INDUCTION OF INTERLEUKIN-8 IN LUNG EPITHELIAL CELLS BY GRAM-NEGATIVE BACTERIA AND ITS MODULATION BY PULMONARY SURFACTANT LIPIDS

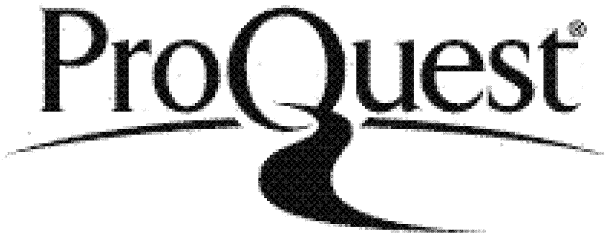
BY

ABDULAZIZ ALGHAITHY

A thesis submitted to Cardiff University

in accordance with the requirement of the degree of Doctor of Philosophy

Department of Medical Microbiology
2005



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed.....(Abdulaziz Alghaithy)

Date.....1/3/2006.....

STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

Signed.....(Abdulaziz Alghaithy)

Date.....1/3/2006.....

Signed.....(Supervisor)

Date.....

STATEMENT 2

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organization.

Signed.....(Abdulaziz Alghaithy)

Date.....1/3/2006.....

To,

My mother

and

in loving memory of my father.

ACKNOWLEDGEMENTS.

I would like to express my heartfelt gratitude to my government and King Khaled University for their financial support and the opportunity that I have been given to pursue my PhD at University of Wales, UK. I would like to thank Dr. Saleh Alghamdy for his unreserved support in facilitating the PhD studentship.

I am very grateful to Dr. Simon Karim Jackson, Senior Lecturer and Research Director, Department of Medical Microbiology, School of Medicine, Cardiff University for his continuous guidance, unlimited support and encouragement during my study.

Many thanks go to Mrs. Joan Parton for her expert advice and technical support during the lab work. I am also indebted to Dr. Henry Ryley, Dr. Amanda Tonks, Mrs. J. Weeks, and Mrs. Lynne Neale for their advice and technical assistance. Also my thanks to Mrs. Monica Angrove and to Miss. Linda Glenys Jones. Special thanks also go to Dr. M. Rittig at University of Nottingham for his invaluable support with electron microscopy.

Many thanks also go to Dr. Wondwossen Abate for his genuine friendship and support during difficult periods, as the saying goes friend in need are friend indeed. I am also indebted to Dr. Hamid Albakistany for his true friendships and to Miss. Sarah Humphrey for her great help in LPS isolation.

This thesis would be incomplete without mentioning the support of my great and dearest mother who kept my spirits up throughout my study. Without her constant encouragement and emotional supports, this project would have not come to completion and to show my appreciation I dedicate this work to her. I would especially like to thank my dearest brothers Ali, Saud and Faisal for covering my duties and responsibilities back home and for their supports and encouragement. Finally, I would like to thank my lovely niece Rascha for her support and standing by me during my difficult days.

SUMMARY

Respiratory epithelial cells, which form a primary interface between the lung and the environment, play a crucial role in innate immunity. Controlled and regulated lung innate immune responses are vital for the clearance of bacterial infections; however, dysregulation of these responses can lead to tissue damage and chronic inflammation. Pulmonary surfactant, in addition to its biophysical function, has been shown to exert anti-inflammatory properties in myeloid cells. In this work, the potential immunoregulatory role of surfactant lipids on innate immune responses of lung epithelial cells to Gram negative respiratory pathogens was investigated. The effect of the pulmonary surfactants Survanta®, Curosurf® and the phospholipid component dipalmitoylphosphatidylcholine (DPPC) on the growth, uptake and subsequent induction of IL-8 in lung epithelial cells by *P. aeruginosa* and *B. cepacia* have been investigated using the A549 cell line as a model system. In addition, the effect of the surfactants on the growth, uptake and IL-8 induction on both clinical and environmental strains of these bacteria was also investigated.

Both surfactant preparations (Survanta® and Curosurf®) as well as DPPC at physiologically relevant concentrations significantly inhibited the growth of *P. aeruginosa* and *B. cepacia* in saline supplemented with either pooled human serum or tryptone soy broth. Utilising fluorescence microscopy and FITC-conjugated bacteria, it was shown that A549 cells readily internalised both *P. aeruginosa* and *B. cepacia* and the internalized bacteria were confirmed by antibiotic protection assays and electron microscopy. The endocytosis of the bacteria by A549 cells was cytoskeleton dependent as it was inhibited by cytochalasin D. Survanta®, Curosurf® and DPPC significantly increased the uptake of *B. cepacia* by the A549 cells whereas Curosurf® and DPPC increased but Survanta® decreased the internalisation of *P. aeruginosa* by A549 cells.

