (JNK inhibitor) (Sigma-Aldrich) or combinations of the inhibitors all in duplicate, for 1 hour at 37°C. These inhibitors have been seen to be effective in other studies and in industry (Davies *et al* 2000;

Ropert 2005). Control wells with no inhibitor were included. LPS was then added at a concentration of 1 μg/ml to all wells and to control wells, the plates were then mixed by gentle tapping and incubated overnight at 37°C, 5% CO_2 in a humidified incubator. Supernatants were collected after centrifugation for 5 min at 2500 rpm and either assayed immediately for TNF-α concentration by ELISA or stored at -80°C until testing.

2.8. Nuclear extracts and assessment of NF-κB activation by Electrophoretic Mobility Shift Assay (EMSA)

2.8.1. Preparation of subcellular fractions

In this procedure PMSF was added to each of the buffers A, C and D just prior to their use at concentrations stated in the appendix. MM6 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated foetal bovine serum, 5ml 200mM L-glutamine, 1% v/v non-essential amino acids, 100U/ml penicillin, 100ug/ml streptomycin, and 1mM sodium pyruvate . Cells were incubated at 37°C with 5% CO_2. Cell viability (>95%) was checked using the trypan blue exclusion method. Cells were dispensed in 1 ml aliquots at a concentration of $1x10^6$ viable cells into 24 well culture plates. Cells were stimulated with 1 μg/ml of LPS from *B.cenocepacia*, *B.multivorans* and *E.coli* at various time intervals (15, 30, 60, and 120 min). Stimulation was terminated by transferring cells into 5 ml ice-cold DPBS and each sample was centrifuged at 1040 xg for 5 min. The pellets were re-suspended in 1 ml buffer A, transferred to Eppendorf tubes and centrifuged at 9155 xg for 1 min. The pellets were then re-suspended in 20 μl buffer A containing 0.1% NP40 and allowed to stand on ice for 5 min to lyse. The cell fractions were then centrifuged at 9155 xg for 5 min at 4°C. After centrifugation the supernatant (cytosolic extract) was removed to a fresh tube and the remaining pellet was re-suspended in 15 μl buffer C containing the PMSF, this was then left on ice for 15 min. The fractions were then centrifuged at 9155 xg for 5 min at 4°C. The supernatants (nuclear extracts) were then removed to fresh tubes and 75 μl of buffer D was added to each tube, extracts were then stored at

-80°C until use. Samples of the extracts were taken before freezing and assayed for protein concentration using the coomassie blue G binding method.

2.8.2. Detection of NF-κB in cell extracts

18x16 cm BioRad Electrophoresis cassettes with 1.5 mm spacers were used. The polyacrylamide gel was prepared (see appendix) and poured slowly into the cassette, a 10 well comb was inserted carefully to avoid trapping any air bubbles. The gel was left to polymerise for a minimum of 1 hour.

Nuclear fractions were kept on ice until used, samples of equal protein (1 μg) were incubated in an Eppendorf tube with 10,000 cpm of a ^{32}P-labelled oligonucleotide containing the consensus sequence for NF-κB in binding buffer and 2 μg of poly (dI-dC) at RT for 30 min. The samples were then run on a 5% polyacrylamide gel at 180 V for ~90 min or until the lower dye is ~20 mm from the bottom of the gel.
The gel was then removed and dried for 2 hours and finally autoradiographed overnight at -80°C on Kodak Biomax film.

2.9. Statistical analysis of ELISA data

For statistical analysis of ELISA data the mean of the number of repeats and the standard error for the read-out of each sample was calculated using Microsoft Excel. P values (with a significance level of 0.05) were then obtained by non-parametric Mann-Whitney analysis using SPSS software. A non-parametric test was chosen as it makes no assumptions about the distribution of the data (normality).

Results

3.1. Comparison of *B.cepacia* LPS-1 (BcLPS-1) and *B.cepacia* LPS-2 (BcLPS-2)

In order to optimise experiments throughout this study and to ensure that a reasonable comparison could be made between LPS extracted from clinical samples and LPS that has been bought already purified, a number of experiments were conducted to ascertain a dose response from LPS stimulated cells and a TNF-α output from cells stimulated with a commercially available LPS that had been further purified.

3.1.1. Effect of the quantity of LPS used to stimulate MM6 cells on the inflammatory activity observed.

A representative *B.cepacia* complex LPS (Cardiff A1, *B.multivorans*) was used to test the TNF-α response from MM6 cells at different doses. This was done primarily to ascertain what concentration of LPS would be most appropriate to use throughout this study. MM6 (10^6 cells) were stimulated with 100 ng, 500 ng, or 1 μg of purified LPS from *E.coli* (0111:B4- Sigma), *P.aeruginosa* (PA01- Sigma) and from Cardiff A1 clinical isolate for 6 hours. TNF-α levels were measured by ELISA.

The results (Figure 3.1.1.) show that the amount of TNF-α produced is proportional to the LPS dose. For all the LPS samples the amount of TNF-α produced at 100 ng and 500 ng was low. At 1 μg there is a profound increase in TNF-α production and it is much easier to see the differences between purified LPS samples when using 1 μg to stimulate cells compared to the lower concentrations, for this reason a concentration of 1 μg LPS/ 10^6 cells was used in the majority of experiments in this study.

The results from this experiment also illustrate that LPS from *P.aeruginosa* (PA01) produces much less TNF-α from MM6 cells compared to LPS from Cardiff A1 (*B.multivorans*) LPS.

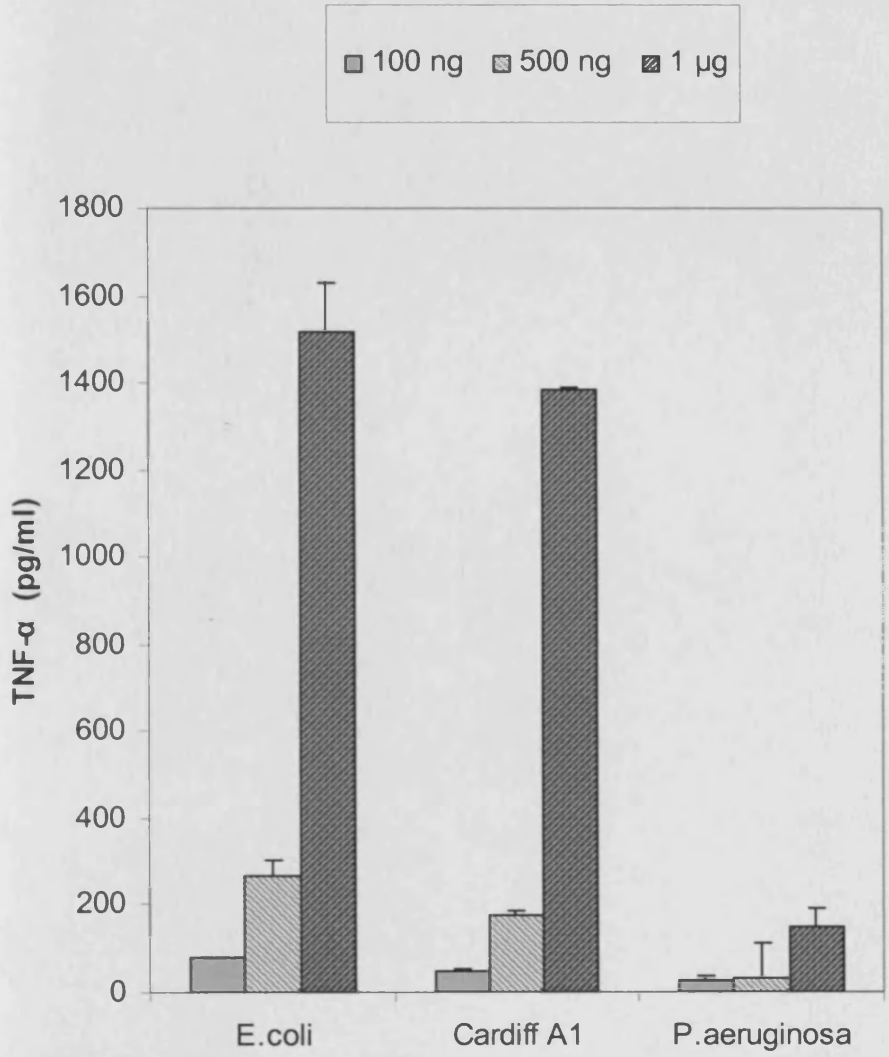

Figure 3.1.1. Dose response from MM6 cells incubated with100 ng, 500 ng and 1µg LPS/10^6 cells (LPS from *E.coli* (0111:B4), *B.multivorans* (Cardiff A1) and *P.aeruginosa* (PA01)). Cells were stimulated for 6 hours and the resulting supernatants were tested for TNF-α by ELISA. Values are means ±SD of three independent experiments.

3.1.2. Testing of control LPS

Commercial *E.coli* LPS (Sigma) which had been purified using PCP extraction was used as a control throughout this study as it is considered to have the most pro-inflammatory LPS structure. It was then subjected to the same enzyme treatments as the *B.cepacia* complex LPS (incubation with DNase, RNase, trypsin and proteinase K), inflammatory response tests were done on both the commercially available *E.coli* LPS and the further purified preparation (Figure 3.2.1.). LPS from a clinical Cardiff *E.coli* isolate was also purified and tested in order to gain a comparison. Levels of TNF-α from MM6 cells stimulated for 6 hours with both preparations of *E.coli* LPS, showed no significant difference (Figure 3.2.1.). Therefore through the rest of this study commercially bought PCP extracted *E.coli* LPS was used without further purification.

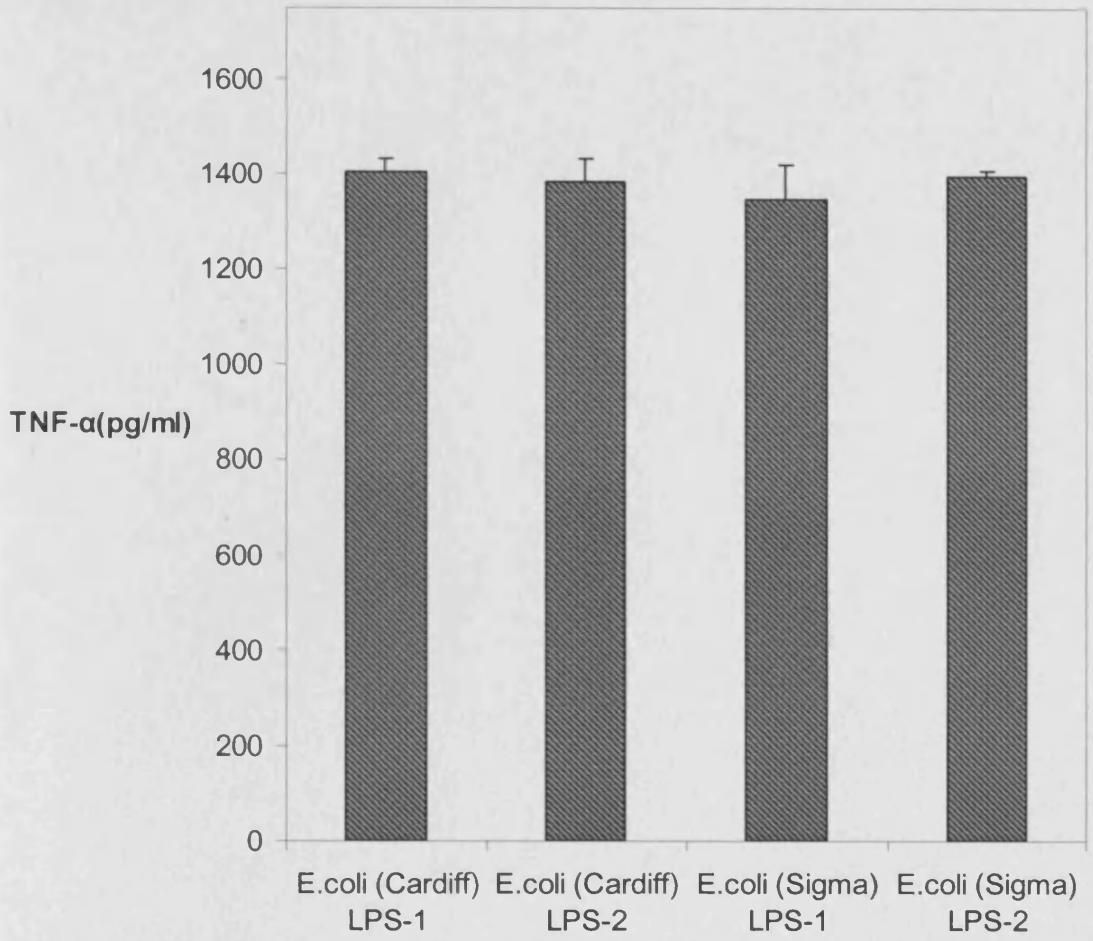

Figure 3.2.1. TNF-α response from MM6 cells (at $1x10^6$ cells/ml) stimulated with 1μg LPS fro 6 hours. LPS purified from a Cardiff *E.coli* isolate used throughout this study extracted to LPS-1 and further purified to LPS-2 compared against LPS from commercial *E.coli* (O111:B4) LPS (Sigma). The commercially bought LPS was used to stimulate cells (LPS-1) and also further purified in the same way as the Cardiff LPS before stimulation (LPS-2).

3.1.3. Stimulation of MM6 cells with LPS extracted by different methods from various *B.cepacia* complex strains.

LPS from *B.cepacia* complex organisms elicits a much stronger inflammatory response from leukocytes compared with that of *P.aeruginosa* LPS (Zughaier *et al*, 1999a and Shaw *et al*, 1995). It has been suggested that this inflammatory potency may be due to protein contamination present in LPS preparations (Shimomura *et al*, 2001) and not the LPS itself.

To determine whether LPS alone can stimulate an inflammatory response independent of any protein or nucleic acid contaminants, LPS from various clinical and environmental isolates of *B.cepacia* complex was first extracted by the conventional phenol-chloroform-petroleum ether (PCP) method of (Galanos *et al*, 1969). The LPS from this initial extraction was designated BcLPS-1. Half the LPS from each such preparation was then further purified using enzyme treatments and sodium deoxycholate, this second extraction was designated BcLPS-2.

Human monocytic (MM6) cells were stimulated with either 1μg LPS/10^6 cells of extracted *B.cepacia* complex LPS or with LPS from *E.coli* 0111:B4 (Sigma). Levels of TNF-α (Figure 3.1.3.) and IL-6 (Figure 3.1.4.) were measured by an ELISA method. TNF-α was measured after 6 hours stimulation with LPS and IL-6 measured after 16 hours stimulation with LPS as these incubation times have been previously shown in our laboratory to yield optimal amounts of these cytokines (data not shown, Zughaier *et al*, 1999a)

The level of cytokine production from the initial PCP extractions and the more highly purified preparations were found to be similar, with no significant differences shown between BcLPS-1 and BcLPS-2. Previous studies have shown that bacterial proteins such as flagellin and bacterial nucleic acids do provoke an inflammatory response (Wagner & Bauer 2006). The results indicate that the inflammatory response seen with human derived MM6 cells is due to LPS and not contamination by protein or nucleic acid.

The results also indicate that there is a difference in inflammatory activity between the LPS extracted from various clinical *B.cepacia* complex strains and the LPS from environmental strains. LPS from *B.cepacia* strains are much less inflammatory than either that of *B.cenocepacia* or *B.multivorans*. LPS from Cardiff A1 (*B.multivorans*) and Cardiff P5 (*B.cenocepacia* ET-12) are both seen to produce levels of TNF-α and IL-6 that are comparable to levels produced by *E.coli* LPS (Figure 3.1.3. and Figure 3.1.4.).

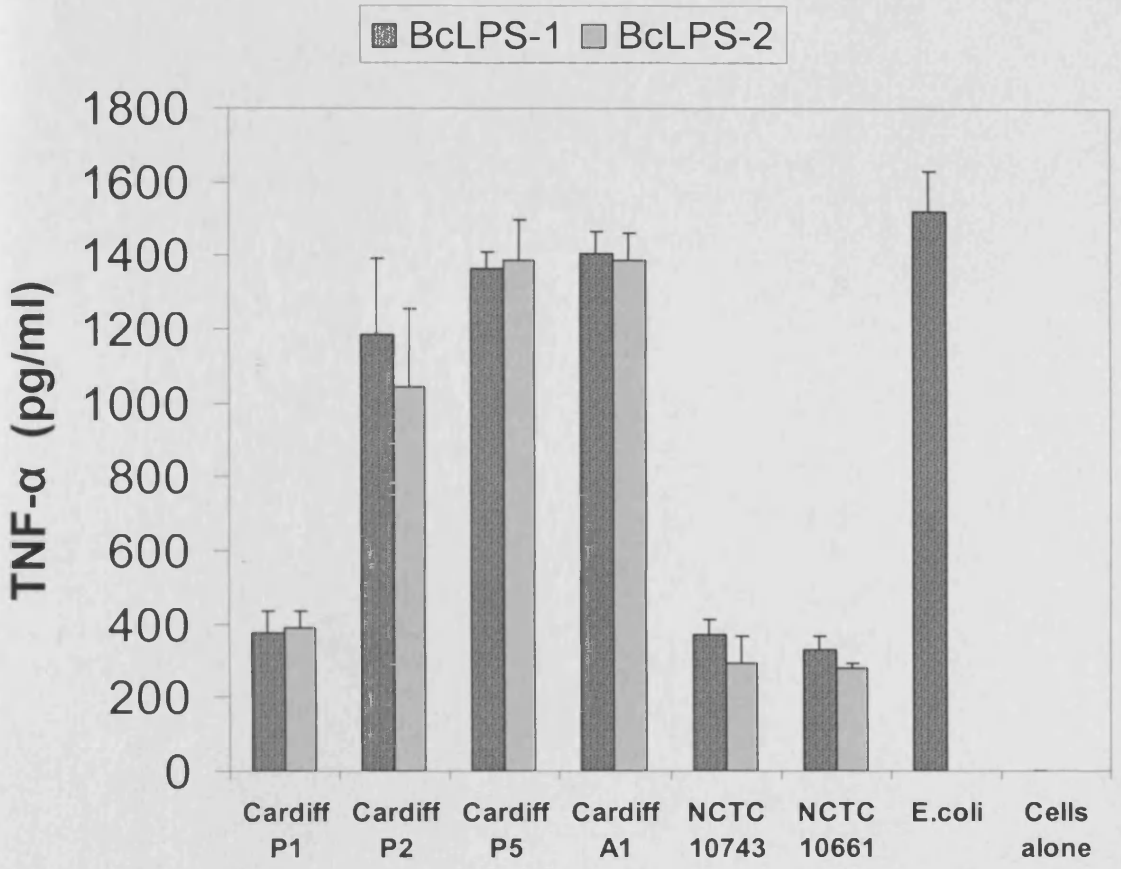

Figure 3.1.3. TNF-α production from MM6 cells stimulated with 1μg/ml BcLPS-1 and BcLPS-2 for 6 hours at 37°C and measured by ELISA. BcLPS-1 vs. BcLPS-2 *P*=0.9103 showing that there is no significant difference between the PCP extracted BcLPS-1 and the more highly purified, enzyme treated BcLPS-2. Values are means ±SD of three independent experiments.

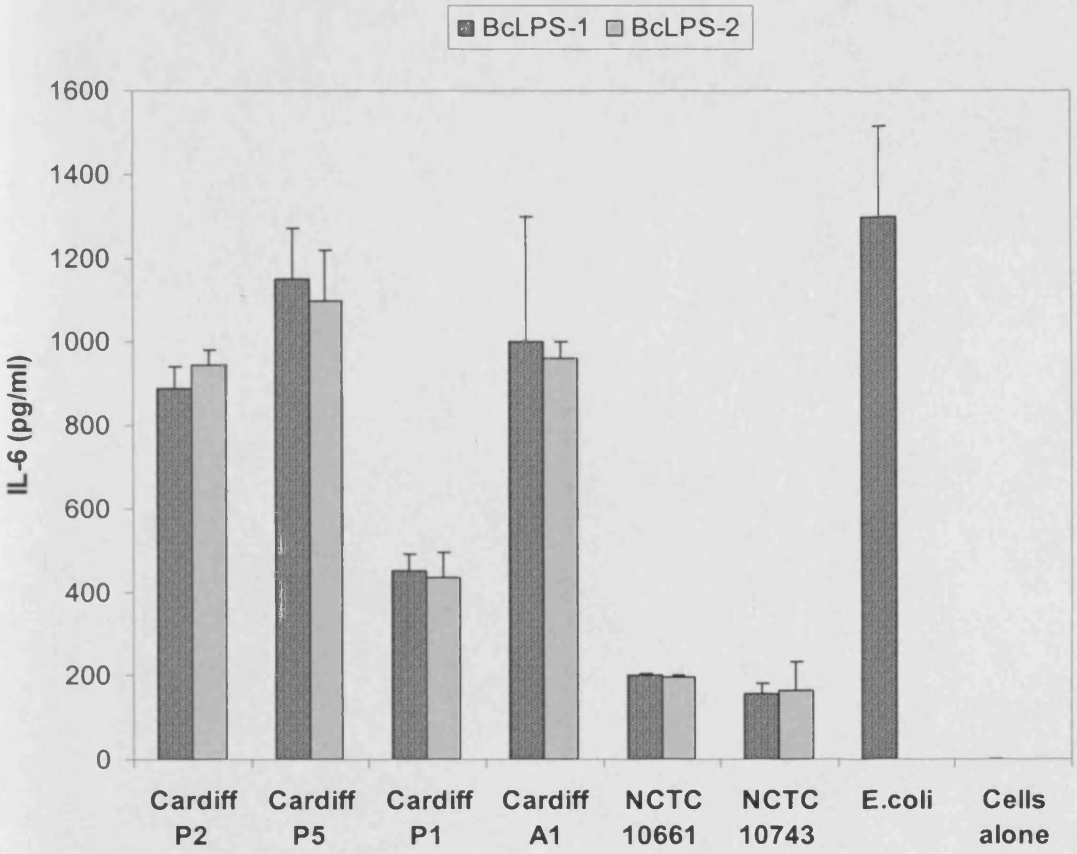

Figure 3.1.4. IL-6 production from MM6 cells stimulated with 1μg/ml BcLPS-1 and BcLPS-2 for 16 hours at 37°C and measured by ELISA. BcLPS-1 vs. BcLPS-2 P=0.9754 showing that there is no significant difference between the PCP extracted BcLPS-1 and the more highly purified, enzyme treated BcLPS-2. Values are means ±SD of three independent experiments.

3.1.4. Stimulation of A549 cells with LPS extracted by different methods from various *B.cepacia* complex strains.

A549 cells are derived from alveolar epithelial cells and so represent the primary interface between invading bacteria and the immune system. Increased viscosity of mucous membranes in the CF lung means that bacteria are not cleared away from these cells efficiently and so their reaction to BcLPS is relevant.
A549 cells have been shown to lack the LPS co-receptor, membrane bound CD14 (mCD14) and so require soluble CD14 (sCD14) present in human serum for response to LPS, this would normally be present *in vivo*.

A549 cells were stimulated with 1μg LPS/10^6 cells of either BcLPS-1 or BcLPS-2 from various *B.cepacia* complex clinical and environmental strains. 1% human serum was included in each experiment to provide sCD14, control experiments were included where A549 were stimulated with LPS without the presence of human serum. IL-8 production was detected by ELISA after the optimal 16 hours stimulation.

Cells that were stimulated with LPS without human serum being present showed very little IL-8 production which indicates that the LPS tested all required a component of human serum, sCD14, in order to produce an inflammatory effect (Figure 3.1.5.)

The results also show that there was no significant difference between BcLPS-1 and BcLPS-2 samples tested. This indicates that IL-8 production is being caused by LPS alone and not any protein or nucleic acid contaminant (Figure 3.1.6.). Environmental isolates tested NCTC 10661 and NCTC 10743 do show a difference in production of

IL-8 between BcLPS-1 and BcLPS-2 (Figure 3.1.5.) when tested without human serum, however the difference is exaggerated because of the small scale used due to the tiny amounts of IL-8 being produced without human serum.

LPS preparations from environmental *B.cepacia* strains were seen to produce lower levels of IL-8 than LPS from either *B.cenocepacia* or *B.multivorans* which are all of clinical origin. LPS preparations from Cardiff P5 (*B.cenocepacia* ET-12) and CardiffA1 (*B.multivorans*) produced levels of IL-8 that were comparable to levels produced by *E.coli* LPS.

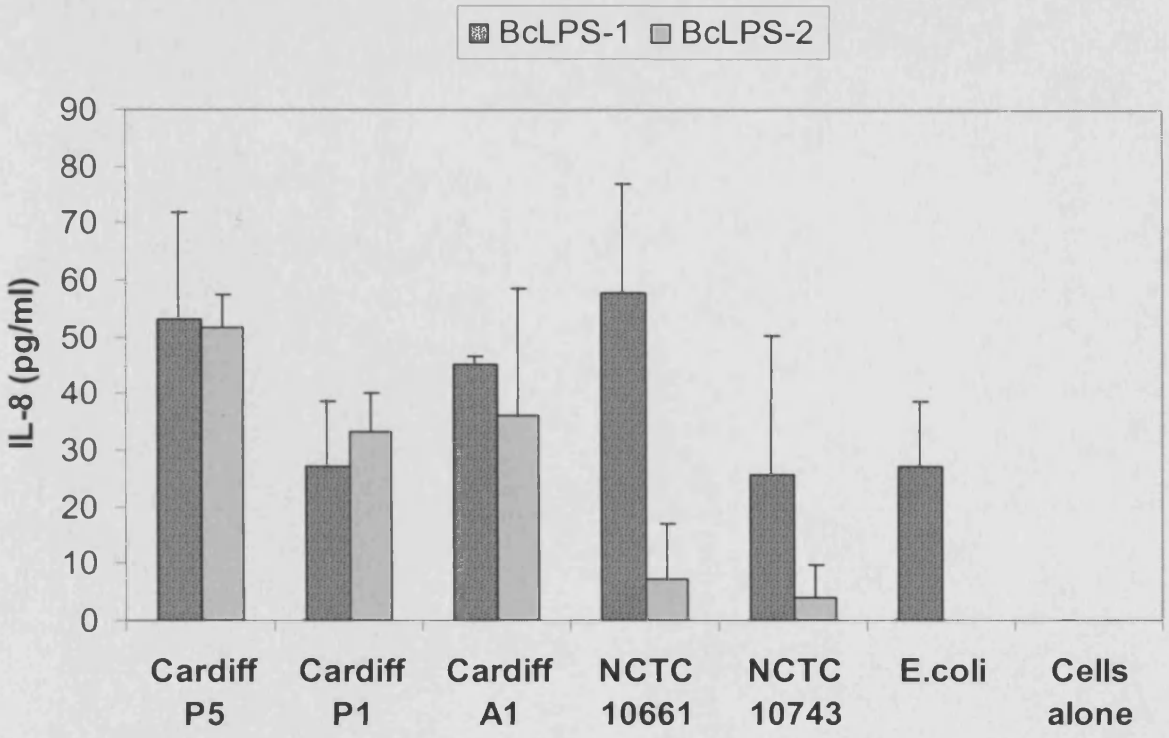

Figure 3.1.5. IL-8 production from A549 cells stimulated with 1μg/ml BcLPS-1 and BcLPS-2 without the presence of human serum for 16 hours at 37°C and measured by ELISA. The quantity of IL-8 produced under serum-free conditions is much reduced compared to incubation with 1% human serum. Values are means ±SD of three independent experiments.

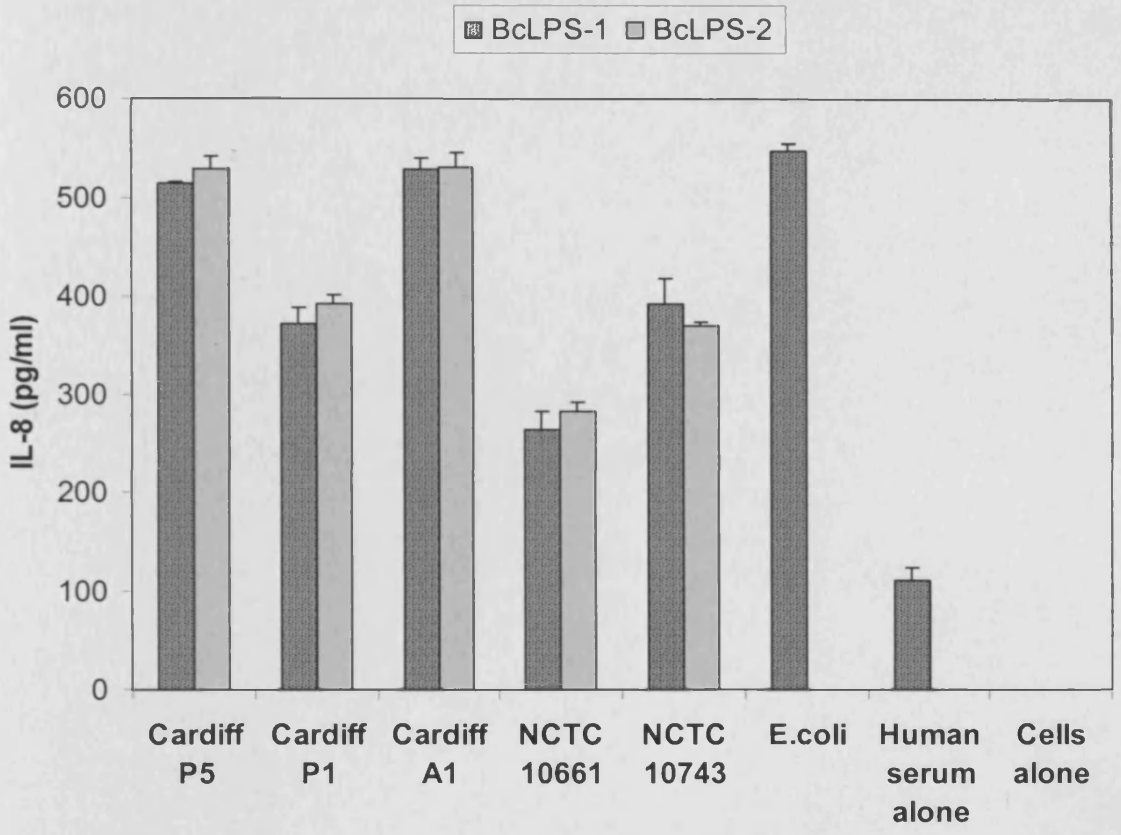

Figure 3.1.6. IL-8 production from A549 cells incubated with 1% human serum and then stimulated with 1μg/ml BcLPS-1 and BcLPS-2 for 16 hours at 37°C and measured by ELISA. BcLPS-1 vs. BcLPS-2 P= 0.9218 showing that there is no significant difference between the PCP extracted BcLPS-1 and the more highly purified, enzyme treated BcLPS-2. Values are means ±SD of three independent experiments.

3.2. Biological activity of *B.cepacia* LPS

3.2.1. TNF-α production in MM6 cells stimulated by LPS

BcLPS-2 is used in all experiments from this point (and is referred to as LPS) although it has been shown that there is no significant difference between inflammatory activity of BcLPS-1and BcLPS-2 in these experiments, further study would be required to make sure there is no contamination each time the samples are prepared, therefore it was logical to continue using BcLPS-2.

In order to assess the biological activity of various *B.cepacia* complex strains' LPS and to compare it to LPS from *E.coli* and *P.aeruginosa*, MM6 cells were stimulated with LPS (1μg/10^6 cells) for 6 hours and resulting TNF-α levels were measured by ELISA. Five different *B.cenocepacia* ET-12 strains, one *B.cenocepacia* Type B, two *B.multivorans* and two environmental *B.cepacia* samples were tested in order to get a broad view of inflammatory activity. Figure 3.2.1. shows the vast difference in inflammatory activity observed not only between different *B.cepacia* complex species but between isolates with the same genotype. Cardiff P1, P2, P3, P4 and P5 are all *B.cenocepacia* ET-12 strains, though phenotypically and phylogenetically identical there were large differences in the amount of TNF-α produced. The LPS from environmental *B.cepacia* strains induced a lower inflammatory response as compared to the LPS from clinical samples. LPS from Cardiff P5 and Cardiff A1 again stimulated a level of TNF-α which was comparable to that from *E.coli*. All *B.cepacia* LPS tested induced significantly higher levels of TNF-α production as compared to LPS from *P.aeruginosa*.

3.2.2. IL-6 production in MM6 cells stimulated by LPS

To investigate the induction of other inflammatory cytokines by LPS, MM6 cells were stimulated with LPS (1μg/10^6 cells) for 16 hours and levels of IL-6 were then measured form the resulting supernatants by ELISA. The same panel of LPS samples were used as for the TNF-α experiments (Figure 3.2.1.). Of the *B.cenocepacia* LPS samples, that from Cardiff P1 showed the lowest level of IL-6 production, with LPS from Cardiff A1 and Cardiff P5 showing levels that are comparable to that of *E.coli*. The LPS from *B.cepacia* environmental strains produced levels of IL-6 more than 5 times lower than that of the highest *B.cenocepacia* and *B.multivorans* LPS samples. As with TNF-α production, LPS from *P.aeruginosa* produced a much lower level of IL-6 than that from any of the clinical *B.cepacia* complex samples (Figure 3.2.2.).

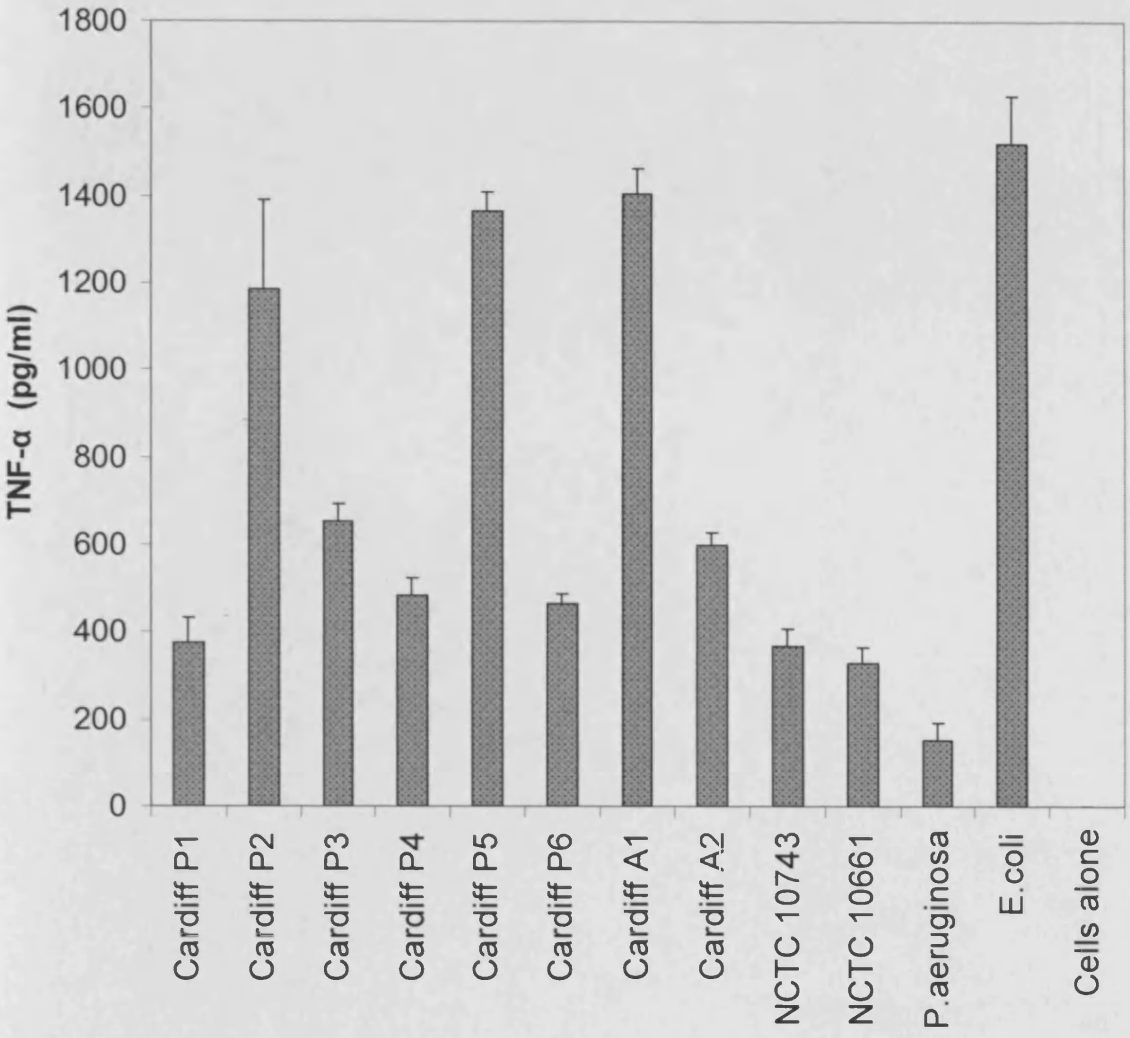

Figure 3.2.1. TNF-α production from MM6 cells stimulated with 1μg LPS/10^6 cells. BcLPS isolated from different strains compared with LPS isolated from *P.aeruginosa* and *E.coli*. Cells were stimulated for 6 hours at 37°C and measured by ELISA. Values are means ±SD of three independent experiments.

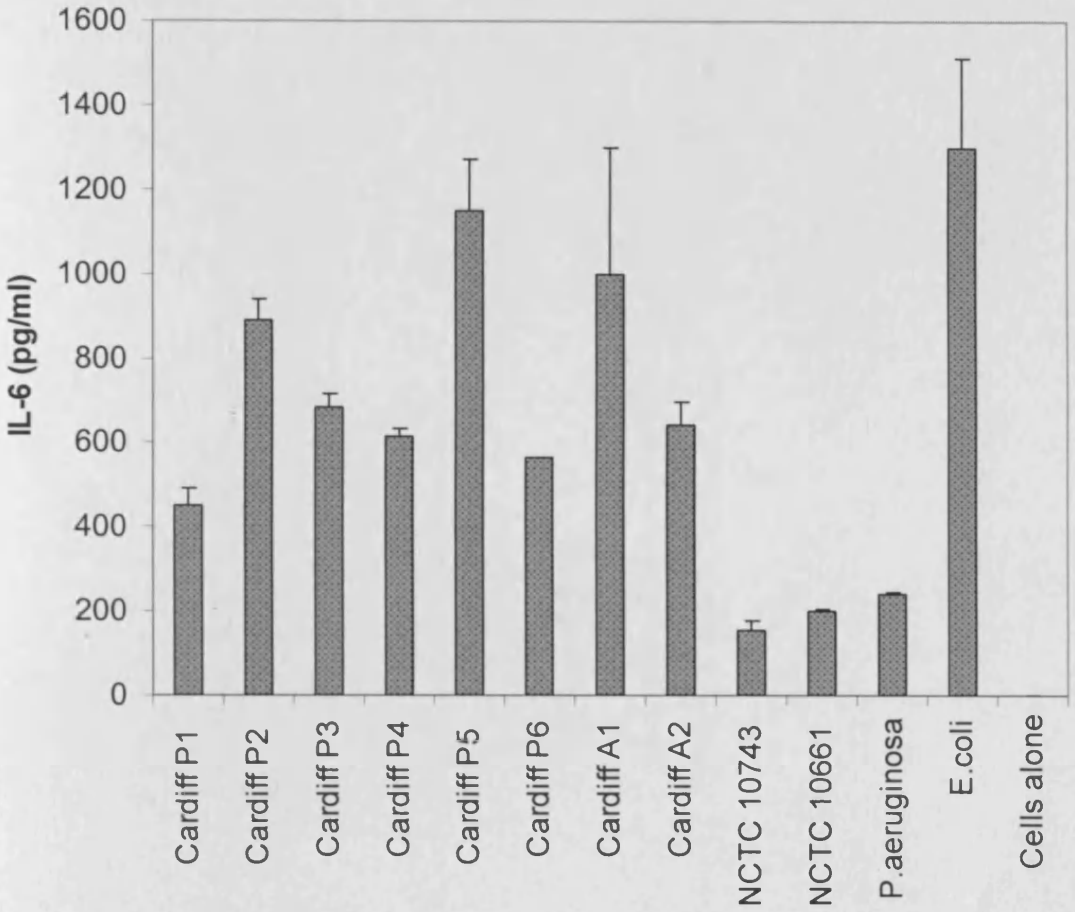

Figure 3.2.2. IL-6 production from MM6 cells stimulated with 1µg LPS/10^6 cells. BcLPS isolated from different strains compared with LPS isolated from *P.aeruginosa* and *E.coli*. Cells were stimulated for 16 hours at 37°C and measured by ELISA. Values are means ±SD of three independent experiments.

3.2.3. Difference in inflammatory response caused by environmental vs clinical strains.

Results from MM6 cells stimulated with 1μg *B.cepacia* complex LPS/10^6 cells for 6 hours and then tested for levels of TNF-α by ELISA, were pooled to ascertain the difference in biological activity between clinical and environmental isolates. LPS from all *B.cenocepacia* (Cardiff P1, P2, P3, P4, P5 and P6), *B.multivorans* (Cardiff A1 and Cardiff A2) and *B.cepacia* (NCTC 10661 and NCTC 10743) samples were used. The results indicate that LPS from clinical samples induced the production of higher levels of TNF-α than environmental LPS, also that the LPS from *B.cenocepacia* samples are not as biologically active as the *B.multivorans* strains (Figure 3.2.3.).

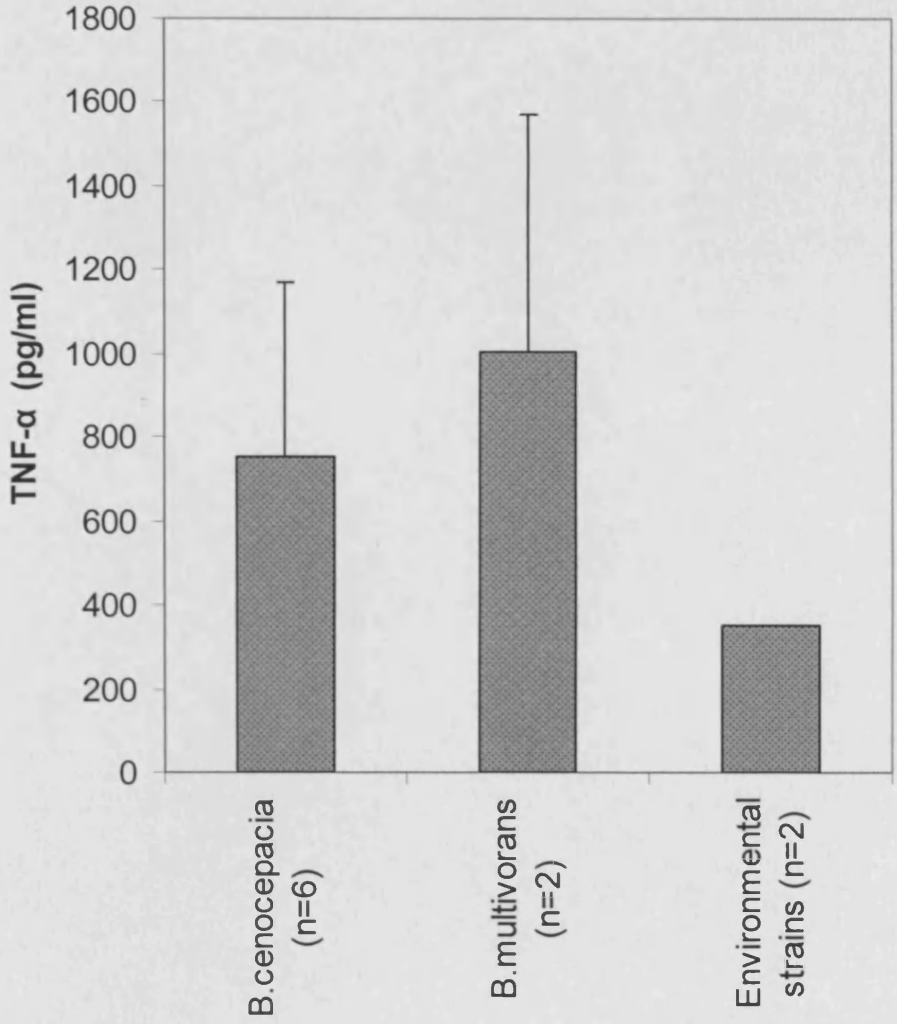

Figure 3.2.3. TNF-α production from MM6 cells stimulated with 1μg LPS/10^6 cells of LPS isolated from *B.cenocepacia* and from *B.multivorans* vs. LPS isolated from Bcc environmental strains. Cells were stimulated for 6 hours at 37°C and measured by ELISA. Values are means ±SD of three independent experiments.

3.2.4. Variability in TNF-α production within *B.cenocepacia* ET-12 strains.

B.cenocepacia ET-12 strains are phylogenetically identical. Variability in inflammatory responses produced by LPS extracted from these samples has been observed earlier in this study (Figures 3.2.1. and 3.2.2.). To observe whether this variability is unique to Cardiff clinical *B.cenocepacia* strains or whether it can be seen in isolates from clinical sources elsewhere, *B.cenocepacia* ET-12 isolates that had been collected from CF clinics around the UK were examined. Each isolate was grown up and subjected to LPS extraction in an identical fashion to the Cardiff samples and MM6 cells were stimulated with 1μg LPS/10^6 cells for 6 hours and the resulting supernatants were tested for levels of TNF-α by ELISA. The results (Figure 3.2.4.) show that there is pronounced variability in the level of TNF-α production from the ET-12 strains. The average TNF-α production from all five Cardiff strains is comparable to levels produced by the Liverpool and Edinburgh strains. However LPS from isolates from Manchester and London were both more inflammatory (Figure 3.2.4.).

3.2.5. Culturing and re-culturing of *B.cenocepacia* ET-12 strains to check for variability.

Variability in the biological activity of LPS extracted from identical *B.cenocepacia* ET-12 strains might be due to the amount of time the organism has been grown up *in vitro*. It is possible that with each re-culturing of the isolate within the laboratory

environment, small structural changes could occur within the LPS, and this may account for the difference in TNF-α, IL-6 and IL-8 production from stimulated cells. In order to ascertain whether this is the case, two Cardiff *B.cenocepacia* ET-12 strains were cultured, and then re-cultured 20 times each, both isolates were initially collected early in the infection of the patient and had not been re-cultured more than four times from their initial isolation from the patient's sputum. Every fifth time the bacteria were cultured, a sample was taken and grown up on a larger scale in order to extract LPS thereby giving an image over time as to how (if at all) the inflammatory activity of LPS changes with re-culturing. The lowest (Cardiff P1) and highest (Cardiff P5) TNF-α producers were chosen in order to show any differences in biological activity. The results show that the level of TNF-α produced in MM6 cells stimulated with *B.cenocepacia* ET-12 LPS does not change with culturing over time (Figure 10). This suggests that re-culturing the bacteria in the laboratory does not affect the structure of LPS and therefore the inflammatory response from the LPS, and that the variability seen within the Cardiff ET-12 strains is not due to re-culturing.

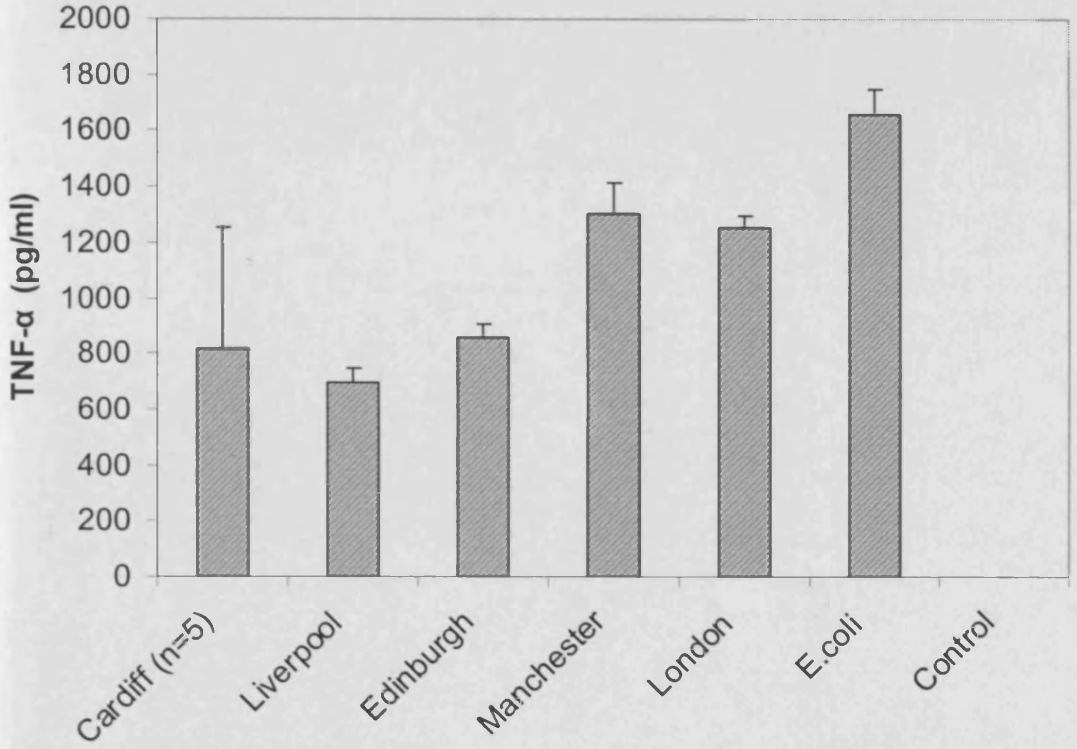

Figure 3.2.4. TNF-α production from MM6 cells stimulated with 1μg/ml of LPS from 5 different Cardiff *B.cenocepacia* ET-12 (P1, P2, P3, P4, P5) strains and from individual *B.cenocepacia* ET-12 strains from clinical sources around the UK. Cells were stimulated for 6 hours at 37°C and measured by ELISA. Values are means ±SD of three independent experiments.

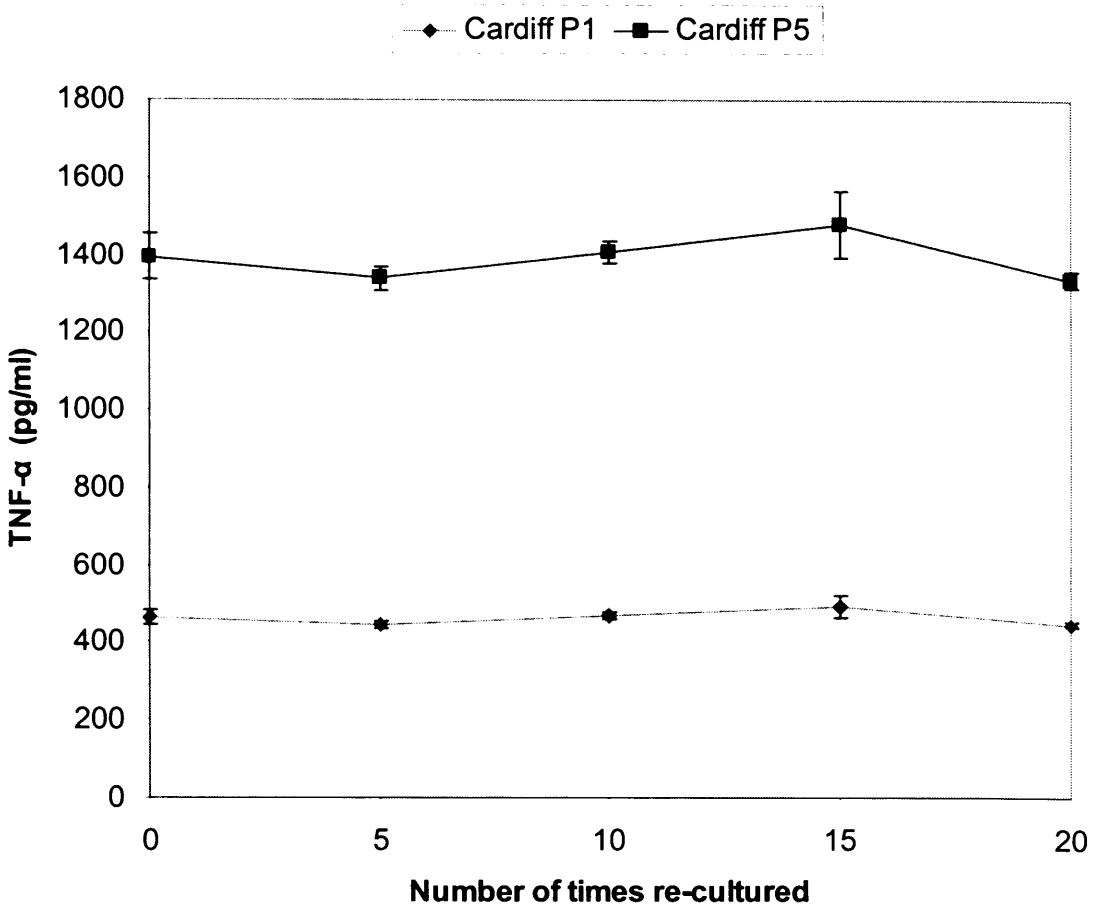

Figure 3.2.5. Representative *B.cenocepacia* ET-12 strains Cardiff P5 and Cardiff P1, were grown up and subbed (from 0-20 times) over time to ensure there were no differences in TNF-α production caused by growing the strains outside the body. LPS was extracted and MM6 cells were stimulated with 1 μg LPS for 6 hours and TNF-α was measured by ELISA. Values are means ±SD of four independent experiments.

3.2.6. Silver staining of purified *B.cepacia* complex LPS

Purified LPS from representative *B.cepacia* complex isolates were characterised by silver staining on SDS-PAGE gels. Figure 3.2.6. shows LPS extracted to BcLPS-1 and BcLPS-2 from three difference *B.cepacia* complex strains. The gel shows that these samples were all rough type LPS distinguished by a complete LPS core at the bottom of the gel. The gel also shows that the LPS extraction procedure does not have any effect on LPS O-antigen. Most of the clinical samples tested were rough type (Figure 3.2.6., lanes: 3,4,5,6,7,8 and Figure 3.2.7., lanes: 5,6). The other samples tested were either smooth type LPS or a mixture of rough and smooth. *B.cepacia* environmental samples were found to be smooth (Figure 3.2.7., lanes: 1,2). For comparison a smooth and rough type LPS from *S.minnesota* was included (Figure 3.2.6.).

3.2.7. TNF-α production from MM6 cells stimulated with rough and smooth type *B.cepacia* complex LPS

Purified representative *B.cepacia* complex LPS samples that were known to be either rough or smooth type, were used to stimulate MM6 cells in order to determine if there was any correlation between inflammatory response and whether the LPS samples is rough or smooth. Cells were stimulated with 1 $\mu g/10^6$ cells LPS for 6 hours and TNF-α levels were measured by ELISA (Figure 3.2.8.). The results show that the smooth type LPS samples produced a lower level of TNF-α than the rough samples. The

smooth LPS samples tested were both from environmental strains. The rough samples were from clinical strains, and induced much higher TNF-α levels than the smooth type, but there was a large difference in TNF-α production between the two rough samples tested. It can be seen that although the smooth type LPSs tested here induce a smaller inflammatory response than the rough type LPSs, the variability in inflammatory responses between different rough samples is so large that the level of TNF-α produced cannot be directly linked to whether the sample is rough or smooth.

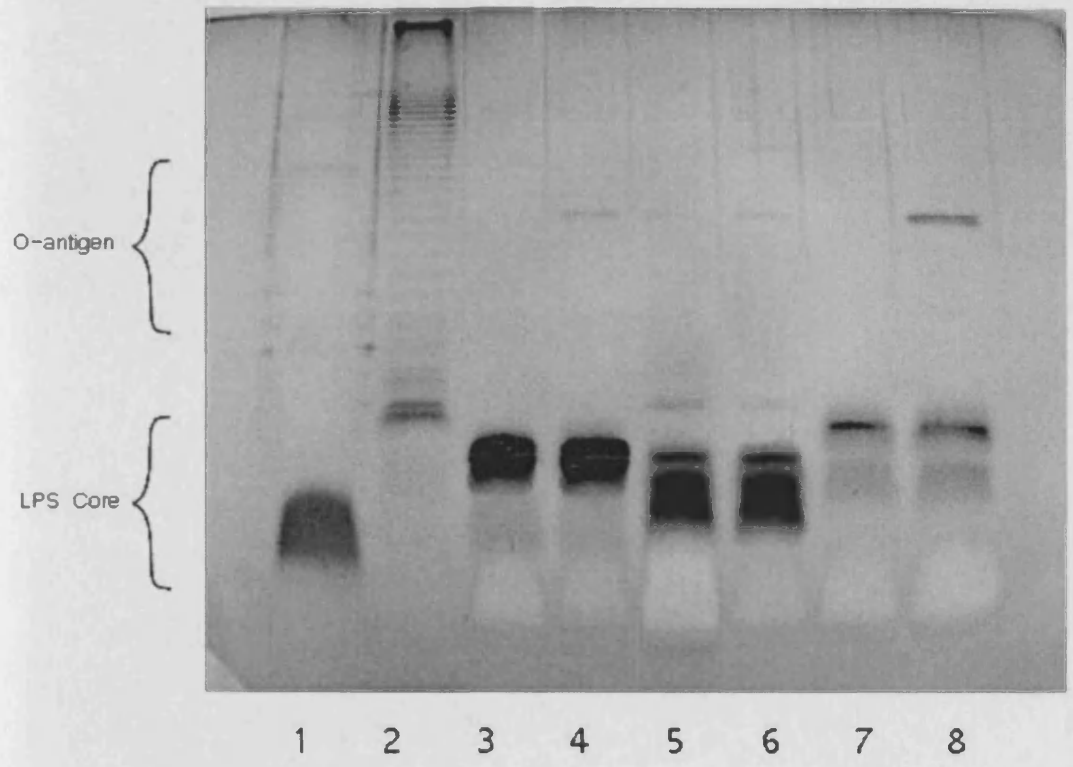

Figure 3.2.6. Silver staining of various LPS samples from *B. cepacia* complex using *S. minnesota* as comparison samples. From left to right Lane 1- Rough sample, *S. minnesota* R7 (Rd), Lane 2- Smooth sample, *S. minnesota* wild-type, Lane 3- Cardiff A3 BcLPS-1, Lane 4- Cardiff A3 BcLPS-2, Lane 5- NCTC 13009 BcLPS- 1, Lane 6- NCTC 13009 BcLPS-2, Lane 7- Cardiff A1 BcLPS-1, Lane 8- Cardiff A1 BcLPS-2.

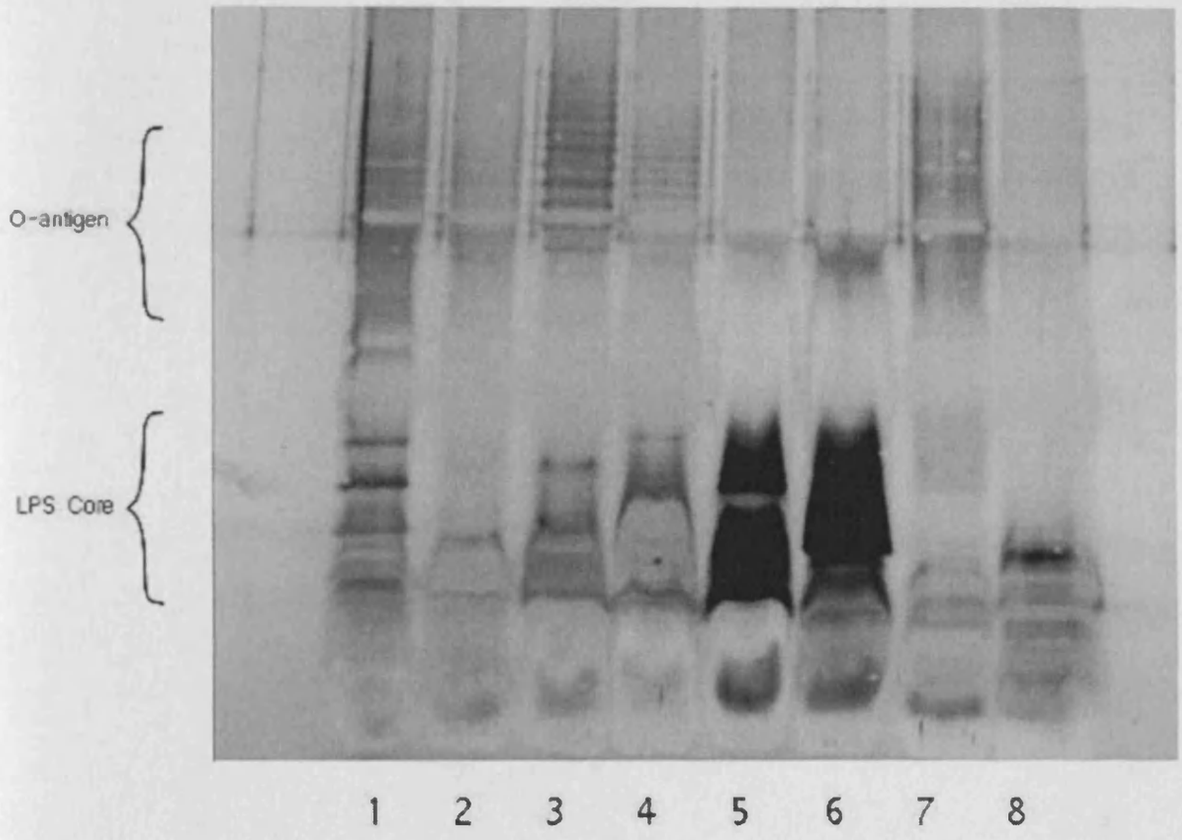

Figure 3.2.7. Silver staining of various LPS samples from *B. cepacia* complex. From left to right, Lane 1- NCTC10743, Lane 2- NCTC 10661, Lane 3-NCTC 10744, Lane 4-NCTC 13011, Lane 5- Cardiff A2, Lane 6- Cardiff A1, Lane 7-Cardiff P4 , Lane 8-Cardiff P5.

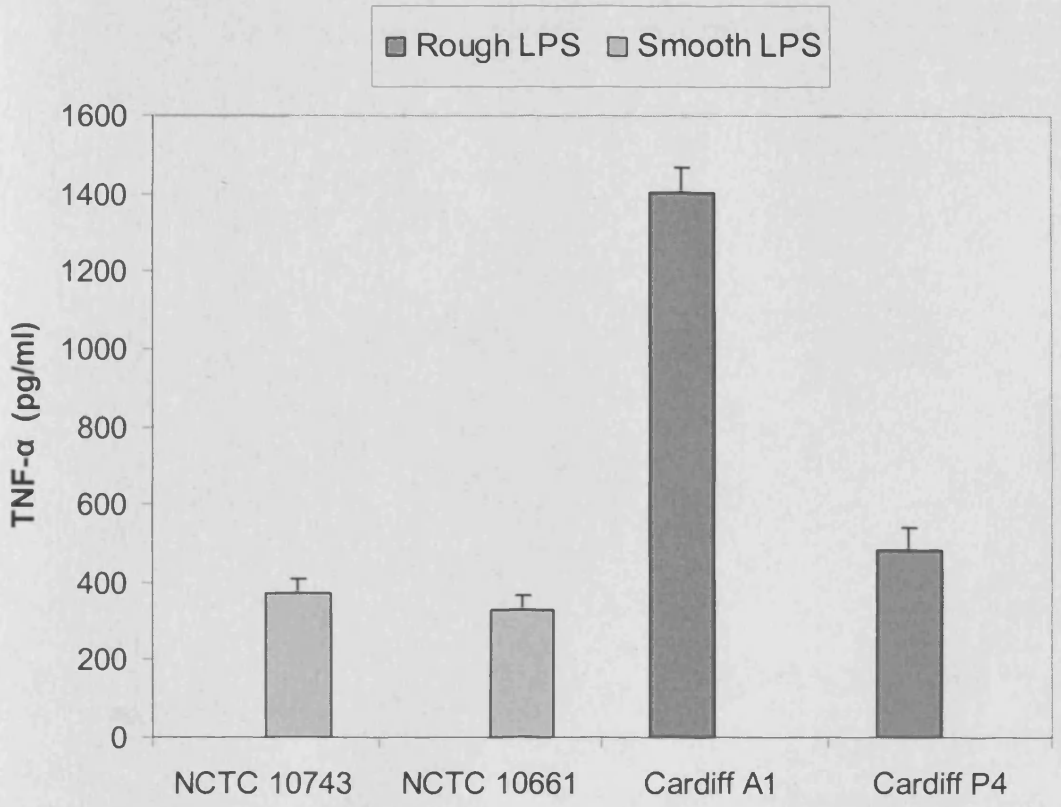

Figure 3.2.8. TNF-α production from MM6 cells suspended at $1x10^6$/ml and stimulated with 1μg/ml of LPS from representative Bcc isolates known to be either rough or smooth. Cells were stimulated for 16 hours at 37°C and measured by ELISA. Values are means ±SD of three independent experiments.

3.2.8. *B.cepacia* complex LPS inactivation by Polymyxin B

Polymyxin B is an antibiotic that has been shown to bind LPS and prevent its ability to induce inflammation. However *B.cepacia* complex bacteria are naturally resistant to the antimicrobial action of Polymyxin B due to the presence of an Ara4N with a positive charge which reduces the electro negativity of the LPS and therefore should reduce binding by Polymyxin B. The question arises if Polymyxin B still inhibitory effects on the inflammatory activity of LPS from *B.cepacia* complex. In order to investigate the binding of representative *B.cepacia* complex LPS to Polymyxin B, MM6 (10^6/ml) cells were stimulated with different doses of LPS from Cardiff A1 (chosen for the potency of its LPS to induce inflammatory cytokines from previous experiments), *E.coli* and *P.aeruginosa* in the presence of Polymyxin B at different concentrations. After 6 hours TNF-α level was measured by ELISA. Polymyxin B was found to effectively neutralize 100 ng Cardiff A1 LPS by more than 50% at concentrations of >5 μg/ml (Figure 3.2.9.) as shown by a reduction in TNF-α production. At an increased LPS dose of 500 ng/ml Polymyxin B also neutralized Cardiff A1 LPS in a dose dependant manner (Figure 3.2.10.). Results for *E.coli* and *P.aeruginosa* showed similar reduction in TNF-α production that was dependant on dose of LPS and concentration of Polymyxin B. This suggests that there is no difference in the binding of Cardiff A1 LPS to Polymyxin B as compared with *E.coli* and *P.aeruginosa*.

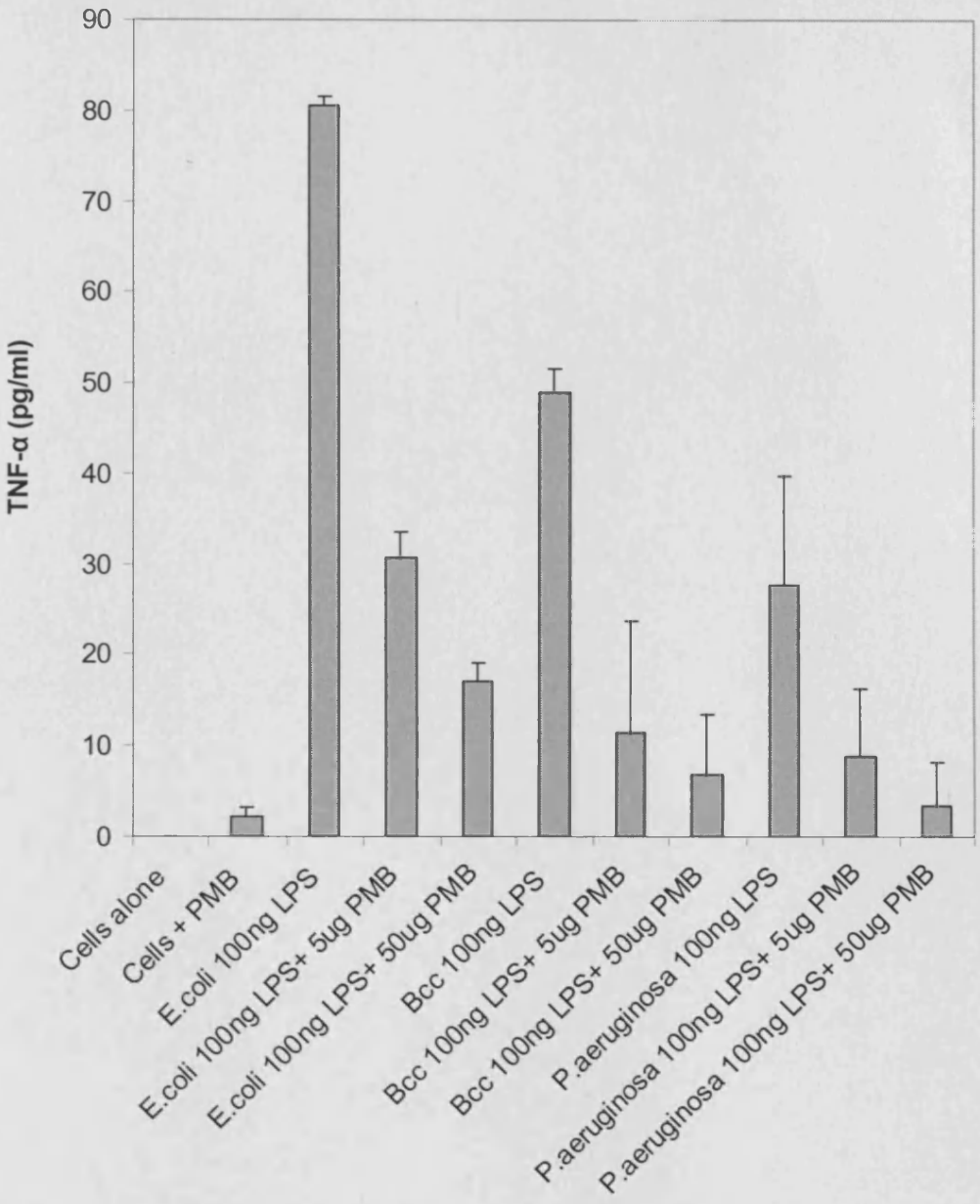

Figure 3.2.9. Effect of Polymyxin B sulphate on the production of TNF-α from LPS stimulated MM6 cells. Cells were suspended at $1x10^6$/ml and LPS from *E.coli*, Bcc (Cardiff A1) and *P.aeruginosa* was added to make a final concentration of 100 ng, at the same time either 5 µg or 50 µg of Polymyxin B (PMB) was added. Control wells containing LPS alone, cells alone and Polymyxin B alone were also included. The incubation was terminated after 6 hours. Values are means ±SD of three independent experiments.

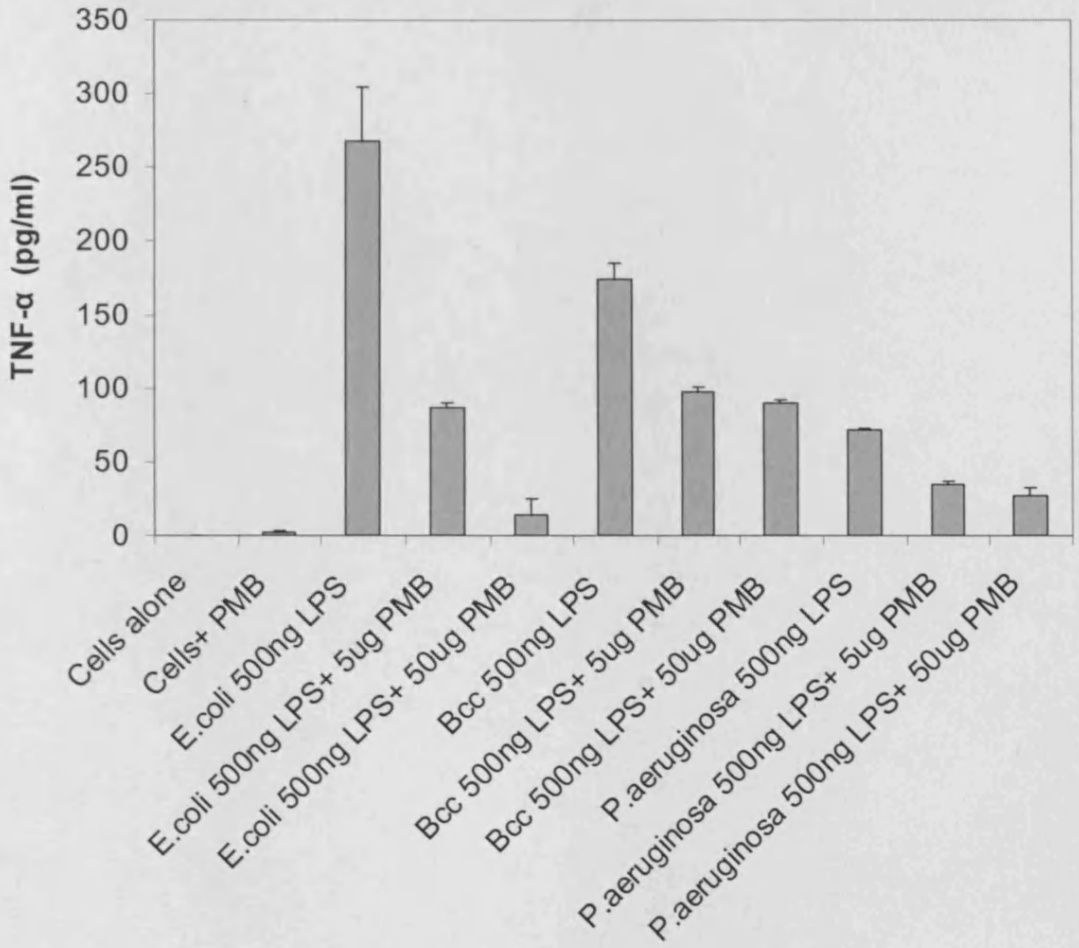

Figure 3.2.10. Effect of Polymyxin B sulphate on the production of TNF-α from LPS stimulated MM6 cells. Cells were suspended at $1x10^6$/ml and LPS from *E.coli*, Bcc (Cardiff A1) and *P.aeruginosa* was added to make a final concentration of 500 ng, at the same time either 5 μg or 50 μg of Polymyxin B (PMB) was added. Control wells containing LPS alone, cells alone and Polymyxin B alone were also included. The incubation was terminated after 6 hours. Values are means ±SD of three independent experiments.

3.3. Respiratory Burst in *B.cepacia* complex LPS primed MM6 cells

3.3.1. MM6 cells respiratory burst responses

Respiratory burst is a critical mechanism in the defence against invading pathogens and this oxidative activity is important in antimicrobial action by cells. Monocytes and macrophages produce superoxide anion in response to bacteria or bacterial products which have been engulfed (Allen *et al*, 1972).

Overproduction of reactive oxygen radicals can cause tissue damage during inflammation. LPS does not directly cause the triggering of a respiratory burst response, however it has been shown to up regulate the response by cells to other stimuli, therefore acting as a priming agent (Schopf *et al*, 1990, Yan *et al*, 2002).

It has been suggested that increased respiratory burst activity in the lungs may contribute to inflammation by the release of tissue damaging proteolytic enzymes and reactive oxygen species and that LPS from *B.cepacia* complex may play a considerable role in lung inflammation in cystic fibrosis (Hughes *et al* 1997).

In order to compare *B.cepacia* complex LPS to that of *P.aeruginosa* and *E.coli* in their ability to prime a respiratory burst response, MM6 cells were primed overnight with 100 ng LPS/10^6 cells with LPS from various representative clinical *B.cepacia* complex isolates which showed different abilities to induce TNF-α in previous experiments or with LPS from *P.aeruginosa* (PA01), or *E.coli* (0111:B4). The

respiratory burst response was triggered using opsonised zymozan and the production of superoxide anions was measured by enhanced chemiluminescence using Luminol.

The respiratory burst activation by opsonised zymozan was maximal at either 15 or 20 minutes for each reaction, and the relative light unit (RLU) levels at the peak of each sample reaction is shown (Figures 3.3.2 and 3.3.4 and 3.3.6.).

B.cepacia complex LPS primed cells to produce varying levels of superoxide anion, with Cardiff A1 (*B.multivorans*) (Figures 3.3.1. and 3.3.5.) and Cardiff P6 (*B.cenocepacia*) (Figure 3.3.3.) producing higher levels than LPS from *E.coli*, Cardiff P5 (*B.ceocepacia* ET-12) or Cardiff A2 (*B.multivorans*)

Cardiff A1 LPS showed the greatest ability to prime for respiratory burst activity (Figures 3.3.1. and 3.3.5.) this strain produced high levels of TNF-α, IL-6 and IL-8 in cytokine stimulation experiments (Figures3.1.5. and 3.2.1. and 3.2.2.).

LPS from Cardiff P5 (*B.cenocepacia* ET-12) was the most inflammatory *B.cepacia* complex LPS in stimulating cytokine production, but was not the most effective at priming MM6 cells for respiratory burst. LPS from Cardiff P6 (*B.cenocepacia*) (Figures 16a and 16b) also produce higher amounts of superoxide anion than LPS from *E.coli* but in cytokine stimulation experiments it produced lower levels of inflammatory cytokines than *E.coli*. These examples show that there is little correlation between ability to produce an inflammatory response directly though cytokine production and the ability to prime monocytes for respiratory burst. LPS from Cardiff P1 (*B.cenocepacia* ET-12) (Figure 3.3.3.) and Cardiff P2 (*B.cenocepacia*

ET-12) (Figure 3.3.5.) were the least effective samples tested with Cardiff P2 showing a lesser ability to prime for respiratory burst than even *P.aeruginosa* (Figure 3.3.6.), The differences seen between these ET-12 samples demonstrates the variability of LPS that is derived from identical *B.cenocepacia* ET-12 isolates in their ability to prime monocytes for respiratory burst.

It is suggested that induction of inflammatory response is separate and dissimilar to the oxidative burst response. The respiratory burst response is a direct method of killing pathogens by host cells, so inducing this response is not advantageous to *B.cepacia* complex organisms as they are cleared more efficiently when the response is induced.

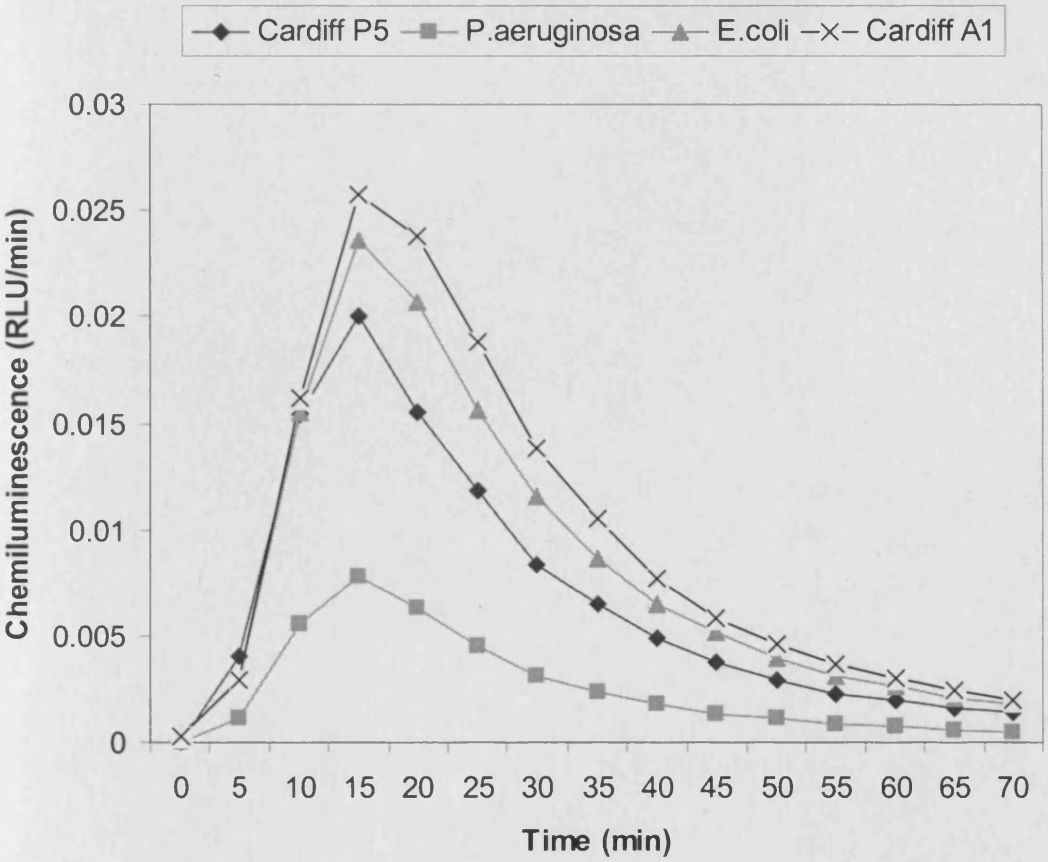

Figure 3.3.1. Typical chemiluminescence plot of MM6 cells primed overnight with 100 ng LPS from representative Cardiff *B.cepacia* strains, *E.coli* and *P.aeruginosa.* Respiratory burst was triggered with opsonised zymozan and detected using luminol.

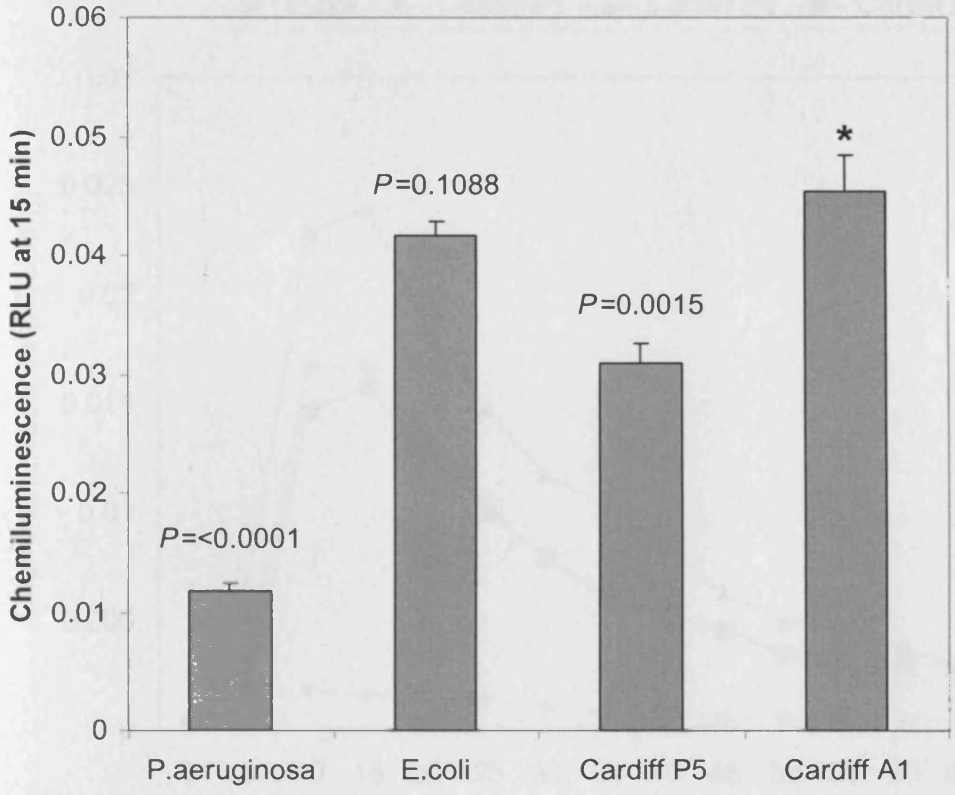

Figure 3.3.2. The peak of chemiluminescence at 15 min from MM6 cells primed overnight with 100 ng LPS from representative Cardiff *B.cepacia* strains, *E.coli* and *P.aeruginosa.* * = *P* values were obtained vs Cardiff A1 LPS. Respiratory burst was triggered with opsonised zymozan and detected using luminol.

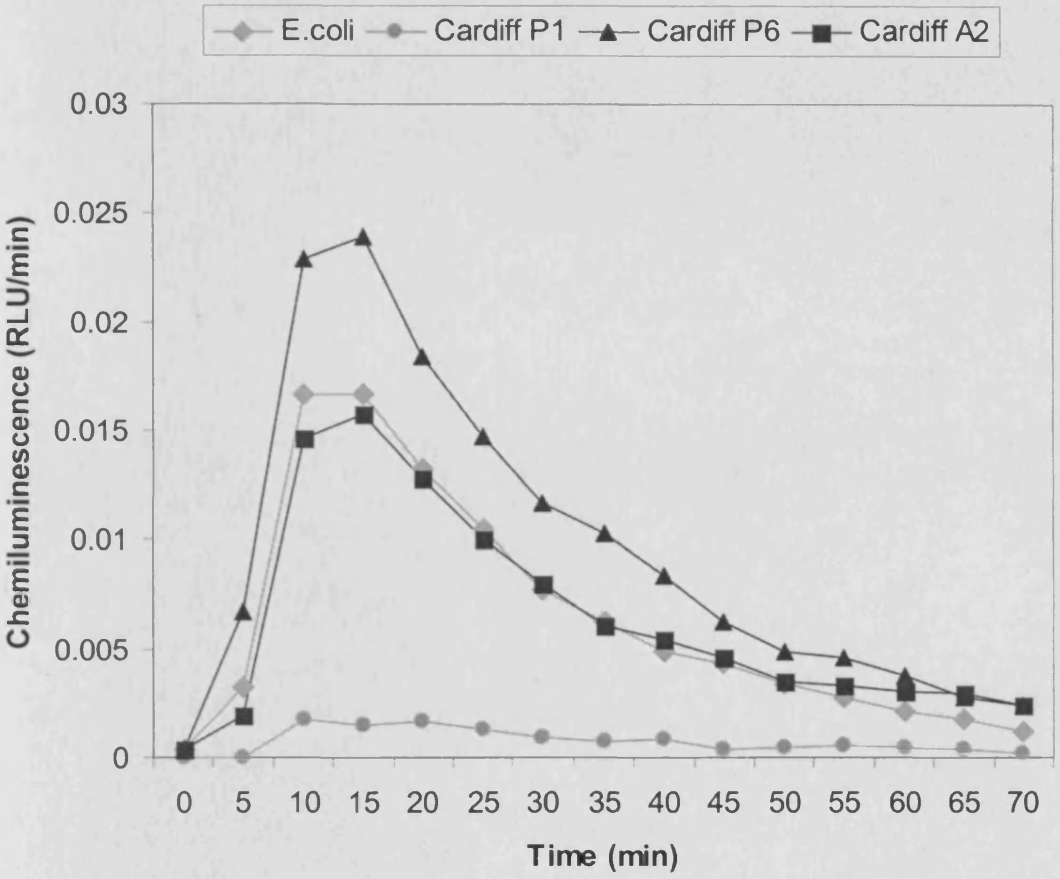

Figure 3.3.3. Typical chemiluminescence plot of MM6 cells primed overnight with 100 ng LPS from Cardiff *B.cepacia* strains and *E.coli.* Respiratory burst was triggered with opsonised zymozan and detected using luminol

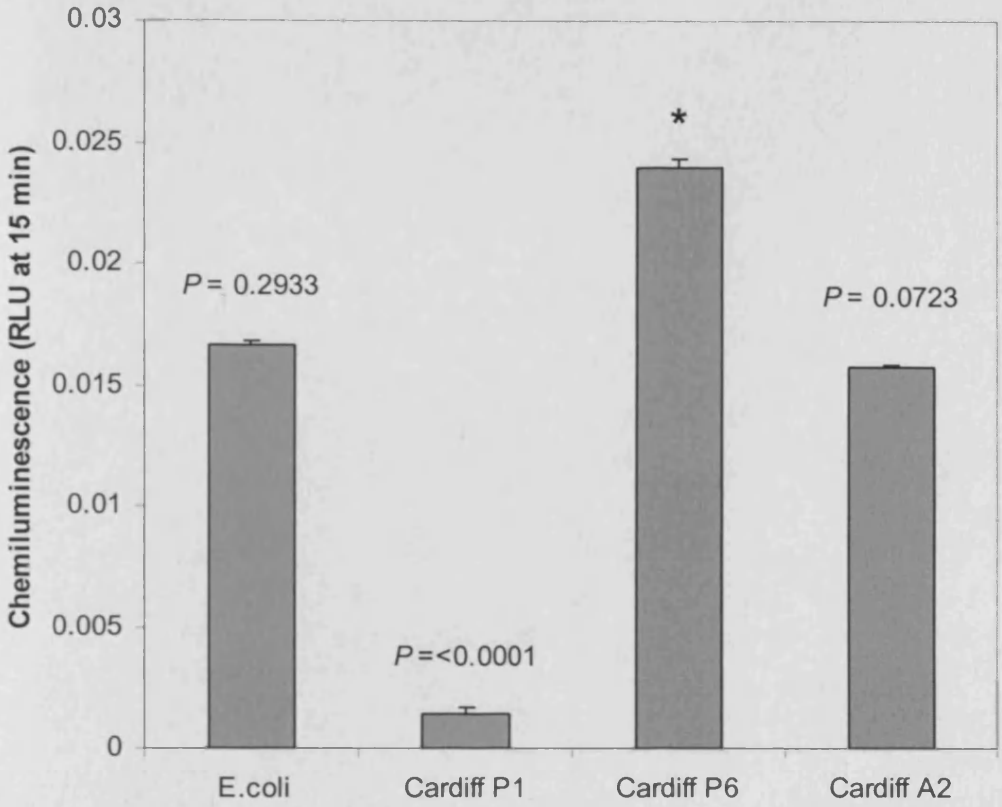

Figure 3.3.4. The peak of chemiluminescence at 15 min from MM6 cells primed overnight with 100 ng LPS from representative Cardiff *B.cepacia* strains and *E.coli.* * = *P* values were obtained vs Cardiff P6 LPS. Respiratory burst was triggered with opsonised zymozan and detected using luminol.