A549 cells released a significant level of IL-8 when stimulated with either clinical or environmental strains of *P. aeruginosa* and *B. cepacia* and this was shown not to require bacterial internalization. However, IL-8 release was markedly suppressed when membrane CD14 and TLR4 receptors were blocked with specific antibody indicating lipopolysaccharide (LPS) is a key initiator of the bacterial-induced IL-8 responses in lung epithelial cells. Survanta®, Curosurf® and DPPC markedly suppressed the release of IL-8 induced by both environmental and clinical strains of *P. aeruginosa* and *B. cepacia* and LPS purified from these strains. Furthermore, semi-quantitative PCR experiments revealed that the surfactants inhibited IL-8 mRNA formation, suggesting that they act on signalling systems upstream of gene transcription.

The surfactants did not inhibit IL-8 induced by IL-1β in A549 cells suggesting that they act at the level of the LPS receptor complex. Disruption of membrane micro-domains ('rafts') with methyl-β-cyclodextrin significantly inhibited bacteria and LPS induced IL-8 in A549 cells. Isolation of membrane raft containing fractions by sucrose density gradient ultra-centrifugation showed that TLR4 was recruited into membrane lipid rafts on cell stimulation with the bacteria or LPS. The surfactants inhibited the bacterial and LPS mediated translocation of TLR4 into raft domains suggesting that their mechanism of action involves inhibition of LPS receptor complex formation in lipid raft domains.

This study has shown that pulmonary surfactant can directly modulate inflammatory responses of lung epithelial cells to Gram-negative bacterial pathogens and suggests the importance of surfactants in regulating potentially damaging inflammatory responses in the lung.

ABBREVIATIONS

AM	Alveolar macrophages
ANOVA	Analysis of variance
ARDS	Adult respiratory distress syndrome
BAL	Bronchoalveolar lavage
CARD	Caspase activating and recruitment domain
CAP	Community acquired pneumonia
cDNA	complementary DNA
CFU	Colony forming unit
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CM	Conditioned media from bacteria
DMSO	Dimethyl sulphoxide
dNTP	Deoxynucleotide triphosphate
DPPC	Dipalmitoyl phosphatidylcholine
EDTA	Ethylaminediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ENA78	Epithelial cell derived neutrophil-chemotactic agent 78
EU	Endotoxin unit
FITC	Fluorescein isothiocyanate
GRO	Growth regulated oncogene
hCM	Heated Conditioned media from bacteria

HK	Heat killed
HRP	Horseradish peroxidase
ICAM-1	Intracellular adhesion molecule 1
IFN	Interferon
IKK	Inhibitory k B kinase
IL	Interleukin
IRAK	IL-1 receptor associated kinase
JNK	c-Jun N-terminal Kinase
Kdo	3-deoxy-D-manno-oct-2-ulopyranosonic acid
LAL	Limulus Amebocyte lysate
LBP	LPS binding protein
LDS	Lithium dodecyl sulphate
LRTI	Lower respiratory tract infection
LTA	Lipoteichoic acid
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MβCD	Methyl-β-Cyclodextrin
MCD14	Membrane bound CD14
MCP	Monocyte chemotactic protein
MDP	Muramyl dipeptide
MIP-1	Macrophage inflammatory protein-1
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid

MOI	Multiplicity of infection
NF-κB	Nuclear factor κB
NIK	NF-κB-inducing kinase
NOD	Nucleotide oligomerization domain
PAGE	polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PGN	Peptidoglycan
PGE_2	Prostaglandin E_2
PI	Phosphatidyinistol
PMPC	Palmitoylmyristoyl-PC
POPC	Palmitoyloleoyl-PC
PI	Phosphatidyinistol
PLPC	Palmitoyllineoyl-PC
PRR	Pathogen recognition receptor
RICK	Receptor interacting protein-like interacting CLARP kinase
rIL-1	Recombinant interleukin-1
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
sCD14	Soluble CD14

SD	Standard deviation
SDS	Sodium dodecyl sulphate
SP	Surfactant associated protein
TAB	Tak-1 binding protein
TIR	Toll/interleukin-1 receptor
TICAM1	TIR-containing adaptormolecule-1
TLR	Toll like receptor
TLRs	Toll like receptors
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor α
TRAF	Tumor necrosis factor-α receptor associated factor
TRAM	TRIF-released adaptor molecule
TRIF	TIR domain-adaptor inducing interferon-β
TSB	Tryptone soy broth

CONTENTS

CHAPTER 1

INTRODUCTION

1 GENERAL INTRODUCTION

The respiratory system consists of two functionally and structurally distinct regions known as the upper and lower respiratory tracts. The upper respirator tract consists of the nasal and paranasal passage, and the pharynx (collectively termed the nasopharyngeal region). The trachea connect the nasopharyngeal region to the lower respiratory tract, and the main bronchi (Fig 1.1A) are often considered as the start of the lower respiratory tract, functioning in the conduction of inspired air though to the gas exchange region of the alveoli.

The alveoli, or the air sacs, are organized as clusters continuous with alveolar ducts. Each alveolus is surrounded by many blood capillaries in such an arrangement that constitutes an extensive air-blood interface separated by a thin tissue barrier and which, therefore, allows for optimal diffusion of gases across the respiratory membrane. The alveolar epithelial-pulmonary capillary barrier comprises mainly the alveolar epithelium and pulmonary capillary endothelium. In part of the barrier the basal membranes of the epithelium and endothelial cells are directly in contact, while in other parts they are separated by interstitium.