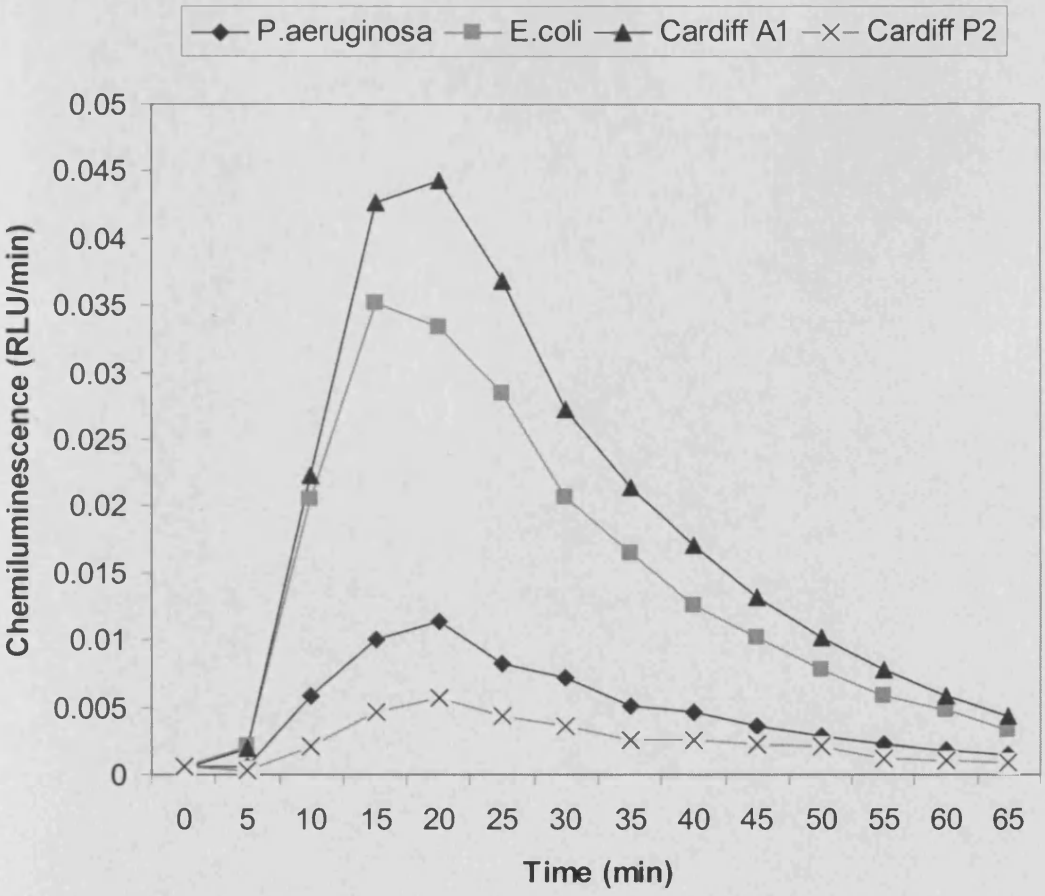

Figure 3.3.5. Typical chemiluminescence plot of MM6 cells primed overnight with 100 ng LPS from Cardiff *B.cepacia* strains *E.coli* and *P.aeruginosa*. Respiratory burst was triggered with opsonised zymozan and detected using luminol

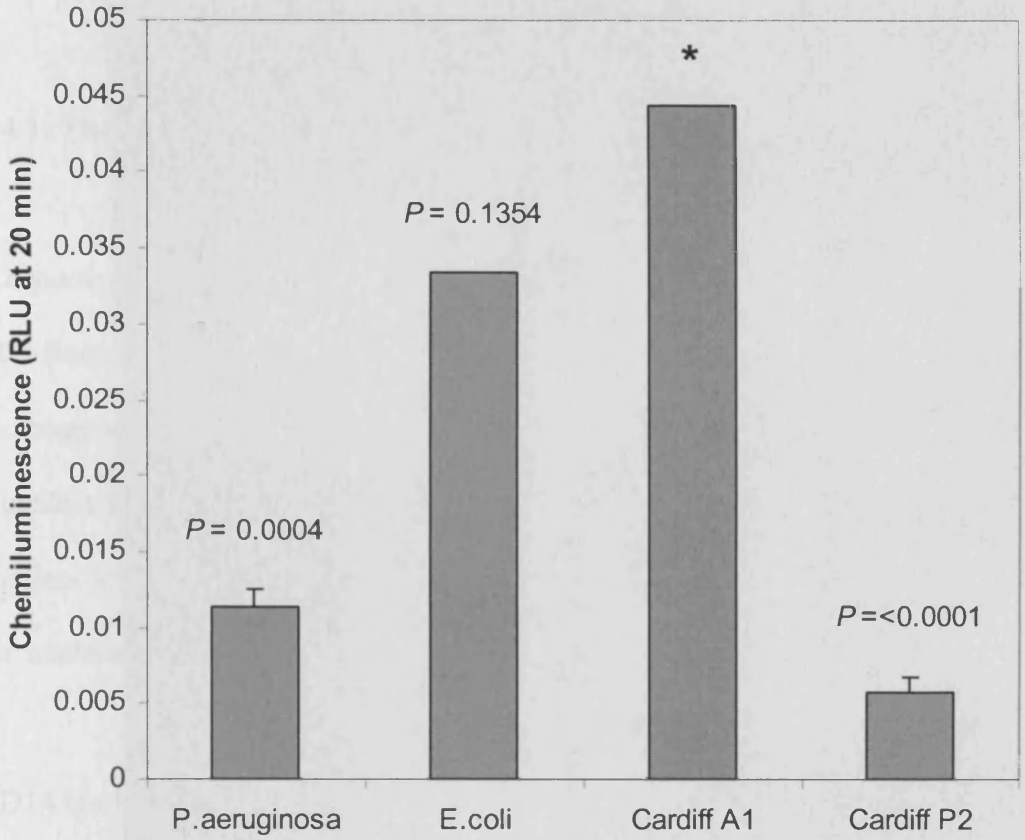

Figure 3.3.6. The peak of chemiluminescence at 20 min from MM6 cells primed overnight with 100 ng LPS from representative Cardiff *B.cepacia* strains, *E.coli* and *P.aeruginosa* * = *P* values were obtained vs Cardiff A1 LPS. Respiratory burst was triggered with opsonised zymozan and detected using luminol.

3.4. Investigating the receptor signalling by *B.cepacia* complex LPS

3.4.1. The role of CD14 in *B.cepacia* complex signalling

B.cepacia complex LPS might activate cells via different receptors and pathways than LPS from other bacterial species, a study has suggested that the closely related *P.aeruginosa* signal through TLR2 (Erridge *et al*, 2004). This work set out to elucidate the receptors that are bound and activated by *B.cepacia* complex LPS and to confirm which signalling pathway is activated between the cell surface receptor and the nucleus.

CD14 has been shown to be the initial cell surface receptor to respond to LPS (Fenton & Golenbrock 1998), on the cell surface it is referred to as mCD14 (membrane bound CD14) but it is also present in serum where it is referred to as sCD14 (solubleCD14).

In order to block the action of both mCD14 and sCD14, monoclonal anti-CD14 antibody (at an optimal 20μg/10^6 cells) was incubated with MM6 cells followed by stimulation with 100ng/10^6 cells of representative *B.cepacia* complex LPS. A lower dose of LPS was used to stimulate cells in all antibody blocking experiments to allow the use of as low a dose of blocking antibody as possible. TNF-α levels were measured by ELISA after 6 hours incubation.

E.coli LPS is already known to require CD14 to mediate an inflammatory response, the results show that the production of TNF-α due to stimulation with *E.coli* LPS is reduced by a total of 75% following pre-treatment with anti-CD14 (Figure 3.4.1.).

TNF-α levels produced by Cardiff A1 (*B.multivorans*) and Cardiff P4 (*B.cenocepacia* ET-12) were both reduced to almost zero when pre-treated with anti-CD14 before stimulation. This strongly indicates that both LPS samples utilize CD14 to activate the signalling pathway that culminates in an inflammatory response. These results are further backed up by cytokine stimulation experiments using purified LPS and A549 cells (Figure 3.1.5.) in the absence of sCD14 which showed a much reduced IL-8 production compares to cells that had been incubated with human serum that provides sCD14.

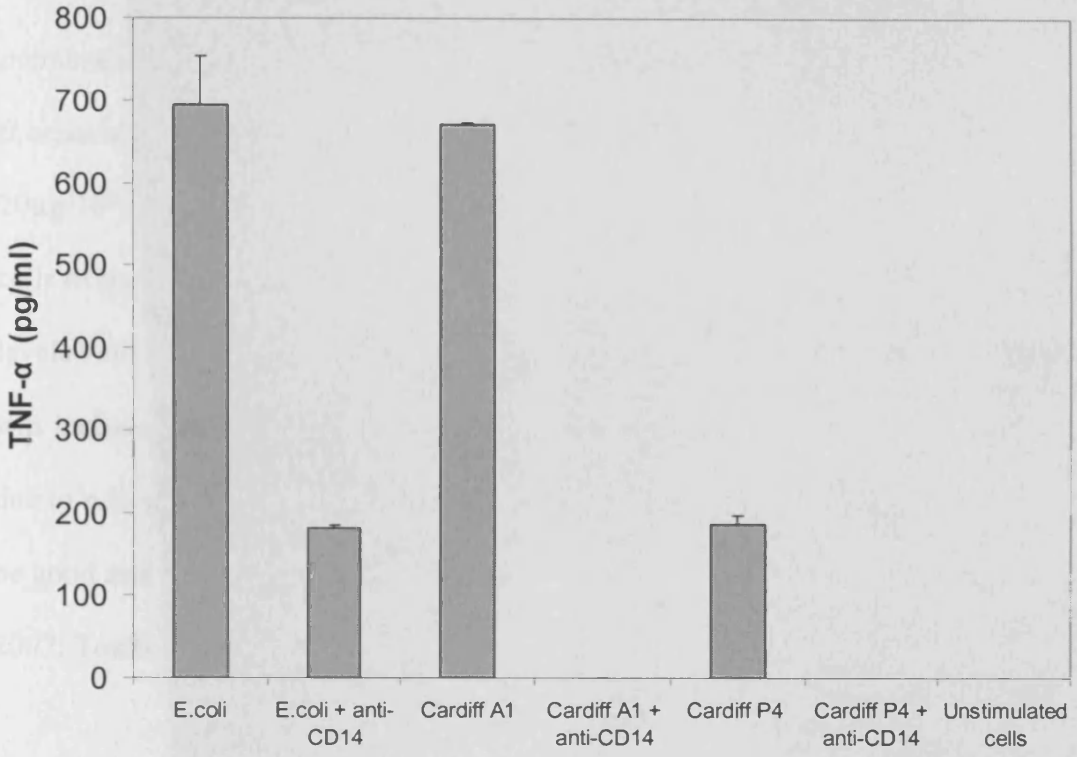

Figure 3.4.1. MM6 cells were pre-incubated with 5µg monoclonal anti-CD14 antibody and then stimulated with 25ng/250µl cells of representative *B.cepacia* LPS and *E.coli* LPS, MM6 cells were also stimulated with LPS without anti-CD14 as a control. Supernatants were harvested after 16 hours and were tested for levels of TNF-α by ELISA. Values are means ±SD of two independent experiments.

3.4.2. Role of TLR4 receptor in *B.cepacia* complex LPS signalling

In *E.coli* when CD14 has bound LPS, the signal is then conveyed through the trans-membrane receptor TLR4. To investigate the role of TLR4 in the signalling of *B.cepacia* complex LPS, monoclonal anti-TLR4 blocking antibody (at an optimal 20μg/10^6 cells) was incubated with MM6 cells before stimulation with 100ng/10^6 cells of purified representative *B.cepacia* complex LPS or LPS from *E.coli*. TNF-α levels were measured by ELISA after 6 hours incubation. An IgG2a isotype antibody was included as a control and was found to not reduce the amount of TNF-α produced due to non-specific binding. The efficacy of anti-TLR4 antibodies has been shown to be good and they have been used for many years, in many different studies (Fan *et al* 2007; Tonks *et al* 2007; Ahmad *et al* 2007).

All samples but one (Cardiff A1- *B.multivorans*) LPS, including the environmental strain NCTC 10743, showed a reduction of more than 80% in TNF-α production when the cells were pre-treated with anti-TLR4 compared to those that were not pre-treated (Figures 3.4.2. and 3.4.3.).

There was a reduction of 40% in TNF-α production when Cardiff A1 (*B.multivorans*) was incubated with anti-TLR4 as compared to LPS alone. Although this is still a significant reduction, the anti-TLR4 did not inhibit TNF-α production as effectively as with the other LPS samples tested. All conditions were the same over the course of these experiments and only the LPS sample used was variable. The TNF-α production from *E.coli* LPS was seen to be reduced to zero when anti-TLR4 was included. The IgG2a isotype antibody had no effect on TNF-α production from *E.coli* LPS

stimulated cells and had no effect on MM6 cells alone. Studies using cells which are deficient in the TLR4 receptor, for example C3h/HEJ mice, would confirm that these LPS samples are indeed signalling through TLR4.

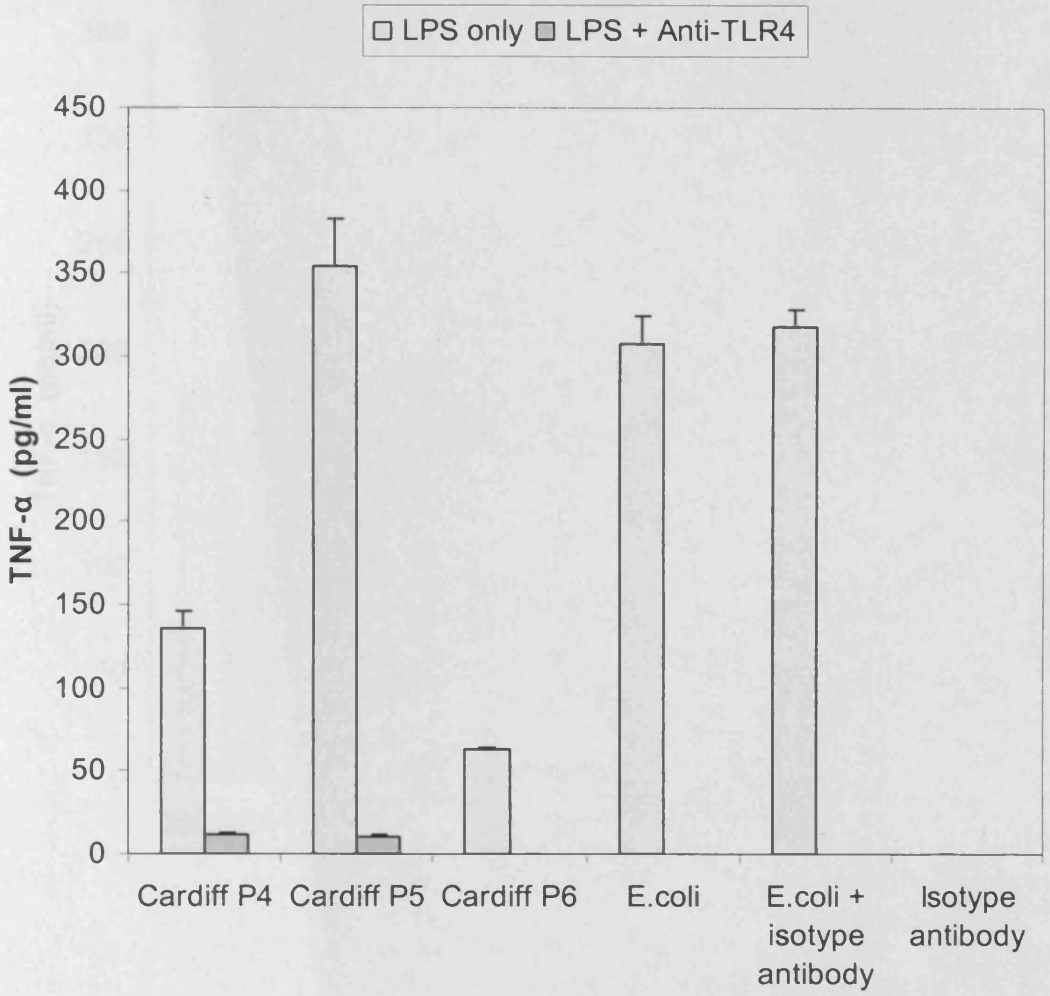

Figure 3.4.2. MM6 cells were pre-incubated with 5µg monoclonal anti-TLR4 antibody and then stimulated with 25ng/250µl cells of representative *B.cenocepacia* LPS and *E.coli* LPS, an IGg2a isotype antibody was included with *E.coli* LPS and by itself to determine any effect. Supernatants were harvested after 16 hours and were tested for levels of TNF-α by ELISA. Values are means ±SD of two independent experiments

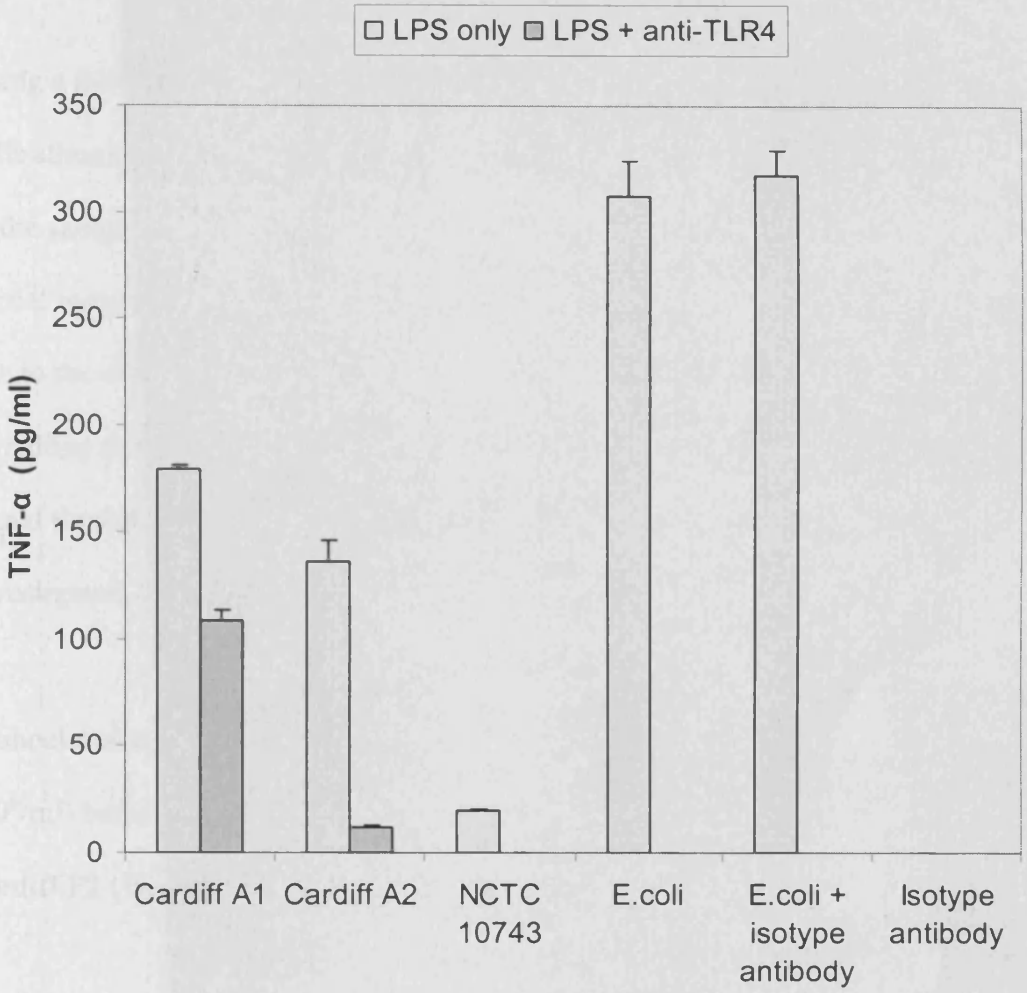

Figure 3.4.3. MM6 cells were pre-incubated with 5µg monoclonal anti-TLR4 antibody and then stimulated with 25ng/250µl of cells of *B.cepacia* LPS and *E.coli* LPS, an IGg2a isotype antibody was included with *E.coli* LPS and by itself to determine any effect. Supernatants were harvested after 16 hours and were tested for levels of TNF-α by ELISA. Values are means ±SD of two independent experiments.

3.4.3. Role of TLR2 receptor in *B.multivorans* LPS signalling

Using a monoclonal antibody to TLR4 reduced the TNF-α production from MM6 cells stimulated with LPS from *B.cepacia* complex LPS dramatically. The exception in the samples tested was the Cardiff A1 (*B.multivorans*) sample which showed only a partial reduction in TNF-α production when pre-treated with anti-TLR4, this could be due to the efficacy of the blocking antibody or because Cardiff A1 is partially signalling through another receptor. Some atypical LPS samples have been shown to signal through TLR2, because of this, the possible role of TLR2 in signalling was investigated. (Werts *et al* 2001; Erridge *et al* 2004).

Monoclonal anti-TLR2 blocking antibody (5μg/250ul) was incubated with MM6 cells (10^6/ml) before stimulation with 100ng/ml of purified Cardiff A1 (*B.multivorans*), Cardiff P2 (B.*multivorans*) LPS or LPS from *E.coli*.

TNF-α levels were measured by ELISA after 6 hours incubation. The results for incubation with anti-TLR2 and treatment with Cardiff A1 LPS (Figure 3.4.4.) show that there was an insignificant reduction in the production of TNF-α, which may indicate that TLR2 is not required for LPS signalling from this strain. Cardiff A2 and *E.coli* LPS also show no difference in inflammatory activity when pre-incubated with anti-TLR2 which may indicate that this receptor is not required for signal transduction for LPS from these strains either.

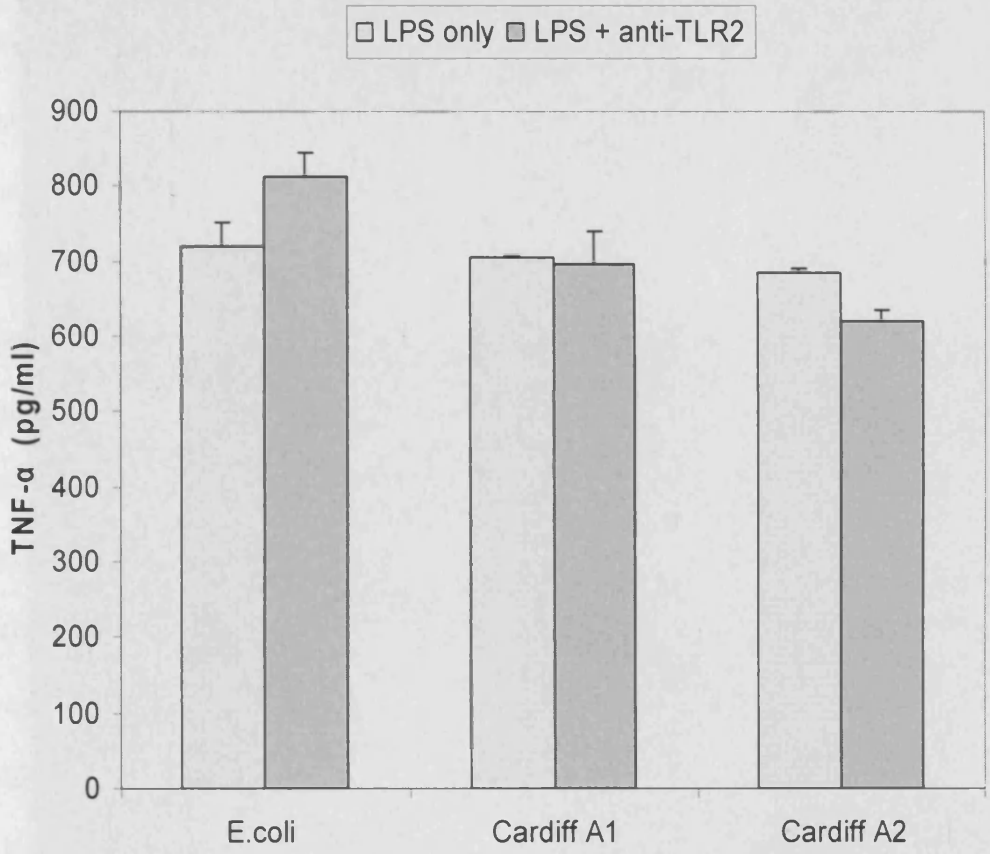

Figure 3.4.4. MM6 cells were pre-incubated with 5μg monoclonal anti-TLR2 antibody and then stimulated with 25ng/250μl of cells of *B.multivorans* LPS and *E.coli* LPS. Supernatants were harvested after 16 hours and were tested for levels of TNF-α by ELISA. Values are means ±SD of two independent experiments.

3.4.4. Role of MyD88 adaptor protein in *B.cepacia* complex LPS signalling

LPS has been shown to signal via MyD88 dependent (Miyake 2004) and independently of MyD88 (Hoebe *et al*, 2003) through TRIF/TRAM. In order to find out whether representative *B.cepacia* complex LPS samples require MyD88 to signal, MM6 cells 10^6/ml were incubated with either 100 μM of MyD88 inhibitor peptide (sequence= DRQIKIWFQNRRMKWKKRDVLPGT) or 100 μM of a control peptide (sequence= DRQIKIWFQNRRMKWKK) for 24 hours. The MyD88 inhibitor peptide is cell permeable and prevents the homodimerization of MyD88 thus preventing signalling through it. The cells were stimulated with 100 ng purified LPS from *E.coli*, Cardiff A1 and Cardiff P5 for 6 hours and TNF-α levels were measured by ELISA. The amount of TNF-α produced by *E.coli* LPS stimulated cells was reduced significantly when MyD88 inhibitor peptide was present (P=<0.0001) (Figure 3.4.5.) and inclusion of the control peptide had no effect.

Cardiff A1 LPS (Figure 3.4.6.) showed a much lower reduction in TNF-α level when MyD88 inhibitor peptide was included although still significant (P=0.0002). This result may indicate that as well as signalling through MyD88, Cardiff A1 LPS may stimulate via the MyD88 independent pathway. This could only be confirmed with use of other techniques and models, for example using a $MyD88^{-/-}$ mouse or by using RNAi to inactivate the MyD88 gene. When the control peptide was included it no effect on TNF-α production.

Cardiff P5 LPS stimulated cells (Figure 3.4.7.) showed a significant reduction in TNF-α production when incubated with MyD88 inhibitor protein when compared

with LPS alone (P=<0.0001) which would suggest that this purified LPS signals only through MyD88. The control peptide again showed no effect on the level of TNF-α produced.

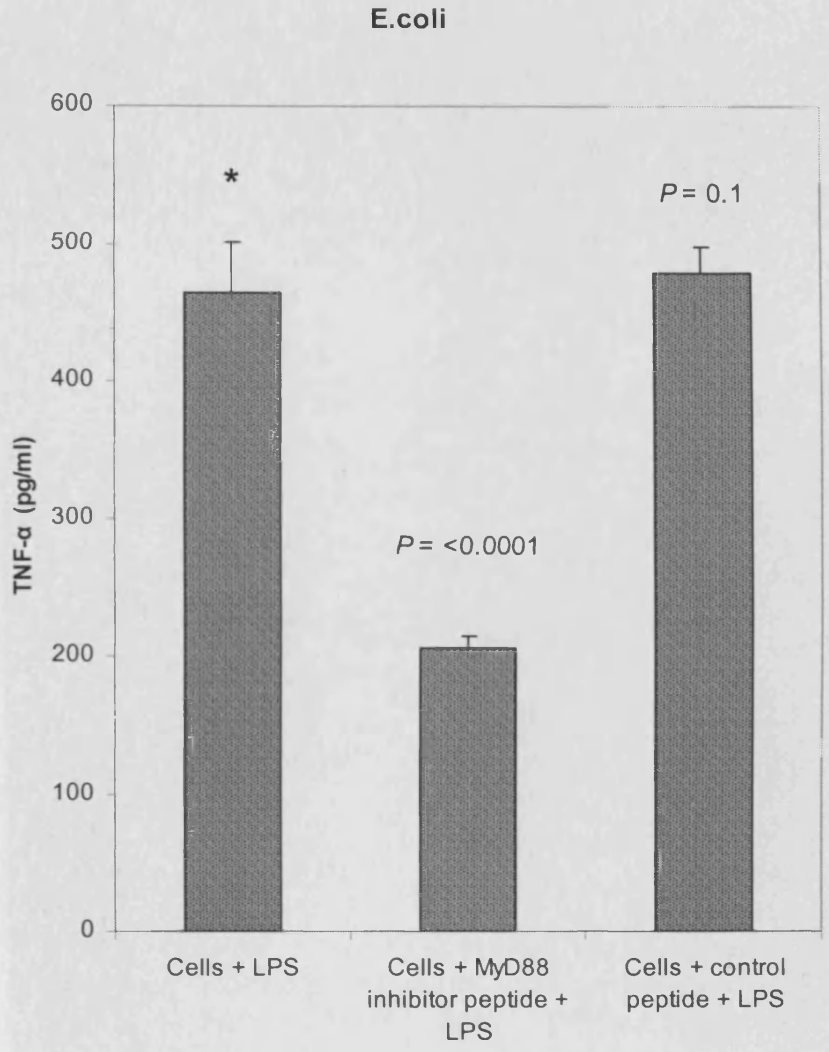

Figure 3.4.5. MM6 cells suspended at $1x10^6$ cells/ml were incubated with either 100 μM of MyD88 inhibitor peptide or 100 μM of a control peptide for 24 hours then stimulated with 100 ng of LPS from *E.coli* for 6 hours, control cells stimulated with LPS alone were included. The supernatants were tested for TNF-α by ELISA.

* = *P* values were obtained vs *E.coli* LPS alone. Values are means ±SD of three independent experiments.

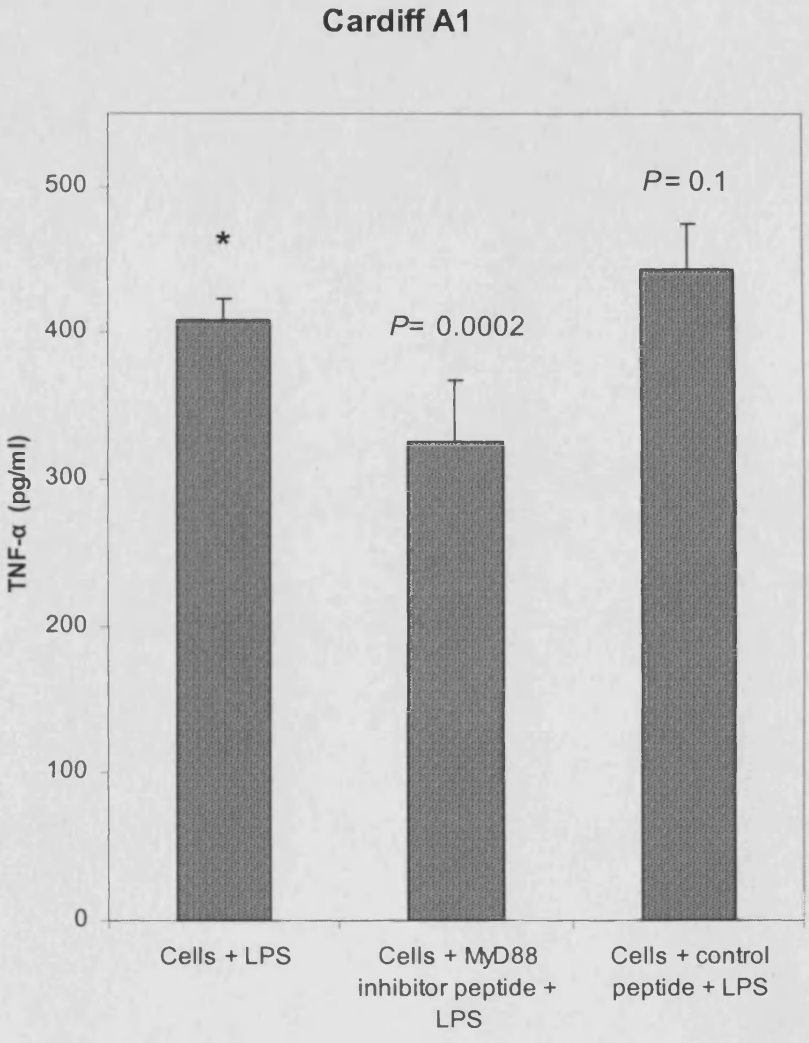

Figure 3.4.6. MM6 cells suspended at $1x10^6$ cells/ml were incubated with either 100 μM of MyD88 inhibitor peptide or 100 μM of a control peptide for 24 hours then stimulated with 100 ng of LPS from *B.multivorans* (Cardiff A1) for 6 hours, control cells stimulated with LPS alone were included. The supernatants were tested for TNF-α by ELISA. * = *P* values were obtained vs Cardiff A1 LPS alone. Values are means ±SD of three independent experiments.

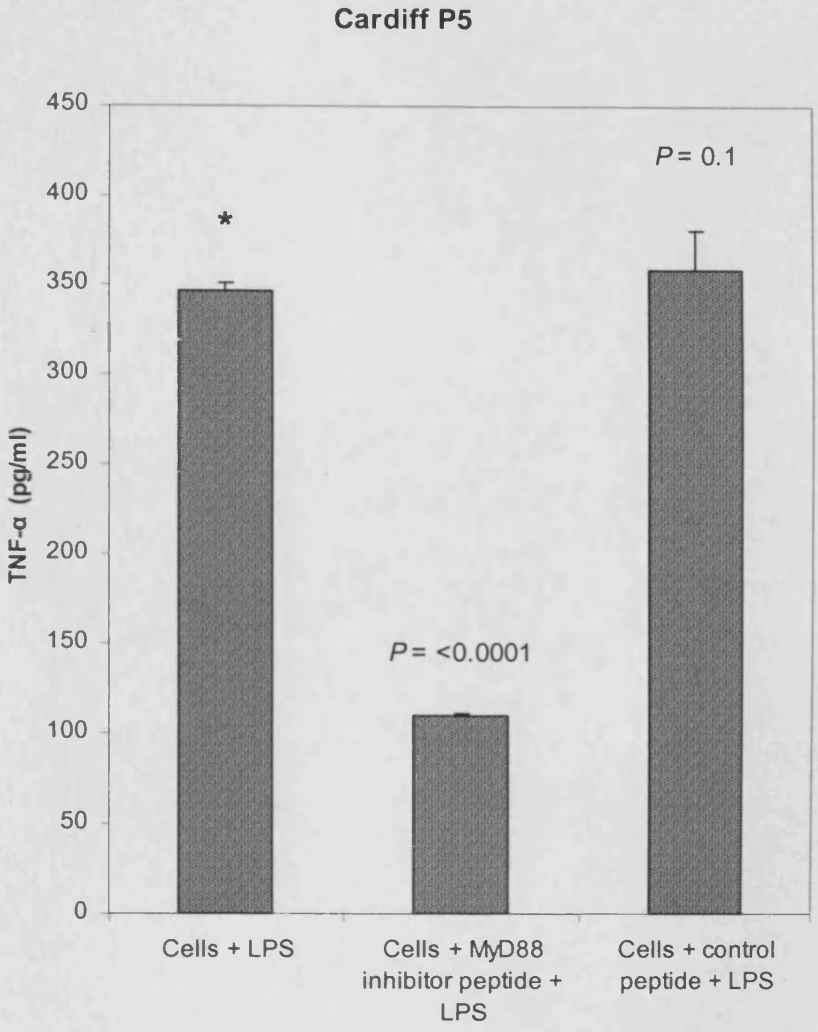

Figure 3.4.8. MM6 cells suspended at $1x10^6$ cells/ml were incubated with either 100 μM of MyD88 inhibitor peptide or 100 μM of a control peptide for 24 hours then stimulated with 100 ng of LPS from *B.cenocepacia* (Cardiff P5) for 6 hours, control cells stimulated with LPS alone were included. The supernatants were tested for TNF-α by ELISA. * = *P* values were obtained vs Cardiff P5 LPS alone. Values are means ±SD of three independent experiments.

3.5. MAP Kinase activation by *B.cepacia* complex LPS

3.5.1. Phosphorylation of P38 by *B.cepacia* complex LPS

Mitogen activated protein (MAP) kinases belong to three families, P38 MAPKs, extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinase (JNK). LPS from various bacteria has been shown to activate all three families, but primarily ERKs and P38 (Raingeaud *et al* 1995; Scherle *et al* 1998).

MAP kinase activation has not been investigated in *B.cepacia* complex bacteria in detail. MAP kinases activate by phosphorylation which can be detected by western blot. To compare whether MAPK activation pathways used by *B.cepacia* complex LPS was similar to that used by *E.coli* LPS, MM6 cells were stimulated with 1 μg LPS to induce a strong response, for 1 hour before protein extraction and Western blot analysis. Whole cell lysate from stimulated and control cell containing equal amounts of protein were loaded onto each lane of a 10% Bis-Tris gel. The antibody used only binds when the MAPK is phosphorylated and therefore active, it does not bind to non-phosphorylated P38. Both Cardiff P5 and Cardiff A1 (Figure 3.5.1.) were shown to phosphorylate P38 MAPK in a similar manner to *E.coli* LPS. There are no bands present with the samples that were not stimulated with LPS.

3.5.2. Phosphorylation of P44/42 by *B.cepacia* complex LPS

P44/42 is also known as ERK 1/2 and is a member of the ERK kinase group. In order to see whether P44/42 is activated when monocytic cells are stimulated with *B.cepacia* complex LPS MM6 cells were stimulated with 1 μg LPS for 1 hour before protein extraction and Western blot analysis. Equal amounts of protein were loaded onto each lane for each blot. It was found that all *B.cepacia* complex LPS samples tested produced bands corresponding to P44/42 MAPK (Figures 3.5.2. and 3.5.3. and 3.5.4.) similar to that of *E.coli* LPS. The antibody used only binds when the MAPK is phosphorylated and therefore active, it does not bind to non-phosphorylated P44/42. Unstimulated control cells showed much weaker banding, which may correspond to less phosphorylation, compared to the stimulated samples, this could be confirmed using densitometry.

3.5.3. Total vs phosphorylated P44/42

MM6 cells were stimulated with 1 μg LPS for 1 hour before protein extraction and Western blot analysis for phosphorylated P44/42 MAPK and total amount of P44/42 MAPK. Equal amounts of protein were loaded onto each lane for each blot. Total P44/42 MAPK showed much denser and more intense bands than the bands for phosphorylated P44/42 alone which may indicate that not all P44/42 MAPK is activated when stimulated with any LPS tested including *E.coli* (Figure 3.5.5.) In order for these results to be quantitative in any way, a loading control antibody in the form of a house-keeping gene (e.g GAPDH) and densitometry would have to be

performed. However the results here indicate that LPS from BCC is activating P44/42 MAPK and indeed P38 MAPK. Signalling through these MAPKs has been further indicated with MAPK inhibition experiments (section 3.6.).

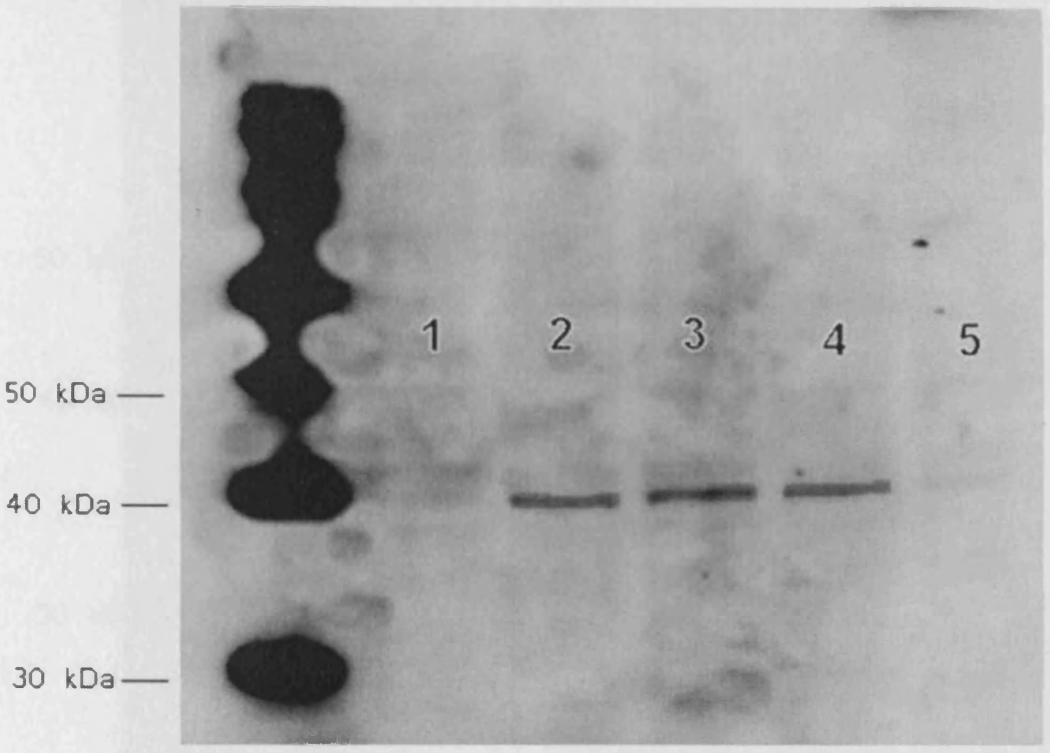

Figure 3.5.1. Western blot of phosphorylated P38 MAPK stimulated by *B.cepacia* complex LPS. MM6 cells ($1x10^6$/ml) were stimulated with 1 μg LPS for 1 hour. Lanes: 1- non-stimulated control, 2-*E.coli*, 3-Cardiff P5, 4-Cardiff A1, 5- non-stimulated control. Bands shown correspond to 43kDa.

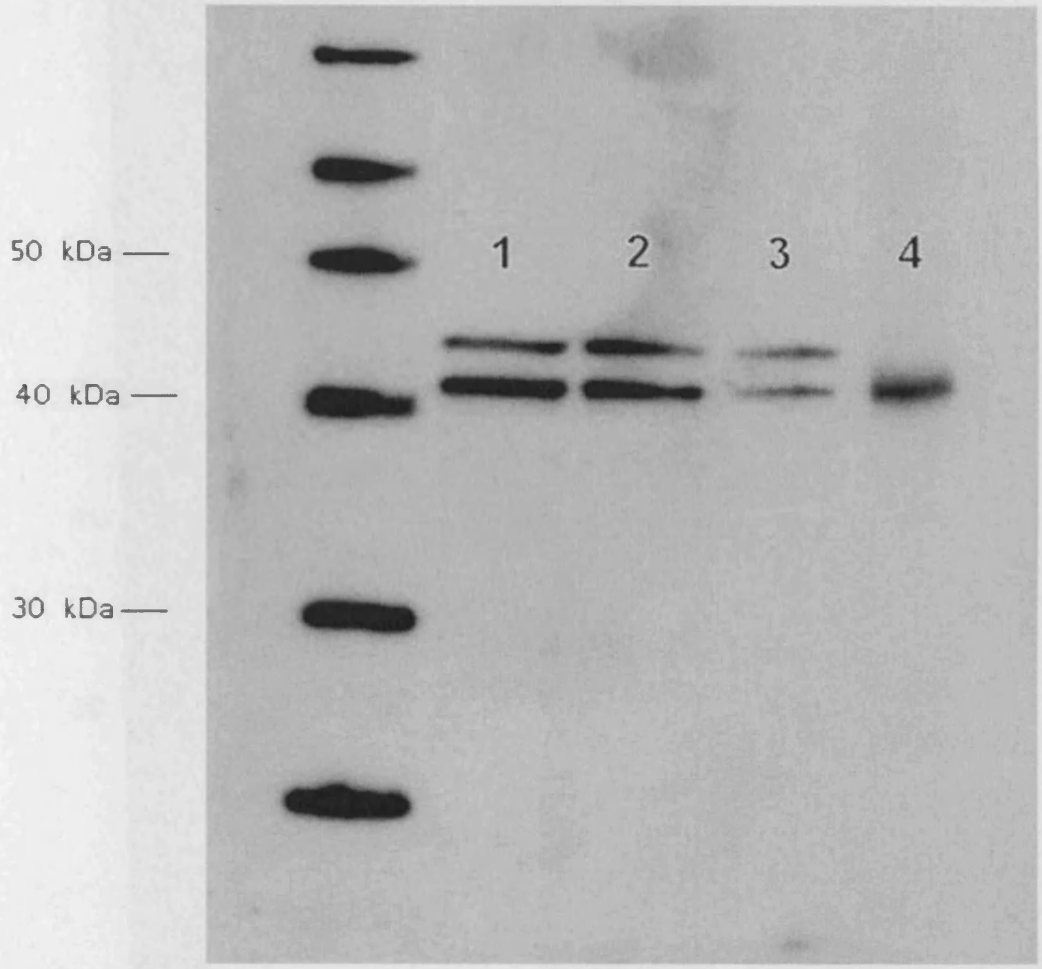

Figure 3.5.2. Western blot of phosphorylated P44/42 MAPK stimulated by *B.cepacia* complex LPS. MM6 cells ($1x10^6$/ml) were stimulated with 1 μg LPS for 1 hour. Lanes: 1- Cardiff A1. 2- Cardiff P5, 3- non-stimulated control, 4- positive control cell extract. Bands shown correspond to 44kDa and 42kDa.