The total alveolar epithelium surface area within an average adult human lung has been estimated, using electron microscopy, to be as large as 140 m^2 (Gehr et al., 1978). The alveolar epithelium surface is covered with a film of surfactant that lowers the surface tension during respiration. The volume of this alveolar film has been calculated at between 7-20 mL for the alveolar surface area (Bastacky et al., 1995, Macklin, 1954)

The alveolar epithelium is comprised predominantly of two specialized epithelial cells types; alveolar epithelial cells type-I (AETI) which constitutes approximately 93% percent of the alveolar epithelial surface area (33% by number) and the surfactant producing alveolar epithelial type-II (AETII) cells comprising the remaining 7% by surface area and 67% by epithelial cell number (Crapo et al., 1982). The epithelial type II cells are considerably smaller than type I cells and a uniform cell thickness of approximately 10 μm (Carpo et al., 1982) and richly endowed with organelles and microvilli on its apical membrane. The alveoli are connected to the bronchioles by

tiny ducts and each alveolar sac is surrouned by a capillary bed, which is a network of tiny blood vessels (Fig 1.1B)

The upper respiratory tract removes the vast majority of inhaled particles, and turbulent airflow deposits most of the particles on the mucus. The highly efficient mucocilliary clearance system acts to return most particles to the posterior pharynx where it is swallowed. Small concentrations of inhaled infectious agents that avoid removal by the mucocilliary apparatus (Chandra et al., 1981) and reach the underlying upper and lower respiratory tract epithelium require processing by the immune system of the lung.

Respiratory diseases account for 25% of all emergency medical admission and 34% of all deaths in UK (Chung et al., 2002). Lower respiratory tract infection (LRTI) caused by a variety of microbial agents including viruses and both Gram-positive and Gram-negative bacteria. Bacterial pathogens account for 40-60% of acute exacerbation of chronic bronchitis. Bacterial pneumonia caused by Gram-negative bacteria are the commonest causes of nosocomial pneumonia (Mims C and Playfair J, 1998). Gram-negative bacteria such as *P. aeruginosa* and *B. cepacia* are frequently isolated from the lung and are considered the most important pathogens in cystic fibrosis patients.

Bacteria may cause injury to the lung epithelial cells and this may contribute to bacterial persistence and progressive airway obstruction. Epithelial cells in the lung play a pivotal role in the initiation of inflammatory responses by producing mediators such as interleukin-8 (IL-8) (Diamond et al., 2000), which is a major chemoattractant for neutrophils. The inflammatory response which is induced by bacterial pathogens or their products is critical for the host defense helping to clear the infectious agent and its products from the area. In most cases the immune response leads to elimination of the infectious agents without producing clinically detectable signs of inflammation (Nelson and Summer, 1998). The increased level of this chemokine has been reported from patients with chronic inflammatory lung diseases (Drost and MacNee, 2002).

The innate immune system in the lung has both a recognition function, which detects bacteria and bacterial products in the tissues and attracts phagocytic cells to the site of infection (Zhang et al., 2000, Zhang P, 2000). The inflammatory mediators that are released by alveolar macrophages and non-myeloid cells assist the migration of neutrophils that destroy the foreign agents.

Lung epithelial cells also secrete pulmonary surfactant, which plays an important role in lung function. Pulmonary surfactant, a complex mixture of lipids and proteins, lines the respiratory epithelium of the lung. It exhibits biophysical functions relating to its ability to confer low surface tension at the air liquid interface and prevent lung collapse (Bastacky et al., 1995,Hills, 1999).

In addition to its biophysical action, pulmonary surfactant has been shown to play a role against infection to a variety of bacterial, viral and fungal pathogens (Coonrod, 1987,Crouch and Wright, 2001). Several reports (Morris et al., 2000, Suwab et al., 1998, Tonks et al., 1999) suggested that pulmonary surfactant lipids play an important anti-inflammatory role in the lung. In addition to its anti-inflammatory role, pulmonary surfactant may also have direct effects on bacterial invasion of the lung

As the respiratory epithelium forms the initial contact with bacteria and bacterial products after pulmonary surfactant, understanding the pulmonary surfactant action on bacteria on the epithelial cells response may provide an important potential tool in the elimination of infection in the lung and modulation of the inflammatory responses in the lung epithelial cells.

The results of this study will increase our knowledge and understanding of the potential role of pulmonary surfactant lipids on bacterial lung infection and on its anti-inflammatory role of within the alveoli. This will be important in the development of new strategies to combat the increasing prevalence of chronic inflammation and bacterial infection in the lung.