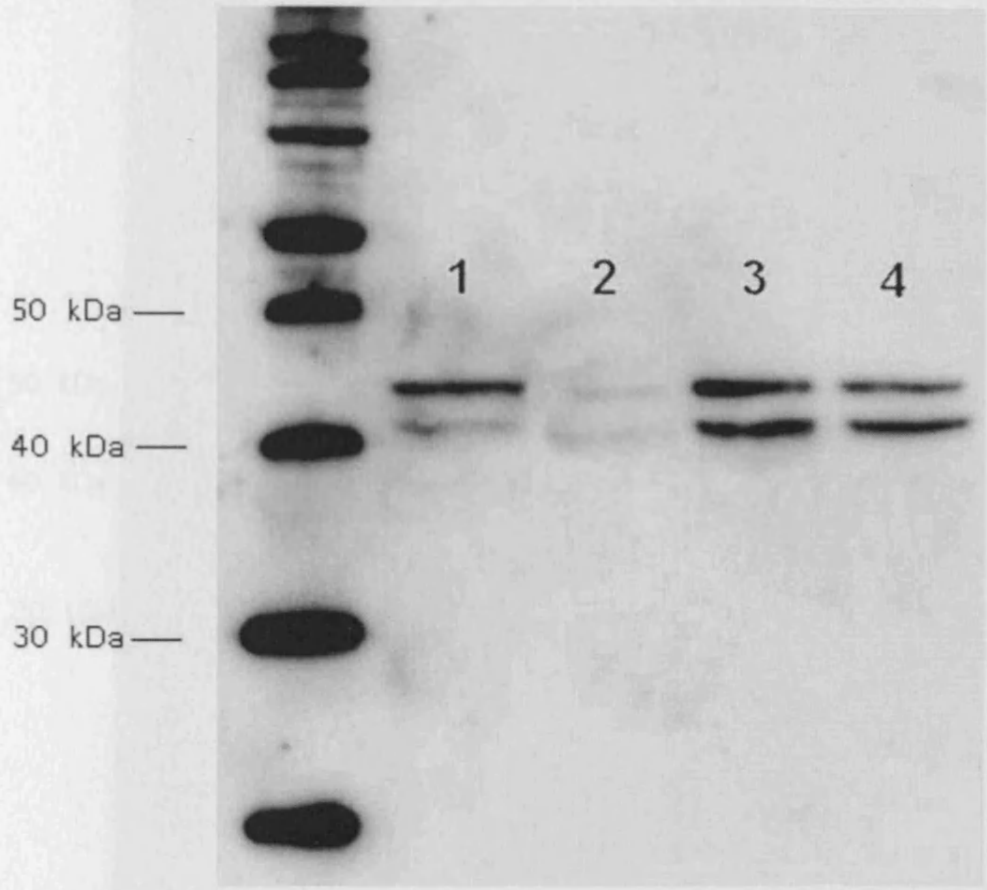

Figure 3.5.3. Western blot of phosphorylated P44/42 MAPK stimulated by *B.cepacia* complex LPS. MM6 cells ($1x10^6$/ml) were stimulated with 1 μg LPS for 1 hour.

Lanes: 1- Cardiff P1, 2- non-stimulated control, 3- Cardiff P6, 4- Cardiff P4

Bands shown correspond to 44kDa and 42kDa.

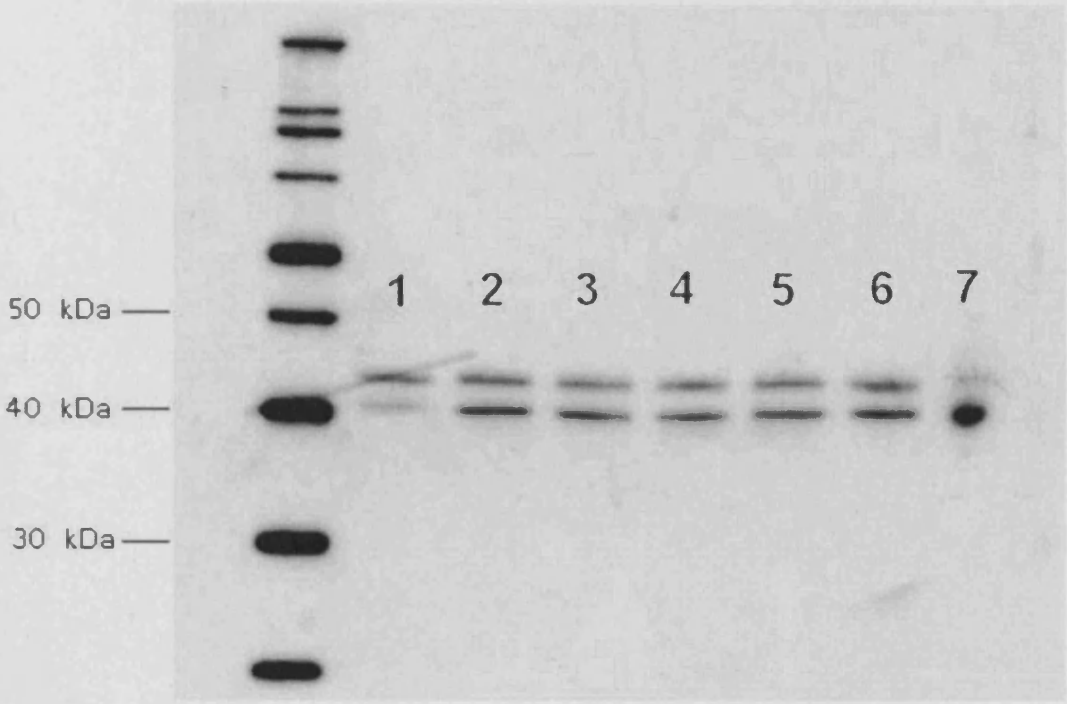

Figure 3.5.4. Western blot of phosphorylated P44/42 MAPK stimulated by *B.cepacia* complex LPS. MM6 cells ($1x10^6$/ml) were stimulated with 1 μg LPS for 1 hour. Lanes: 1- non-stimulated control, 2- Cardiff P1, 3- Cardiff P2, 4- Cardiff P3, 5- Cardiff P4, 6- Cardiff P5, 7- phosphorylated control sample.

Bands shown correspond to 44kDa and 42kDa.

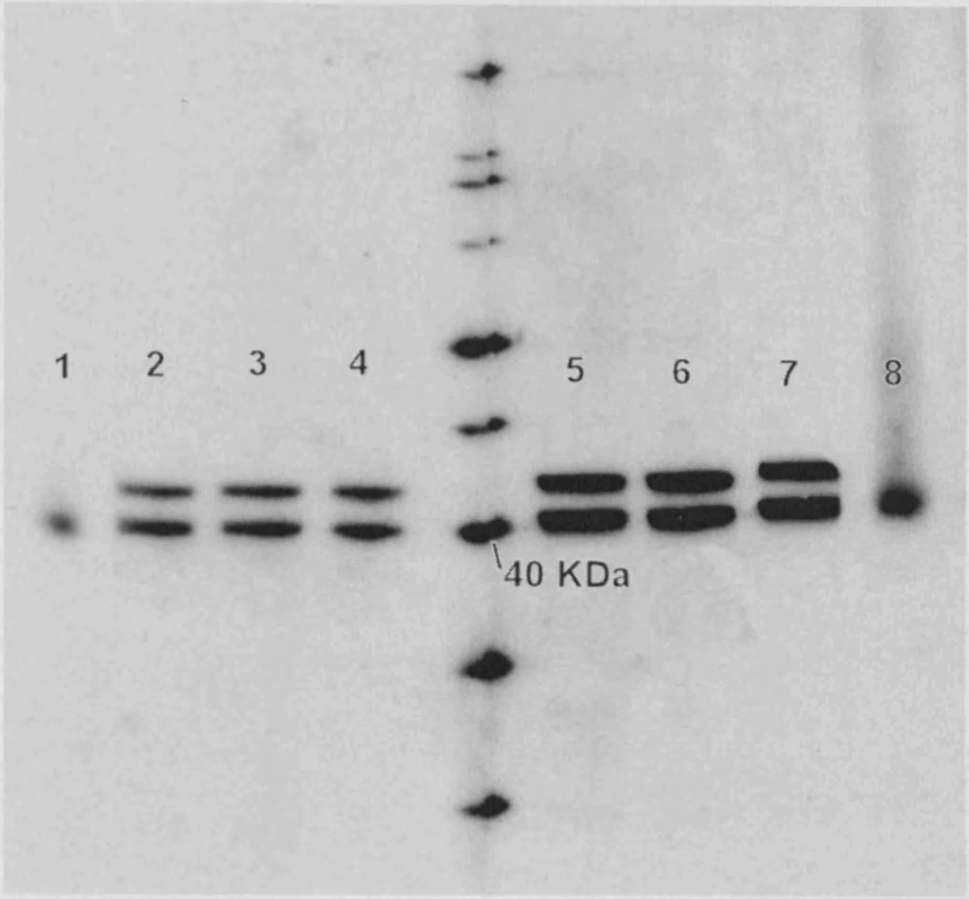

Figure 3.5.5. Western blot of phosphorylated and total P44/42 MAPK stimulated by *B.cepacia* complex LPS. MM6 cells ($1x10^6$/ml) were stimulated with 1 μg LPS for 1 hour.

Lanes: 1- phosphorylated P44/42 control extract, 2- phosphorylated *E.coli*, 3- phosphorylated Cardiff A1, 4- phosphorylated Cardiff P5, 5- total *E.coli*, 6- total Cardiff A1, 7- total Cardiff P5, 8- total P44/42 control extract.

3.6. Effect of MAPK inhibition on inflammatory response by *B.cepacia* complex LPS

3.6.1. Effect of specific MAP kinase inhibitors on TNF-α production stimulated by LPS

To assess the effect of inhibiting each of three MAP kinases through which LPS signalling is known to occur, and the effect of inhibiting more than one of these at a time, MM6 (10^6 /ml) cells were incubated with 10 μM of PD98059 (P42/44 inhibitor), SB203580 (P38 inhibitor) or SP600125 (JNK inhibitor) or a combination of inhibitors for 1 hour before 6 hours stimulation with *B.cepacia* complex LPS or *E.coli* LPS. Non-stimulated controls and controls with the MAPK inhibitors alone were included. These MAPK inhibitors are cell permeable and specific, they have been used successfully in many studies prior to this (Ropert 2005) and are used as therapeutic agents in disease, for example Crohn's disease (van den blink et al 2002).
MAPK inhibitors alone were seen to have no effect on MM6 cells. In *E.coli* (Figure 3.6.1.) each inhibitor was seen to reduce the amount of TNF-α produced with inhibition of JNK causing a 40% reduction TNF-α production. Combinations of inhibitors were all more effective with the combination of P38 and P44/42 inhibitors being the most effective and totally inhibiting TNF-α production. The effect of MAPK inhibition on Cardiff P5 (Figure 3.6.2.) and Cardiff A1 (Figure 3.6.3.) showed similar results to *E.coli*, with Cardiff P5 LPS showing slightly less of a reduction in TNF-α production with all of the individual MAPK inhibitors than *E.coli*. In the presence of JNK inhibitor TNF-α production was not as greatly reduced when stimulated by LPS from Cardiff A1 compared to LPS from *E.coli* or Cardiff P5 but

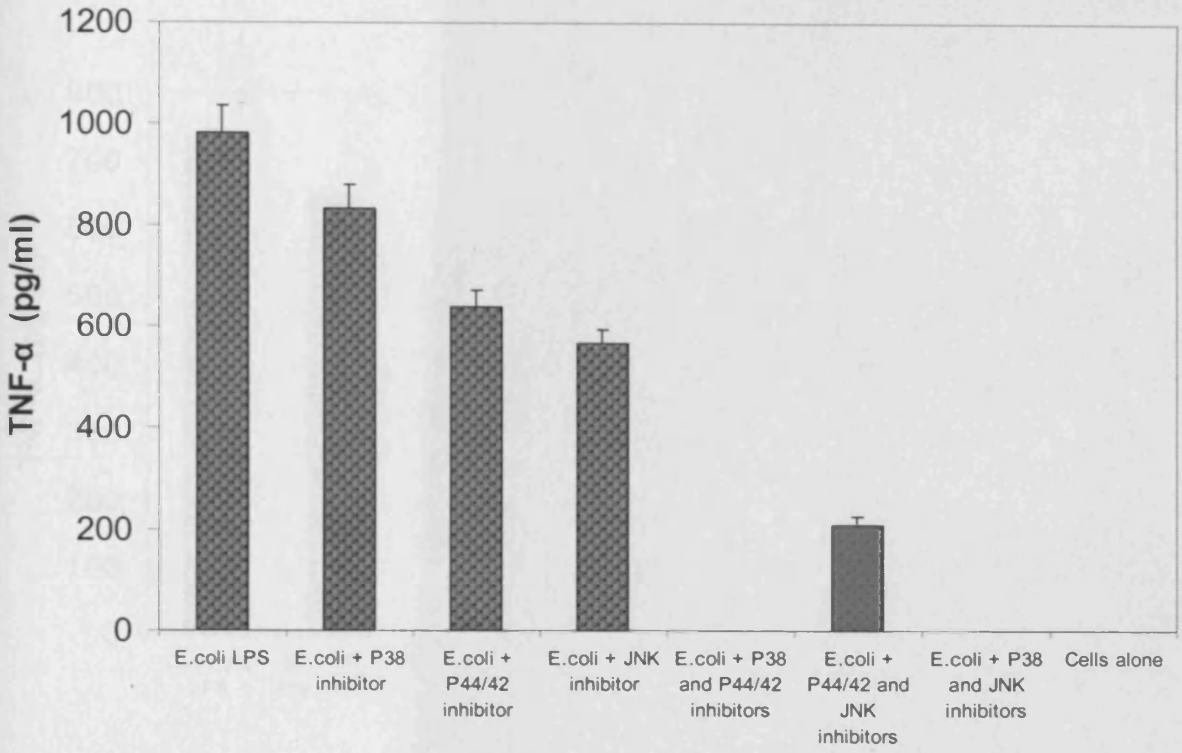

Figure 3.6.1. MM6 cells suspended at $1x10^6$ cells/ml were incubated with either 10 μM of the MAPK inhibitors PD98059 (P42/44 inhibitor), SB203580 (P38 inhibitor) or SP600125 (JNK inhibitor) or a combination of inhibitors, for 1 hour before stimulation with LPS for 6 hours. The level of TNF-α was measured by ELISA. Values are means ±SD of three independent experiments.

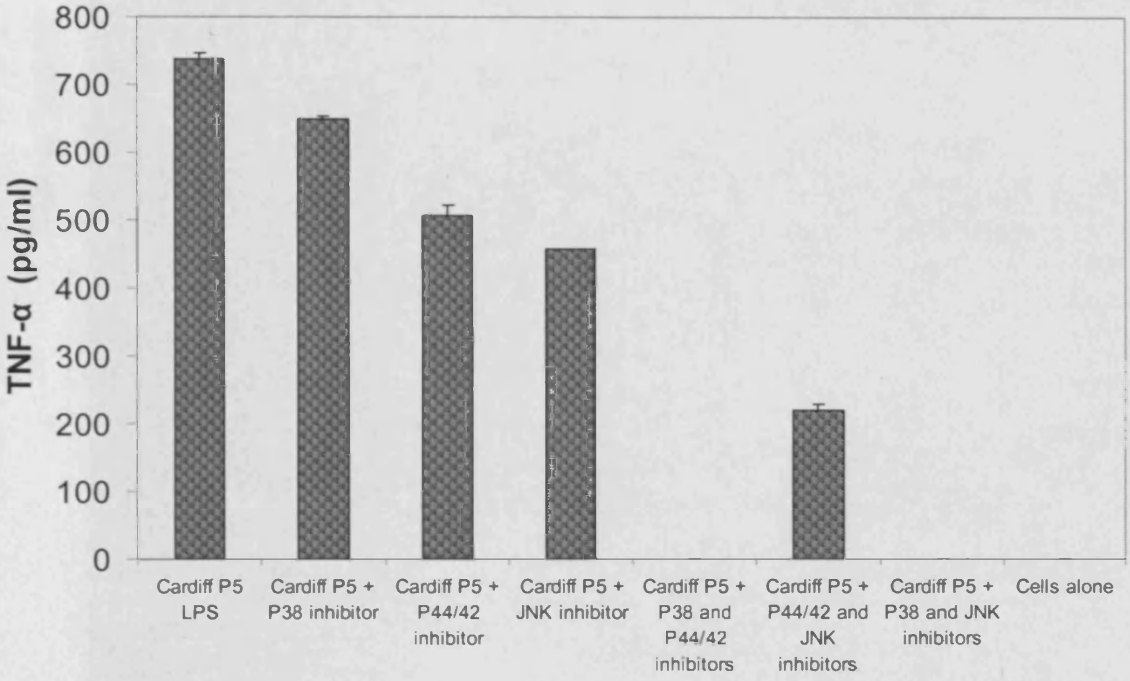

Figure 3.6.2. MM6 cells suspended at $1x10^6$ cells/ml were incubated with either 10 μM of the MAPK inhibitors PD98059 (P42/44 inhibitor), SB203580 (P38 inhibitor) or SP600125 (JNK inhibitor) or a combination of inhibitors, for 1 hour before stimulation with LPS for 6 hours. The level of TNF-α was measured by ELISA. Values are means ±SD of three independent experiments.

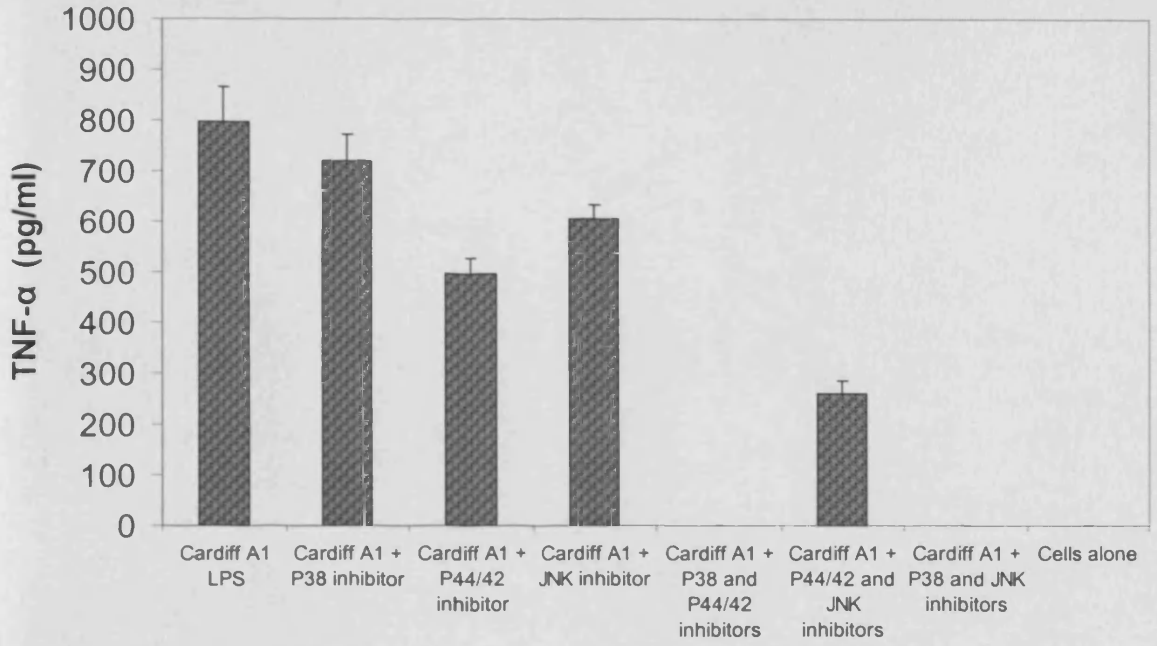

Figure 3.6.3. MM6 cells suspended at $1x10^6$ cells/ml were incubated with either 10 μM of the MAPK inhibitors PD98059 (P42/44 inhibitor), SB203580 (P38 inhibitor) or SP600125 (JNK inhibitor) or a combination of inhibitors, for 1 hour before stimulation with LPS for 6 hours. The level of TNF-α was measured by ELISA. Values are means ±SD of three independent experiments.

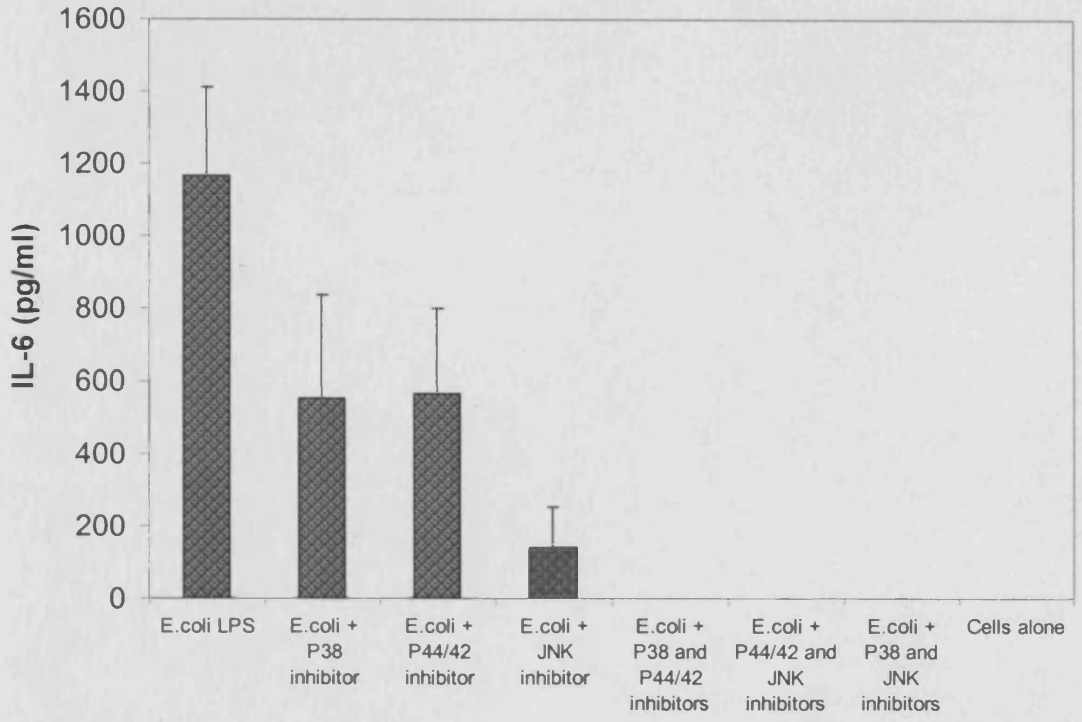

Figure 3.6.4. MM6 cells suspended at $1x10^6$ cells/ml were incubated with either 10 μM of the MAPK inhibitors PD98059 (P42/44 inhibitor), SB203580 (P38 inhibitor) or SP600125 (JNK inhibitor) or a combination of inhibitors, for 1 hour before stimulation with LPS for 6 hours. The level of IL-6 was measured by ELISA. Values are means ±SD of three independent experiments.

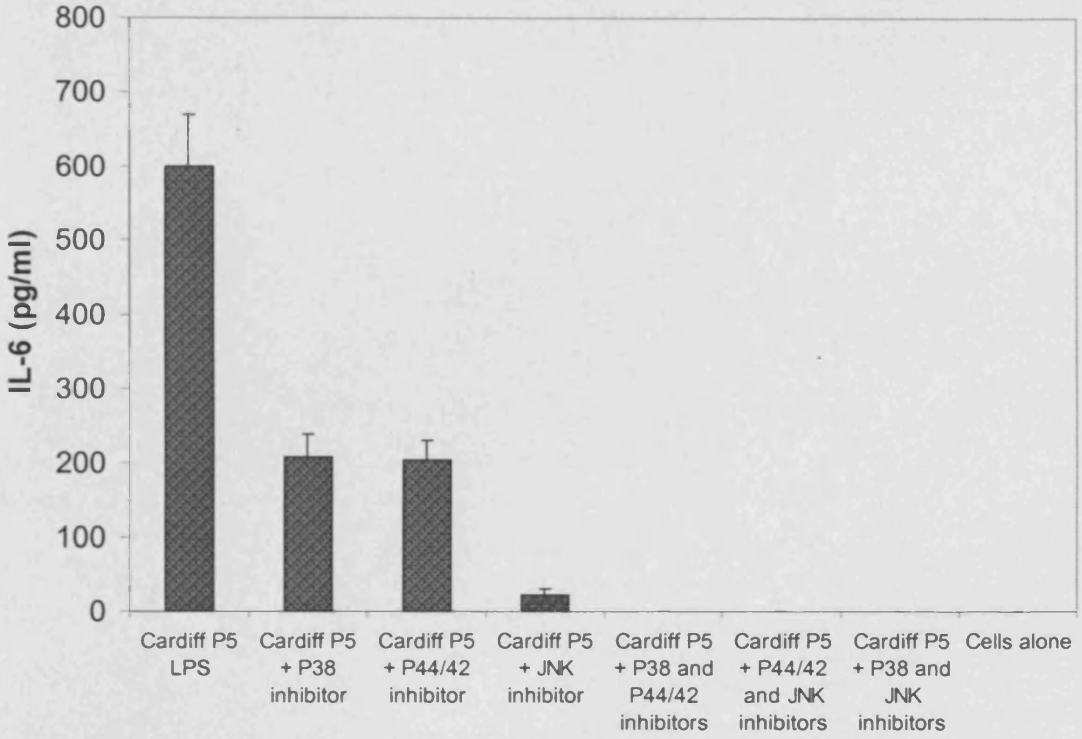

Figure 3.6.5. MM6 cells suspended at $1x10^6$ cells/ml were incubated with either 10 μM of the MAPK inhibitors PD98059 (P42/44 inhibitor), SB203580 (P38 inhibitor) or SP600125 (JNK inhibitor) or a combination of inhibitors, for 1 hour before stimulation with LPS for 6 hours. The level of IL-6 was measured by ELISA. Values are means ±SD of three independent experiments.

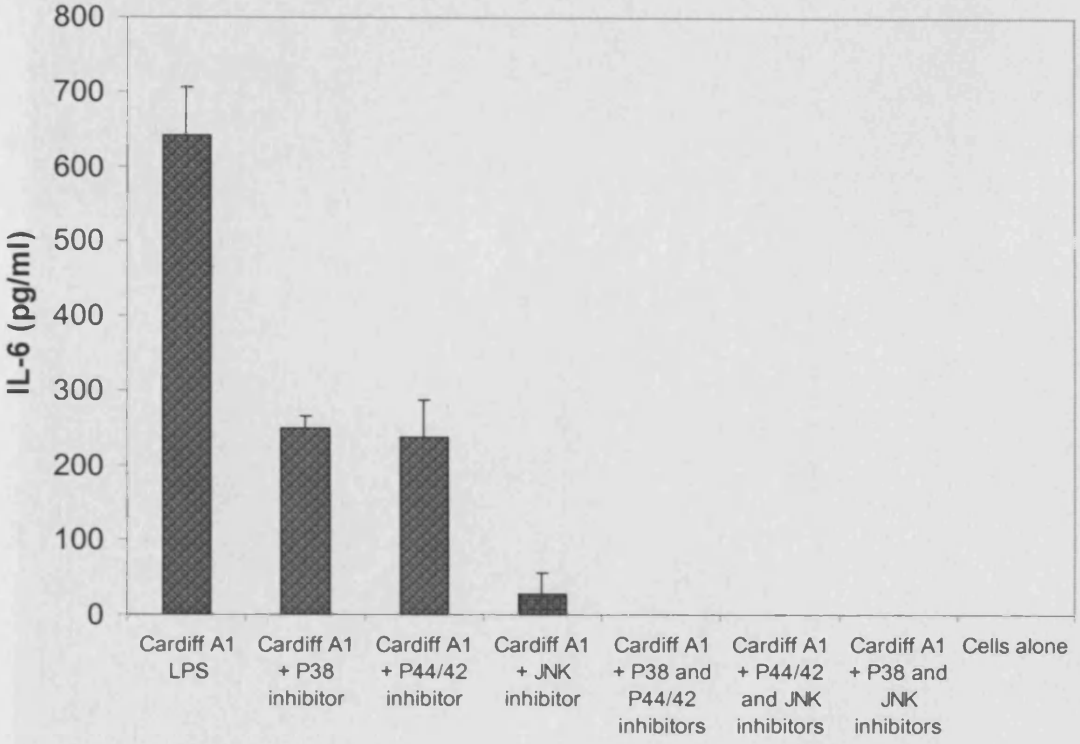

Figure 3.6.6. MM6 cells suspended at $1x10^6$ cells/ml were incubated with either 10 μM of the MAPK inhibitors PD98059 (P42/44 inhibitor), SB203580 (P38 inhibitor) or SP600125 (JNK inhibitor) or a combination of inhibitors, for 1 hour before stimulation with LPS for 6 hours. The level of IL-6 was measured by ELISA. Values are means ±SD of three independent experiments.

3.7. Assessment of IκB and NF-κB activation by *B.cepacia* complex LPS

3.7.1. IκB activation by *B.cepacia* complex LPS

IκB is an inhibitor protein for the transcription factor NF-κB. When IκB dissociates NF-κB translocates into the nucleus, therefore activation of IκB by LPS would suggest that NF-κB is also being activated.

In order to find out whether *B.cepacia* complex LPS activates IκB MM6 cells were stimulated with 1 μg LPS for 1 hour before protein extraction and Western blot analysis for IκB, the IκB antibody only bound when proteolysis and phosphorylation of the molecule had not occurred. Equal amounts of protein were loaded onto each lane for each blot. *E.coli*, Cardiff A1 and Cardiff P5 all show less intense bands (Figures 3.7.1. and 3.7.2.) than the non-stimulated control cell bands, this may indicate that when the cells are stimulated with LPS from all the bacterial isolates tested, IκB undergoes proteolysis and phosphorylation and is therefore in an activated state. As IκB is the inhibitor protein for NF-κB it is reasonable to assume that if these purified LPS samples are activating IκB then they are also activating NF-κB. This theory was tested using electrophoretic mobility shift assay (EMSA) analysis (section 3.7.2.).

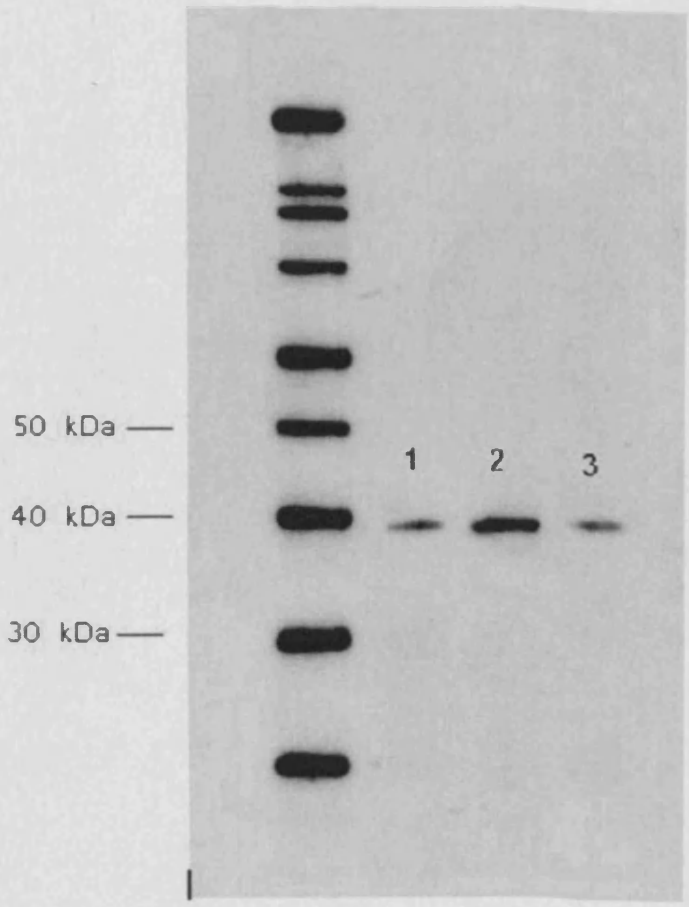

Figure 3.7.1. Western blot of IκB in MM6 ($1x10^6$/ml) cells stimulated by 1 μg *B.cepacia* complex LPS for 1 hour. Lanes: 1- E.coli. 2- non-stimulated control, 3- Cardiff P5. Bands correspond to 37 kDa.

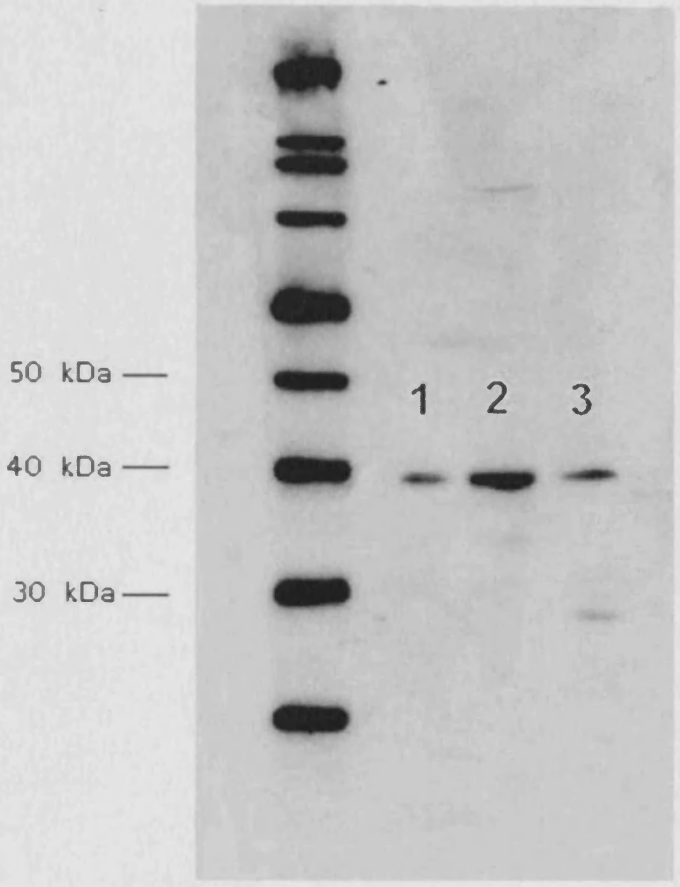

Figure 3.7.2. Western blot of IκB in MM6 ($1x10^6$/ml) cells stimulated by 1 μg *B.cepacia* complex LPS for 1 hour. Lanes: 1- Cardiff A1, 2- non-stimulated control, 3- E.coli. Bands correspond to 37 kDa.

3.7.2. NF-κB by *B.cepacia* complex LPS

NF-κB activation by stimulation with *B.cepacia* complex LPS was tested using an electrophoretic mobility shift assay (EMSA, gel shift). MM6 cells were stimulated with 1 μg/ml of LPS from *B.cenocepacia*, *B.multivorans* at various time intervals (15, 30, 60, and 120 min), subcellular (nuclear) fractions were then prepared and incubated with a ^{32}P-labelled oligonucleotide containing the consensus sequence for NF-κB. The fractions were then run on a 5% polyacrylamide gel and autoradiographed overnight at -80°C. Figures 3.7.3. and 3.7.2. clearly show both the Cardiff P5 and Cardiff A1 stimulated cell samples have NF-κB in the nucleus, and that there is a progressive increase in level of activation over the time period tested.

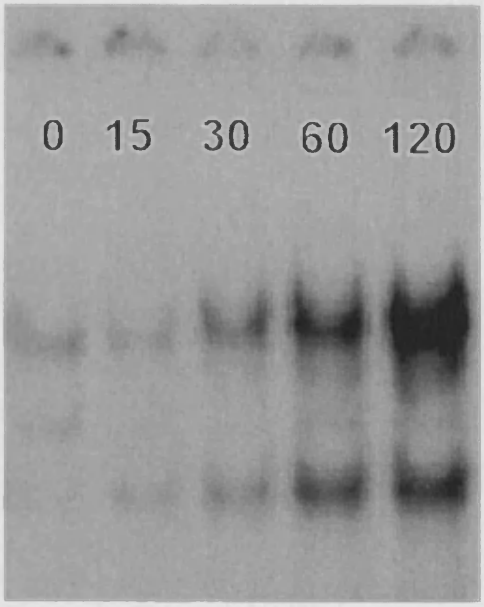

Figure 3.7.3. MM6 cells (10^6/ml) stimulated with 1 μg Cardiff P5 LPS over 0-120 minutes and tested for NF-κB activation by electrophoretic mobility shift assay (EMSA). Upper bands shown are NF-κB present in the nuclear extract that has interacted with probe, the lower bands are free DNA.

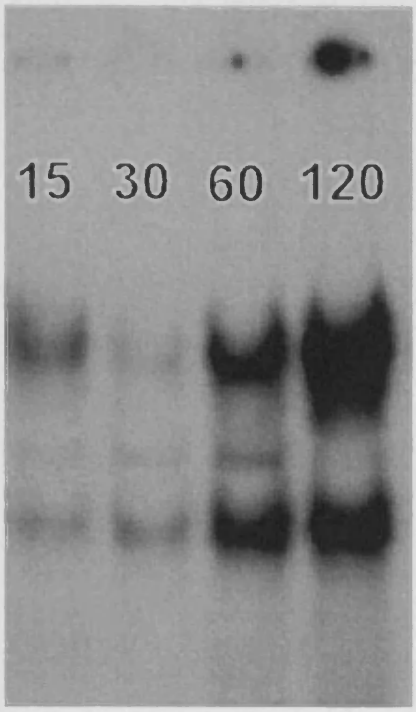

Figure 3.7.4. MM6 cells (10^6/ml) stimulated with 1 μg Cardiff A1 LPS over 0-120 minutes and tested for NF-κB activation by EMSA. Upper bands shown are NF-κB present in the nuclear extract that has interacted with probe, the lower bands are free DNA.

Discussion

4.1. Extraction of LPS and optimisation of experiments.

Bcc species are recognised as opportunistic pathogens in humans, with the majority of infection found in CF patients. Although they are not as commonly isolated as other CF pathogens such as *P.aeruginosa*, organisms from the Bc complex have a major clinical impact. The clinical course following Bcc infection follows a variable pattern which ranges from no discernable change in clinical status and lung function, to a rapid decline in lung function which is associated with necrotizing pneumonia, sepsis and death- this is known as 'cepacia syndrome'.

Although pulmonary inflammation is a critical element of host defence, it also contributes significantly to the lung damage that is a hallmark of CF. Pathogens that are capable of inducing an intense and sustained inflammatory response are likely to add to the severity of lung disease in CF. Bacterial products from both *P. aeruginosa* and members of the Bcc stimulate an excessive and prolonged pro-inflammatory response in epithelial cells and/or monocytes; however, products, specifically LPS, from Bcc species are more potent inducers of this response than *P. aeruginosa* (Shaw *et al*; 1995; Zughaier *et al*; 1999a; Kube *et al* 2001).

Little is known about the molecular mechanisms involved during Bcc infection. This study aimed to elucidate the role of BcLPS as a virulence factor in infection by investigating the inflammatory response it invokes and the intracellular signalling pathway that it triggers.

LPS samples were purified using phenol-chloroform petroleum ether (PCP) extraction, these samples were then split into two groups, one of which was purified further using DNase, RNase, trypsin and proteinase K. These two LPS preparations named BcLPS-1 and BcLPS-2 were then compared against each other in their capacity to elicit an inflammatory response from monocytes and airway epithelial cells.

A study by Shimomura *et al* (2001) showed that there was a significant difference between enzyme treated and non-enzyme treated Bcc LPs preparations ability to produce an inflammatory response in C3H/HeN murine macrophages and suggested that the large response seen from Bcc LPS was due to protein contamination. It was important to eliminate the possibility that inflammatory response from cells was due to contamination by bacterial components other than LPS such as DNA or proteins like flagellin, which have been shown to stimulate a cytokine response (Hayashi *et al* 2001; Urban *et al* 2004).

This study did not find any significant difference in production of TNF-α or IL-6 from human monocytes or IL-8 from human epithelial cells when stimulated with BcLPS-1 compared to BcLPS-2. This would suggest that purification using the standard PCP method alone is enough to remove any protein or nucleic acid contaminants that may

cause an inflammatory response and that the subsequent enzyme treatments may not be necessary to obtain a pure LPS preparation.

The differences seen in the reduction in inflammatory response, when LPS is further purified, seen between the study by Shimomura *et al* (2001) and this study may be attributed to the species origin of the cells used. It has been shown that bacterial DNA, which is a probable contaminant in PCP purified LPS preparations, can provoke an inflammatory response and that this can be attributed to the presence of unmethylated CpG dinucleotides (Chuang *et al* 2002; Hartman & Krieg 2000). Responses to CpG motifs are mediated by toll-like receptor 9 (TLR9) (Coenye & Vandamme 2005) and differences in human and murine TLR9 specificity might explain why murine cells are stimulated by small amounts of Bcc DNA that is present in LPS preparations whereas human cells are not. Also it has been shown that human alveolar macrophages and several other human cell types express very little TLR9 making them unresponsive to bacterial DNA CpG motifs (Suzuki *et al* 2005).

During this study, commercial preparations of *E.coli* O111:B4 LPS were used as a comparison to BcLPS because *E.coli* LPS has been investigated intensively as a "typical" LPS that has high endotoxic activity. The lyophilised commercial *E.coli* LPS is purified using the standard PCP method, in order to assure a meaningful comparison the LPS was also further treated with the same enzymes used to purify BcLPS. The original LPS preparation was then compared against the further purified sample and again, there were no significant difference in the ability of the two samples to stimulate TNF-α production from MM6 cells. This ensured that the commercial LPS was indeed free of protein and nucleic acid contaminants and

therefore a suitable comparison to the highly purified BcLPS. The use of highly purified LPS preparations was fundamental to this study and ensured that the inflammatory responses being seen were the result of BcLPS alone, however in order to ensure purity, mass spectrometry would have to be conducted on all of the samples purified.

4.2. Biological activity of BcLPS

Exposure to LPS results in an inflammatory response *in vivo* and involves the activation of macrophages and dendritic cells with release of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 (Broad *et al* 2006). Considerable evidence is now emerging that LPS from Bcc species is an important virulence factor that stimulates the inflammatory response in Bcc infections (Hughes *et al* 1997; Zughaier *et al* 1999a,b; Ryley 2003; Mahenthiralingam *et al* 2005). Some studies have shown that Bcc bacteria in the form of whole cell lysates, or purified LPS can elicit up to 10 times more TNF-α from immune cells as equivalent treatments with LPS from other lung pathogens including *P.aeruginosa* (Shaw *et al* 1995; Hughes *et al* 1997; Zughaier *et al* 1999a). Although it has been argued that these high levels of cytokine production maybe due to contaminants present due to the LPS purification procedure, this study has used a highly purified LPS preparations, treated to remove nucleic acid and protein contaminants, and has found that BcLPS from certain strains can produce nearly as much TNF-α, IL-6 and IL-8 as the highly potent LPS from enteric *E.coli*.

Response to BcLPS in this study was carried out using well characterised human cell lines- MM6 (human monocytic cell line) and A549 (human airway epithelial cells) which have been previously been shown to be good models (Ziegler-Heitbrock 1994; Zughaier *et al* 1999a), this is discussed in more detail below. Many studies have used murine models and cell lines which may not be validly comparable to human cells as recent studies have shown that murine cells show differences in Toll-like receptor expression compared with human cells (Rehli 2002). In detail, the current differences in TLR4 and TLR2 expression are as follows: the predominant TLR4 expressing cells

in both species are of the myeloid origin monocytes, macrophages, microglia, myeloid dendritic cells and granulocytes (Lehnardt *et al* 2002; Hornung *et al* 2002). In human tissues, TLR4 is expressed most strongly in the spleen and on PBLs, weaker levels of expression are detected in most other tissues with the exception of liver, in which TLR4 mRNA levels are low to undetectable (Zarember & Godowski 2002). Murine TLR4 mRNA appears most strongly expressed in the lung, heart and spleen, with strong presence in muscle, liver and kidney (Qureshi *et al* 1999), indicating that the basal tissue expression might not be identical. In addition, human and murine TLR4 genes acquired different exons during evolution, which are subject to alternative splicing. Both exons theoretically induce an early stop codon, probably leading to non-functional or very short peptide chains in human and murine cells, respectively. Expression levels of TLR4 transcripts appear to be modified differently on stimulation with the major TLR4 agonist LPS: in human monocytes and macrophages, TLR4 expression increases on activation (Muzio *et al* 2000), whereas in murine macrophages, TLR4 transcripts are down-regulated following cellular activation with LPS (Matsuguchi *et al* 2000; Poltorak *et al* 1998). There are also differences seen in TLR2 expression, for instance its presence in murine, but not in human, thymus and T-cells (Haehnel *et al* 2002). TLR2 mRNA expression is low or undetectable in murine blood cells and is strongly induced by pro-inflammatory cytokines or microbial activators (e.g LPS) (Matsuguchi *et al* 2000). In humans however the highest constitutive levels of TLR2 mRNA expression have been observed in peripheral blood leukocytes (PBLs) (Rock *et al* 1998). In human monocytes, TLR2 mRNA is further up-regulated after adherence to tissue culture plates but no additional induction is observed after short-term stimulation with bacterial products (Muzio *et al* 2000). Induction of TLR2 mRNA is weak or non-

existant after stimulation of human macrophages or macrophage cell lines differentiated *in vitro* (Zarember & Godowski 2002). These differences in expression may explain the variability in inflammatory responses seen in cells of human and murine origin. Therefore in order to gain a relevant view on the responses seen in human disease it is important to utilize human cells.