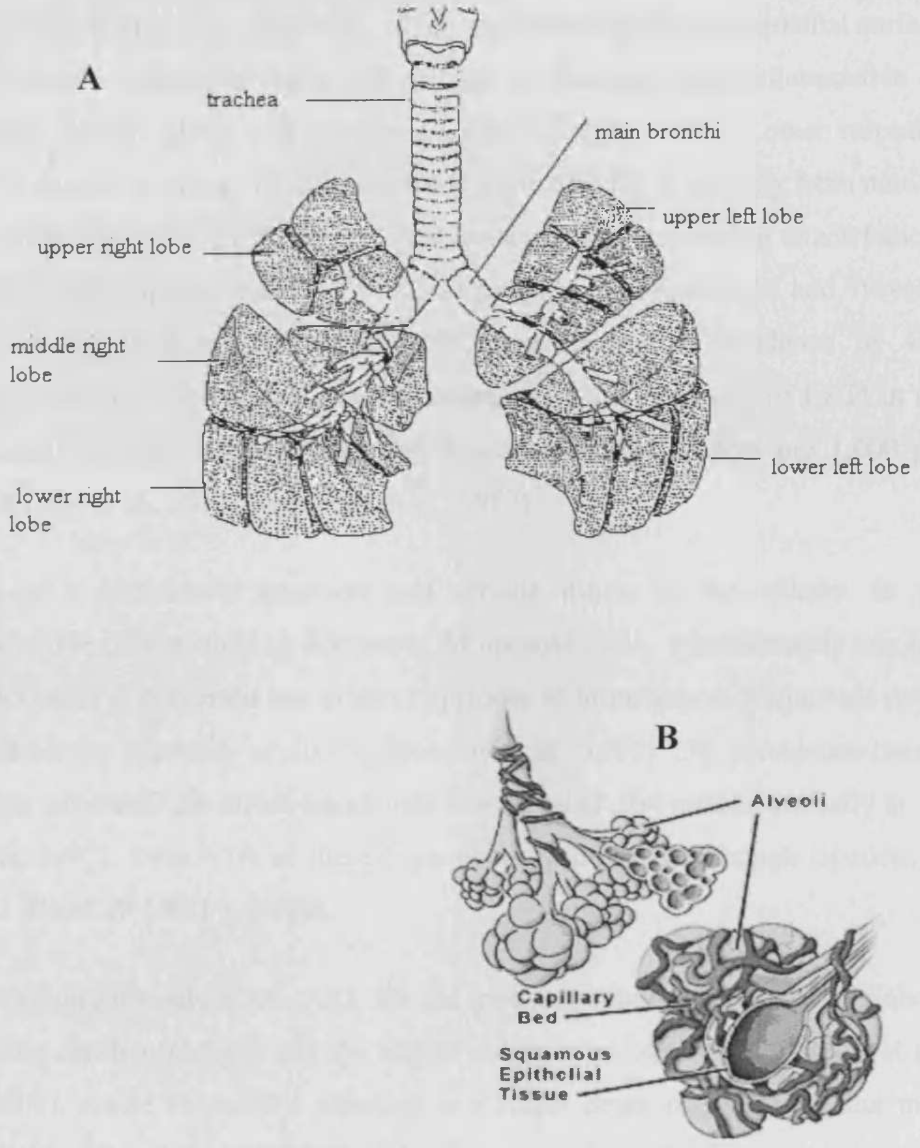


Figure 1.1: Schematic diagram of the human lung. Complete gross anatomy of the lung (A). Each bronchiole supplies air to a cluster of alveoli (B), called an alveolar sac. The alveoli are connected to the bronchioles by tiny ducts and surrounded by many blood capillaries of tiny blood vessels (B). (Modified from: Fuchs et al., 2002).

1.1 LUNG INFECTIONS

Respiratory diseases account for 25% of all emergency medical admission and 34% of all deaths in UK (Chung et al., 2002). The lung, which represents the largest epithelial surface area of the body, is a uniquely vulnerable organ and exposed to damages from inflammation caused by airborne particles, oxidant gases and infectious agents (Wright, 2003). Lower respiratory tract infection (LRTI) describes a range of symptoms and signs, varying in severity from non-pneumonic LRTI in the young healthy adult through to pneumonia or life-threatening exacerbation in a patient with severe COPD. Approximately 1.7 million people aged between 16 and 79 years were treated for LRTI in England and Wales in 1991, representing an incidence of 46/1,000 population (Macfarlane et al., 1993). UK studies documenting the frequency of LRTI in the adult population in general practice suggest an overall incidence of 44-84 cases per 1,000 population per year (Macfarlane et al., 1993,Woodhead et al., 1987).

LRTI is a particularly common and serious illness in the elderly. In a population-based retrospective cohort study in Rochester, Minnesota, USA, approximately one in 18 residents older than 65 years experienced one or more episodes of bronchitis or pneumonia over one year, with an overall 30-day mortality of 10.7% (Houston et al., 1995). UK prevalence-based burden of illness analysis estimated the direct health care cost to be £1,364 million annually at 1992/93 (Guest and Morris, 1997). Over 60% of these costs occur in hospital, although inpatient care accounted for only 1.4% of all LRTI episodes.

Acute respiratory infections (ARI) are the most important single cause of global burden of disease in young children globally and the largest single cause of mortality (Black et al., 2003,Mathers et al., 2001). Acute respiratory infection is a major cause of morbidity and mortality in children worldwide. Four million children under five years of age die from respiratory infections each year (Leowski, 1986,Garenne et al., 1992) and most of the deaths are due to acute lower respiratory infections (ALRI), primarily pneumonia (Garenne et al., 1992). Pneumonia and chronic lung diseases such as COPD account nearly two thirds of deaths from respiratory disease (Chung et al., 2002).

Pneumonia is an acute infection of the parenchyma of the lung resulting from the infection of bronchioles and alveoli in the lung. Pneumonia caused by a variety of microbial agents including both Gram-positive and Gram-negative bacteria, which are the commonest cause of nosocomial pneumonia (Mims C and Playfair J, 1988).

The airway epithelium is remarkably resistant to bacterial invasion. Epithelia provide a formidable mucosal barrier and contribute mucociliary clearance functions. The tight junctions between cells help to prevent microbial invasion (Knowles and Boucher, 2002,Whitsett, 2002,Kagnoff and Eckmann, 1997). Normal epithelial cells secrete antimicrobial peptides (Nakamura et al., 1992,Singh et al., 1998), such as ß-defensin and lactoferrins, which directly contribute to host defense (Harder et al., 2000). There are few bacterial receptors displayed on the apical surface of airway epithelial cells, which may serve to prevent inadvertent activation by transient contamination of the lower airways. However, in response to a significant bacterial challenge or exposure to bacterial products, airway cells actively mobilize signaling components to the apical surface of the airway cell to initiate inflammatory responses (Soong et al., 2004).

The invasion of host cells and tissues by microorganisms is a critical step in the series of events that lead to a successful infection. Bacterial invasion of the respiratory epithelial cells and progress infections to the lung cause lung injury of the epithelial cells (Dreyfuss and Ricard, 2005) and this contribute to bacteria persistence and causes progressive airway obstruction (Gelb et al., 1993).
Many reports found that bacteria such as *P. aeruginosa* and *B. cepacia* adhere and bind to lung epithelial cells (Azghani et al., 2002). Invasion and replication within the respiratory epithelial cells was associated with some bacteria like *B. cepacia* (Burns et al., 1996) and *S. agalactiae* (Doran et al., 2002, Mikamo et al., 2004).

Bacterial pathogens have been shown to induce the elaboration of pro-inflammatory cytokines such as tumor necrosis factor TNF-α, IL-6 and IL-8 in the lung (Sethi and Murphy, 2001), which are potential mechanisms that upregulate inflammation in the lung. Bacterial secreted components also play a role in lung inflammation by inducing inflammatory cytokines such as IL-8 from lung epithelial cells (Fink et al., 2003,Leidal et al., 2001,Palfreyman et al., 1997).

Bacterial components like LPS, a major cell wall component of Gram-negative bacteria, (Rietschel and Brade, 1992) contribute to the lung inflammation and induce different cytokines and inflammatory products in lung epithelial cells (Palfreyman et al., 1997,Pechkovsky et al., 2000). There are also a few reports showing that peptidoglycan (PGN) induces inflammatory cytokines from lung epithelial cells (Saraf et al., 1999). Lipoteichoic acid (LTA) is a surface-associated adhesion amphiphile from Gram-positive bacteria and regulator of autolytic wall enzymes (muramidases). It is released from the bacterial cells mainly after bacteriolysis induced by lysozyme, cationic peptides from leucocytes, or beta-lactam antibiotics. Although there were some reports of cycooxugenase-2 (Cox-2) release from A549 cells (Lin et al., 2002,Lin et al., 2001) in response to lipoteichoic acid of Gram-positive bacteria.

1.2 INNATE IMMUNITY OF THE LUNG

The upper respiratory tract removes the vast majority of inhaled particles, and turbulent airflow deposits most of the particles on the mucus. The highly efficient mucocilliary clearance system acts to return most particles to the posterior pharynx where it is swallowed. Small concentrations of inhaled infectious agents that avoid removal by the mucocilliary apparatus (Chandra at al 1981) and reach the underlying upper and lower respiratory tract epithelium require processing by the immune system of the lung. The lung is an anatomically complex organ with at least three distinct compartments, including the airways and the airspace, the interstitium, and vasculature.

The cells in lung control the inflammatory response and do not respond to all inhaled particles but it has selective response to pathogens. This process depends on the interaction between conserved patterns on microorganisms, pathogen-associated molecular patterns (PAMPs), and host Toll like receptors. Also dose of infection, type of the stimuli and components released are control the response in the lung. This specifity of this response give rationale for anti-inflammatory regulation in the lung. The integrity of the respiratory tract critically depends on a tightly regulated host defence apparatus. The innate immune system provides initial protection against microorganisms and stimulates the adaptive immune response (Medzhitove and Janeway, Jr., 2000).

The infectious agents that reach the alveoli first come in contact with the epithelium and the resident phagocytes (most notably alveolar macrophages). Cellular components of the innate immune system include phagocytic cells such as neutrophils or macrophages, natural killer cells, basophils, mast cells, eosinophils and others. Phagocytes as well as lung epithelial cells release cytokines and chemokines to upregulate the cellular response. These cells initiate and coordinate the host response to infection, including adaptive immunity (Bals et al., 1999,Medzhitov and Janeway, Jr., 2000,McCormack and Whitsett, 2002).