The cell lines chosen in this study may have had an impact on the results seen, they were chosen for several reasons as explained below, but further work on other cells would have to be conducted to draw further conclusions from the results seen here.

MM6 cells are human peripheral blood monocytes derived from acute monocytic leukaemia, they were chosen for this study as they are a robust well characterised cell line and have been shown to be a good model for cytokine production when stimulated with bacterial cell wall products (Ziegler-Heitbrock et al 1992; Quentmeier et al 1996). They are relevant to the response seen in cepacia syndrome as the condition culminates in sepsis.

A549 cells are carcinomic human alveolar basal epithelial cells. In addition to its barrier function, the airway epithelium plays an important role for the immune response in the lung. The lung epithelium is a major source of cytokine, chemokines and other inflammatory mediators that affect the adaptive and innate immune response and therefore modulate inflammatory. The species origin was an important factor in choosing the cell line used, murine cells are often used as a model for LPS signalling, this may be unrepresentative of what would happen in a human cell line due to differential expression of TLR receptors in murine cells.

In order to gain a greater understanding of the inflammatory responses seen in CF patients it would be necessary to use a CF cell line, these are available as immortalised cells, for example CFT43 cells which are immortalised airway epithelial cells.

The responses seen in these cells could be compared to 'normal' non-CF airway cells in order to observe any difference in response to BcLPS.

Limitations of cell lines are primarily that they are derived from an isolated subpopulation and may not always express phenotypic characteristics of the principal cell type of origin. Primary cell cultures may be more representative of cells *in vivo* but they have a limited life span, will not have been characterised as well as immortalised lines and are more difficult to obtain

During this study LPS from 5 different *B.cenocepacia* ET12 strains, 1 *B.cenocepacia* Type B, 2 *B.multivorans* and 2 environmental *B.cepacia* strains were initially tested in their ability to stimulate a cytokine response from MM6 and from A549 cells to gain a broad view of their inflammatory activity. The quantity of samples tested was dictated by the amount of time it took to purify the LPS from these species to a satisfactory level. LPS derived from clinical samples of Bcc were seen to elicit a much greater inflammatory response in both cell lines tested, than LPS derived from *P.aeruginosa* and from environmental *B.cepacia* strains, this is in agreement with several other studies (Shaw *et al* 1995; Zughaier *et al* 1999a; DeSoyza *et al* 2004). The same pattern of inflammatory activity from BcLPS was seen in TNF-α and IL-6 production from MM6 cells and in IL-8 production from A549 cells.

There was seen to be a great variability in the inflammatory activity of LPS purified from different Bcc isolates tested in this study. LPS derived from clinical isolates of Bcc (*B.cenocepacia* and *B.multivorans*) induced more TNF-α and IL-6 from stimulated monocytes, than the environmental *B.cepacia* strain. Outside of *B. cenocepacia* and *B. multivorans*, the remaining Bcc species account for less than 10% of all CF infections caused by the complex (Reik *et al* 2005) and so study of these species is of particular relevance.

A study by Ernst *et al* (1999) found structural differences in the lipid A component of LPS, the key determinant of pro-inflammatory activity, from *P.aeruginosa* isolated from the airways of CF patients compared with LPS from isolates from other patients and sites. A recent study (DeSoyza *et al* 2004) found different acylation patterns between *B.cenocepacia* and *B.multivorans* which they used to explain the differences in inflammatory activities between the whole cell lysates of these species, however only one lipid A was analysed from each strain. It has been shown that acylation status of lipid A can determine the inflammatory activity of *P.aeruginosa* LPS, human monocytes respond to very low levels of hexa-acylated lipid A but require much higher concentrations of penta-acylated lipidA (Hajjar *et al* 2002). The same may be true of Bcc lipid A and this may need further investigation by mass spectrometry of purified BcLPS, this would be an important next step for further study as it may shed light on the difference in inflammatory response seen through infection with different strains of Bcc bacteria.

B.cenocepacia is seen as the most virulent species of the Bc complex and is more frequently associated with 'cepacia syndrome' than any other strain. Typically

survival rates for patients infected with *B.cenocepacia* are lower than those infected with *B.multivorans* (LiPuma *et al* 2001). Mahenthiralingam *et al* reported a higher mortality among CF patients with *B cenocepacia* in Vancouver than patients with *B multivorans*. (Mahenthiralingam *et al* 2001) We found that a *B.multivorans* strain produced as high a TNF-α and IL-6 level as the most potent *B.cenocepacia* strain tested, these results are in contrast to a report by DeSoyza and coworkers (DeSoyza *et al* 2004) who found that whole cell lysates of *B.cenocepacia* were more potent cytokine stimulators than lysates of *B.multivorans* when used to stimulate U937 monocytes. However the whole cell lysates used in this study may stimulate cytokine release in monocytes by multiple non-LPS factors, some of which have been shown to bind to CD14 (Ryley 2004; Urban *et al* 2004)

Although pre-transplant infection with *B.cenocepacia* is reported to have a worse outcome than *B.multivorans* in patients undergoing pulmonary transplant (DeSoyza *et al* 2001), growing evidence shows that *B.multivorans* infection is also associated with poor clinical prognoses in CF patients (Baldwin *et al* 2008) in addition *B.cenocepacia* has been seen to the causative agent of 'cepacia syndrome' (Zahariadis *et al* 2003).

During this study LPS from 6 *B.cenocepacia* strains 5 of which were ET12 strains, were tested for inflammatory activity. There was variability in cytokine induction capability between the LPS isolated from within these identical strains. The ET12 strain of *B.cenocepacia* has been associated with the transmission of Bcc infections between patients in different CF clinics and a poor clinical outcome (Ledson *et al* 1998b; Ryley & Doull 2003). All ET12 samples produced high levels of both TNF-α and IL-6 as compared to *P.aeruginosa*, however there was seen to be 2 isolates that

produced almost twice as much inflammatory cytokine than the others. The LPS from Cardiff P1 was seen to be a less effective cytokine producer than the other ET12 strains. This isolate was the index case from which the *B.cenocepacia* ET12 strain spread to nine other patients over a 10 year period. This patient acquired the infection following a stay in a summer camp in Ontario, Canada in 1987. Molecular typing by *recA* gene sequence variation analysis and pulsed field gel electrophoresis (PFGE) has confirmed that there are no genetic differences between these isolates from different patients and from the original environmental strain from which they have arisen (Mahenthiralingam *et al* 2005).

Variability in cytokine producing ability of BcLPS isolated from the clonal *B.cenocepacia* ET12 strains may have been due to passaging over time in the laboratory. Gram negative bacteria including *P.aeruginosa* can modulate the structure of their LPS on invasion of host tissues in order to avoid detection and reduce inflammatory response, therefore inflammatory potential of LPS may revert when the bacteria is cultured *ex vivo* over time (Ernst *et al* 1999).

In order to see whether this was the case 2 *B.cenocepacia* ET12 samples were cultured and re-cultured in the laboratory a total of 20 times, the cytokine producing ability was tested every 5 passages. No difference in the ability of the LPS to produce inflammatory cytokines was seen over this time. This suggests that the inflammatory activity of the LPS samples were not affected by laboratory culturing and that the variability seen between these samples may not be attributed to culturing in a laboratory environment.

The variation in the pro-inflammatory activity of the LPS isolated from this strain and the variable clinical outcome in different patients carrying this strain may suggest that there are patient- or centre-derived factors and/or subtle patient-specific phenotypic changes in the LPS that mediate these clinical and inflammatory differences. The results seen in this study would also suggest that it is not possible to conclude that all ET12 clones will behave in the same way when tested in experimental models. The non ET12 strain of *B.cenocepacia* tested was relatively potent but did not produce as much TNF-α and IL-6 as the top ET12 producers. This theory would have to be tested by including a much larger panel of bacterial strains including more ET12 clones.

In order to assess whether the variability in the inflammatory response seen from purified ET12 BcLPS was isolated to Cardiff, other ET12 clones sourced from around the UK were investigated. The differences seen in the *B.cenocepacia* ET12 clones were not limited to isolates from the Cardiff CF clinic. ET12 isolates were collected from CF clinics from around the UK and LPS was purified using the same method as the Cardiff samples. TNF-α levels were measured from monocytes and again there was seen to be variability in the pro-inflammatory activity of the LPS preparations. The Manchester ET12 strain showed a TNF-α output almost as great as *E.coli* whereas the Liverpool ET12 showed a much lower level. The data suggests that there is no relation between the pro-inflammatory activity of LPS isolated and the proximity of the CF clinics that the samples were isolated from, a wide variation in inflammatory activity was seen from these samples collected from around the country.

Presence of an intact O-antigen on LPS indicates a smooth-type LPS whereas lack of O-antigen indicates a rough-type LPS. Environmental BcLPS samples were tested by silver staining along with LPS derived from a *B.cenocepacia* ET12 strain and a *B.multivorans* to ascertain whether the LPS was of a rough or smooth chemotype BcLPS1 and BcLPS2 preparations were both included which proved that the extra purification process of LPS had no effect on the O-antigen. The environmental samples were seen to smooth (intact O-antigen) and the clinical samples were rough (O-antigen lacking). These samples were then tested for their capacity to elicit a TNF-α response in monocytes. The smooth samples produced a much lower level of TNF-α than the rough samples tested. This has been seen in a study by Bäckhed *et al* (2003) where smooth LPS isolated from *E.coli* was seen to be 100-fold less potent than rough LPS at inducing an IL-8 response from the human epithelial cell line T24. In another study by Rittig *et al* (2003), *brucellae* organisms with a rough LPS type were seen to induce considerably higher amounts of cytokines and chemokines in stimulated human monocytes than *brucellae* with a smooth LPS type, however this effect could not be replicated with purified LPS. However due to the sample size in this study and the variability seen in the TNF-α producing ability of our rough BcLPS samples, no relationship is indicated between the rough and smooth samples of BcLPS and their ability to incite an inflammatory response. *In vivo*, shedding the O-antigen may be a response by Bcc organisms to avoid the adaptive immune response by the host, rough LPS may therefore been seen as an advantage. The lipid A component of LPS is essential to the integrity of the bacterial membrane whereas the O-antigen is not, another reason for shedding this component may be that it is metabolically demanding and non-essential for viability.

4.3. *B.cepacia* complex LPS inactivation by Polymyxin B (Pmx B)

Polymyxin is a cationic peptide protein which has similarities in its structure to defensins which are the host antimicrobial peptides. Resistance to PMB in Bcc could be an important virulence factor for these bacteria. Polymyxin is bactericidal for Gram-negative bacteria and the mode of action comprises two steps, binding and permeabilisation of the outer membrane and subsequent induction and leakage of cytoplasmic components across the cytoplasmic membrane. It has been well established that PMB has a high affinity for negatively charged cell surface components and that it's binding to LPS plays an essential role in its bactericidal activity (Vaara 1992).

Typical LPSs have one to three molecules of 3-deoxy-D*manno*-octulosonic acid (KDO) in the inner core region attached to the lipid A moiety, and the lipid A-KDO-KDO structure is well conserved among a wide range of bacterial species. In the inner core region of the LPS of *B. cepacia*, a unique component, D-*glycero*-D-*talo*-2-octulosonic acid (KO), has been found in the structure of lipid A-KDO-KO, and it has been shown that the KO is partially replaced at the C-8 position with 4-amino-4-deoxy-L-arabinose (Ara4N) (Shimomura *et al* 2003). PMB is also known as a potent LPS antagonist that suppresses various biological activities of LPS, such as lethal toxicity and induction of cytokines, by forming PMB-LPS complexes with electrostatic and hydrophobic interactions. The presence of Ara4N with a positive charge that reduces the electronegativity of LPS is considered to be responsible for the decreased binding of PMB, causing LPS to be resistant to the antagonistic action of PMB (Coyne & Fenwick 1993).

Binding of PMB was investigated in a bioassay system where the effect of stimulating TNF-α from MM6 cells was neutralized by PMB, the cells were checked for viability before and after the procedure to ensure that the PMB was not killing the cells and reducing TNF-α output. The results indicated that PMB neutralized Bcc LPS and inhibited TNF-α production in a dose dependant manner which was similar to that seen in *E.coli* and *P.aeruginosa*. This suggests that BcLPS and LPS from the PMB sensitive bacterial strains have a similar binding affinity to PMB and that PMB resistance in Bcc species may not be due to a decreased binding of the antibiotic to LPS. Coyne and Fenwick (1993) showed similar results which indicated that LPS induced TNF-α production in alveolar macrophages was inhibited by PMB. These results may suggest that the resistance of Bcc species to PMB is not due to the binding of the antibiotic to LPS, and that there is another system in place such as efflux pumps or enzymatic inactivation that allows the bacteria resistance to PMB.

When the LPS concentration used to stimulate MM6 cells was increased there was an interesting effect seen with the *B.multivorans* LPS, TNF-α was no longer inhibited in a dose dependant manner and the highest doses of PMB did not inhibit cytokine production as efficiently as had been expected. It may be that this *B.multivorans* strain has a more complete replacement of KO with Ara4N in the lipid A region of LPS rendering it less negatively charged than the LPS from other species, this may inhibit the binding of PMB in a more complex manner than other structures of lipid A. This theory could only be confirmed with mass spectrometry.

4.4. Respiratory Burst in BcLPS primed monocytes

Macrophages and monocytes produce reactive oxygen species (ROS) in the respiratory burst in response to engulfed bacteria or bacterial products. The generated reactive oxygen radicals which help to kill phagocytosed bacteria are transformed to water by host detoxifying enzymes (Zughaier *et al* 1999b). However, excess production of reactive oxygen radicals can cause tissue damage, which may be particularly important in the lungs (Brown & Kelly 1994). LPS, while not triggering the respiratory burst directly, has been shown to up regulate this oxidative response to other stimuli and therefore to act as a priming agent. The potent priming activity of Bcc LPS for neutrophils has been described by Hughes *et al* (1997). They suggested that the increased neutrophil recruitment and respiratory burst responses in the lungs may contribute to inflammation by the release of tissue-damaging proteolytic enzymes and reactive oxygen species. In an attempt to further characterise the inflammatory activity of BcLPS, its ability to prime monocytes for respiratory burst was investigated. Release of ROS causes tissue damage and may account for the ability of BcLPS to cause the massive inflammatory reaction that distinguishes it from *P.aeruginosa* LPS.

In this study monocytes were primed with BcLPS, *P.aeruginosa* and *E.coli* LPS and then stimulated with opsonised zymozan, which is a yeast cell wall product, to trigger oxidative burst, superoxide anion is then detected. BcLPS samples which had previously produced very high levels of TNF-α when monocytes were directly stimulated did not produce the highest levels of ROS. It was found that the *B.multivorans* LPS and Cardiff P6 (*B.cenocepacia* non-ET12) tested produced higher

levels of superoxide anion than *E.coli* LPS, these were the only times in this study that BcLPS isolates were found to produce a greater response than *E.coli* LPS.

It is suggested that, from the samples tested in this study, the induction of the inflammatory response from BcLPS stimulated monocytes is separate and in some cases, may be opposite to the superoxide anion release seen from BcLPS primed monocytes. Respiratory burst is a mechanism that is aimed at directly and rapidly killing the invading pathogen and provoking this response may be considered disadvantageous to the survival of the bacteria in the host, bacteria that did not strongly provoke this response may have a chance to colonise whereas those that due are eradicated.

The interesting discrepancy between the ability of BcLPS to stimulate a cytokine response directly and its ability to prime for respiratory burst would benefit from future investigation; this would have to include a much larger sample size to allow statistical analyses to be conducted. There are several methods available to measure respiratory burst in primed cells superoxide production is usually quantified spectrophotometrically by measuring ferricytochrome c reduction however this method lacks sensitivity. The chemiluminescent detection method that was utilised in this study to investigate respiratory burst is much more sensitive but may lack a high specificity for superoxide and the sensitivity can be reduced further by redox cycling reactions which themselves generate superoxide. There are several other methods in development, that can be employed but which also have their drawbacks including the need for specialised machinery and cost (Filatov *et al* 1995; Tan & Berridge 2000).

4.5. Signalling Pathway activated by BcLPS

Due to the inherent variation in inflammatory response produced by LPS from different Bcc species and strains used in this study, it was important to analyse the molecular mechanisms utilized by the different LPS isolates in stimulating these responses. The signalling pathways utilized by LPS from enteric organisms such as *E.coli* has been well documented, at the cell surface, LPS from classical enterobacteria first binds to CD14, a GPI anchor or soluble protein that delivers the LPS to TLR4 associated with an accessory molecule MD2. Subsequent recruitment of the intracellular adaptor protein MyD88 through homotypic Toll/IL-1R domains leads to intracellular signalling through NF-κB and MAPKs activation (Nahori *et al* 2005). The ultimate result of this LPS recognition is a pro-inflammatory cytokine and chemokine response.

Lipid A is the anchor moiety of LPS in the bacterial membrane and is the active component of LPS responsible for its toxic activity and functions. Lipid A from *E. coli* or *Salmonella* species stimulates the TLR4 receptor complex. However, lipid A structures show some variability between bacterial species; the degree of acylation, phosphorylation, or the length of acyl chains of lipid A can be responsible for modified proinflammatory capacities of LPS (Darveau 1998). There is a growing list of atypical LPSs that utilize different receptors and pathways to the classical one stimulated by enterobacterial species, these include *Helicobacter pylori, Leptospira interoggans, Rhizobium spp*, *Legionella spp*, and *Porphyromonas gingivalis* (Nahori *et al* 2005). Little is known about the molecular pathways that underlie cell stimulation by LPS from Bcc strains.

Cytokine stimulation experiments using A549 cells tested for IL-8 production when stimulated with BcLPS, had shown that without the presence of human serum there was no IL-8 produced. A549 cells lack membrane bound CD14 (mCD14) which is the co-receptor needed for signal transduction when LPS is present (Antal-Szalmás 2000). Experiments which did not have serum present (therefore no CD14 present) were included as a control and to show that there was no other inflammatory reaction was being caused in the system. Contaminants such as flagellin, signal through TLR5 and do not require CD14 and as such would cause a reaction even when CD14 was not present. These results may indicate that BcLPS required CD14 to signal. In order to test this, CD14 blocking experiments were performed.

Blocking mCD14 on the surface of MM6 cells before stimulation with BcLPS from *B.multivorans* and *B.cenocepacia* completely inhibited the production of TNF-α, this would suggests that the signalling pathways of these LPS isolates are similar to that of enteric organisms. A valuable next step would be to use cells from mice lacking CD14 due to a targeted disruption of the CD14 gene (CD14-deficient knockout (CD14KO), this would more definitively show that BcLPS is signalling through CD14. CD14 has been shown to bind non-LPS ligands (Kurt-Jones *et al* 2000; Esen & Kielian 2005) and so it was important to investigate the LPS signalling pathway further.

The TLR4 receptor has been demonstrated to be almost exclusively used by LPS in innate immune responses (Beutler 2000; Hajjar et al 2002). However some groups have suggested that LPS from certain organisms including *Bacteroides fragilis*, *Chlamydia trachomatis* and *Pseudomonas aeruginosa*, activate cells via binding to

TLR2 (Kirschning *et al* 1998; DaSilva Correia *et al* 2001; Werts *et al* 2001; Erridge *et al* 2004)

Blocking experiments using monoclonal anti-TLR4 antibodies showed that the inflammatory activity of all *B.cenocepacia* ET12 strains tested (Cardiff P4,P5 and P6) was almost completely reduced when TLR4 was blocked. Incubation with *E.coli* LPS and with an isotype control antibody, there seen to be no decrease in TNF-α production which indicated that the anti-TLR4 antibody was specific. TNF-α production by an environmental *B.cepacia* strain and a clinical *B.multivorans* was also blocked. However inflammatory activity induced by LPS from another clinical *B.multivorans* strain was seen to be reduced only by 50% which was unexpected; however this was still a significant reduction, the result may be due to the efficacy of the blocking antibody, although the antibodies used in this study have been shown to be effective in many studies previously (Hornef *et al* 2002; Schaefer *et al* 2005; Lagoumintzis *et al* 2008). This result would have to be tested by other means, using this strain's LPS, cells from TLR4 deficient mice (for instance CH3/HeJ mice) could be stimulated and a cytokine response measure. If there is no response in $TLR4^{-/-}$ mice then this would be a much stronger indication that TLR4 is required. Blocking of the TLR2 receptor showed that the LPS from this strain had the same level of TNFα production whether TLR2 was blocked or not, which would indicate that TLR2 is not being used as a receptor for this strain or for the other BcLPS tested. Including a TLR ligand such as lipoteichoic acid would demonstrate the efficacy of the anti-TLR2 antibody. This work would benefit from being backed up with other techniques, as mentioned previously this could be done using knockout mice, the disadvantage of this would be differential expressions and sensitivities of TLRs between humans and

mice, another way of investigating the role of TLR4 and TLR2 in BcLPS signalling would be to transiently transfect HEK293T cells with the Toll receptor that was to be tested and then stimulate with BcLPS. HEK293T cells lack expression of TLRs and they can be transfected with the TLR of interest to see whether it is required for the secretion of pro-inflammatory cytokines.

4.6. MyD88 Inhibition

The next stage of the classical LPS signalling cascade is through cytosolic adaptor molecule myeloid differentiation protein 88 (MyD88)-dependent signaling, MyD88 is recruited to the TLR4 receptor through interaction with the TIR domain of TLR4 (O'Neil *et al* 2003). This complex in turn facilitates the recruitment of IRAK1 and IRAK4 via a death domain present in both molecules (Li *et al* 2002). Upon LPS stimulation, TIRAP also associates with TLR4 via a TIR–TIR interaction, and is essential for MyD88-dependent signaling (Fitzgerald *et al* 2001).

The MyD88-independent signaling pathway was identified in studies using MyD88 deficient mice. Activation of NF-κB and MAP kinases was observed in these LPS-stimulated MyD88-deficient cells with the delayed kinetics (Kawai *et al* 1999; Kawai *et al* 2001). These results suggested the existence of a MyD88-independent pathway downstream of ligand engagement of the TLR4 receptor. To account for a MyD88-independent pathway of NF-κB activation, a novel MyD88 homologue termed MyD88 adaptor-like (Mal) protein (Fitzgerald *et al* 2001) or TIR domain-containing adaptor protein (TIRAP) (Horng *et al* 2001) was described. This was shown to act as an adaptor protein specifically involved in TLR4 but not other TLRs or IL-1R–induced NF-κB activation. As Mal/TIRAP does not contain the death domain (DD) found in MyD88, this was proposed to induce an alternative signalling pathway that also involves RNA-dependent protein kinase (PKR) and leads to the induction of IFNß and interferon-inducible protein-10 (IP-10) (Fitzgerald *et al* 2001; Horng *et al* 2001) .

In this study inhibition of MyD88 in monocytes was achieved using a cell permeable inhibitor peptide, which was added before cells were stimulated with test LPS, this inhibitor mimics the MyD88 BB loop heptapeptide and has been found to very effective (Rosenberg 2007). A significant reduction in TNF-α output was seen from cells stimulated with control *E.coli* LPS and with *B.cenocepacia* ET12 LPS however the reduction was not significant when our *B.multivorans* LPS was tested.

MyD88 deficient cells from mice fail to produce inflammatory cytokines in response to LPS although these cells do show activation of NF-κB and MAP kinases in response to LPS (Kawai *et al* 1999), however a study by Hoebe *et al* (2003) showed that MyD88$^{-/-}$ murine macrophages do produce TNFα in response to LPS at lower levels and a recent study has demonstrated that LPS can indeed induce a cytokine response through the MyD88-independent pathway in human macrophages (Andreakos *et al* 2004).

The results here may suggest that LPS from *B.cenocepacia* activates these cells through MyD88 dependant pathways while LPS isolated from *B.multivorans* can activate cells through MyD88-dependent and through MyD88-independent pathways. In this regard, LPS from Bcc organisms may be similar to lipooligosaccharide from *Neisseria meningitidis* which has been found to activate both MyD88-dependant and MyD88-independent pathways in monocytes (Zughaier *et al* 2005). Such differential activation of cells might explain the differences in clinical outcomes observed in patients infected with *B.multivorans* or *B.cenocepacia* (LiPuma *et al* 2001). However the relevance of MyD88-independent signalling in human cells has yet to be established. This is of key importance given the major role of LPS in the pathology of

sepsis. Further work would have to be done here to firmly establish whether this BcLPS sample does indeed signal through the MyD88-independent pathway, this could be done using a MyD88 knockout mouse or another suggested way of doing this would be using RNA interference (RNAi), this technique inhibits gene expression at the stage of translation or can hinder the transcription of specific genes. Small interfering RNA strands (siRNA) could be used to inhibit gene expression of MyD88, this would allow testing of BcLPS in cells of human origin and give a clearer result as to whether the MyD88 independent pathway is being triggered. The disadvantage to this technique would be time taken to optimise and cost of the procedure.

4.7. Activation of MAPK pathways and NF-κB activation

Members of the mitogen activated protein kinase (MAPK) family are critically involved in intracellular signal transduction, mediating cell responses to a variety of different infectious and inflammatory stimuli, including LPS. In vitro, typical LPS from *E.coli* has been shown to activate p38 MAPK, c-jun N-terminal kinase (JNK and p42/44 MAPK (Hambleton *et al* 1996; Huh *et al* 2007; Lim *et al* 2002). One study found that LPS from *E.coli* activated p38 MAPK and p42/44 MAPK but not JNK in peripheral blood mononuclear cells (PBMCs) (van den Blink *et al* 2001).

Activation of MAPKs was initially investigated using Western blotting to ascertain whether specific MAPKs were being phosphorylated when cells were stimulated with LPS samples, the results from this indicated that P38 and P44/42 were being activated by all the BcLPS samples tested. However this technique just indicated whether activation was occurring and comparison of amount of activation could not be ascertained without densitometry. Also finding an effective antibody was difficult with all three MAPK families and expensive. Therefore signalling pathways involved in cell activation were investigated by analysing the roles played by these three MAP kinases that are known to be activated by stimulation with LPS by using specific inhibitors of MAP kinases. These compounds have been extensively tested and used in the last few years as research tool to elucidate intracellular signalling pathways and have been found to be effective and specific (Davies *et al* 2000; Ropert 2005). MAPK inhibitors are now even used in a clinical setting as a treatment for Crohn's disease (van denBlink 2002).

The IC_{50} (median inhibition concentration) for all the MAPK inhibitors used here ranged between 40nM and 1μM and so they are very efficient.

Inhibition of these MAP kinases using selective MAPK inhibitors individually was found to reduce but not completely inhibit TNF-α production from LPS stimulated monocytes. This was true for *E.coli* LPS and BcLPS. Inhibition using two out of three of the combinations of MAPK inhibitors reduced the TNF-α production from *E.coli* and Bcc LPS stimulated cells to zero. This suggests that Bcc LPS activates these MAP kinases in monocytes and that co-ordinate activation of MAPK pathways is required for TNF-α production. These findings are consistent with a report by Reddi *et al* (2003) which showed that whole bacterial cells of a clinical isolate of *B.cenocepacia* induced IL-8 secretion in alveolar epithelial cells through CD14 and MAPK signalling.

Inhibition using p42/44 and JNK inhibitors did not reduce TNF-α output from stimulated cells to zero as with the other combinations tested, which could be indicative of a compensation mechanism within the MAPK signalling cascade. Various investigators have observed an enhancement of p42/44 phosphorylation when specific p38 inhibitors have been used (Feng *et al* 1999; Ropert *et al* 2001) along with an increase of JNK activation. These finding may indicate a certain level of cross-talk and a compensation mechanism between the MAPKs. The results from our MAPK inhibition would suggest that there is also compensation by p38 when p42/44 MAPK and JNK are blocked. In future work the activation of various MAPKs could be ascertained by ELISA using antibodies that are specific to the phosphorylated forms of the MAPK, this may allow a quantitative result to be produced.

In order to gain a more complete view of the signalling series triggered by BcLPS, the activation of NF-κB was studied. The transcription factor, NF-κB is generally present in an inactive, cytoplasmic form bound to the inhibitory protein IκB. Cell stimulation causes dissociation of this protein and allows translocation of NF-κB to the nucleus (May & Ghosh 1998). NF-κB mediates production of numerous pro-inflammatory genes, including TNF-α.

Investigation through Western blotting showed that the BcLPS isolates tested including *B.cenocepacia* and *B.multivorans*, activated IκB. This strongly suggested that NF-κB was also being activated, to confirm this EMSA analysis was performed on the BcLPS stimulated cell samples. Results confirmed that NF-κB was being translocated to the nucleus when the cells were stimulated with BcLPS and the strength of the bands increased over the times tested. NF-κB activation was seen for the LPS from *B.cenocepacia* and for LPS from the *B.multivorans* isolate.

This study demonstrates that LPS isolated from strains of Bc complex bacteria and then highly purified, provokes a high production the inflammatory cytokines TNF-α and IL-6 from a monocytic cell line and IL-8 from an epithelial cell line, and that these levels are in some cases much greater than those stimulated by *P.aeruginosa* and comparable to the prototypical, highly inflammatory *E.coli* LPS. Conclusions and comparisons drawn from the work here are based on a small panel of bacterial samples and this is due to the lengthy, and often failure prone, process of purification to ensure that minimal contaminants are present. This is a process that is often missing from studies on bacterial products. It can be seen that the samples tested are

highly inflammatory and that there is variation in this activity between the Cardiff isolates tested and between isolates from various UK CF centres.

The work done was conducted in non-CF cells of monocytic and epithelial origin and it may be of benefit in future studies to conduct work in CF cell lines or from primary cells of CF and non-CF origin, although it may be difficult to obtain these cells.

The cell signalling work conducted in this study indicates that BcLPS signals through TLR4 and not TLR2 and that the intracellular signalling cascade is similar to that of *E.coli*. As mentioned previously, further studies would have to be conducted to back up the evidence presented here, ideally utilizing a knockout or RNAi system and these methods may indicate more definitively the pathway being triggered, and may shed more light as to whether there are any BcLPS samples that utilize the MyD88-independent pathway.

The development of novel therapies for sepsis depends on the understanding of the basic mechanisms of the disease including elucidating any signalling cascades that the causative agent, LPS, activates. The results from this study indicate that the LPS from all Bcc species studied activate monocytes by conventional LPS signalling pathways and there is no difference that would explain the enhanced inflammatory activity of BcLPS. However LPS from Bcc species is much more inflammatory than LPS isolated from *P.aeruginosa*. A study by Ernst *et al* (1999) found structural differences in the lipid A component of LPS, the key determinant of pro-inflammatory activity, from *P.aeruginosa* isolated from the airways of CF patients compared with LPS from isolates from other patients and sites Human monocytes respond to very low levels of

hexa-acylated lipid A but require much higher concentrations of penta-acylated lipid A (Hajjar *et al* 2002). The same may be true of Bcc lipid A and this may need further investigation. A recent study (DeSoyza *et al* 2004) found different acylation patterns between *B.cenocepacia* and *B.multivorans* which they used to explain the differences in inflammatory activities between the whole cell lysates of these species, however only one lipid A was analysed from each strain. It is possible that the differences in inflammatory activity from different purified BcLPS samples may be down to structural differences in the molecule and further study would be aimed at elucidating the relationship between lipid A structure and inflammatory activity in Bcc organisms. This would involve purifying many more strains of Bcc LPS sourced from different centres around the UK, then detailed mass spectrometry of the BcLPS samples would have to be compared to the inflammatory activity of the sample, this may allow a way of predicting which strains are likely to be the most inflammatory and therefore the most damaging in the host.

Conclusion

LPS extracted for various clinical and environmental Bcc isolates confirmed that LPS elicits a massive inflammatory response from monocytes and epithelial cells. The use of highly purified LPS confirmed that the effect seen was not due to contaminants present in the LPS preparation such as flagellin or bacterial DNA.

BcLPS stimulated the release of greater amounts of cytokine on a weight for weight basis than LPS from *P.aeruginosa*, BcLPS from certain isolates were seen to be as inflammatory as *E.coli* LPS.

Variability in pro-inflammatory potential of LPS from different Bcc species and even within clonal strains was seen and this difference was not due to prolonged culturing in the laboratory. It is possible that modifications made by the bacteria, particularly structurally to the lipid A region, are responsible for the differences seen. Polymyxin B was seen to inhibit the inflammatory effect of BcLPS which strongly suggests that PMB is binding to BcLPS, therefore the PMB resistance that is seen in Bcc species may not be due to the lack of PMB binding the unusual structure of BcLPS.

Bcc species LPS was found to have a higher priming activity in monocytes for the respiratory burst response in comparison to LPS from *P.aeruginosa*. It was found that the ability to prime for respiratory burst was not related to the direct pro-inflammatory activity of LPS and in some cases was opposite. This may be due to the respiratory burst being a direct killing mechanism by the host and would be considered disadvantageous to bacterial colonisation.

The results from this study indicate that LPS from all species studied activate monocytes by conventional LPS signalling pathways, utilizing CD14 and TLR4, by activating all three families of MAP kinases and prompting the translocation of NF-κB to the nucleus. It was found that the *B.multivorans* LPS tested, may activate monocytes via a MyD88-independent pathway whereas the *B.cenocepacia* LPS tested, activated through a MyD88-dependant pathway.

The results of this investigation support the argument that BcLPS is an important virulence factor in CF lung disease. It provokes a large inflammatory response from human monocytes and epithelial cells and there is great variability in this response between species and even within different clonal strains. This may suggest that there are structural differences in the lipid A region such as differences in acylation status and other factors or pathways involved in the response to BcLPS (Trent 2004) which are responsible for the inflammatory effect. Therefore future studies should be aimed at elucidating the structure of BcLPS and relating this to the inflammatory activity of the molecule.

References

ADAMO, R., SOKOL, S., SOONG, G., GOMEZ, M. & PRINCE, A. (2004) Pseudomonas aeruginosa flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor 5. *Am J Respir Cell Mol Biol,* 30, 627-34.

ADEREM, A. & ULEVITCH, R. (2000) Toll-like receptors in the induction of the innate immune response. *Nature,* 406, 782-7.

AHMAD, R., SYLVESTER, J., ZAFARULLAH, M. (2007) MyD88, IRAK1 and TRAF6 knockdown in human chondrocytes inhibits interleukin-1-induced matrix metalloproteinase-13 gene expression and promoter by impairing MAP kinase activation. *Cellular signalling.* 19 , 2549-2557.

AKASHI, S., SHIMAZU, R., OGATA, H., NAGAI, Y., TAKEDA, K., KIMOTO, M. & MIYAKE, K. (2000) Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. *J Immunol,* 164, 3471-5.

AKIRA, S., TAKEDA, K. & KAISHO, T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol,* 2, 675-80.

AKIRA, S., YAMAMOTO, M. & TAKEDA, K. (2003) Role of adapters in Toll-like receptor signalling. *Biochem Soc Trans,* 31, 637-42.

ALDALLAL, N., MCNAUGHTON, E., MANZEL, L., RICHARDS, A., ZABNER, J., FERKOL, T. & LOOK, D. (2002) Inflammatory response in airway epithelial cells isolated from patients with cystic fibrosis. *Am J Respir Crit Care Med,* 166, 1248-56.

ALESSI, B. (1995) PD098059 is a specific inhibitor of the activation of mitogen

activated protein kinase kinase in vitro and in vivo. *J Biol Chem.* 270, 27489-27494.

ALLEN, R., STJERNHOLM, R. & STEELE, R. (1972) Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. *Biochem Biophys Res Commun,* 47, 679-84.

ANDERSON, D., KUHNS, J., VASIL, M., GERDING, D. & JANOFF, E. (1991) DNA fingerprinting by pulsed field gel electrophoresis and ribotyping to distinguish Pseudomonas cepacia isolates from a nosocomial outbreak. *J Clin Microbiol,* 29, 648-9.

ANDREAKOS, E., SACRE, S., SMITH, C., LUNDBERG, A., KIRIAKIDIS, S., STONEHOUSE, T., MONACO, C., FELDMANN, M. & FOXWELL, B. (2004) Distinct pathways of LPS-induced NF-kappa B activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. *Blood,* 103, 2229-37.

ANTAL-SZALMÁS, P. (2000) Evaluation of CD14 in host defence. *Eur J Clin Invest,* 30, 167-79.

ANTAL-SZALMAS, P., POPPELIER, M., SUMEGI, A., VAN DER BRUGGEN, T., VERHOEF, J., VAN KESSEL, K. & VAN STRIJP, J. (2004) Spare CD14 molecules on human monocytes enhance the sensitivity for low LPS concentrations. *Immunol Lett,* 93, 11-5.

ANWAR, H., BROWN, M., COZENS, R. & LAMBERT, P. (1983) Isolation and characterization of the outer and cytoplasmic membranes of Pseudomonas cepacia. *J Gen Microbiol,* 129, 499-507.

ARIS, R., ROUTH, J., LIPUMA, J., HEATH, D. & GILLIGAN, P. (2001) Lung transplantation for cystic fibrosis patients with Burkholderia cepacia complex. Survival linked to genomovar type. *Am J Respir Crit Care Med,* 164, 2102-6.

ARMSTRONG, D., GRIMWOOD, K. & CARLIN, J. (1998) Severe viral respiratory infections in infants with cystic fibrosis. *Pediatr Pulmonol* 26, 371-9.

BACKHED, F., NORMARK, S. & RICHTER-DAHLFORS, A. (2002) TLR4-dependent lipopolysaccharide signalling in epithelial cells is independent of extracellular protease activity. *Cell Microbiol.*, 4, 297-303.

BACKHED, F., NORMARK, S., SCHWEDA, E., OSCARSON, S. & RICHTER-DAHLFORS, A. (2003) Structural requirements for TLR4-mediated LPS signalling: a biological role for LPS modifications. *Microbes Infect,* 5, 1057-63.

BALDURSSON, O., OSTEDGAARD, L., ROKHLINA, T., COTTEN, J. & WELSH, M. (2001) Cystic fibrosis transmembrane conductance regulator Cl- channels with R domain deletions and translocations show phosphorylation-dependent and -independent activity. *J Biol Chem,* 276, 1904-10.

BALDWIN, A., MAHENTHIRALINGAM, E., DREVINEK, P. & POPE, C. (2008) Elucidating global epidemiology of Burkholderia multivorans in cases of cystic fibrosis by multilocus sequence typing. *J Clin Microbiol,* 46, 290-5.

BARTON, G. & MEDZHITOV, R. (2003) Toll-like receptor signaling pathways. *Science.*, 300, 1524-5.

BAUERNFEIND, A., BERTELE, R., HARMS, K., HÖRL, G., JUNGWIRTH, R., PETERMÜLLER, C., PRZYKLENK, B. & WEISSLEIN-PFISTER, C. (1987) Qualitative and quantitative microbiological analysis of sputa of 102 patients with cystic fibrosis. *Infection,* 15, 270-7.

BAZIL, V. & STROMINGER, J. (1991) Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. *J Immunol,* 147, 1567-74.

BECKMAN, W. & LESSIE, T. (1979) Response of Pseudomonas cepacia to beta-Lactam antibiotics: utilization of penicillin G as the carbon source. *J Bacteriol,* 140, 1126-8.

BERGER, M. (2002) Lung inflammation early in cystic fibrosis: bugs are indicted, but the defense is guilty. *Am J Respir Crit Care Med,* 165, 857-8.

BEUTLER, B. (2000) Tlr4: central component of the sole mammalian LPS sensor. *Curr Opin Immunol,* 12, 20-6.

BEUTLER, B. (2001) Sepsis begins at the interface of pathogen and host. *Biochem Soc Trans.,* 29, 853-9.

BEUTLER, B., HOEBE, K., DU, X. & ULEVITCH, R. (2003a) How we detect microbes and respond to them: the Toll-like receptors and their transducers. *J Leukoc Biol,* 74, 479-85.

BEUTLER, B. & RIETSCHEL, E. (2003b) Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol.,* 3, 169-76.

BEVIVINO, A., DALMASTRI, C., TABACCHIONI, S., CHIARINI, L., BELLI, M., PIANA, S., MATERAZZO, A., VANDAMME, P. & MANNO, G. (2002) Burkholderia cepacia complex bacteria from clinical and environmental sources in Italy: genomovar status and distribution of traits related to virulence and transmissibility. *J Clin Microbiol.,* 40, 846-51.

BOCHUD, P. & CALANDRA, T. (2003) Pathogenesis of sepsis: new concepts and implications for future treatment. *BMJ,* 326, 262-6.

BONFIELD, T., KONSTAN, M. & BERGER, M. (1999) Altered respiratory epithelial cell cytokine production in cystic fibrosis. *J Allergy Clin Immunol,* 104, 72-8.

BONFIELD, T., KONSTAN, M., BURFEIND, P., PANUSKA, J., HILLIARD, J. &

BERGER, M. (1995) Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis. *Am J Respir Cell Mol Biol,* 13, 257-61.

BORASCHI, D. & TAGLIABUE, A. (2006) he interleukin-1 receptor family. *Vitam Horm,* 74.

BROAD, A., JONES, D. & KIRBY, J. (2006) Toll-like receptor (TLR) response tolerance: a key physiological "damage limitation" effect and an important potential opportunity for therapy. *Curr Med Chem,* 13, 2487-502.

BROWN, R. & KELLY, F. (1994) Evidence for increased oxidative damage in patients with cystic fibrosis. *Pediatr Res,* 36, 487-93.

BURKHOLDER, W. (1949) Sour skin, a bacterial rot of onion bulbs. *Phytopathology,* 40, 115-117.

BYLUND, J., CAMPSALL, P., MA, R., CONWAY, B. & SPEERT, D. (2005) Burkholderia cenocepacia induces neutrophil necrosis in chronic granulomatous disease. *J Immunol,* 174, 3562-9.

CAMPANA, S., TACCETTI, G., RAVENNI, N., FAVARI, F., CARIANI, L., SCIACCA, A., SAVOIA, D., COLLURA, A., FISCARELLI, E., DE INTINIS, G. & BUSETTI, M. (2005) Transmission of Burkholderia cepacia complex: evidence for new epidemic clones infecting cystic fibrosis patients in Italy. *J Clin Microbiol,* 43, 5136-42.

CARIO, E., ROSENBERG, I., BRANDWEIN, S., BECK, P., REINECKER, H. & PODOLSKY, D. (2000) Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol,* 164, 966-72.

CARSON, L., FAVERO, M., BOND, W. & PETERSEN, N. (1973) Morphological, biochemical, and growth characteristics of pseudomonas cepacia from distilled

water. *Appl Microbiol,* 25, 476-83.

CAZZOLA, G., AMALFITANO, G., TONOLLI, E., PERAZZOLI, C., PIACENTINI, I. & MASTELLA, G. (1996) Burkholderia (Pseudomonas) cepacia epidemiology in a cystic fibrosis population: a genome finger-printing study. *Acta Paediatr,* 85, 554-7.

CFF, U. (2007) About cystic fibrosis- cystic fibrosis foundation. http://www.cff.org/AboutCF

CHAUDRY, G., NAVARRO, O., LEVINE, D. & OUDJHANE, K. (2006) Abdominal manifestations of cystic fibrosis in children. *Pediatr Radiol,* 36, 233-40.

CHEN, Z., GIBSON, T., ROBINSON, F., SILVESTRO, L., PEARSON, G., XU, B., WRIGHT, A., VANDERBILT, C. & COBB, M. (2001) MAP kinases. *Chem Rev,* 101, 2449-76.

CHENG, H. & LESSIE, T. (1994) Multiple replicons constituting the genome of Pseudomonas cepacia 17616. *J Bacteriol,* 176, 4034-42.

CHERNISH, R. & SD, A. (2003) Approach to resistant gram-negative bacterial pulmonary infections in patients with cystic fibrosis. *Curr Opin Pulm Med,* 9, 509-515.

CHIARINI, L., BEVIVINO, A., DALMASTRI, C., TABACCHIONI, S. & VISCA, P. (2006) Burkholderia cepacia complex species: health hazards and biotechnological potential. *Trends Microbiol,* 14, 277-86.