The innate immune system in the lung has a recognition function, which detects bacteria and bacterial products in the tissues and attracts phagocytic cells to the site of infection (Zhang et al., 2000). The inflammatory mediators that are released by alveolar macrophages and non-myeloid cells assist the migration of neutrophils that destroy the foreign agents. These mediators include complement, and chemotactic proteins such as IL-8, macrophage inflammatory proteins such as (MIP-2), and related cytokines (Zhang et al., 2000,Nelson and Summer, 1998a).

The inflammatory response, which is induced by bacterial pathogens or their products, is critical for the host defense helping to clear the infectious agent and its product from the area. In most cases the immune response lead to eliminate of the infectious agents without producing clinically detectable sign of inflammation (Nelson and Summer 1998a).

1.2.1 THE ROLE OF LUNG EPITHELIUM IN INNATE RESPONSES IN THE RESPIRATORY TRACT

The epithelial cells lining the airways and the alveoli act as the primary interface between pathogens and the environment. Through the interaction of pathogens with the epithelium, the protective barrier of the respiratory epithelium will be disrupted and compromised following infectious insults and this will initiate an inflammatory response in the lung (Nelson and Summer, 1998,Diamond et al., 2000,Bals and Hiemstra, 2004). Epithelial surfaces are the most important route of entry of microbial pathogens (Ganz, 2002). A few studies have provided evidence for the presence of complement components at the alveolar epithelium where inhaled particles and microorganisms deposited. The epithelium cells in the lung also synthesize and secreted specific

components of the classical (C2, C3, C4 and C5) and alternative pathways (C5) (Strunk et al., 1988,Zhao et al., 2000).

As a part of the innate immune response, pattern recognition receptors mediate the interaction between conserved patterns on microorganisms, pathogen-associated molecular patterns (PAMPs), and the host. Toll-like receptors (TLRs) have been demonstrated to participate in this process since they are involved in the recognition of microbial-derived molecules from Gram-positive and Gram-negative bacteria (Lien et al., 1999,Schwandner et al., 1999b). To date, at least 11 TLR family members have been identified in mammalian cells (Takeda and Akira, 2005).

The epithelial cells in the lung express pathogen recognition receptors (PPR) and are therefore, capable of detecting the pathogen associated molecular patterns (PAMPs) for LPS (Schulz et al., 2002), peptidoglycan (PGN) and lipoteichoic acid (LTA) (Droemann et al., 2003). The innate immune functions of the respiratory epithelium therefore play a significant role in the pathogenesis of a variety of human diseases. Failure of the mucosal host defense may result in bacterial colonisation and subsequently infection of the airways and the lung parenchyma, which may lead to the access of bacteria to the alveoli.

Stimulation of respiratory epithelial cells with infectious stimuli causes the increase expression and secretion of a number of cytokines. Lung epithelial cells express and secrete chemoattractant cytokines IL-8, growth regulated oncogenes GRO-α, GRO-β, GRO-γ and epithelial cell derived neutrophil chemoattractant (ENA-78) upon stimulation with LPS and supernatant from *S. aureus* (Sachse et al., 2005) that have the ability to activate neutrophil (Zlotnik and Yoshie, 2000). Activation of the epithelial cells also secrete monocyte chemotactic protein (MCP-1) (Krakauer, 2000), and regulated upon activation, normal T-cell expressed, and presumably secreted (RANTES) (Ray et al., 1997) which act as chemoattractant for macrophages, eosinophils (Diamond et al.,. 2000). During inflammation, lung epithelial cells respond to a range of signals from neighboring cells like macrophages, T helper cells (Th) and other cells, as suggested by expression of receptors for several cytokines including IL-1, TNF-α, IL-4.

1.2.2 THE ROLE OF CHEMOKINES IN INFLAMMATION IN THE LUNG

Chemokines constitute a large family of chemotactic cytokines, which are divided into four families based on the position of conserved cysteines: CXX, CC, C and CXC chemokines (Zlotnik and Yoshie, 2000). As a further illustration, the archetypal CXC chemokine and neutrophil chemoattractant, IL-8 has been redesignated CXCL8 and signals *via* receptors CXCR1 and CXCR2 (Rossi and Zlotnik, 2000). The chemotaxis activity is a well-established function of chemokines, and several other responses are characteristic of the activation of leukocytes by chemokines. These include the production of reactive oxygen intermediates (ROI) and the release of cytoplasmic storage granules, such as proteases from neutrophils. Other functions of chemokine include dendritic cell maturation and macrophage and T cells activation. Chemokines have multifunction on the development of the immune responses by being involved in the maturation, differentiation, activation, and chemotactic and trafficking of leukocytes (Wong and Fish, 2003).

Chemokines are thought to play an important role at the site of inflammation in the lung. The human lung epithelial cell line A549 secretes different chemokines including Epithelial Neutrophil Activating Peptide-78 (ENA-78) (Arenberg et al., 1998) a member of the CXC chemokine family, which is defined as a family of leukocyte chemoattractant (Miller and Krangel, 1992). Activation and migration of inflammatory cells are regulated by cytokines called Chemokines, which are specialized cytokines with a very important role providing the directional step for trafficking of leukocytes to the site of inflammation (Lloyd, 2002,Wong and Fish, 2003,Wong et al., 2004).

IL-8 is a non-glycosylated protein that belongs to CXC chemokine subfamily. IL-8/CXCL8 is the most extensively studied member of this group, and its concentration increases during inflammation. The concentrations of IL-8 have been noted to be increased in the sputum and BAL of patients with cystic fibrosis (CF) (Kronborg et al., 1993). IL-8 is released by a variety of cells in the lung including pulmonary epithelial cells (Nakamura et al., 1992). Also Gram-negative bacteria such as *B. cepacia* and *P. aeruginosa* stimulate IL-8 release from lung epithelial cells (Palfreyman et al., 1997, Fink et al., 2003, Reddi et al., 2003). Gram-positive bacteria such *S. aureus*, *streptococcus agalactiae* and *streptococcus pneumonia* also stimulate lung epithelial cells to secrete IL-8 (Doran et al., 2002,Madsen et al., 2000,Mikamo et al., 2004b). Bacterial products such as LPS activate the release of IL-8 from human lung epithelial cells (Koyama et al., 2000). The

migration of neutrophils to the lung and airways is not completely understood. IL-8 or related CXC chemokines lead to rapid neutrophil accumulation. IL-8 is substantially elevated in bronchoalveolar lavage from patients with acute lung injury, and expression levels are positively correlated to neutrophil recruitment (Lamblin et al., 1998).

The gene that transcribes IL-8 in humans is located on chromosome 4, q12-q21 (Luster, 1998, Rollins et al., 1997), and consists of four exons and three introns. The 5'-flanking region of IL-8/CXCL8 contains the usual "CCAAT" and "TATA" box-like structures. In addition, this region has a number of potential binding sites for several nuclear factors (Luster, 1998a,Luster, 1998,Rollins, 1997,Mori et al., 1999).

Previous studies have shown induction of IL-8 mRNA and increased protein level in response to mediators or oxidative stress, including hydrogen peroxide, TNF-α, IL-1 (DeForge et al., 1993,Kwon et al., 2001) and bacteria or their product such LPS (Eckmann et al., 1995,Kunkel et al., 1991).

Regulation expression of IL-8 is beneficial for the lung immune response as it aids lung immune responses in recruiting neutrophils into the lung and thus helping to clear infection. Over production of IL-8 chemokine may lead to high amount of neutrophils recruitment and this may lead to tissue damage by protease and elastase. Pulmonary surfactant may play on regulation of IL-8 gene expression induced by bacteria and bacterial products such LPS. This study will investigate the role of such possible regulation by surfactant lipids.

1.2.3 THE ROLE OF ALVEOLAR MACROPHAGES AND NEUTROPHILS IN LUNG IMMUNITY

AMs play a critical role in immune defence by protecting the lung from infectious agents that evade the removal by the upper respiratory system and gain access to the lower airways. The sources of pulmonary macrophages in the lung are generally peripheral blood monocytes that have migrated into the lung generally or into the alveolus. AM are one unique class of macrophage that function primarily in lung defense against inhaled particulate matter, microorganisms, and environmental toxins (Dorger and Krombach, 2000). Macrophages are versatile in bactericidal activity due to their phagocytic and secretary products capability. The alveolar macrophages are capable of phagocytosing large numbers of infectious agents. The mechanism of phagocytosis is a dynamic process in which the bacteria are attached to the macrophage membrane in the process of ingestion. Besides their phagocytic and microbicidal functions, AM also secrete numerous chemical mediators upon stimulation, thereby playing and initiating inflammatory responses (Twigg, III, 1998) and elaborate a number of proinflammatory cytokines (Miles et al., 1999) .

It has been shown in several lung injury models that activated AM release the cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) as well as the chemokines monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1β (MIP-1β) (Monton and Torres, 1998). Alveolar macrophages phagocytose invading bacteria and kill bacteria such as *P. aeruginosa* and *B. cepacia*, *S. pneumoniae*, *S. aureus*. The phagocytic defence is essential for the clearance of small microbial challenges and maintaining the sterility of the alveolar surface in the lung (Zhang, 2000).

Neutrophils are also one of the key effectors of innate immunity in the lung, and recruiting them into the lung is important in clearance of infections. A variety of stimuli induce neutrophil migration into the lung. LPS of Gram-negative bacteria stimulate the response to chemoattractants and increases neutrophil migration at sites of inflammation. In the lung, the response to LPS is most likely regulated by epithelial cells or alveolar macrophages.