CHMIEL, J. & DAVIS, P. (2003) State of the art: why do the lungs of patients with cystic fibrosis become infected and why can't they clear the infection? *Respir Res,* 4.

CHOW, J., YOUNG, D., GOLENBOCK, D., CHRIST, W. & F, G. (1999) Toll-like

receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem.,* 274, 10689-92.

CHUANG, T., LEE, J., KLINE, L., MATHISON, J. & ULEVITCH, R. (2002) Toll-like receptor 9 mediates CpG-DNA signaling. *J Leukoc Biol,* 71, 538-44.

CLODE, F., KAUFMANN, M., MALNICK, H. & PITT, T. (2000) Distribution of genes encoding putative transmissibility factors among epidemic and nonepidemic strains of Burkholderia cepacia from cystic fibrosis patients in the United Kingdom. *J Clin Microbiol,* 38, 1763-6.

COENYE, T., VANDAMME P. (2005) Overrepresentation of immunostimulatory CpG motifs in Burkholderia genomes. *J Cyst Fibros,* 4, 193-6.

COENYE, T., VANDAMME, P (2007) *Burkholderia: Molecular Microbiology and Genomics*, Horizon Bioscience.

COENYE, T., VANDAMME, P., GOVAN, J. & LIPUMA, J. (2001) Taxonomy and identification of the Burkholderia cepacia complex. *J Clin Microbiol.,* 39, 3427-36.

COHEN, J. (2002) The immunopathogenesis of sepsis. *Nature,* 420, 885-91.

CONESE, M. & ASSAEL, B. (2001) Bacterial infections and inflammation in the lungs of cystic fibrosis patients. *Pediatr Infect Dis J,* 20, 207-213.

COURTNEY, J., ENNIS, M. & ELBORN, J. (2005) Cytokines and inflammatory mediators in cystic fibrosis. *J Cyst Fibros,* 3, 223-31.

COX, A., WRIGHT, J., LI, J., HOOD, D., MOXON, E. & RICHARDS, J. (2003) Phosphorylation of the lipid A region of meningococcal lipopolysaccharide: identification of a family of transferases that add phosphoethanolamine to lipopolysaccharide. *J Bacteriol.,* 185, 3270-7.

COYNE, C. & FENWICK, B. (1993) Inhibition of lipopolysaccharide-induced

macrophage tumor necrosis factor-alpha synthesis by polymyxin B sulfate. *Am J Vet Res,* 54, 305-14.

CUSCHIERI, J. (2004) Implications of lipid raft disintegration: enhanced anti-inflammatory macrophage phenotype. *Surgery.,* 136, 169-75.

DA SILVA CORREIA, J., SOLDAU, K., CHRISTEN, U., TOBIAS, P. & ULEVITCH, R. (2001) Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. *J Biol Chem,* 276, 21129-35.

DANIELS, C., GRIFFITHS, C., COWLES, B. & LAM, J. (2002) Pseudomonas aeruginosa O-antigen chain length is determined before ligation to lipid A core. *Environ Microbiol.,* 4, 883-97.

DARVEAU, R. (1998) Lipid A diversity and the innate immune response to bacterial infection. *Curr Opin Microbiol.* 1, 36-42.

DAVIES, S., REDDY, H., CAIVANO, M. & COHEN, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J,* 351, 95-105.

DE MAAGD, R. & LUGTENBERG, B. (1987) Outer membranes of gram-negative bacteria. *Biochem Soc Trans,* 15, 54s-62s.

DE SOYZA, A., ELLIS, C., KHAN, C., CORRIS, P. & DEMARCO DE HORMAECHE, R. (2004) Burkholderia Cenocepacia Lipopolysaccharide, Lipid A and Pro-inflammatory Activity. *Am J Respir Crit Care Med,* 170, 70-77.

DE SOYZA, A., MCDOWELL, A., ARCHER, L., DARK, J., ELBORN, S., MAHENTHIRALINGAM, E., GOULD, K. & CORRIS, P. (2001) Burkholderia cepacia complex genomovars and pulmonary transplantation outcomes in patients with cystic fibrosis. *Lancet,* 358, 1780-1.

DEVANEY, J., GREENE, C., TAGGART, C., CARROLL, T., O'NEILL, S. & MCELVANEY, N. (2003) Neutrophil elastase up-regulates interleukin-8 via toll-like receptor 4. *FEBS Lett,* 544, 129-32.

DIMANGO, E., RATNER AJ, BRYAN R, TABIBI S, PRINCE A (1998) Activation of NF-kappaB by adherent Pseudomonas aeruginosa in normal and cystic fibrosis respiratory epithelial cells. *J Clin Invest,* 101, 2598-605.

DIMANGO, E., ZAR, H., BRYAN, R. & PRINCE, A. (1995) Diverse Pseudomonas aeruginosa gene products stimulate respiratory epithelial cells to produce interleukin-8. *J Clin Invest,* 96, 2204-10.

DING, A., PORTEU, F., SANCHEZ, E. & NATHAN, C. (1990) Shared actions of endotoxin and taxol on TNF receptors and TNF release. *Science,* 248, 370-2.

DIXON, D. & DARVEAU, R. (2005) Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid a structure. *J Dent Res.,* 84, 584-95.

DUNZENDORFER, S., LEE, H., SOLDAU, K. & TOBIAS, P. (2004) TLR4 Is the Signaling but Not the Lipopolysaccharide Uptake Receptor. *J Immunol,* 173, 1166-70.

EGAN, T., DETTERBECK, F., MILL, M., BLEIWEIS, M., ARIS, R., PARADOWSKI, L., RETSCH-BOGART, G. & MUELLER, B. (2002) Long term results of lung transplantation for cystic fibrosis. *Eur J Cardiothorac Surg,* 22, 602-9.

ERNST, R., YI, E., GUO, L., LIM, K., BURNS, J., HACKETT, M. & MILLER, S. (1999) Specific lipopolysaccharide found in cystic fibrosis airway Pseudomonas aeruginosa. *Science,* 286, 1561-5.

ERRIDGE, C., PRIDMORE, A., ELEY, A., STEWART, J. & POXTON, I. (2004)

Lipopolysaccharides of Bacteroides fragilis, Chlamydia trachomatis and Pseudomonas aeruginosa signal via Toll-like receptor 2. *J Med Microbiol,* 53, 735-40.

ESEN, N. & KIELIAN, T. (2005) Recognition of Staphylococcus aureus-derived peptidoglycan (PGN) but not intact bacteria is mediated by CD14 in microglia. *J Neuroimmunol,* 170, 93-104.

EUZEBY, J. (2008) List of Prokaryotic names with Standing in Nomenclature Formerly List of Bacterial names with Standing in Nomenclature (LBSN). http://www.bacterio.cict.fr/

FAN, J., KAPUS, A., MARSDEN, P., LI, Y., OREOPOULOS, G., MARSHALL, J., FRANTZ, S., KELLY, R., MEDZHITOV, R. & ROTSTEIN, O. (2002) Regulation of Toll-like receptor 4 expression in the lung following hemorrhagic shock and lipopolysaccharide. *J Immunol.,* 168, 5252-9.

FAN, J., LI, Y, LEVY, R., FAN, J. (2007) Hemorrhagic shock induces NAD(P)H oxidase activation in neutrophils: role of HMGB1-TLR4 signaling. *Journal of Immunology.* 178, 6573-6580.

FARINA, C., THEIL, D., SEMLINGER, B., HOHLFELD, R. & MEINL, E. (2004) Distinct responses of monocytes to Toll-like receptor ligands and inflammatory cytokines. *Int Immunol,* 16, 799-809.

FENG, G., GOODRIDGE, H., HARNETT, M., WEI, X., NIKOLAEV, A., HIGSON, A. & LIEW, F. (1999) Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. *J Immunol,* 163, 6403-12.

FENTON, M. & GOLENBOCK, D. (1998) LPS-binding proteins and receptors. *J Leukoc Biol,* 64, 25-32.

FERRERO, E., JIAO, D., TSUBERI, B., TESIO, L., RONG, G., HAZIOT, A. & GOYERT, S. (1993) Transgenic mice expressing human CD14 are hypersensitive to lipopolysaccharide. *Proc Natl Acad Sci U S A,* 6.

FERRERO, R. & PHILPOTT, D. (2005) Nod1 signaling to extracellular mucosal bacteria in epithelial cells. *International congress series* 1285, 68-77.

FILATOV, M., VARFOLOMEEVA, E., IVANOV, E. (1995) Flow cytofluorometric detection of inflammatory processes by measuring respiratory burst reaction of peripheral blood neutrophils. *Biochemical and molecular medicine.* 55, 116-121.

FIORE, A., LAEVENS, S., BEVIVINO, A., DALMASTRI, C., TABACCHIONI, S., VANDAMME, P. & CHIARINI, L. (2001) Burkholderia cepacia complex: distribution of genomovars among isolates from the maize rhizosphere in Italy. *Environ Microbiol,* 3, 137-43.

FITZGERALD, K., PALSSON-MCDERMOTT, E., BOWIE, A., JEFFERIES, C., MANSELL, A., BRADY, G., BRINT, E., DUNNE, A., GRAY, P., HARTE, M., MCMURRAY, D., SMITH, D., SIMS, J., BIRD, T. & O'NEILL, L. (2001) Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature.,* 413, 78-83.

GALANOS, C., LÜDERITZ, O. & WESTPHAL, O. (1969) A new method for the extraction of R lipopolysaccharides. *Eur J Biochem,* 9, 245-9.

GAZVANI, R. & LEWIS-JONES, D. (2004) Cystic fibrosis mutation screening before assisted reproduction. *Int J Androl,* 27, 1-4.

GESSNER, A. & MORTENSEN, J. (1990) Pathogenic factors of Pseudomonas cepacia isolates from patients with cystic fibrosis. *J Med Microbiol,* 33, 115-20.

GIARD, D., AARONSON, S., TODARO, G., ARNSTEIN, P., KERSEY, J., DOSIK, H. & PARKS, W. (1973) In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst,* 51, 1417-23.

GIBSON, R., BURNS, J. & RAMSEY, B. (2003) Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med,* 168, 918-51.

GILLIGAN, P. (1991) Microbiology of airway disease in patients with cystic fibrosis. *Clin Microbiol Rev,* 4, 35-51.

GOVAN, J., DOHERTY C, GLASS S. (1987) Rational parameters for antibiotic therapy in patients with cystic fibrosis. *Infection,* 15, 300-7.

GOVAN, J., BROWN PH, MADDISON J, DOHERTY CJ, NELSON JW, DODD M, GREENING AP, WEBB AK. (1993) Evidence for transmission of Pseudomonas cepacia by social contact in cystic fibrosis. *Lancet,* 342, 15-19.

GOVAN, J., DERETIC V. (1996) Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiol Rev,* 60, 539-74.

GOVAN, J. & HARRIS, G. (1985) Typing of Pseudomonas cepacia by bacteriocin susceptibility and production. *J Clin Microbiol,* 22, 490-4.

GOYERT, S., FERRERO, E., SEREMETIS, S., WINCHESTER, R., SILVER, J. & MATTISON, A. (1986) Biochemistry and expression of myelomonocytic antigens. *J Immunol,* 137, 3909-14.

GREALLY, P., HUSSEIN, M., COOK, A., SAMPSON, A., PIPER, P. & PRICE, J. (1993) Sputum tumour necrosis factor-alpha and leukotriene concentrations in cystic fibrosis. *Arch Dis Child,* 68, 389-92.

GUHA, M. & MACKMAN, N. (2001) LPS induction of gene expression in human monocytes. *Cell Signal,* 13, 85-94.

GUILLOT, L., MEDJANE, S., LE-BARILLEC, K., BALLOY, V., DANEL, C., CHIGNARD, M. & SI-TAHAR, M. (2004) Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J Biol Chem.,* 279, 2712-8.

GUO L, LIM, K., PODUJE, C., DANIEL, M., GUNN, J., HACKETT, M. & MILLER, S. (1998) Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell,* 95, 189-98.

GUO, L., LIM, K., GUNN, J., BAINBRIDGE, B., DARVEAU, R., HACKETT, M. & MILLER, S. (1997) Regulation of lipid A modifications by Salmonella typhimurium virulence genes phoP-phoQ. *Science,* 276, 250-3.

HAEHNEL, V., SCHWARZFISCHER, L., FENTON, M. & REHLI, M. (2002) Transcriptional regulation of the human toll-like receptor 2 gene in monocytes and macrophages. *J Immunol,* 168, 5629-37.

HAJJAR, A., ERNST RK, TSAI JH, WILSON CB, MILLER SI. (2002) Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nat Immunol.,* 3, 354-9.

HAJJAR, A., O'MAHONY, D., OZINSKY, A., UNDERHILL, D., ADEREM, A., KLEBANOFF, S. & WILSON, C. (2001) Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. *J Immunol.,* 166, 15-9.

HAMANN, L., STAMME, C., ULMER, A. & SCHUMANN, R. (2002) Inhibition of LPS-induced activation of alveolar macrophages by high concentrations of LPS-binding protein. *Biochem Biophys Res Commun.,* 295, 553-60.

HAMBLETON, J., WEINSTEIN, S., LEM, L. & DEFRANCO, A. (1996) Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. *Proc Natl Acad Sci U S A,* 93, 2774-8.

HAN, J., LEE, J., BIBBS, L. & ULEVITCH, R. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science.,* 265, 808-11.

HAN, J. & ULEVITCH, R. (2005) Limiting inflammatory responses during activation of innate immunity. *Nat Immunol,* 6, 1198-205.

HART, C. & WINSTANLEY, C. (2002) Persistent and aggressive bacteria in the lungs of cystic fibrosis children. *Br Med Bull,* 61, 81-96.

HARTMANN, G. & KRIEG, A. (2000) Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J Immunol,* 164, 944-53.

HAYASHI, F., SMITH, K., OZINSKY, A., HAWN, T., YI, E., GOODLETT, D., ENG, J., AKIRA, S., UNDERHILL, D. & ADEREM, A. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature,* 410, 1099-103.

HAZIOT, A., CHEN, S., FERRERO, E., LOW, M., SILBER, R. & GOYERT, S. (1988) The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. *J Immunol,* 141, 547-52.

HAZIOT, A., FERRERO, E., LIN, X., STEWART, C. & GOYERT, S. (1995) CD14-deficient mice are exquisitely insensitive to the effects of LPS. *Prog Clin Biol Res. 1995;392:349-51,* 392, 349-51.

HE, Y., LI, H., ZHANG, J., HSU, C., LIN, E., ZHANG, N., GUO, J., FORBUSH, K. & BEVAN, M. (2004) The extracellular matrix protein mindin is a pattern-recognition molecule for microbial pathogens. *Nat Immunol.,* 5, 88-97.

HEINE, H., KIRSCHNING, C., LIEN, E., MONKS, B., ROTHE, M. &

GOLENBOCK, D. (1999) Cutting edge: cells that carry A null allele for toll-like receptor 2 are capable of responding to endotoxin. *J Immunol,* 162, 6971-5.

HIKI, N., BERGER, D., PRIGL, C., BOELKE, E., WIEDECK, H., SEIDELMANN, M., STAIB, L., KAMINISHI, M., OOHARA, T. & BEGER, H. G. (1998) Endotoxin Binding and Elimination by Monocytes: Secretion of Soluble CD14 Represents an Inducible Mechanism Counteracting Reduced Expression of Membrane CD14 in Patients with Sepsis and in a Patient with Paroxysmal Nocturnal Hemoglobinuria. *Infect. Immun.,* 66, 1135-1141.

HIRSCHFELD, M., WEIS, J., TOSHCHAKOV, V., SALKOWSKI, C., CODY, M., WARD, D., QURESHI, N., MICHALEK, S. & VOGEL, S. (2001) Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. *Infect Immun,* 69, 1477-82.

HITCHCOCK, P. & BROWN, T. (1983) Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol,* 154, 269-77.

HOEBE, K., DU, X., GEORGEL, P., JANSSEN, E., TABETA, K., KIM, S., GOODE, J., LIN, P., MANN, N., MUDD, S., CROZAT, K., SOVATH, S., HAN, J. & BEUTLER, B. (2003) Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature,* 424, 743-8.

HOFFMANN, J., KAFATOS, F., JANEWAY, C. & EZEKOWITZ, R. (1999) Phylogenetic perspectives in innate immunity. *Science,* 284, 1313-8.

HØIBY, N., GIWERCMAN, B., JENSEN, E., PEDERSEN, S., KOCH, C. & KHARAZMI, A. (1994) Mechanism of action of antibiotics in chronic pulmonary pseudomonas infection. *Adv Pharmacol,* 30, 53-84.

HOLLSING, A., GRANSTRÖM, M., VASIL, M., WRETLIND, B. & STRANDVIK, B. (1987) Prospective study of serum antibodies to Pseudomonas aeruginosa

exoproteins in cystic fibrosis. *J Clin Microbiol,* 25, 1868-74.

HOLMES, A., GOVAN, J. & GOLDSTEIN, R. (1998) Agricultural use of Burkholderia (Pseudomonas) cepacia: a threat to human health? *Emerg Infect Dis,* 4, 221-7.

HORNEF, M., FRISAN, T., VANDEWALLE, A., NORMARK, S., RICHTER-DAHLFORS, A. (2002) Toll-like receptor 4 resides in the golgi apparatus and colocalises with internalised lipopolysaccharide in intestinal epithelial cells. *Journal of experimental medicine.* 195, 559-570.

HORNG, T., BARTON, G. & MEDZHITOV, R. (2001) TIRAP: an adapter molecule in the Toll signaling pathway. *Nat Immunol,* 2, 835-41.

HORNUNG, V., ROTHENFUSSER, S., BRITSCH, S., KRUG, A., JAHRSDÖRFER, B., GIESE, T., ENDRES, S. & HARTMANN, G. (2002) Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol,* 168, 4531-7.

HOSHINO, K., TAKEUCHI, O., KAWAI, T., SANJO, H., OGAWA, T., TAKEDA, Y., TAKEDA, K. & AKIRA, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol,* 162, 3749-52.

HU, J., JACINTO, R., MCCALL, C. & LI, L. (2002) Regulation of IL-1 receptor-associated kinases by lipopolysaccharide. *J Immunol,* 168, 3910-4.

HU, L., WONG, P., MOORE, P. & BIAN, J. (2007) Hydrogen sulfide attenuates lipopolysaccharide-induced inflammation by inhibition of p38 mitogen-activated protein kinase in microglia. *J Neurochem,* 100, 1121-8.

HUGHES, J., STEWART, J., BARCLAY, G. & GOVAN, J. (1997) Priming of neutrophil respiratory burst activity by lipopolysaccharide from Burkholderia

cepacia. *Infect Immun.*, 65, 4281-7.

ICHIKAWA, J., NORRIS, A., BANGERA, M., GEISS, G., VAN 'T WOUT, A., BUMGARNER, R. & LORY, S. (2000) Interaction of pseudomonas aeruginosa with epithelial cells: identification of differentially regulated genes by expression microarray analysis of human cDNAs. *Proc Natl Acad Sci U S A,* 97, 9659-64.

IMLER, J. & HOFFMANN, J. (2000) Toll and Toll-like proteins: an ancient family of receptors signaling infection. *Rev Immunogenet,* 2, 294-304.

ISLES, A., MACLUSKY, I., COREY, M., GOLD, R., PROBER, C., FLEMING, P. & LEVISON, H. (1984) Pseudomonas cepacia infection in cystic fibrosis: an emerging problem. *J Pediatr,* 104, 206-10.

ISSHIKI, Y., ZAHRINGER, U. & KAWAHARA, K. (2003) Structure of the core-oligosaccharide with a characteristic D-glycero-alpha-D-talo-oct-2-ulosylonate-(2-->4)-3-deoxy-D-manno-oct-2-ulosonate [alpha-Ko-(2-->4)-Kdo] disaccharide in the lipopolysaccharide from Burkholderia cepacia. *Carbohydr Res,* 338, 2659-66.

ISSIKI, Y., KAWAHARA, K. & ZÄHRINGER, U. (1998) Isolation and characterisation of disodium (4-amino-4-deoxy-ß-L-arabinopyranosyl)-(1 8)-(D-glycero- -D-talo-octo-2-ulopyranosylonate)-(2 4)-(methyl-3-deoxy-D-manno-octo-2-ulopyranoside) onate from the lipopolysaccharide of Burkholderia cepacia. *Carbohydr. Res.* , 313, 21-27.

JACK, R., FAN, X., BERNHEIDEN, M., RUNE, G., EHLERS, M., WEBER, A., KIRSCH, G., MENTEL, R., FÜRLL, B., FREUDENBERG, M., SCHMITZ, G., STELTER, F. & SCHÜTT, C. (1997) Lipopolysaccharide-binding protein is required to combat a murine gram-negative bacterial infection. *Nature,* 389, 742-5.

JAFFÉ, A. & BUSH, A. (2001) Cystic fibrosis: review of the decade. *Monaldi Arch*

Chest Dis, 56, 240-7.

JAVIER MALLOL, D. S. I. A. T. C. R. S. M. S.-Q. (2001) The authors reply. *Pediatric Pulmonology,* 32, 264.

JOHNSON, W., TYLER, S. & ROZEE, K. (1994) Linkage analysis of geographic and clinical clusters in Pseudomonas cepacia infections by multilocus enzyme electrophoresis and ribotyping. *J Clin Microbiol,* 32, 924-30.

JONES, A. & WEBB, A. (2003) Recent advances in cross-infection in cystic fibrosis: Burkholderia cepacia complex, Pseudomonas aeruginosa, MRSA and Pandoraea spp. *J R Soc Med,* 96, 66-72.

KALIS, C., KANZLER, B., LEMBO, A., POLTORAK, A., GALANOS, C. & FREUDENBERG, M. (2003) Toll-like receptor 4 expression levels determine the degree of LPS-susceptibility in mice. *Eur J Immunol.,* 33, 798-805.

KAWAI, T., ADACHI, O., OGAWA, T., TAKEDA, K. & AKIRA, S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity,* 11, 115-22.

KAWAI, T. & AKIRA, S. (2006) TLR signaling. *Cell Death Differ,* 13, 816-25.

KAWAI, T., TAKEUCHI, O., FUJITA, T., INOUE, J., MÜHLRADT, P., SATO, S., HOSHINO, K. & AKIRA, S. (2001) Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol,* 167, 5887-94.

KEIG, P., INGHAM, E. & KERR, K. (2001) Invasion of human type II pneumocytes by Burkholderia cepacia. *Microb Pathog,* 30, 167-70.

KEIG, P., INGHAM, E., VANDAMME, P. & KERR, K. (2002) Differential invasion of respiratory epithelial cells by members of the Burkholderia cepacia

complex. *Clin Microbiol Infect,* 8, 47-49.

KENNA, D., BARCUS, V., LANGLEY, R., VANDAMME, P. & GOVAN, J. (2003) Lack of correlation between O-serotype, bacteriophage susceptibility and genomovar status in the Burkholderia cepacia complex. *FEMS Immunol Med Microbiol,* 35, 87-92.

KHAN, S., EVEREST, P., SERVOS, S., FOXWELL, N., ZÄHRINGER, U., BRADE, H., RIETSCHEL, E., DOUGAN, G., CHARLES, I. & MASKELL, D. (1998) A lethal role for lipid A in Salmonella infections. *Mol Microbiol,* 29, 571-9.

KHAN, T., WAGENER, J., BOST, T., MARTINEZ, J., ACCURSO, F. & RICHES, D. (1995) Early pulmonary inflammation in infants with cystic fibrosis. *Am J Respir Crit Care Med,* 151, 1075-82.

KIRSCHNING, C., WESCHE, H., MERRILL AYRES, T. & ROTHE, M. (1998) Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J Exp Med,* 11.

KOEHLER, D., SAJJAN, U., CHOW, Y., MARTIN, B., KENT, G., TANSWELL, A., MCKERLIE, C., FORSTNER, J. & HU, J. (2003) Protection of Cftr knockout mice from acute lung infection by a helper-dependent adenoviral vector expressing Cftr in airway epithelia. *Proc Natl Acad Sci U S A,* 100, 15364-9.

KÖHLER, D., APP, E., SCHMITZ-SCHUMANN, M., WÜRTEMBERGER, G. & MATTHYS, H. (1986) Inhalation of amiloride improves the mucociliary and the cough clearance in patients with cystic fibroses. *Eur J Respir Dis Suppl,* 146, 319-26.

KÖHLER, T., KOK, M., MICHEA-HAMZEHPOUR, M., PLESIAT, P., GOTOH, N., NISHINO, T., CURTY, L. & PECHERE, J. (1996) Multidrug efflux in intrinsic resistance to trimethoprim and sulfamethoxazole in Pseudomonas

aeruginosa. *Antimicrob Agents Chemother,* 40, 2288-90.

KONSTAN, M., HILLIARD, K., NORVELL, T. & BERGER, M. (1994) Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. *Am J Respir Crit Care Med,* 150, 448-54.

KONSTAN, M., WALENGA, R., HILLIARD, K. & HILLIARD, J. (1993) Leukotriene B4 markedly elevated in the epithelial lining fluid of patients with cystic fibrosis. *Am Rev Respir Dis,* 148, 896-901.

KOSOROK, M. R., L. ZENG, S. E. WEST, M. J. ROCK, M. L. SPLAINGARD, A. LAXOVA, C. G. GREEN, J. COLLINS, AND P. M. FARRELL. (2001) Acceleration of lung disease in children with cystic fibrosis after Pseudomonas aeruginosa acquisition. . *Pediatr. Pulmonol,* 32.

KOTLYAROV, A., NEININGER, A., SCHUBERT, C., ECKERT, R., BIRCHMEIER, C., VOLK, H. & GAESTEL, M. (1999) MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. *Nat Cell Biol,* 1, 94-7.

KOWALSKI, M. & PIER, G. (2004) Localization of cystic fibrosis transmembrane conductance regulator to lipid rafts of epithelial cells is required for Pseudomonas aeruginosa-induced cellular activation. *J Immunol.,* 172, 418-25.

KREJCÍ, E. & KROPPENSTEDT, R. (2006) Differentiation of species combined into the Burkholderia cepacia complex and related taxa on the basis of their fatty acid patterns. *J Clin Microbiol,* 44, 1159-64.

KUBE, D., SONTICH, U., FLETCHER, D. & DAVIS, P. (2001) Proinflammatory cytokine responses to P. aeruginosa infection in human airway epithelial cell lines. *Am J Physiol Lung Cell Mol Physiol,* 280, L493-502.

KURT-JONES, E., POPOVA, L., KWINN, L., HAYNES, L. & JONES, L. (2000)

Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol,* 1, 398-401.

LAEMMLI, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227, 680-5.

LAGOUMINTZIS, G., XAPLANTERI, P., DIMITRACOPOULOS, G. (2008) TNF-alpha induction by Pseudomonas aeruginosa lipopolysaccharide or slime-glycoprotein in human monocytes is regulated at the level of mitogen activated protein kinase activity: a distinct role of toll-like receptor 2 and 4. *Clinical immunology.* 67, 193-203.

LAKHANI, S. & BOGUE, C. (2003) Toll-like receptor signaling in sepsis. *Curr Opin Pediatr.,* 15, 278-82.

LAUFER, P., FINK, J., BRUNS, W., UNGER, G. & KALBFLEISCH, J. (1984) Allergic bronchopulmonary aspergillosis in cystic fibrosis. *J Allergy Clin Immunol,* 73, 44-8.

LEDSON, M., GALLAGHER, M., CORKILL, J., HART, C. & WALSHAW, M. (1998b) Cross infection between cystic fibrosis patients colonised with Burkholderia cepacia. *Thorax,* 53, 432-6.

LEDSON MJ, G. M., WALSHAW MJ. (1998a) Chronic Burkholderia cepacia bronchiectasis in a non-cystic fibrosis individual. *Thorax.,* 53, 430-2.

LEHNARDT, S., LACHANCE, C., PATRIZI, S., LEFEBVRE, S., FOLLETT, P. & JENSEN, F. (2002) The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *J Neurosci,* 22, 2478-86.

LI, S., STRELOW, A., FONTANA, E. & WESCHE, H. (2002) IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc Natl Acad Sci U S A,* 99, 5567-72.

LIM, W., MA, W., GEE, K., AUCOIN, S., NANDAN, D., DIAZ-MITOMA, F., KOZLOWSKI, M. & KUMAR, A. (2002) Distinct role of p38 and c-Jun N-terminal kinases in IL-10-dependent and IL-10-independent regulation of the costimulatory molecule B7.2 in lipopolysaccharide-stimulated human monocytic cells. *J Immunol,* 168, 1759-69.

LIOU, T., ADLER, F., FITZSIMMONS, S., CAHILL, B., HIBBS, J. & MARSHALL, B. (2001) Predictive 5-year survivorship model of cystic fibrosis. *Am J Epidemiol,* 153, 345-52.

LIPUMA, J. (1998) Burkholderia cepacia. Management issues and new insights. *Clin Chest Med,* 19, 473-86.

LIPUMA, J. (2005) Update on the Burkholderia cepacia complex. *Curr Opin Pulm Med,* 11, 528-33.

LIPUMA, J., SPILKER, T., GILL, L., CAMPBELL, P. R., LIU, L. & MAHENTHIRALINGAM, E. (2001) Disproportionate distribution of Burkholderia cepacia complex species and transmissibility markers in cystic fibrosis. *Am J Respir Crit Care Med,* 164, 92-6.

LIU, S., TOBIAS, R., MCCLURE, S., STYBA, G., SHI, Q. & JACKOWSKI, G. (1997) Removal of endotoxin from recombinant protein preparations. *Clin Biochem.,* 30, 455-63.

LLOYD-STILL, J. (1994) Crohn's disease and cystic fibrosis. *Dig Dis Sci,* 39, 880-5.

LUCHI, M. & MORRISON, D. (2000) Comparable endotoxic properties of lipopolysaccharides are manifest in diverse clinical isolates of gram-negative bacteria. *Infect Immun.,* 68, 1899-904.

LYCZAK, J., CANNON, C. & PIER, G. (2002) Lung infections associated with cystic fibrosis. *Clin Microbiol Rev,* 15, 194-222.

MAHENTHIRALINGAM, E., SIMPSON, D. & SPEERT, D. (1997) Identification and characterization of a novel DNA marker associated with epidemic Burkholderia cepacia strains recovered from patients with cystic fibrosis. *J Clin Microbiol,* 35, 808-16.

MAHENTHIRALINGAM, E., VANDAMME, P. & CAMPBELL, M. (2001) Infection with Burkholderia cepacia complex genomovars in patients with cystic fibrosis: virulent transmissible strains of genomovar III can replace Burkholderia multivorans. *Clin Infect Dis,* 33, 1469-75.

MAHENTHIRALINGAM E, B. J., BYRNE SK, RADOMSKI C, DAVIES JE, AV-GAY Y, VANDAMME P (2000) DNA-Based diagnostic approaches for identification of Burkholderia cepacia complex, Burkholderia vietnamiensis, Burkholderia multivorans, Burkholderia stabilis, and Burkholderia cepacia genomovars I and III. *J Clin Microbiol,* 38, 3165-73.

MAHENTHIRALINGAM E, U. T., GOLDBERG JB. (2005) The multifarious, multireplicon Burkholderia cepacia complex. *Nat Rev Microbiol.* , 3, 144-56.

MANNIELLO, J., HEYMANN, H. & ADAIR, F. (1979) Isolation of atypical lipopolysaccharides from purified cell walls of Pseudomonas cepacia. *J Gen Microbiol. 1979 Jun;112(2):397-400,* 112, 397-400.

MANTHEY, C. & VOGEL, S. (1994) Elimintation of trace endotoxin protein from rough chemotype LPS. *Journal of Endotoxin Research,* 1, 84-91.

MAROLDA, C., HAURODER, B., JOHN, M., MICHEL, R. & VALVANO, M. (1999) Intracellular survival and saprophytic growth of isolates from the Burkholderia cepacia complex in free-living amoebae. *Microbiology.,* 145, 1509-17.

MARSHALL, B. & CARROLL, K. (1991) Interaction between Pseudomonas aeruginosa and host defenses in cystic fibrosis. *Semin Respir Infect,* 6, 11-18.

MARTIN, D. & MOHR, C. (2000) Invasion and intracellular survival of Burkholderia cepacia. *Infect Immun,* 68, 24-9.

MATHEWS, L., SPECTOR, S., LEMM, J. & POTTER, J. (1963) STUDIES ON PULMONARY SECRETIONS. I. THE OVER-ALL CHEMICAL COMPOSITION OF PULMONARY SECRETIONS FROM PATIENTS WITH CYSTIC FIBROSIS, BRONCHIECTASIS, AND LARYNGECTOMY. *Am Rev Respir Dis,* 88, 199-204.

MATSUGUCHI, T., MUSIKACHAROEN, T., OGAWA, T. & YOSHIKAI, Y. (2000) Gene expressions of Toll-like receptor 2, but not Toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages. *J Immunol,* 165, 5767-72.

MATSUI, H., GRUBB, B., TARRAN, R., RANDELL, S., GATZY, J., DAVIS, C. & BOUCHER, R. (1998) Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell,* 95, 1005-15.

MAY, M. & GHOSH, S. (1998) Signal transduction through NF-kappa B. *Immunol Today,* 19, 80-8.

MCCLOSKEY, M., MCCAUGHAN, J., REDMOND, A. & ELBORN, J. (2001) Clinical outcome after acquisition of Burkholderia cepacia in patients with cystic fibrosis. *Ir J Med Sci,* 170, 28-31.

MCCOY, S., KURTZ, S., HAUSMAN, F., TRUNE, D., BENNETT, R. & HEFENEIDER, S. (2004) Activation of RAW264.7 macrophages by bacterial DNA and lipopolysaccharide increases cell surface DNA binding and internalization. *J Biol Chem.,* 279, 17217-23.

MCDOWELL, A., MAHENTHIRALINGAM, E., DUNBAR, K., MOORE, J., CROWE, M. & ELBORN, J. (2004) Epidemiology of Burkholderia cepacia complex species recovered from cystic fibrosis patients: issues related to

patient segregation. *J Med Microbiol,* 53, 663-8.

MEANS, T., LIEN, E., YOSHIMURA, A., WANG, S., GOLENBOCK, D. & FENTON, M. (1999) The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. *J Immunol,* 163, 6748-55.

MEDVEDEV, A., LENTSCHAT, A., WAHL, L., GOLENBOCK, D. & VOGEL, S. (2002) Dysregulation of LPS-induced Toll-like receptor 4-MyD88 complex formation and IL-1 receptor-associated kinase 1 activation in endotoxin-tolerant cells. *J Immunol,* 169, 5209-16.

MEDZHITOV, R., PRESTON-HURLBURT, P. & JANEWAY, C. J. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature,* 388, 394-7.

MILLER, S., LIPUMA, J. & PARKE, J. (2002) Culture-based and non-growth-dependent detection of the Burkholderia cepacia complex in soil environments. *Appl Environ Microbiol,* 68, 3750-8.

MIYAKE, K. (2004) Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. *Trends Microbiol,* 12, 186-92.

MOCHIDA-NISHIMURA, K., SUREWICZ, K., CROSS, J. & HEJAL, R. (2001) Differential activation of MAP kinase signaling pathways and nuclear factor-kappaB in bronchoalveolar cells of smokers and nonsmokers. *Mol Med,* 7, 177-85.

MOORE, E. & ELBORN, S. (2003) Burkholderia cepacia and cystic fibrosis [online]. Northern Ireland Regional Adult Cystic Fibrosis Centre, Belfast City Hospital, Northern Ireland, United Kingdom, Northern Ireland Public Health Laboratory, *. http://www.cysticfibrosismedicine.com

MOORE, R. & HANCOCK, R. (1986) Involvement of outer membrane of

Pseudomonas cepacia in aminoglycoside and polymyxin resistance. *Antimicrob Agents Chemother.*, 30, 923-6.

MORAN, A., HARDIN, D., RODMAN, D. & ALLEN, H. (1999) Diagnosis, screening and management of cystic fibrosis related diabetes mellitus: a consensus conference report. *Diabetes Res Clin Pract.*, 45, 61-73.

MORRISON, D. & RYAN, J. (1987) Endotoxins and disease mechanisms. *Annu Rev Med,* 38, 417-32.

MOSKWA, P., LORENTZEN, D., EXCOFFON, K., ZABNER, J. & MCCRAY, P. J. (2007) A novel host defense system of airways is defective in cystic fibrosis. *Am J Respir Crit Care Med,* 175, 174-83.

MOYNAGH, P. (2003) Toll-like receptor signalling pathways as key targets for mediating the anti-inflammatory and immunosuppressive effects of glucocorticoids. *J Endocrinol,* 179, 139-44.

MUHDI, K., EDENBOROUGH, F., GUMERY, L., O'HICKEY, S., SMITH, E., SMITH, D. & STABLEFORTH, D. (1996) Outcome for patients colonised with Burkholderia cepacia in a Birmingham adult cystic fibrosis clinic and the end of an epidemic. *Thorax,* 51, 374-7.

MUHLEBACH, M. & NOAH, T. (2002) Endotoxin activity and inflammatory markers in the airways of young patients with cystic fibrosis. *Am J Respir Crit Care Med.,* 165, 911-5.

MUIR, A., SOONG, G., SOKOL, S., REDDY, B., GOMEZ, M., VAN HEECKEREN, A. & PRINCE, A. (2003) Toll like receptors in normal and cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol*

MUZIO, M., BOSISIO, D., POLENTARUTTI, N., D'AMICO, G. & STOPPACCIARO, A. (2000) Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in

dendritic cells. *J Immunol,* 164, 5998-6004.

NAHORI, M., FOURNIE-AMAZOUZ, E., QUE-GEWIRTH, N. (2005) Differential TLR recognition of leptospiral lipid A and lipopolysaccharide in murine and human cells. *Journal of Immunology.* 175, 6022-6031.

NETEA, M., VAN DEUREN, M., KULLBERG, B., CAVAILLON, J. & VAN DER MEER, J. (2002) Does the shape of lipid A determine the interaction of LPS with Toll-like receptors? *Trends Immunol,* 23, 135-9.

NHS (2008) Cystic fibrosis screening programme. www.gosh.nhs.uk/newborn/cf/screening

NICHOLS, D., CHMIEL, J. & BERGER, M. (2008) Chronic Inflammation in the Cystic Fibrosis Lung: Alterations in Inter- and Intracellular Signaling. *Clin Rev Allergy Immunol,* 34, 146-162.

NIKAIDO, H. & NAKAE, T. (1979) The outer membrane of Gram-negative bacteria. *Adv Microb Physiol,* 20, 163-250.

NIXON, G., ARMSTRONG, D., CARZINO, R. & CARLIN, J. (2001) Clinical outcome after early Pseudomonas aeruginosa infection in cystic fibrosis. . *J. Pediatr.* , 138, 699-704.

NUNLEY, D., OHORI, P., GRGURICH, W., IACONO, A., WILLIAMS, P., KEENAN, R. & DAUBER, J. (1998) Pulmonary aspergillosis in cystic fibrosis lung transplant recipients. *Chest,* 114, 1321-9.

O'CARROLL, M., KIDD, T., COULTER, C., SMITH, H., ROSE, B., HARBOUR, C. & BELL, S. (2003) Burkholderia pseudomallei: another emerging pathogen in cystic fibrosis. *Thorax.,* 58, 1087-91.

OGAWA, H., RAFIEE, P., HEIDEMANN, J., FISHER, P., JOHNSON, N., OTTERSON, M., KALYANARAMAN, B., PRITCHARD, K. J. & BINION,

D. (2003) Mechanisms of endotoxin tolerance in human intestinal microvascular endothelial cells. *J Immunol.,* 170, 5956-64.

OLIVER, A., CANTÓN, R., CAMPO, P., BAQUERO, F. & BLÁZQUEZ, J. (2000) High frequency of hypermutable Pseudomonas aeruginosa in cystic fibrosis lung infection. *Science,* 288, 1251-4.

O'NEILL, L. (2003) SIGIRR puts the brakes on Toll-like receptors. *Nat Immunol,* 4, 823-4.

PALFREYMAN, R., WATSON, M., EDEN, C. & SMITH, A. (1997) Induction of biologically active interleukin-8 from lung epithelial cells by Burkholderia (Pseudomonas) cepacia products. *Infect Immun.,* 65, 617-22.

PÅLSSON-MCDERMOTT, E. & O'NEILL, L. (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology,* 113, 153-62.

PAMUKCU, A., BUSH, A. & BUCHDAHL, R. (1995) Effects of pseudomonas aeruginosa colonization on lung function and anthropometric variables in children with cystic fibrosis. *Pediatr Pulmonol,* 19, 10-5.

PEDERSEN, S., HØIBY, N., ESPERSEN, F. & KOCH, C. (1992) Role of alginate in infection with mucoid Pseudomonas aeruginosa in cystic fibrosis. *Thorax,* 47, 6-13.

PEGUES, D., CARSON, L., ANDERSON, R., NORGARD, M., ARGENT, T., JARVIS, W. & WOERNLE, C. (1993) Outbreak of Pseudomonas cepacia bacteremia in oncology patients. *Clin Infect Dis,* 16, 407-11.

PETSCH, D. & ANSPACH, F. (2000) Endotoxin removal from protein solutions. *J Biotechnol.,* 76, 97-119.

PIER, G. (2007) Pseudomonas aeruginosa lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. *Int J Med Microbiol,* 297, 277-95.

PIER, G., TAKEDA, S., GROUT, M. & MARKHAM, R. (1993) Immune complexes from immunized mice and infected cystic fibrosis patients mediate murine and human T cell killing of hybridomas producing protective, opsonic antibody to Pseudomonas aeruginosa. *J Clin Invest,* 91, 1079-87.

PINSKY, M. (2004) Dysregulation of the immune response in severe sepsis. *Am J Med Sci,* 328, 220-9.

PITT , T., KAUFMANN, M., PATEL, P., BENGE, L., GASKIN, S. & LIVERMORE, D. (1996) Type characterisation and antibiotic susceptibility of Burkholderia (Pseudomonas) cepacia isolates from patients with cystic fibrosis in the United Kingdom and the Republic of Ireland. *J Med Microbiol,* 44, 203-10.

POLTORAK, A., HE, X., SMIRNOVA, I., LIU, M., VAN HUFFEL, C., DU, X. & BIRDWELL, D. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science.,* 282, 2085-8.

PUGIN, J., SCHÜRER-MALY, C., LETURCQ, D., MORIARTY, A., ULEVITCH, R. & TOBIAS, P. (1993) Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc Natl Acad Sci U S A,* 90, 2744-8.

QURESHI, N., TAKAYAMA, K. & RIBI, E. (1982) Purification and structural determination of nontoxic lipid A obtained from the lipopolysaccharide of Salmonella typhimurium. *J Biol Chem,* 257, 11808-15.

QURESHI, S., LARIVIÈRE, L., LEVEQUE, G., CLERMONT, S., MOORE, K., GROS, P. & MALO, D. (1999) Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med,* 189, 615-25.

QURESHI, S. & MEDZHITOV, R. (2003) Toll-like receptors and their role in experimental models of microbial infection. *Genes Immun.,* 4, 87-94.

RAINGEAUD, J., GUPTA, S., ROGERS, J., DICKENS, M., HAN, J., ULEVITCH, R. & DAVIS, R. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem,* 270, 7420-6.

RAMSEY, B. (1996) Management of pulmonary disease in patients with cystic fibrosis. *N Engl J Med,* 18, 3.

RAO, K. (2001) MAP kinase activation in macrophages. *J Leukoc Biol,* 69, 3-10.

RAO, S. & GRIGG, J. (2006) New insights into pulmonary inflammation in cystic fibrosis. *Arch Dis Child,* 91, 786-8.

REDDI, K., PHAGOO, S., ANDERSON, K. & WARBURTON, D. (2003) Burkholderia cepacia-induced IL-8 gene expression in an alveolar epithelial cell line: signaling through CD14 and mitogen-activated protein kinase. *Pediatr Res.,* 54, 297-305.

REHLI, M. (2002) Of mice and men: species variations of Toll-like receptor expression. *Trends Immunol,* 23, 375-8.

REIK, R., SPILKER, T. & LIPUMA, J. (2005) Distribution of Burkholderia cepacia complex species among isolates recovered from persons with or without cystic fibrosis. *J Clin Microbiol,* 43, 2926-8.

RESCIGNO, M., URBANO, M., RIMOLDI, M., VALZASINA, B., ROTTA, G., GRANUCCI, F. & RICCIARDI-CASTAGNOLI, P. (2002) Toll-like receptor 4 is not required for the full maturation of dendritic cells or for the degradation of Gram-negative bacteria. *Eur J Immunol.,* 32, 2800-6.

RICH, D., BERGER, H., CHENG, S., TRAVIS, S., SAXENA, M., SMITH, A. & WELSH, M. (1993) Regulation of the cystic fibrosis transmembrane conductance regulator Cl- channel by negative charge in the R domain. *J Biol Chem,* 268, 20259-67.

RICHMAN-EISENSTAT, J. (1996) Cytokine soup: making sense of inflammation in cystic fibrosis. *Pediatr Pulmonol,* 21, 3-5.

RIORDAN, J., ROMMENS, J., KEREM, B., ALON, N., ROZMAHEL, R. & GRZELCZAK, Z. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science,* 245, 1066-73.

RITTER, M., MENNERICH, D., WEITH, A., SEITHER, P. (2005) Characterisation of Toll-like receptors in primary lung epithelial cells: strong impact on TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response. *J Inflamm.* 2, 16.

RITTIG, M., KAUFMANN, A., ROBINS, A., SHAW, B., SPRENGER, H. & GEMSA, D. (2003) Smooth and rough lipopolysaccharide phenotypes of Brucella induce different intracellular trafficking and cytokine/chemokine release in human monocytes. *J Leukoc Biol,* 74, 1045-55.

ROCK, F., HARDIMAN, G., TIMANS, J., KASTELEIN, R. & BAZAN, J. (1998) A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A,* 95, 588-93.

ROPERT, C. (2005) Translating MAPK inhibitors to anti-inflammatory compounds. *Current enzyme inhibition,* 1, 75-84.

ROPERT, C., ALMEIDA, I., CLOSEL, M., TRAVASSOS, L., FERGUSON, M., COHEN, P. & GAZZINELLI, R. (2001) Requirement of mitogen-activated protein kinases and I kappa B phosphorylation for induction of proinflammatory cytokines synthesis by macrophages indicates functional similarity of receptors triggered by glycosylphosphatidylinositol anchors from

parasitic protozoa and bacterial lipopolysaccharide. *J Immunol,* 166, 3423-31.

ROSENBERG, H. (2007) Rational design of MyD88 inhibitors- new pathways to inflammatory control: an interview with Dr. Claudio Sette. *Journal of leukocyte biology.* 82, 811-812.

RYLEY , H. & DOULL, J. (2003) Burkholderia cepacia complex infection in patients with cystic firbrosis: laboratory investigations, epidemiology and clinical management. *Reviews in Medical Microbiology,* 14, 15-24.

RYLEY, H. C. (2004) Burkholderia cepacia complex infection in cystic fibrosis patients: mechanisms of pathogenesis. . *Reviews in Medical Microbiology,* 15, 93-101.

SAINI, L., GALSWORTHY, S., JOHN, M. & VALVANO, M. (1999) Intracellular survival of Burkholderia cepacia complex isolates in the presence of macrophage cell activation. *Microbiology.,* 145, 3465-75.

SAJJAN, S. & FORSTNER, J. (1992) Identification of the mucin-binding adhesin of Pseudomonas cepacia isolated from patients with cystic fibrosis. *Infect Immun,* 60, 1434-40.

SAJJAN, U., THANASSOULIS G, CHERAPANOV V, LU A, SJOLIN C, STEER B, WU YJ, ET AL (2001) Enhanced susceptibility to pulmonary infection with Burkholderia cepacia in Cftr(-/-) mice. *Infect Immun,* 69, 5138-50.

SAJJAN, U., LIU L, LU A, SPILKER T, FORSTNER J, LIPUMA JJ. (2002) Lack of cable pili expression by cblA-containing Burkholderia cepacia complex. *Microbiology,* 148, 3477-84.

SAJJAN, U., SUN, L., GOLDSTEIN, R. & FORSTNER, J. (1995) Cable (cbl) type II pili of cystic fibrosis-associated Burkholderia (Pseudomonas) cepacia: nucleotide sequence of the cblA major subunit pilin gene and novel morphology of the assembled appendage fibers. *J Bacteriol,* 177, 1030-8.

SCHAEFER, L., BABELOVA, A., KISS, E. (2005) The matrix component biglycan is proinflammatory and signals through toll-like receptors 2 and 4 in macrophages. *J Clin Invest.* 155, 2223-2233.

SCHERLE, P., JONES, E., FAVATA, M., DAULERIO, A. & COVINGTON, M. (1998) Inhibition of MAP kinase kinase prevents cytokine and prostaglandin E2 production in lipopolysaccharide-stimulated monocytes. *J Immunol,* 161, 5681-6.

SCHOPF, R., KELLER, R., REHDER, M., BENES, P., KALLINOWSKI, F. & VAUPEL, P. (1990) TNF alpha primes polymorphonuclear leukocytes for an enhanced respiratory burst to a similar extent as bacterial lipopolysaccharide. *J Invest Dermatol,* 95, 216S-218S.

SCHOREY, J. & COOPER, A. (2003) Macrophage signalling upon mycobacterial infection: the MAP kinases lead the way. *Cell Microbiol,* 5, 133-42.

SCHULZ, C., FARKAS, L., WOLF, K. & KRATZEL, K. (2002) Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type II-like pneumocytes (A-549). *Scand J Immunol,* 56, 294-302.

SCHUMANN, R., LEONG, S., FLAGGS, G., GRAY, P., WRIGHT, S. & MATHISON, J. (1990) Structure and function of lipopolysaccharide binding protein. *Science,* 249, 1429-31.

SCHWAB, U., LEIGH, M., RIBEIRO, C., YANKASKAS, J., BURNS, K., GILLIGAN, P., SOKOL, P. & BOUCHER, R. (2002) Patterns of epithelial cell invasion by different species of the Burkholderia cepacia complex in well-differentiated human airway epithelia. *Infect Immun.,* 70, 4547-55.

SHAW, D., POXTON, I. & GOVAN, J. (1995) Biological activity of Burkholderia (Pseudomonas) cepacia lipopolysaccharide. *FEMS Immunol Med Microbiol,* 11, 99-106.

SHIMAZU, R., AKASHI, S., OGATA, H., NAGAI, Y., FUKUDOME, K., MIYAKE, K. & KIMOTO, M. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med,* 189, 1777-82.

SHIMOMURA, H., MATSUURA, M., SAITO, S., HIRAI, Y., ISSHIKI, Y. & KAWAHARA, K. (2001) Lipopolysaccharide of Burkholderia cepacia and its unique character to stimulate murine macrophages with relative lack of interleukin-1beta-inducing ability. *Infect Immun.,* 69, 3663-9.

SHIMOMURA, H., MATSUURA, M., SAITO, S., HIRAI, Y., ISSHIKI, Y. & KAWAHARA, K. (2003) Unusual interaction of a lipopolysaccharide isolated from Burkholderia cepacia with polymyxin B. *Infect Immun.,* 71, 5225-30.

SIMMONS, D., TAN, S., TENEN, D., NICHOLSON-WELLER, A. & SEED, B. (1989) Monocyte antigen CD14 is a phospholipid anchored membrane protein. *Blood,* 73, 284-9.

SMITH, J., TRAVIS, S., GREENBERG, E. & WELSH, M. (1996) Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell,* 85, 229-236.

SOONG, G., REDDY, B., SOKOL, S., ADAMO, R. & PRINCE, A. (2004) TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. *J Clin Invest,* 113, 1482-9.

SORENSEN, R., KLINGER, J., CASH, H., CHASE, P. & DEARBORN, D. (1983) In vitro inhibition of lymphocyte proliferation by Pseudomonas aeruginosa phenazine pigments. *Infect Immun,* 41, 321-30.

SPEERT, D., HENRY, D., VANDAMME, P., COREY, M. & MAHENTHIRALINGAM, E. (2002) Epidemiology of Burkholderia cepacia complex in patients with cystic fibrosis, Canada. *Emerg Infect Dis,* 8, 181-7.

SPEERT, D. P. (2001) Understanding Burkholderia cepacia: epidemiology, genomovars, and virulence. *Infect. Med,* 18, 49-56.

STANIER, R., PALLERONI, N. & DOUDOROFF, M. (1966) The aerobic pseudomonads: a taxonomic study. *J Gen Microbiol,* 43, 159-271.

STEERE, A., TENNEY, J., MACKEL, D., SNYDER, M., POLAKAVETZ, S., DUNNE, M. & DIXON, R. (1977) Pseudomonas species bacteremia caused by contaminated normal human serum albumin. *J Infect Dis,* 13, 729-35.

STRAUS, D., LONON, M., WOODS, D. & GARNER, C. (1989) Production of an extracellular toxic complex by various strains of Pseudomonas cepacia. *J Med Microbiol,* 30, 17-22.

STRAUS, D., WOODS, D., LONON, M. & GARNER, C. (1988) The importance of extracellular antigens in Pseudomonas cepacia infections. *J Med Microbiol,* 26, 269-80.

STRIETER, R. (2002) Interleukin-8: a very important chemokine of the human airway epithelium. *Am J Physiol Lung Cell Mol Physiol,* 283, L688-9.

STRIZ, I., MIO, T., ADACHI, Y., BAZIL, V. & RENNARD, S. (1998) The CD14 molecule participates in regulation of IL-8 and IL-6 release by bronchial epithelial cells. *Immunol Lett.,* 62, 177-81.

SUZUKI, K., SUDA, T., NAITO, T., IDE, K., CHIDA, K. & NAKAMURA, H. (2005) Impaired toll-like receptor 9 expression in alveolar macrophages with no sensitivity to CpG DNA. *Am J Respir Crit Care Med,* 171, 707-13.

SUZUKI, N., SUZUKI, S., DUNCAN, G., MILLAR, D. & WADA, T. (2003) Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature,* 416, 750-6.

TAKEDA, K., KAISHO, T. & AKIRA, S. (2003) Toll-like receptors. *Annu Rev Immunol,* 21, 335-76.

TAKEUCHI, O., HOSHINO, K., KAWAI, T., SANJO, H. & TAKADA, H. (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity,* 11, 443-51.

TAPLIN, D., BASSETT, D. & MERTZ, P. M. (1971) Foot lesions associated with Pseudomonas cepacia. *Lancet* 2, 568-571.

TAN, A. & BERRIDGE, M. (2000) Superoxide produced by activated neutrophils efficiently reduces tetrazolium salt, WST-1 to produce formazan: a simple colorimetric assay for measuring respiratory burst activation and for screening anti-inflammatory agents. (2000). *Journal of immunological methods*. 238, 59-68.

TAYLOR, C. & ASWANI, N. (2002) The pancreas in cystic fibrosis. *Paediatr Respir Rev. 2002 Mar;3(1):77-81,* 3, 77-81.

THOMASSEN, M., DEMKO, C., DOERSHUK, C., STERN, R. & KLINGER, J. (1986) Pseudomonas cepacia: decrease in colonization in patients with cystic fibrosis. *Am Rev Respir Dis,* 134, 669-71.

TOMICH, M., HERFST, C., GOLDEN, J. & MOHR, C. (2002) Role of flagella in host cell invasion by Burkholderia cepacia. *Infect Immun,* 70, 1799-806.

TONKS, A., DUDLEY, E., PORTER, NG., PARTON, J., BRAZIER, J., SMITH, E., TONKS, A. (2007) A 5.8-kDa component of manuka honey stimulates immune cells via TLR4. *J Leukoc Biol*, 82, 1147-55.

TRIANTAFILOU, K., TRIANTAFILOU, M., LADHA, S., MACKIE, A., DEDRICK, R., FERNANDEZ, N. & CHERRY, R. (2001) Fluorescence recovery after photobleaching reveals that LPS rapidly transfers from CD14 to hsp70 and hsp90 on the cell membrane. *J Cell Sci,* 114, 2535-45.

TRIANTAFILOU, M., BRANDENBURG, K., KUSUMOTO, S., FUKASE, K., MACKIE, A., SEYDEL, U. & TRIANTAFILOU, K. (2004a) Combinational clustering of receptors following stimulation by bacterial products determines lipopolysaccharide responses. *Biochem J.,* 381, 527-36.

TRIANTAFILOU, M., MANUKYAN, M., MACKIE, A., MORATH, S., HARTUNG, T., HEINE, H. & TRIANTAFILOU, K. (2004b) Lipoteichoic acid and toll-like receptor 2 internalization and targeting to the Golgi are lipid raft-dependent. *J Biol Chem,* 279, 40882-9.

TRIANTAFILOU, M., MIYAKE, K., GOLENBOCK, D. & TRIANTAFILOU, K. (2002b) Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. *J Cell Sci.,* 115, 2603-11.

TRIANTAFILOU, M. & TRIANTAFILOU, K. (2002a) Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. *Trends Immunol,* 23, 301-4.

TSUI, L. (1995) The cystic fibrosis transmembrane conductance regulator gene. *Am J Respir Crit Care Med,* 151, S47-53.

ULMER, A., RIETSCHEL, E., ZAHRINGER, U. & HEINE, H. (2002) Lipopolysaccharide: structure, bioactivity, receptores and signal transduction. *Trends in Glycoscience and Glycotechnology,* 14, 53-68.

UNDERHILL, D. (2004) Toll-like receptors and microbes take aim at each other. *Curr Opin Immunol,* 16, 483-7.

URBAN, T., GRIFFITH, A., TOROK, A., SMOLKIN, M., BURNS, J. & GOLDBERG, J. (2004) Contribution of Burkholderia cenocepacia flagella to infectivity and inflammation. *Infect Immun,* 72, 5126-34.

VAARA, M. (1992) Agents that increase the permeability of the outer membrane. *Microbiol Rev,* 56, 395-411.

VAN AMERSFOORT, E., VAN BERKEL, T. & KUIPER, J. (2003) Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. *Clin Microbiol Rev.,* 16, 379-414.

VAN DEN BLINK, B., BRANGER, J., WEIJER, S., DEVENTER, S., VAN DER POLL, T. & PEPPELENBOSCH, M. (2001) Human endotoxemia activates p38 MAP kinase and p42/44 MAP kinase, but not c-Jun N-terminal kinase. *Mol Med,* 7, 755-60.

VAN DEN BLINK, B., HOVE, T., VAN DEN BRINK, G., PEPPELENBOSCH, MP., VAN DEVENTER, S. (2002) From extracellular to intracellular targets, inhibiting MAP kinases in treatment of Crohn's disease. *Ann N Y Acad Sci.* 973, 349-58.

VANDAMME, P., HOLMES, B., VANCANNEYT, M., COENYE, T., HOSTE, B. & COOPMAN, R. (1997) Occurrence of multiple genomovars of Burkholderia cepacia in cystic fibrosis patients and proposal of Burkholderia multivorans sp. nov. *Int J Syst Bacteriol,* 47, 1188-200.

VERMIS, K., BRACHKOVA, M., VANDAMME, P. & NELIS, H. (2003) Isolation of Burkholderia cepacia complex genomovars from waters. *Syst Appl Microbiol,* 26, 595-600.

VICTOR, V. & DE LA FUENTE, M. (2003) Several functions of immune cells in mice changed by oxidative stress caused by endotoxin. *Physiol Res.,* 52, 789-96.

VILLUNGER, A., O'REILLY, L., HOLLER, N., ADAMS, J. & STRASSER, A. (2000) Fas ligand, Bcl-2, granulocyte colony-stimulating factor, and p38 mitogen-activated protein kinase: Regulators of distinct cell death and survival pathways in granulocytes. *J Exp Med,* 192, 647-58.

VINION-DUBIEL, A. & GOLDBERG, J. (2003) Lipopolysaccharide of Burkholderia cepacia complex. *J Endotoxin Res.,* 9, 201-13.

VORACHEK-WARREN, M., RAMIREZ, S., COTTER, R. & RAETZ, C. (2002) A triple mutant of Escherichia coli lacking secondary acyl chains on lipid A. *J Biol Chem,* 277, 14194-205.

WAGNER, H. & BAUER, S. (2006) All is not Toll: new pathways in DNA recognition. *J Exp Med,* 203, 265-8.

WANG, X., DIENER, K., MANTHEY, C. & WANG, S. (1997) Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase. *J Biol Chem.,* 272, 23668-74.

WEBB, A. & GOVAN, J. (1998) Burkholderia cepacia: another twist and a further threat. *Thorax,* 53, 333-334.

WELSH, M. & SMITH, A. (1993) Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell,* 73, 1251-4.

WERTS, C., TAPPING, R., MATHISON, J. & CHUANG, T. (2001) Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat Immunol,* 2, 346-52.

WHEELER, A. & BERNARD, G. (1999) Treating patients with severe sepsis. *N Engl J Med.,* 340, 207-14.

WHITBY, P., CARTER, K., HATTER, K., LIPUMA, J. & STULL, T. (2000) Identification of members of the Burkholderia cepacia complex by species-specific PCR. *J Clin Microbiol,* 38, 2962-5.

WHITEFORD, M., WILKINSON, J., MCCOLL, J., CONLON, F., MICHIE, J., EVANS, T. & PATON, J. (1995) Outcome of Burkholderia (Pseudomonas)

cepacia colonisation in children with cystic fibrosis following a hospital outbreak. *Thorax,* 50, 1194-8.

WILSCHANSKI, M., DUPUIS, A., ELLIS, L., JARVI, K. & ZIELENSKI, J. (2006) Mutations in the cystic fibrosis transmembrane regulator gene and in vivo transepithelial potentials. *Am J Respir Crit Care Med,* 174, 787-94.

WORLITZSCH, D., TARRAN, R., ULRICH, M., SCHWAB, U. & CEKICI, A. (2002) Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. *J Clin Invest,* 109, 317-25.

YAMAMOTO, M., SATO, S., HEMMI, H., UEMATSU, S., HOSHINO, K. & KAISHO, T. (2003) TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol,* 4, 1144-50.

YAN, S., AL-HERTANI, W., BYERS, D. & BORTOLUSSI, R. (2002) Lipopolysaccharide-binding protein- and CD14-dependent activation of mitogen-activated protein kinase p38 by lipopolysaccharide in human neutrophils is associated with priming of respiratory burst. *Infect Immun,* 70, 4068-74.

YAN, W., ZHAO, K., JIANG, Y., HUANG, Q., WANG, J. & KAN, W. (2002) Role of p38 MAPK in ICAM-1 expression of vascular endothelial cells induced by lipopolysaccharide. *Shock.,* 17, 433-8.

YANG, C., CHIEN, C., HSIAO, L., LUO, S. & WANG, C. (2002) Interleukin-1beta-induced cyclooxygenase-2 expression is mediated through activation of p42/44 and p38 MAPKS, and NF-kappaB pathways in canine tracheal smooth muscle cells. *Cell Signal,* 14, 899-911.

YANG, R., MARK, M., GRAY, A., HUANG, A., XIE, M. & ZHANG, M. (1998) Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature,* 395, 284-8.

YANG, S., GALANIS, A. & SHARROCKS, A. (1999) Targeting of p38 mitogen-activated protein kinases to MEF2 transcription factors. *Mol Cell Biol.,* 19, 4028-38.

ZAHARIADIS, G., LEVY, M. & BURNS, J. (2003) Cepacia-like syndrome caused by Burkholderia multivorans. *Can J Infect Dis,* 14, 123-5.

ZAREMBER, K. & GODOWSKI, P. (2002) Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol,* 168, 554-61.

ZHANG, W. & LIU, H. (2002) MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res,* 12, 9-18.

ZIEGLER-HEITBROCK, H., SCHRAUT, W. & WENDELGASS, P. (1994) Distinct patterns of differentiation induced in the monocytic cell line Mono Mac 6. *J Leukoc Biol,* 55, 73-80.

ZIELENSKI, J., ROZMAHEL, R., BOZON, D., KEREM, B. & GRZELCZAK, Z. (1991) Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics,* 10, 214-28.

ZUELZER, W. & NEWTON, W. J. (1949) The pathogenesis of fibrocystic disease of the pancreas; a study of 36 cases with special reference to the pulmonary lesions. *Pediatrics,* 4, 53-69.

ZUGHAIER, S., RYLEY, H. & JACKSON, S. (1999a) Lipopolysaccharide (LPS) from Burkholderia cepacia is more active than LPS from Pseudomonas aeruginosa and Stenotrophomonas maltophilia in stimulating tumor necrosis factor alpha from human monocytes. *Infect Immun.,* 67, 1505-7.

ZUGHAIER, S., RYLEY, H. & JACKSON, S. (1999b) A melanin pigment purified from an epidemic strain of Burkholderia cepacia attenuates monocyte respiratory burst activity by scavenging superoxide anion. *Infect Immun.,* 67,

908-13.

ZUGHAIER, S., ZIMMER, S., DATTA, A., CARLSON, R. & STEPHENS, D. (2005) Differential induction of the toll-like receptor 4-MyD88-dependent and -independent signaling pathways by endotoxins. *Infect Immun,* 73**,** 2940-50.

Appendix 1

Bcc LPS preparation for analysis

Lysis buffer

Sodium dodecyl sulphate	200 mg
2-mercaptoethanol	400 µl
Bromophenol blue	10 mg
Stacking gel buffer	3 ml
Glycerol	1 ml
Water	6 ml

The SDS, glycerol and stacking gel buffer were added to 6 ml of sterile pyrogen free water. 400 µl of mercaptoethanol was added and the solution mixed thoroughly, finally 10 mg of bromophenol blue was added. The mixing was done entirely in a fume cupboard and the solution filtered through a 0.2 µm Millipore filter, the solution was then aliquoted into 1 ml amounts and stored at -20°C.

Electrophoresis of LPS on 14% SDS PAGE

Stacking gel buffer: 0.5 M Tris-HCl (pH 6.8)

6 g of Tris was dissolved in 40 ml water, titrated with 2 M HCl to pH 6.8 and the volume was made up to 100 ml with water.

Resolving gel buffer: 3.0 M Tris-HCl (pH 8.8)

Tris	36.2 g
HCl (2 M)	24 ml

Water up to 100 ml

Stock reservoir buffer: 0.25 M Tris, 1.92 M Glycine, 1% SDS (pH 8.3)

Tris	30.3 g
Glycine	144 g
SDS	10 g

Distilled water up to 1000 ml

This buffer was diluted 1:10 before use

Stock acrylamide bis solution

Acrylamide	30 g
Bisacrylamide	0.8 g
Dowex AG1 resin	1 g

This solution was stored in a dark bottle at 4°C and the resin was filtered off prior to use.

Ammonium persulphate solution: 1.5%

150 mg of ammonium persulphate was dissolved in 10 ml of distilled water freshly prior to use.

Sodium dodecyl sulphate (SDS) solution: 10%

1 g of SDS was dissolved in 10 ml distilled water and kept at RT.

Resolving gel (30 ml), 14% polyacrylamide

Acrylamide/Bis solution	14 ml
Rsolving gel buffer	3.75 ml

10 % SDS	0.3 ml
Ammonium persulphate	1.5 ml
Distilled water	10.4 ml
TEMED	30 µl

Stacking gel (20 ml), 2.5% polycrylamide

Acrylamide/Bis solution	2.5 ml
Stacking gel buffer	5 ml
10% SDS	0.2 ml
Ammonium persulphate	1 ml
Distilled water	11.3 ml
TEMED	15 µl

Silver staining of LPS

Fixing reagent

Isopropanol	50 ml
Acetic acid	14 ml
Water	186 ml

Oxidising reagent

Periodic acid	1.05 g
Isopropanol	2 ml
Acetic acid	200 µl
Water	150 ml

This reagent was made up freshly, just prior to use.

Silver nitrate solution

Silver nitrate	2 g
Water	10 ml

This reagent was stored in the dark.

Alkaline reagent

Sodium hydroxide (0.1% solution)	28 ml
Ammonium hydroxide (35%)	1 ml
Water	115 ml

This reagent was made up freshly prior to use.

Staining solution

To the total alkaline solution, approximately 4 ml of Silver nitrate solution was added drop-wise with continual shaking until a permanent silver precipitate was just visible. Excess precipitation was reversed by adding ammonium hydroxide drop-wise. This solution was made up just prior to use.

Developing solution

Citric acid	12.5 mg
Formaldehyde (37%)	125 µl
Water	250 ml

Citric acid was first dissolved in water and the formaldehyde was added to the solution just prior to use.

Stopping solution

Acetic acid	1 ml
Water	250 ml

ELISA:

General reagents

TMB microwell peroxidase substrate	KPL, UK
Horse-radish peroxidase streptavidin	Zymed

Coating buffer	DPBS
Blocking buffer	1% BSA
	5% sucrose in PBS
Wash buffer	0.05% Tween 20 in PBS (pH 7.4)
Diluent solution	0.1% BSA
	0.05% Tween 20 in Tris bufferes saline (pH 7.3)
Stop solution	0.5 M H_2SO_4

Specific TNF-α Reagents

Monoclonal mouse anti-human TNF-α antibody	R&D Systems, UK
Biotinylated goat anti-human TNF-α antibody	R&D Systems, UK
Recombinant human TNF-α standard	R&D Systems, UK

Specific IL-6 Reagents

Monoclonal mouse anti-human IL-6 antibody	R&D Systems, UK
Biotinylated goat anti-human IL-6 antibody	R&D Systems, UK
Recombinant human IL-6 standard	R&D Systems, UK

Specific IL-8 Reagents

Monoclonal mouse anti-human IL-8 antibody	R&D Systems, UK
Biotinyated goat anti-human IL-8 antibody	R&D Systems, UK
Recombinant human IL-8 standard	R&D Systems, UK

Respiratory Burst measurement by enhanced chemiluminescence

Reagents

Opsonised Zymosan: stock 2.5 mg/ml, diluted 1:5 (500 μg/ml) in standard buffer for experimental use.

20 mM Luminol (5-amino-2,3-dihydro-1,4-phthalozinedione): stock solution was prepared at 3.5 mg/ml in DMSO and stored in the dark at 4°C for no more than 1 week. For experimental use it was diluted to 3.5 mM with distilled water prior to use.

Standard buffer

For 100 ml (made up in water)

KH_2PO_4	4.58 mM
Na_2HPO_4	8.03 mM
NaCl	0.76%
KCl	0.033%
Glucose	0.1%
Endotoxin free albumin	0.1%
$MgCl_2$	0.5mM
$CaCl_2$	0.45mM

This buffer was adjusted to pH 7.3 and aliquoted into 20 ml aliquots, it was then stored a -20°C until use.

Western blotting of MAPK proteinsP44/42 (ERK 1/2)

Cell fractionation

Reagents

RIPA buffer base

To 100 ml water add:

NaCl	150 mM
Tris-HCl	10 mM
EDTA	1 mM
EGTA	1 mM

RIPA buffer working solution

To 20 ml RIPA buffer base add:

1 Protease inhibitor cocktail tablet (Sigma)

Na_3VO_4	200 mM

Protein estimation- Coomassie Blue G binding

Stock dye solution

Coomassie Blue G	330 mg
Phosphoric Acid/Ethanol	
(2:1 v/v)	100 ml

Working dye solution

Stock dye reagent	3 ml
Phosphoric acid	8 ml
Ethanol	3.75 ml

Water to 100 ml. This solution was made up freshly on the day of use.

Electrophoresis using Novex system and NuPAGE gels

NuPAGE running buffer

50 ml NuPAGE stock running buffer added to 950 ml ultrapure water.

Reducing buffer

500 µl NuPAGE antioxidant added to 200 ml NuPAGE running buffer.

Transfer to nitrocellulose membrane

NuPAGE transfer buffer

Ultrapure water	750 ml
20x NuPAGE transfer stock buffer	50 ml
NuPAGE antioxidant	1 ml
Methanol	200 ml

Electrophoretic Mobility Shift Assay (EMSA)

Preparation of subcellular fractions

Buffer A

Hepes	10mM (pH 7.9)
$MgCl_2$	1.5 mM
KCl	10 mM
Water	10 ml
Added just before use:	
PMSF	100 mM
DTT	1M

Buffer C

Hepes	20 mM (pH 7.9)
NaCl	420 mM
$MgCl_2$	1.5 mM
EDTA	0.2 mM
Glycerol (25%)	2.5 ml
Water	10 ml
Added just prior to use:	
PMSF	05mM

Buffer D

Hepes	10 mM (pH 7.9)
KCl	25 mM
EDTA	0.1mM
Glycerol (10%)	1 ml
Water	10 ml

Added just prior to use:

PMSF	0.5 mM
DTT	0.5 mM

Detection of NF-κB

10X Binding reaction buffer

Glycerol (40%)	20 µl
EDTA	10 mM
DTT	50 mM
Tris (pH 7.5)	100 mM
NaCl	1 M
Nuclease free BSA	1 mg/ml

Band shift gel

40% acrylamide mix	5 ml
10X TBE	5 ml
Water	40 ml
Ammonium persulphate	0.1 g
TEMED	2 µl

Appendix 2

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Highly purified lipopolysaccharides from *Burkholderia cepacia* complex clinical isolates induce inflammatory cytokine responses via TLR4-mediated MAPK signalling pathways and activation of NFκB

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Summary

In cystic fibrosis (CF), bacteria of the *Burkholderia cepacia* complex (Bcc) can induce a fulminant inflammation with pneumonitis and sepsis. Lipopolysaccharide (LPS) may be an important virulence factor associated with this decline but little is known about the molecular pathogenesis of Bcc LPS. In this study we have investigated the inflammatory response to highly purified LPS from different Bcc clinical isolates and the cellular signalling pathways employed. The inflammatory response (TNFα, IL-6) was measured in human MonoMac 6 monocytes and inhibition experiments were used to investigate the Toll-like receptors and associated adaptor molecules and pathways utilized. LPS from all clinical Bcc isolates induced significant pro-inflammatory cytokines and utilized TLR4 and CD14 to mediate activation of mitogen-activated protein kinase pathways, IκB-α degradation and NFκB activation. However, LPS from different clinical isolates of the same clonal strain of *Burkholderia cenocepacia* were found to induce a varied inflammatory response. LPS from clinical isolates of *Burkholderia multivorans* was found to activate the inflammatory response via MyD88-independent pathways. This study suggests that LPS alone from clinical isolates of Bcc is an important virulence factor in CF and utilizes TLR4-mediated signalling pathways to induce a significant inflammatory response.

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Introduction

A feature of cystic fibrosis (CF) is the unusual respiratory inflammation that results from the interaction between bacterial pathogens and host cells. Despite intense research efforts, the detailed pathogenic mechanisms underlying these CF infections remain poorly understood. Members of the *Burkholderia cepacia* complex (Bcc) cause nosocomial infections in immunocompromised hosts and are particularly feared among CF patients as they can induce a rapid decline in lung function, with pneumonitis and sepsis ('cepacia syndrome') (Isles *et al.*, 1984; Vinion-Dubiel and Goldberg, 2003). In addition, many strains of Bcc are highly transmissible and once acquired are impossible to eradicate from the lungs due to antibiotic resistance.

A distinguishing feature of infections with Bcc compared with other major CF pathogens, such as *Pseudomonas aeruginosa*, is an increase in morbidity and mortality in patients with only mild or moderate lung disease (Muhdi *et al.*, 1996). The pathogenic mechanisms involved in severe Bcc infections are poorly understood; however, it has been shown that lipopolysaccharide (LPS) from Bcc elicits a much stronger inflammatory response from leucocytes compared with that of *P. aeruginosa* (Shaw *et al.*, 1995; Zughaier *et al.* 1999a).

Clinically, *Burkholderia cenocepacia* (formerly genomovar III) is often associated with a rapid decline in lung function and the ET12 clonal strain has been responsible for multiple transmission and infections in North America and the United Kingdom (Govan *et al.*, 1996). In a recent study, De Soyza and colleagues demonstrated that whole cell lysates from *B. cenocepacia* induced more TNFα and IL-1 compared with other Bcc genomovars (De Soyza *et al.*, 2004). This activity was assumed to be due to the LPS from these isolates. In many European CF centres, however, *Burkholderia multivorans* (formerly genomovar II) is the most common species isolated. Furthermore, although LPS is an important inflammatory stimulus in Gram-negative organisms, Bcc strains exhibit many potential virulence factors that could contribute to clinical outcome. Indeed, it has been suggested that LPS-associated proteins may be responsible for the

inflammatory responses (Shimomura *et al.*, 2001) and that signalling mechanisms to Bcc LPS may be different from that for LPS from enteric species (Shimomura *et al.*, 2003). Although details of the signal transduction pathways elicited by LPS in innate immune cells are emerging (Karin and Ben-Neriah, 2000; Rao, 2001), there have been no studies on the signalling mechanisms by which Bcc LPS activates inflammatory responses in these cells. Moreover, some reports suggest that LPS from certain non-enteric organisms may signal through TLR2 (Werts *et al.*, 2001; Erridge *et al.*, 2004).

Due to the importance of Bcc in CF and the potential for LPS to induce the severe inflammatory decline associated with these infections, more information on the molecular mechanisms by which Bcc LPS can elicit inflammatory responses is required. Identification of the receptors and signalling pathways utilized by Bcc LPS will potentially allow new therapeutic approaches to the treatment of these important CF infections. Therefore, we undertook the current study to identify the pro-inflammatory signalling pathways activated by LPS purified from a number of clinical isolates of different Bcc strains from CF patients.

Results

Cytokine production by phenol-chloroform-petroleum ether-purified and enzyme-treated LPS

An initial objective of this work was to compare the inflammatory potential of LPS derived from isolates of different clinical Bcc strains with LPS from a non-clinical source and from an unrelated enteric organism (*Escherichia coli*). We also sought to compare differences in these responses from LPS prepared from clinical isolates from the same Bcc strain. Additionally, we wished to confirm that the inflammatory activity associated with the purified LPS was not due to contaminating protein or nucleic acids. Thus we prepared LPS from three *B. cenocepacia* strains, all belonging to the ET12 epidemic strain, from a clinical isolate of *B. multivorans* and from an environmental *B. cepacia* strain. Each LPS preparation (Bcc LPS-1) was further treated with protease and DNAase to give a purified LPS preparation (Bcc LPS-2).

Treatment of the human monocyte cell line MonoMac 6 (MM6) with LPS preparations from representative Bcc isolates induced the production of TNFα and IL-6 inflammatory cytokines as shown in Fig. 1. As a control, LPS purified by the same method from an *E. coli* strain was used to induce TNFα. The TNF-inducing activity of the purified extracted *E. coli* LPS and *E. coli* LPS from a commercial source gave similar results and the commercial preparation was routinely used (data not shown). Figure 1A and B show that there was no difference in cytokine induction between the phenol-chloroform-petroleum ether (PCP)-extracted LPS (Bcc LPS1) and the further purified LPS (Bcc LPS2) indicating that it is the LPS that is stimulating the cytokine responses and not other contaminants. We were particularly keen to investigate differences in inflammatory potential of purified LPS extracted from different Bcc strains and from within the same clonal strain. Figure 1C shows the TNFα-inducing ability of highly purified LPS preparations from a larger number of clinical Bcc isolates, including ET12 epidemic strains of *B. cenocepacia* derived both from patients in Cardiff and from other UK centres. Figure 1C shows that LPS from environmental *B. cepacia* strains are much weaker TNFα inducers than LPS from Bcc clinical isolates. Furthermore, LPS from *B. multivorans* strains induced similar levels of TNFα to the *B. cenocepacia* strains and there was considerable variation in TNFα-inducing ability between LPS purified from isolates of the same *B. cenocepacia* clonal strain. Moreover, the TNFα-inducing ability of LPS from many clinical Bcc isolates was comparable with LPS purified from the enteric *E. coli*.

Antibody blocking of TLR4, TLR2 and CD14 cell surface receptors

Having described the inflammatory cytokine responses induced by the LPS from different clinical strains of Bcc, we wished to investigate the receptors and signalling mechanisms by which the monocytes were responding to the LPS. In particular, are there differences in LPS recognition and signalling that might explain the differences in cytokine responses seen both between Bcc strains and within the same epidemic Bcc strain. There are reports in the literature that LPS derived from some non-enteric pathogens might be recognized and signal via receptors other than TLR4 (Werts *et al.*, 2001; Erridge *et al.*, 2004). Thus, we first used antibodies to TLR4 to block this LPS receptor and monitored the TNFα responses as in the previous experiments.

In order to ascertain the receptor(s) utilized by Bcc LPS, specific antibodies were used to block a receptor in MM6 cells, the cells were then stimulated with LPS from representative strains of Bcc. The reduction in the level of TNFα after receptor blocking, compared with a non-blocked control, indicates the level of signalling through that receptor. Isotype control antibody did not significantly inhibit LPS-induced responses and confirmed the specificity for blocking TLR4 (data not shown).

Anti-human TLR4 monoclonal antibody was incubated with MM6 cells for 1 h before stimulation with LPS. TNFα levels were then measured by enzyme-linked immunosorbent assay (ELISA) after 6 h. In *E. coli* and in the *B. cenocepacia* LPS samples an almost total reduction in TNFα production was seen. The *B. multivorans* samples also showed a large reduction (60%) in TNFα production

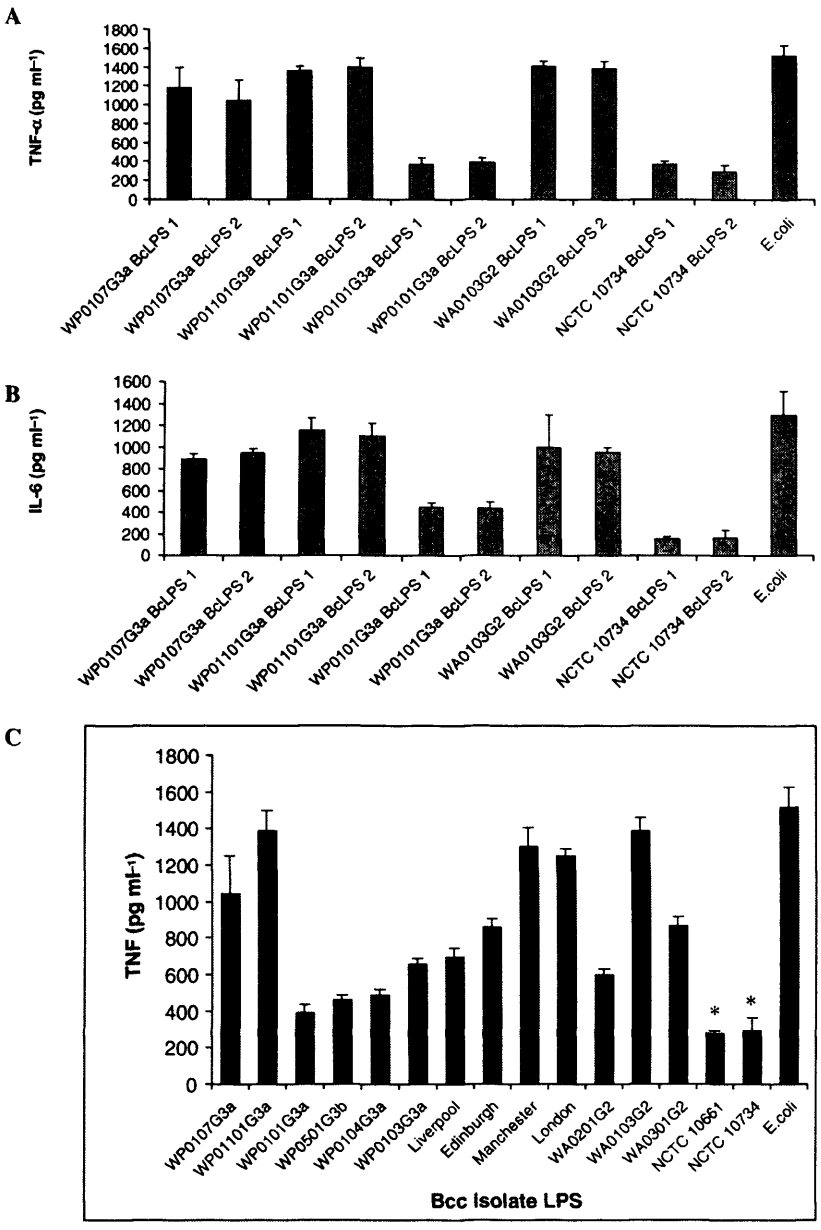

Fig. 1. ELISA experiments showing inflammatory cytokine induction in MM6 cells by Bcc LPS.
A. TNFα production after 6 h of stimulation with Bcc LPS from different sources.
B. IL-6 production after 6 h of stimulation with Bcc LPS from different sources. BcLPS-1 is the PCP-extracted LPS and BcLPS-2 is the more highly purified enzyme-treated preparation.
C. ELISA showing TNFα production from LPS purified from clinical epidemic and non-epidemic strains of Bcc, with LPS from environmental Bcc strains and from *E. coli* shown for comparison. Values are means ± SD of three independent experiments. For Bcc strain details see Table 1 and the text. $^*P < 0.01$ compared with the average TNFα value for the Bcc clinical isolates.

when TLR4 was blocked, although not as much as for *B. cenocepacia* (Fig. 2A).

MM6 cells were also incubated with anti-CD14 antibody as above and similar results were seen with TNFα production. *B. multivorans* and *B. cenocepacia*-stimulated cells' TNFα production was reduced to almost zero and production by *E. coli* LPS was reduced by 79%. (Fig. 2B). In order to ascertain whether the LPS signal was being transmitted through TLR2 as well as TLR4, cells were also treated with anti-TLR2 and then stimulated with the same LPS samples. There was minimal or no reduction in TNFα being produced for all three samples (Fig. 2C). The anti-TLR2 antibody inhibited TNFα production from MM6 cells stimulated with the TLR2 ligand peptidoglycan (data not shown).

Mitogen-activated protein kinase inhibition in MM6 cells stimulated with LPS

Various mitogen-activated protein kinase (MAPK) group members are activated by LPS, specific MAPK inhibitors prevent signal transduction and indicate the level to which that particular MAPK is involved in the signalling.

Specific MAPK inhibitors PD98059 (P42/44 inhibitor), SB03580 (P38 inhibitor) and SP600125 (JNK inhibitor) were incubated singly and in combination at a concentra-

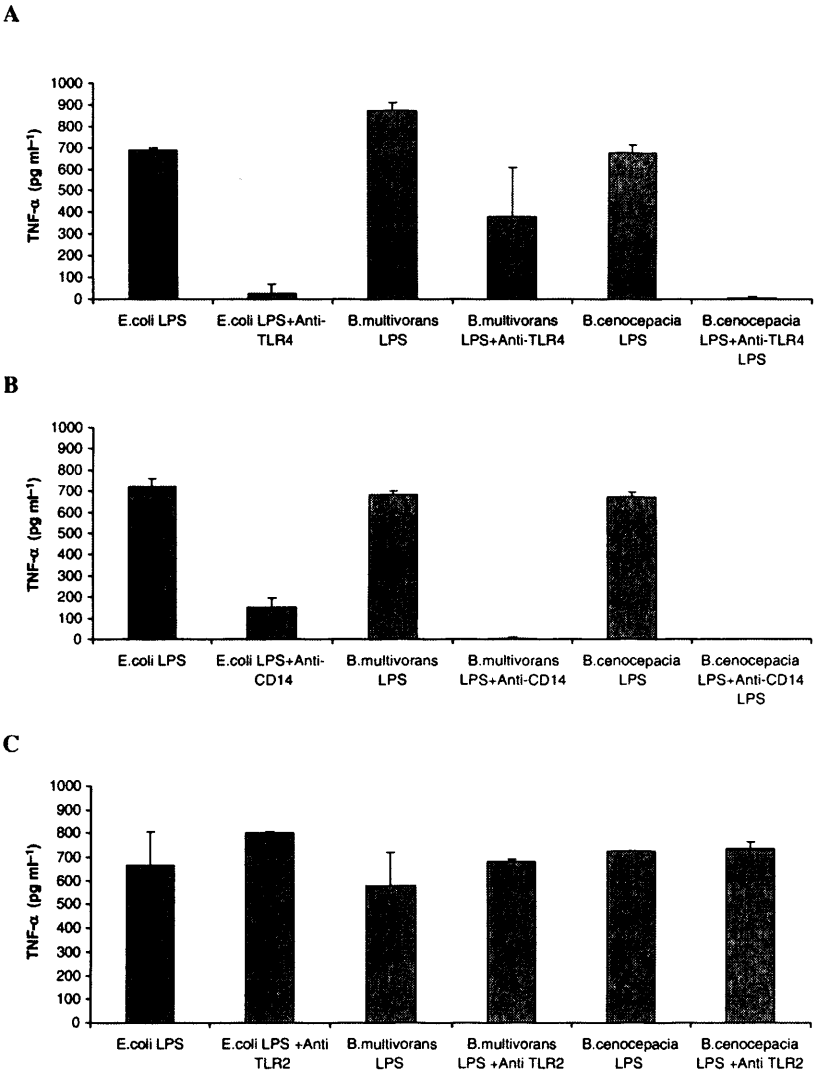

Fig. 2. ELISA experiments showing TNFα production from human MM6 cells treated with Bcc LPS in the presence of blocking antibody to LPS receptors.
A. Anti-TLR4.
B. Anti-CD14.
C. Anti-TLR2.
ELISA results shown are mean values ± SD from three independent experiments for LPS from *B. cenocepacia* (strains WP01101G3a, WP0103G3a) and *B. multivorans* (strains WA0103G2, WA0301G2).

tion of 10 μM for 1 h before stimulation with LPS samples. Control samples using the inhibitors alone were included and the inhibitors were found to cause no reaction or significant reduction in viability (data not shown).

The most dramatic reduction in TNFα production from a single MAPK inhibitor came from the JNK inhibitor, this was the case with all the LPS samples. The JNK inhibitor reduced TNFα production by an average of 63%. P42/44 inhibitor reduced TNFα production by an average of 47% and the least effective was the P38 inhibitor, which reduced production by an average of 37%. (Fig. 3). However, combinations of the inhibitors completely abolished TNFα production from the monocytes (Fig. 3).

Activation of MyD88-independent pathways

Recent reports demonstrate that LPS can activate cells via TLR4 and the recruitment of the adapter protein MyD88, or via MyD88-independent pathways via activation of the adaptors TRIF and TRAM (Palsson-McDermott and O'Neill, 2004; Jiang *et al.*, 2005). We were keen to investigate if LPS from different Bcc isolates required MyD88 for cellular activation. Monocytes were therefore stimulated with LPS in the presence of either a specific MyD88 inhibitor peptide or a control peptide with no effects on cell signalling pathways and the production of TNFα measured. The results (Fig. 4) show that LPS from *E. coli* and from strains of *B. cenocepacia* predominantly utilize MyD88 for cell activation (Fig. 4A and B). However, TNFα production in cells stimulated with LPS from strains of *B. multivorans* was not significantly reduced by MyD88 inhibition even when the concentration of inhibitory peptide was increased to 300 μM (Fig. 4C). This surprising result suggests that LPS from *B. multivorans* can activate cells by the MyD88-independent pathway that could yield different cellular responses and affect clinical outcome.

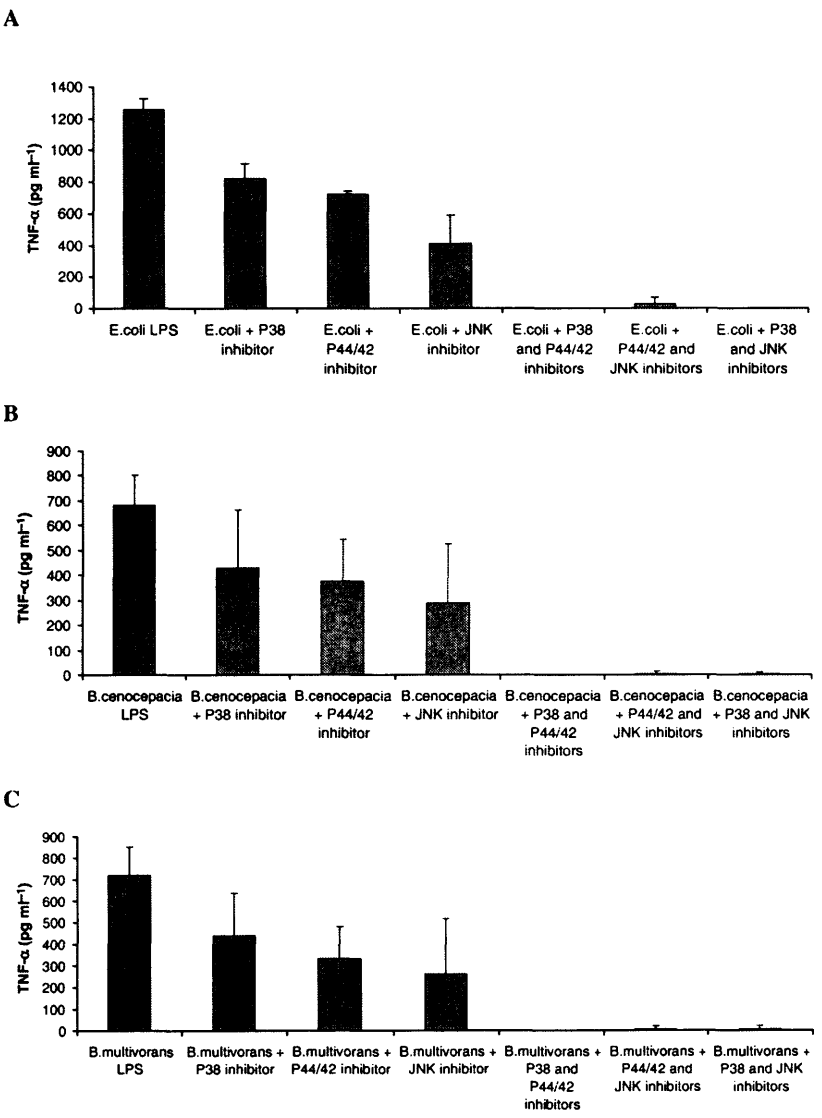

Fig. 3. ELISA experiments showing TNFα production from human MM6 cells treated with LPS with and without the MAPK inhibitors PD98059 (P42/44 inhibitor) SB203580 (P38 inhibitor) or SP600125 (JNK inhibitor).
A. LPS from *E. coli.*
B. LPS from *B. cenocepacia.*
C. LPS from *B. multivorans.*
Cells were incubated with one or more inhibitors for 1 h before stimulation with LPS for 6 h. Results are mean values ± SD from three independent experiments for LPS from representative Bcc strains [*B. cenocepacia* (strains WP01101G3a, WP0103G3a) and *B. multivorans* (strains WA0103G2, WA0301G2)].

Dissociation of IκB-α and activation of NFκB as measured by electrophoretic mobility shift assay

Lipopolysaccharide signalling causes the translocation of the transcription factor NFκB into the nucleus. In order to test whether Bcc LPS activated NFκB, dissociation of its inhibitor protein IκB was tested for after LPS stimulation. Then NFκB translocation was seen using an electrophoretic mobility shift assay (EMSA).

MM6 (1×10^6) cells were treated with LPS from *E. coli* and *B. cenocepacia* (strain WP01101G3a) for 1 h then washed and lysed. The lysates were then separated by electrophoresis and incubated with IκB-α polyclonal antibody followed by detection by a chemiluminescent detection kit (see above). Stimulation with LPS from *E. coli* and *B. cenocepacia* caused IκB-α to dissociate and degrade, this can be seen as narrower less intense bands compared with an unstimulated sample (Fig. 5A). Measurement of NFκB activation was done by EMSA and a gradual but steady increase in translocation to the nucleus was seen over a 120 min period (Fig. 5B).

Discussion

Pathogens belonging to the Bcc are a particular problem in patients with CF due to their antibiotic resistance, their patient-to-patient spread and their ability to cause severe inflammation and fatal invasive disease ('cepacia syndrome'). However, the identity of the bacterial molecules associated with this poor outcome in CF patients has remained elusive. We and others have been investigating the role of Bcc LPS in stimulating the inflammatory

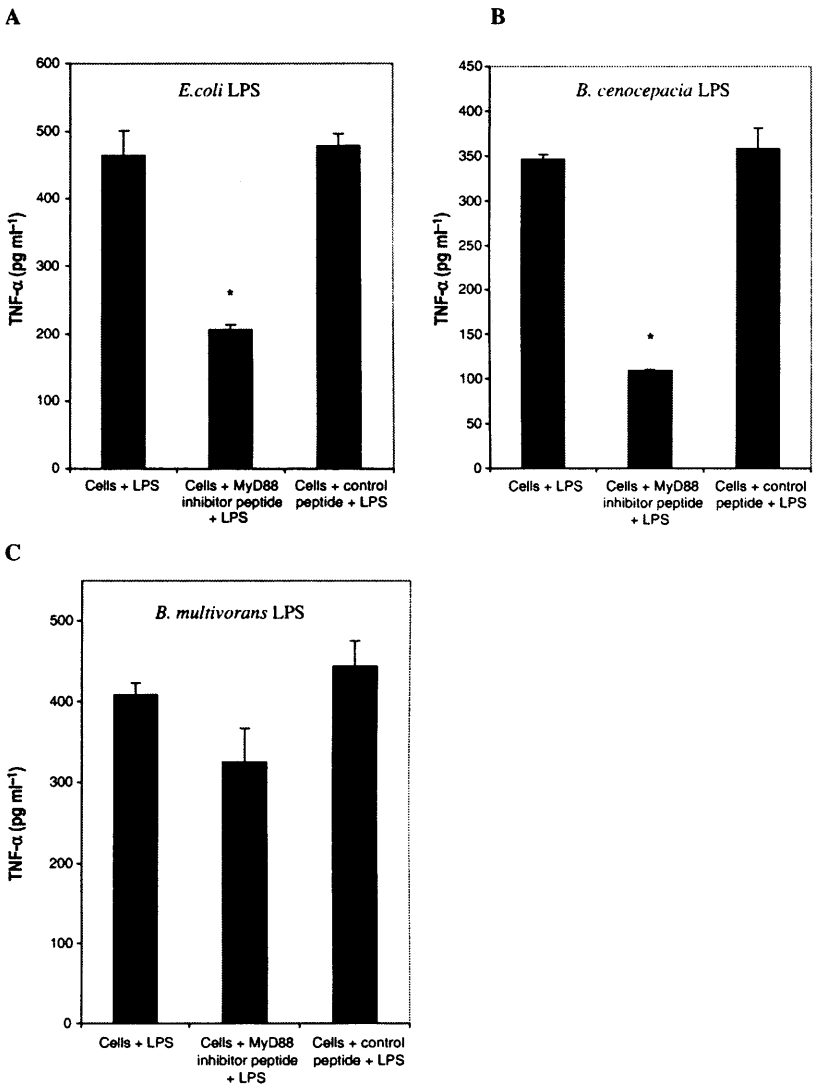

Fig. 4. LPS stimulation of the MyD88-dependent and -independent pathways of cellular activation. ELISA data of TNFα production from MM6 cells in the presence and absence of a MyD88 inhibitor peptide.
A. Cells stimulated with LPS from *E. coli*.
B. Cells stimulated with LPS from *B. cenocepacia* (strains WP01101G3a, WP0103G3a).
C. Cells stimulated with LPS from *B. multivorans* (strains WA0103G2, WA0301G2).
Representative data are shown for each LPS type (mean ± SD) from three independent experiments. $^*P < 0.001$ versus cells treated with control peptide and LPS.

response in monocytes and macrophages (Shaw *et al.*, 1995; Hughes *et al.*, 1997; Zughaier *et al.*, 1999a,b).

In this study, we report that LPS purified from clinical strains of Bcc induce greater inflammatory cytokine responses compared with an environmental strain. There was variation in cytokine-inducing capacity of LPS from the different Bcc strains used, with LPS from some strains eliciting as much cytokine as LPS from an enteric *E. coli* strain. Furthermore, we show that the cytokine induction by all the purified Bcc LPS utilizes CD14 and TLR4 to stimulate MAPK and activate NFκB. Moreover, our use of highly purified LPS indicates that this activity is not due to contaminating protein or nucleic acids. Thus, we have demonstrated that Bcc LPS have significant pro-inflammatory cytokine-inducing capacity and that they stimulate gene expression via common TLR4-mediated LPS signalling pathways.

Considerable evidence is now emerging that LPS is an important virulence factor that stimulates the inflammatory response in Bcc infections (Hughes *et al.*, 1997; Zughaier *et al.*, 1999a,b; Ryley, 2004; Mahenthiralingam *et al.*, 2005). Such evidence comes from studies showing that Bcc bacteria or the LPS derived from them can induce significant pro-inflammatory cytokine production (Zughaier *et al.*, 1999a; Shimomura *et al.*, 2001; Reddi *et al.*, 2003). Some studies have shown that Bcc bacteria or their LPS can elicit up to 10 times more TNFα from these cells as equivalent treatments with LPS from other lung pathogens including *P. aeruginosa* (Shaw *et al.*, 1995; Hughes *et al.*, 1997; Zughaier *et al.*, 1999a). However, it has been argued that the high levels of cytokine production observed in such studies may be due to protein contamination of the LPS and that a conventional phenol-water extraction may not remove

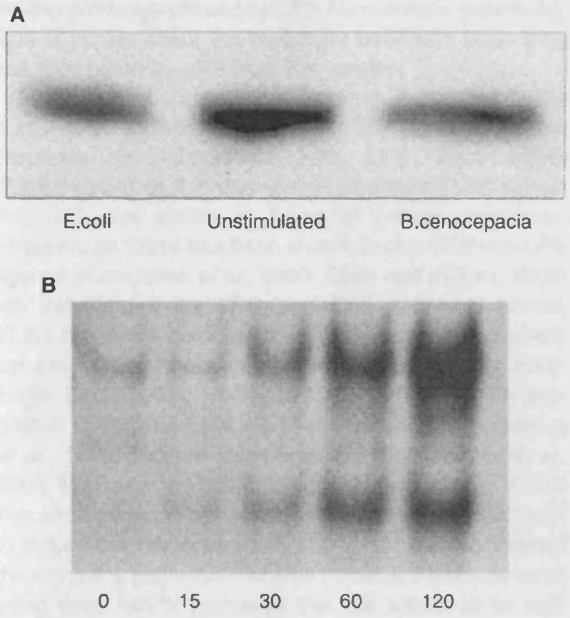

Fig. 5. Activation of NFκB.
A. Representative Western Blot showing dissociation of IκB from cells treated with LPS from *E. coli* (left panel), *B. cenocepacia* (strain WP01101G3a) (right panel) or untreated (centre panel).
B. Measurement of NFκB activation in nuclear fractions from MM6 cells treated with Bcc LPS as measured by EMSA. Samples were taken at 0, 15, 30, 60 and 120 min.

protein contaminants (Shimomura *et al.*, 2001). In the current study therefore, each LPS was prepared by both the conventional phenol-water extraction and a further round of proteinase, DNase and RNase treatment followed by deoxycholate-phenol-water extraction. This latter treatment was developed for the removal of trace protein from LPS preparations and was previously reported to be effective in eliminating 'non-LPS' activity from purified LPS (Shimomura *et al.*, 2001). We found no significant difference between either of the LPS preparations in their ability to induce pro-inflammatory cytokines in MM6 cells and conclude that, at least for the Bcc strains and cells used here, the conventional phenol-water extraction is sufficient. These conclusions were further endorsed by the fact that blocking the LPS receptors CD14 and TLR4 completely inhibited the LPS-induced pro-inflammatory responses.

Our study has shown that there is great variability in the inflammatory activity of LPS purified from different Bcc isolates. LPS derived from clinical isolates of Bcc (*B. cenocepacia* and *B. multivorans*) induced more TNFα and IL-6 production than the environmental strain [*B. cepacia* (genomovar I)] and as much as was induced by LPS purified from the enteric *E. coli* strain. Although *B. cenocepacia* is seen as the most virulent strain and is more frequently associated with 'cepacia syndrome' than other strains, we found *B. multivorans* was also a potent cytokine stimulator, which is interesting as, typically, survival rates for patients infected with *B. cenocepacia* are lower than patients infected with *B. multivorans* (LiPuma *et al.*, 2001). These results are in contrast to a recent report by De Soyza and coworkers (De Soyza *et al.*, 2004) who found that whole cell lysates of *B. cenocepacia* were more potent than lysates of *B. multivorans* in inducing cytokines from U937 monocytes. Although pre-transplant infection with *B. cenocepacia* is reported to have a worse outcome than *B. multivorans* in patients undergoing pulmonary transplants (De Soyza *et al.*, 2001), growing evidence shows that *B. multivorans* is also associated with poor clinical prognoses in patients with CF not undergoing pulmonary transplant (Ryley and Doull, 2003). Furthermore, whole cell lysates, as used in De Soyza's study, may stimulate cytokine release in monocytes by multiple non-LPS factors, some of which have been shown to bind to CD14 (Ryley, 2004; Urban *et al.*, 2004).

Surprisingly, we found that there was also variability in cytokine induction capability between the LPS isolated from within the *B. cenocepacia* strains. The *B. cenocepacia* strains used in our study belong to the ET12 lineage – a virulent strain that has been associated with transmission of Bcc infections between patients in different CF clinics and a poor clinical outcome (Ledson *et al.*, 1998; Ryley and Doull, 2003). All ET12 samples produced high levels of both TNFα and IL-6 (data not shown) with the exception of WP0101G3a which is also an ET12 clone but was a much less effective cytokine producer. This isolate was the index case from which ET12 spread to nine other patients over a 10 year period. The patient acquired it following a stay in a summer camp in Ontario in 1987. Molecular typing has confirmed that there are no genetic differences between these isolates from different patients and the original environmental strain from which they have arisen (Mahenthiralingam *et al.*, 2005). The variation in the pro-inflammatory activity of the LPS from this strain and the variable clinical outcome in different patients carrying this strain, suggests that there are other patient- or centre-derived factors and/or subtle patient-specific phenotypic changes in the LPS that mediate these clinical and inflammatory differences. Our results also suggest that it is not possible to conclude that all ET12 clones will behave the same way in experimental models.

Due to the inherent variation in inflammatory response produced by LPS from the different Bcc strains used in our study, it was important to analyse the molecular mechanisms utilized by the different LPS in stimulating these responses. In contrast to the well-documented sig-

nalling pathways utilized by LPS from enteric organisms, little is known about the molecular pathways underlying cell stimulation by LPS from Bcc strains.

Initial studies showed that blocking membrane CD14 completely inhibited monocyte TNF production in response to stimulation with LPS from either *B. multivorans* or *B. cenocepacia*, suggesting LPS signalling pathways similar to those of enteric organisms. However, as CD14 has been shown to also bind non-LPS ligands (Kurt-Jones *et al.*, 2000; Esen and Kielian, 2005) we investigated the LPS signalling pathways further. TLR4 has been demonstrated to be almost exclusively used by LPS in innate immune responses (Beutler, 2000; Hajjar *et al.*, 2002). However, some LPS has been suggested to activate cells via binding to TLR2 (Kirschning *et al.*, 1998; DaSilva Correia *et al.*, 2001; Werts *et al.*, 2001; Erridge *et al.*, 2004). We found that, at least for the Bcc strains used in our study, LPS from Bcc utilizes TLR4 to induce cell responses and not TLR2. We investigated the signalling pathways involved in cell activation by analysing three MAPK pathways that are known to be activated by LPS stimulation; p42/p44 MAPK (Erk1/2), transcription factor c-Jun and p38 MAPK (Nick *et al.*, 1999; Guha and Mackman, 2001; Palsson-McDermott and O'Neill, 2004). Inhibition of each of these MAPK individually was found to reduce but not completely inhibit TNFα expression in response to either *E. coli* or Bcc LPS. However, inhibition of more than one MAPK was seen to completely inhibit TNFα expression. This suggests that Bcc LPS activates these MAPK in monocytes and that co-ordinate activation of MAPK pathways are required for TNFα production. These findings are consistent with a recent report by Reddi *et al.* (2003), showing that whole bacterial cells of a clinical isolate of *B. cenocepacia* induced IL-8 secretion in alveolar epithelial cells through CD14 and MAPK signalling. Bcc LPS used in our study also activated NFκB via dissociation of the IκB complex, in a similar manner to *E. coli* LPS. NFκB mediates production of numerous pro-inflammatory genes, including TNFα, highlighting that Bcc LPS from all the species studied activates monocytes by conventional LPS pathways. Furthermore, a recent study showed that *P. aeruginosa* LPS is extracted by the CFTR in epithelial cells and activates NFκB (Schroeder *et al.*, 2002). However, Bcc LPS can induce a much greater inflammatory response than *P. aeruginosa* LPS (Shaw *et al.*, 1995; Zughaier *et al.*, 1999a), suggesting that structural differences in the lipid A and other factors or pathways are also involved in the inflammatory response to LPS (Brandenburg and Wiese, 2004; Trent, 2004).

Interestingly, our results with the inhibition of MyD88 suggest that LPS from *B. cenocepacia* activates cells through MyD88-dependent pathways while LPS from *B. multivorans* can activate cells through MyD88-independent pathways. In this regard, LPS from Bcc organisms might be similar to lipooligosaccharide from *Neisseria meningitidis* which was found to activate both MyD88-dependent and -independent pathways in monocytes (Zughaier *et al.*, 2005). Activation of the MyD88-independent pathway is associated with expression of interferon-β, and activation of STAT 1 and production of type 1 interferon-α/β, interferon-α-inducible protein 10, monocyte chemoattractant protein 5, nitric oxide and late activation of NFκB (Kawai *et al.*, 2001; Rhee *et al.*, 2003). Such differential activation of cells might explain the differences in clinical outcomes observed in patients infected with *B. multivorans* or *B. cenocepacia*, particularly when acquired before lung transplantation (LiPuma *et al.*, 2001).

A recent study has found structural differences in the lipid A component of LPS from *P. aeruginosa* isolated from the airways of CF patients compared with LPS from isolates from other patients and sites (Ernst *et al.*, 1999). Interestingly, the study by De Soyza and coworkers (De Soyza *et al.*, 2004) found different acylation patterns between *B. cenocepacia* and *B. multivorans* which they used to explain differences in inflammatory activities between whole cell lysates of these species. However, they only analysed one lipid A from each strain and it may be premature to draw conclusions from that. Indeed, caution should be exercised when trying to apply results obtained in one cell type to another as it is expected that there will be host cell-specific variations in response to LPS. For example, recent studies have shown that murine cells show differences in Toll-like receptor expression and responses compared with human cells (Rehli, 2002). Poorly differentiated cell lines may also not be representative of host responses *in vivo*. In this study, we utilized the well-characterized human monocyte cell line MM6, previously shown to be a good model for human blood monocytes (Ziegler-Heitbrock, 1994; Zughaier *et al.*, 1999a). Preliminary results with our purified Bcc LPS using several epithelial cell lines also show similar results (data not shown) indicating the variation in inflammatory response we see is real.

Other studies have found no correlation between Bcc species and lipid A fatty acid profile and no apparent correlation between lipid A composition and O-antigen (Vinion-Dubiel and Goldberg, 2003). Distinguishing features of Bcc LPS include the 4-amino-4-deoxy arabinose bound to phosphate in the lipid A (Vinion-Dubiel and Goldberg, 2003) that probably mediates the intrinsic resistance to antimicrobial peptides displayed by this species (LiPuma, 1998). Our results suggest that 4-amino-4-deoxyarabinose does not alter the binding or cellular activation pathways of LPS from Bcc. There is no complete information available on the chemical structure of Bcc lipid A. However, a recent report by Silipo and coworkers (Silipo *et al.*, 2005) showed that lipid A purified from a

Table 1. *Burkholderia cepacia* complex strains used in the study.

Designation	Bcc strain	Previous genomovar	Origin
NCTC 10743	*B. cepacia*	Genomovar I	Environmental
NCTC 10661	*B. cepacia*	Genomovar I	Environmental
WA0103G2	*B. multivorans*	Genomovar II	Cardiff CF Clinical Transmissible strain
WA0201G2	*B. multivorans*	Genomovar II	Cardiff CF Clinical non-epidemic strain
WP0301G2	*B. multivorans*	Genomovar II	Cardiff CF Clinical non-epidemic strain
WP0103G3a	*B. cenocepacia* (ET12)	Genomovar IIIa	Cardiff CF Clinical epidemic strain
WP0104G3a	*B. cenocepacia* (ET12)	Genomovar IIIa	Cardiff CF Clinical epidemic strain
WP0107G3a	*B. cenocepacia* (ET12)	Genomovar IIIa	Cardiff CF Clinical epidemic strain
WP01101G3a	*B. cenocepacia* (ET12)	Genomovar IIIa	Cardiff CF Clinical epidemic strain
WP0101G3a	*B. cenocepacia* (ET12)	Genomovar IIIa	Cardiff CF Clinical epidemic strain
WP0501G3b	*B. cenocepacia* Type b	Genomovar IIIb	Cardiff CF Clinical non-epidemic strain
LiverpoolG3a	*B. cenocepacia* (ET12)	Genomovar IIIa	Liverpool CF Clinical epidemic strain
EdinburghG3a	*B. cenocepacia* (ET12)	Genomovar IIIa	Edinburgh CF Clinical epidemic strain
ManchesterG3a	*B. cenocepacia* (ET12)	Genomovar IIIa	Manchester CF Clinical epidemic strain
LondonG3a	*B. cenocepacia* (ET12)	Genomovar IIIa	London CF Clinical epidemic strain

B. cepacia (genomovar I) clinical isolate had a penta-acylated tetrasaccharide backbone containing two phosphoryl-arabinosamine residues in addition to the archetypal glucosamine disaccharide. It remains to be seen if these features are common to all Bcc clinical isolates or only for *B. cepacia* (genomovar I). In addition, it would be interesting to analyse the chemical structures of lipid A from the clinical and environmental Bcc strains to see if there are clear structural differences that might explain the variation in pro-inflammatory activity observed between these strains.

In summary, we have found that purified LPS from Bcc clinical isolates can induce a strong inflammatory response and this is mediated by binding to TLR4 and activation of MAPK and NFκB. LPS from *B. cenocepacia* and *B. multivorans* were equally effective in inducing an inflammatory response although LPS from *B. multivorans* may activate cells via the MyD88-independent pathway and this could be linked to clinical outcome in some patients. There was variation in the inflammatory response elicited by LPS from different clinical isolates of a single clonal epidemic strain of *B. cenocepacia*. This suggests that patient-specific or centre-specific factors might modulate the LPS and detailed structural analyses of lipid A and LPS from these strains would be important. We are currently investigating these structures and other pathways that might be activated by Bcc LPS. Further understanding of the molecular mechanisms and pathways activated by Bcc LPS coupled with detailed structural analyses will allow a greater understanding of the mechanisms that contribute to 'cepacia' syndrome and potentially the development of novel therapeutic strategies.

Experimental procedures

Bacteria isolates

The clinical Bcc isolates used in this work were obtained from the sputum of patients with CF attending either the Paediatric CF Clinic, University Hospital of Wales, Cardiff or the Adult CF Clinic, Llandough Hospital, Penarth. The species of the isolates within the Bcc was determined using restricted endonuclease digestion of the PCR products from amplification of the RecA gene as described by Mahenthiralingam *et al.* (2000) with confirmation by the restriction endonuclease digestion of the 16SrRNA PCR product. All isolates were genotyped by PCR of 16S-23S rRNA intergenic zone followed by *Taq1* digestion of the PCR products (Ryley *et al.*, 1995). The presence of clonal strains that were identified by the above method was confirmed by another genotyping method, Randomly Amplified Polymorphic DNA (RAPD) PCR (Mahenthiralingam *et al.*, 1996). The ET12 clonal strain found in our patients was the strain described previously as the Cardiff Paediatric Epidemic Strain (Ryley *et al.*, 1995). It had an identical genotype to the type strain, NCTC13008, as well as ET12 isolates from other UK centres (kindly supplied by Mrs Fiona Clode, Health Protection Agency, Laboratory of Health Care Associated Diseases, Colindale, UK)

Cell culture

The human monocytic cell line MonoMac 6 [obtained from the German Collection of Microorganisms and Cell Cultures (DSM ACC 124; Braunschweig, Germany)] was used. We have previously validated this cell line as a useful model for human peripheral blood monocytes (Zughaier *et al.*, 1999a). For culture, MM6 cells were maintained in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Labtech International, Ringmer, East Sussex, UK), 1% 2 mM L-glutamine, 1% nonessential amino acids, 1% penicillin (50 IU ml^{-1})/streptomycin (100 μg ml^{-1}), and 1% sodium pyruvate. Cells were cultured in a humidified incubator at 37°C with 5% CO_2.

Lipopolysaccharide preparations

The Bcc strains from which LPS was isolated in this study together with their previous genomovar designations are outlined in Table 1.

Bacterial cells were obtained by culture in tryptone soya broth (Oxoid, Basingstoke, UK) at 37°C for 48 h. LPS was extracted by conventional PCP extraction (Govan *et al.*, 1996). This first preparation was referred to as BcLPS-1. For further purification,

BcLPS-1 was treated with 1 μg of DNase ml^{-1} (from bovine pancreas; Sigma-Aldrich, Poole, UK) plus 1 μg RNase ml^{-1} (from bovine pancreas; BDH, Poole, UK) at 30°C for 90 min at pH 7.5, followed by the addition of 1 μg ml^{-1} of trypsin (from bovine pancreas, Sigma-Aldrich, Poole, UK) and incubation at 30°C for 90 min. Finally 1 μg of proteinase K ml^{-1} (from fungi; Sigma-Aldrich, Poole, UK) was added and incubated at 30°C for 90 min.

Following these enzymatic treatments the solution was dialysed against distilled water overnight and lyophilized. This preparation was then treated with 45% phenol containing triethylamine and sodium deoxycholate and the LPS, extracted in the aqueous phase, was precipitated with ethanol by the deoxycholate-phenol-water extraction method to obtain a second LPS preparation, BcLPS-2. The extracted BcLPS-2 preparations were found to be essentially free from protein and DNA contamination. Lyophilized LPS preparations were stored at −80°C in depyrogenated glass vials. For use, LPS was weighed and reconstituted in pyrogen-free ultra-pure water and diluted to the desired concentration in pyrogen-free PBS. The LPS preparations were examined for induction of inflammatory cytokines in human cells. As a control enterobacterial LPS, *E. coli* O111:B4 LPS was purified by the methods outlined above and was also obtained commercially (Sigma-Aldrich, Poole, UK).

MM6 stimulation and cytokine assay

For experimental use, MM6 cells were suspended at 10^6 ml^{-1} in media in 24 well plates and stimulated with purified LPS for 6 h (TNFα) or 14–16 h (IL-6). Supernatants were collected after centrifugation at 2000 rpm for 3 min and were stored in aliquots at −80°C until assay. Our previous studies (Zughaier *et al.*, 1999a) and dose–response experiments (data not shown) showed that 100 ng ml^{-1} LPS gave optimal cytokine expression in MM6 cells and this concentration was routinely used. In some experiments, such as NFκB activation, higher doses of LPS (1 μg ml^{-1}) gave more significant results.

TNFα and IL-6 levels were assessed by a specific ELISA using mouse anti-TNFα/anti-IL-6 capture antibodies and a biotin-conjugated goat polyclonal secondary antibody, and were detected with a streptavidin-peroxidase-*o*-phenylenediamine substrate. Cytokine levels were determined by using duplicates for each supernatant sample and compared with a standard curve for human recombinant TNFα or IL-6.

Blocking TLR4, TLR2 and CD14

MM6 cells were suspended at 10^6 ml^{-1} in media and dispensed in 250 μl aliquots into 96 well plates. The cells were then pre-incubated with 5 μl/250 μl of anti-human TLR4 monoclonal antibody (HTA125) or IgG2 isotype control antibody (Insight Biotechnology, Wembley, UK) for 1 h at 37°C. Our previous studies had demonstrated that this dilution of antibody and incubation time gave optimal blocking activity. LPS was then added at a concentration of 100 ng ml^{-1} to pre-incubated wells and to control wells, then incubated overnight at 37°C, 5% CO_2 in a humidified incubator. Supernatants were collected after centrifugation for 3 min at 2000 rpm and assayed for TNFα by ELISA. The same procedure was carried out using anti-human TLR2 (TL2.1) (AbCAM, Cambridge, UK) and using anti-human CD14 (R and D Systems, Abingdon, UK) monoclonal antibodies.

MAPK inhibition

MM6 cells were suspended at 10^6 ml^{-1} in media and dispensed in 1 ml aliquots into 24 well plates. The cells were then pre-incubated with 10 μM of either PD98059 (P42/44 inhibitor), SB203580 (P38 inhibitor) or SP600125 (JNK inhibitor) (all from Sigma-Aldrich, Poole, UK) or combinations of the inhibitors, for 1 h at 37°C. LPS was then added at a concentration of 100 ng ml^{-1} to pre-incubated wells and to control wells, then incubated overnight at 37°C, 5% CO_2 in a humidified incubator. Supernatants were collected after centrifugation for 3 min at 2000 rpm and assayed for TNFα by ELISA.

MyD88 pathway activation

MM6 cells (1×10^6 cells ml^{-1}) were dispensed in 250 μl aliquots into 96 well culture plates and pre-incubated with 100 μM MyD88 homodimerization inhibitory peptide or a control peptide (Imgenex, USA) for 24 h. Test LPS was then added to each well at a concentration of 100 ng ml^{-1}. All experiments were conducted in duplicate, and a control with LPS alone and an unstimulated control were included. The cells were incubated at 37°C, 5% CO_2 for 6 h and then harvested, centrifuged at 2500 rpm for 5 min and the supernatants collected. The supernatants were then either stored at −80°C until use or tested immediately for TNFα concentration by ELISA.

IκB activation

MM6 cells (1.5×10^6 ml^{-1}) were stimulated with LPS (as described above) for 1 h, stimulation was then terminated by centrifuging each sample at 2500 rpm and adding 10 ml ice cold PBS. The cells were then washed twice more in ice cold PBS, then they were resuspended in 300 μl ice cold RIPA buffer working solution [150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1 protease cocktail tablet (Roche, Welwyn, UK), 200 mM Na_3VO_4, 1% Triton 100] and left on ice for 30 min. Cells were then sonicated and centrifuged at 2500 rpm for 5 min at 4°C. Cell lysates were either assayed straight away or stored at −80°C.

Cell lysates were examined for equal amounts of protein by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford, 1976). Samples of equal protein content were loaded onto 10% NuPAGE Tris-Bis precast gels (Invitrogen, Paisley, UK) and electrophoresed at 200 V for 1 h. Proteins were then transferred to a nitrocellulose membrane and detection was carried out using IκB-α antibody (1:1000) (kind gift from Dr P. Brennan, School of Medicine, Cardiff University) in conjunction with an anti-rabbit Western Breeze chemiluminescent immunodetection kit according to the manufacturer's instructions (Invitrogen, Paisley, UK).

Preparation of subcellular fractions

MM6 cells (1×10^6 ml^{-1}) were treated with 1 μg ml^{-1} LPS at different time intervals (15, 30, 60, 120 min). Stimulation was terminated by adding 5 ml ice-cold PBS. Each sample was centrifuged at 2500 rpm and then resuspended in 1 ml buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 10 mM PMSF) and centrifuged at 13 000 rpm for 1 min at 4°C. Cells

were then lysed by addition to 20 µl of buffer A containing 0.1% Nonident P40 and left on ice for 10 min, after which time they were centrifuged at 13 000 rpm for 5 min. The nuclear extract was prepared by adding 15 µl buffer C (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.5 mM PMSF) to the supernatant. The mixture was left on ice for 15 min. It was then centrifuged at 13 000 rpm at 4°C for 5 min and the supernatant was added to 75 µl buffer D (10 mM Hepes, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF). The protein in these extracts was measured using the Bradford method (Bradford, 1976). The extracts were stored at −80°C until NFκB assay.

Nuclear extracts and assessment of NFκB activation by EMSA

Samples of 1 µg protein were incubated with 10 000 cpm of a ^{32}P-labelled oligonucleotide containing the consensus sequence for NFκB in binding buffer (40% glycerol, 1 mM EDTA, 10 mM TRIS, pH 7.5, 100 mM NaCl, 0.1 mg ml^{-1} nuclease free BSA, 50 mM dithiothreitol) and 2 µg of poly-(dI-dC) at room temperature for 30 min (Brennan and O'Neill, 1995). The samples were then subjected to electrophoresis on a 5% polyacrylamide gel that was then dried and autoradiographed overnight at −80°C.

References

Beutler, B. (2000) TLR4: central component of the sole mammalian LPS sensor. *Curr Opin Immunol* **12:** 20–26.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. *Anal Biochem* **72:** 248.

Brandenburg, K., and Wiese, A. (2004) Endotoxins: relationships between structure, function, and activity. *Curr Top Med Chem* **4:** 1127–1146.

Brennan, P., and O'Neill, L.A. (1995) Effects of oxidants and antioxidants on nuclear factor kappa B activation in three different cell lines: evidence against a universal hypothesis involving oxygen radicals. *Biochim Biophys Acta* **1260:** 167–175.

DaSilva Correia, J., Soldau, K., Christen, U., Tobias, P.S., and Ulevitch, R.J. (2001) Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex: transfer from CD14 to TLR4 and MD-2. *J Biol Chem* **276:** 21129–21135.

De Soyza, A., McDowell, A., Archer, L., Dark, J.H., Elborn, S.J., Mahenthrialingam, E., *et al.* (2001) *Burkholderia cepacia* complex genomovars and pulmonary transplantation outcomes in patients with cystic fibrosis. *Lancet* **358:** 1780–1781.

De Soyza, A., Ellis, C.D., Khan, C.M., Corris, P.A., and Demarco de Hormaeche, R. (2004) *Burkholderia cenocepacia* lipopolysaccharide, lipid A, and proinflammatory activity. *Am J Respir Crit Care Med* **170:** 70–77.

Ernst, R.K., Yi, E.C., Guo, L., Lim, K.B., Burns, J.L., Hackett, M., and Miller, S.I. (1999) Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science* **286:** 1561–1565.

Erridge, C., Pridmore, A., Eley, A., Stewart, J., and Poxton, I.R. (2004) Lipopolysaccharides of *Bacteroides fragilis, Chlamydia trachomatis* and *Pseudomonas aeruginosa* signal via toll-like receptor 2. *J Med Microbiol* **53:** 735–740.

Esen, N., and Kielian, T. (2005) Recognition of *Staphylococcus aureus*-derived peptidoglycan (PGN) but not intact bacteria is mediated by CD14 in microglia. *J Neuroimmunol* **170:** 93–104.

Govan, J.R., Hughes, J.E., and Vandamme, P. (1996) *Burkholderia cepacia*: medical, taxonomic and ecological issues. *J Med Microbiol* **45:** 395–407.

Guha, M., and Mackman, N. (2001) LPS induction of gene expression in human monocytes. *Cell Signal* **13:** 85–94.

Hajjar, A.M., Ernst, R.K., Tsai, J.H., Wilson, C.B., and Miller, S.I. (2002) Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nat Immunol* **3:** 354–359.

Hughes, J.E., Stewart, J., Barclay, G.R., and Govan, J.R.W. (1997) Priming of neutrophil respiratory burst activity by lipopolysaccharide from *Burkholderia cepacia*. *Infect Immun* **65:** 4281–4287.

Isles, A., Maclusky, I., Corey, M., Gold, R., Probe, R.C., Fleming, P., and Levison, H. (1984) *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. *J Pediatr* **104:** 206–210.

Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., *et al.* (2005) CD14 is required for MyD88-independent LPS signalling. *Nat Immunol* **6:** 565–570.

Karin. M., and Ben-Neriah, Y. (2000) Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annu Rev Immunol* **18:** 621–663.

Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P.F., Sato, S., *et al.* (2001) Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of interferon-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* **167:** 5887–5894.

Kirschning, C.J., Wesche, H., Merrill, A.T., and Rothe, M. (1998) Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J Exp Med* **188:** 2091–2097.

Kurt-Jones, E.A., Popova, L., Kwinn, L., Haynes, L.M., Jones, L.P., Tripp, R.A., *et al.* (2000) Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* **1:** 398–401.

Ledson, M.J., Gallagher, M.J., Corkill, J.E., Hart, C.A., and Walshaw, M.J. (1998) Cross-infection between cystic fibrosis patients colonised with *Burkholderia cepacia*. *Thorax* **53:** 432–436.

LiPuma, J.J. (1998) *Burkholderia cepacia*. Management issues and new insights. *Clin Chest Med* **19:** 473–486.

LiPuma, J.J., Spilker, T., Gill, L.H., Campbell, P.W., Liu, L., and Mahenthiralingam, E. (2001) Disproportionate distribution of *Burkholderia cepacia* complex species and transmissibility markers in cystic fibrosis. *Am J Respir Crit Care Med* **164:** 92–96.

Mahenthiralingam, E., Campbell, M.E., Henry, D.A., and Speert, D.P. (1996) Epidemiology of *Burkholderia cepacia* Infection in patients with cystic fibrosis: analysis by randomly amplified polymorphic DNA fingerprinting. *J Clin Microbiol* **34:** 2914–2920.

Mahenthiralingam, E., Bischof, J., Byrne, S.K., Radomski, C., Davies, J.E., Av-Gay, Y., and Vandamme, P. (2000) DNA-

based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis, Burkholderia multivorans, Burkholderia stabilis,* and *Burkholderia cepacia* genomovars I and III. *J Clin Microbiol* **38:** 3165–3173.

Mahenthiralingam, E., Urban, T.A., and Goldberg, J.B. (2005) The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat Rev Microbiol* **3:** 144–156.

Muhdi, K., Edenborough, F.P., Gumery, L., O'Hickey, S., Smith, E.G., Smith, D.L., and Stableforth, D.E. (1996) Outcome for patients colonised with *Burkholderia cepacia* in a Birmingham adult cystic fibrosis clinic and the end of an epidemic. *Thorax* **51:** 374–377.

Nick, J.A., Avdi, J.N., Young, S.K., McDonald, P.P., Billstrom, M.A., Henson, P.M., *et al.* (1999) An intracellular signaling pathway linking lipopolysaccharide stimulation to cellular responses human neutrophil. *Chest* **116:** 54S–55S.

Palsson-McDermott, E.M., and O'Neill, L.A.J. (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor 4. *Immunology* **113:** 153–162.

Rao, K.M. (2001) MAPkinase activation in macrophages. *J Leukoc Biol* **69:** 3–10.

Reddi, K., Phagoo, S.B., Anderson, K.D., and Warburton, D. (2003) *Burkholderia cepacia*-induced IL-8 gene expression in an alveolar epithelial cell line: signaling through CD14 and mitogen-activated protein kinase. *Pediatr Res* **54:** 297–305.

Rehli, M. (2002) of mice and men: species variations of Toll-like receptor expression. *Trends Immunol* **23:** 375–378.

Rhee, S.H., Jones, B.W., Toshchakov, V., Vogel, S.N., and Fenton, M.J. (2003) Toll-like receptors 2 and 4 activate STAT 1 serine phosphorylation by distinct mechanisms in macrophages. *J Biol Chem* **278:** 22506–22512.

Ryley, H.C. (2004) *Burkholderia cepacia* complex infection in cystic fibrosis patients: mechanisms of pathogenesis. *Rev Med Microbiol* **15:** 93–101.

Ryley, H.C., and Doull, J.M. (2003) *Burkholderia cepacia* complex infection in patients with cystic fibrosis: laboratory investigations, epidemiology and clinical management. *Rev Med Microbiol* **14:** 15–24.

Ryley, H.C., Millar-Jones, L., Paull, A., and Weeks, J. (1995) Characterisation of *Burkholderia cepacia* from cystic fibrosis patients living in Wales by PCR ribotyping. *J Med Microbiol* **43:** 436–441.

Schroeder, T.H., Lee, M.M., Yacono, P.W., Cannon, C.L., Gerceker, A.A., Golan, D.E., and Pier, G.B. (2002) CFTR is a pattern recognition molecule that extracts *Pseudomonas aeruginosa* LPS from the outer membrane into epithelial cells and activates NF-kappa B translocation. *Proc Natl Acad Sci USA* **99:** 6907–6912.

Shaw, D., Poxton, I.R., and Govan, J.R. (1995) Biological activity of *Burkholderia (Pseudomonas) cepacia* lipopolysaccharide. *FEMS Immunol Med Microbiol* **11:** 99–106.

Shimomura, H., Matsuura, M., Saito, S., Hirai, Y., Isshiki, Y., and Kawahara, K. (2001) Lipopolysaccharide of *Burkholderia cepacia* and its unique ability to stimulate murine macrophages with a relative lack of interleukin-1β-inducing ability. *Infect Immun* **69:** 3663–3669.

Shimomura, H., Matsuura, M., Saito, S., Hirai, Y., Isshiki, Y., and Kawahara, K. (2003) Unusual interaction of a lipopolysaccharide isolated from *Burkholderia cepacia* with polymyxin B. *Infect Immun* **71:** 5225–5230.

Silipo, A., Molinaro, A., Cescutti, P., Bedini, E., Rizzo, R., Parrilli, M., and Lanzetta, R. (2005) Complete structural characterization of the lipid A fraction of a clinical strain of *B. cepacia* genomovar I lipopolysaccharide. *Glycobiology* **15:** 561–570.

Trent, M.S. (2004) Biosynthesis, transport, and modification of lipid A. *Biochem Cell Biol* **82:** 71–86.

Urban, T.A., Griffith, A., Torok, A.M., Smolkin, M.E., Burns, J.L., and Goldberg, J.B. (2004) Contribution of *Burkholderia cenocepacia* flagella to infectivity and inflammation. *Infect Immun* **72:** 5126–5134.

Vinion-Dubiel, A.D., and Goldberg, J.B. (2003) Lipopolysaccharide of the *Burkholderia cepacia* complex. *J Endotoxin Res* **9:** 201–213.

Werts, C., Tapping, R.I., Mathison, J.C., Chuang, T.H., Kravchenko, V., Saint Girons, I., *et al.* (2001) Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat Immunol* **2:** 346–352.

Ziegler-Heitbrock, H.W. (1994) Distinct patterns of differentiation induced in the monocytic cell line Mono Mac 6. *J Leukoc Biol* **55:** 73–80.

Zughaier, S., Ryley, H.C., and Jackson, S.K. (1999a) LPS from *B. cepacia* is more active than LPS from *P. aeruginosa* and *St.maltophilia* in stimulating TNFα from human monocytes. *Infect Immun* **67:** 1505–1507.

Zughaier, S.M., Ryley, H.C., and Jackson, S.K. (1999b) A melanin pigment purified from an epidemic strain of *Burkholderia cepacia* attenuates monocyte respiratory burst activity by scavenging superoxide anion. *Infect Immun* **67:** 908–913.

Zughaier, S.M., Zimmer, S.M., Datta, A., Carlson, R.W., and Stephens, D.S. (2005) Differential induction of the toll-like receptor 4-MyD88-dependent and -independent signaling pathways by endotoxins. *Infect Immun* **73:** 2940–2950.