The pattern of migration of neutrophils appears to be unique within the lung. The alveolar epithelial surface contains high concentrations of adhesion molecules, such as intercellular

adhesion molecule-1, which are more concentrated near the junction of alveolar type I and type II epithelial cells (Kang et al., 1993). Under normal conditions it is rare to find neutrophils in pulmonary airways, and neutrophils that reach the alveolar surface are rapidly cleared, suggesting that under normal conditions the lung is designed to exclude rapidly neutrophils from the its alveolar capillary membrane. Regulation of neutrophil migration could be a useful mechanism for allowing neutrophils to conduct surveillance functions. Mechanisms of neutrophils trafficking to the lung, through the lung, and out of the lung are not well defined. Trafficking of neutrophils involves a seemingly number of adhesion molecules and chemoattractants, although the IL-8 chemokine has emerged as a major determinant of neutrophils recruitment in the lung.

Also the recruited neutrophils become functionally activated through stimulation by proinflammatory cytokines such as IL-8 and TNF and other mediators released within the surrounded area in the infected compartment. Antimicrobial factors such as defensins, proteases, lysozymes, reactive oxygen species and other mediators are crucial for the killing of the ingested microorganism inside the neutrophils (Borregaard and Cowland, 1997)

Neutrophils are secondary phagocytic defence when AM fails to protect the lung in adult. Neutrophils may also be important in ingesting and cleaning damaged epithelium from the airways (Hyde et al., 1999). The recent observation that neutrophils undergo apoptosis rather than necrosis in the airways and alveoli is essential to defining pulmonary infections and repair (Haslett, 1999). The average life span of neutrophils is approximately 24 hours and neutrophils infiltrating inflammatory milieu are programmed to die (Cox et al., 1992). Pulmonary surfactant has been noted to modulate the survival of neutrophils in the lung following recruitment (Suwabe et al., 1998).

Clearance of apoptotic cells by phagocytes also plays a role in survival and persistence of inflammation during acute lung injury (Savill et al., 1989). Macrophages and other phagocytic cells recognize apoptotic cells via a number of membrane surface molecules. One of these membrane molecules, namely CD44, appears to play an important role in the clearance of apoptotic neutrophils *in vivo* and *in vitro* (Teder et al., 2002,Hart et al., 1997). It was reported that some bacterial product like *B. cepacia* lipopeptide caused could reduce the protective capacity of pulmonary neutrophils by causing pore formation and cellular apoptosis (Hutchison et al., 1998)

1.3 BACTERIAL LOWER RESPIRATORY TRACT INFECTIONS

Bacteria and viruses may cause infection of the lower respiratory tract. Bacterial infection of the lower respiratory tract can be caused by Gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, group B *streptococcus* (GBS), and by Gram-negative bacteria such as *Klebsiella pneumonia*, *Burkholderia cepacia*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*. Many bacteria are associated with serious opportunistic infection of the lower respiratory airways in many diseases like the association of *Pseudomonas aeruginosa* as an important pulmonary pathogen with cystic fibrosis (CF) (McDowell et al., 2004,Manno et al., 2004). Over the past decade or more however, *Burkholderia cepacia* has emerged as an important lung pathogen in CF patients (Govan and Deretic, 1996). Bacterial products such as LPS also cause lung inflammation and contribute to the lung infections.

Lung and epithelial cells injury by bacteria may occur during infection (Read et al., 1991) and this will contribute to bacterial persistence. Chronic airway inflammation with recurrent *P. aeruginosa* infections is the major cause of morbidity and mortality in patients with CF (Khan et al., 1995). Bacterial factors contribute to the development of chronic infections in CF, particularly the ability of *P. aeruginosa* to form biofilms (Costerton et al., 1999,Shirtliff et al., 2002). Alginate production not only impeded pulmonary clearance but also resulted in more severe lung damage (Song et al., 2003).

P. aeruginosa can cause community acquired pneumonia (CAP). In the community, the incidence of *P. aeruginosa* pneumonia is increased in nursing home residents, patients with chronic obstructive pulmonary disease, and patients recently discharged from the hospital. *P. aeruginosa* rarely causes infection in previously healthy individuals. When *P. aeruginosa* infection occurs in these patients, it has been associated with heavy exposure to aerosols of contaminated water and can be rapidly progressive, with a reported mortality of 33% (Hatchette et al., 2000).

The pathogenesis of *P. aeruginosa* pneumonia is complex, and the outcome of an infection depends on the virulence factors displayed by the bacteria as well as the host response. The large genome of *P. aeruginosa* (Stover et al., 2000) provides a tremendous amount of flexibility and the metabolic capabilities to thrive in environments that are inhospitable to most other organisms.

Surface appendages, such as flagella and pili, which are critical for the initial colonization phase of infection, function as ligands for phagocytic cells or stimulate the recruitment of neutrophils. Thus, mutants that fail to express pili or flagella are less immunogenic (Feldman et al., 1998).

Gram-positive and Gram-negative bacteria may cause infection to the lower respiratory tract. The severity of inflammation is dependant on the immunity in the lung and on the bacteria causing inflammation. Bacterial cell wall components such as lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA) and secretory products such as hemolysin and pyocyanin participate in pathogenicity and inflammation in the lung.

Gram-negative bacteria remain an important cause of morbidity and mortality and have become the leading cause of hospital-acquired pneumonia accounting for 40-60% of the pathogens associated with nosocomial pneumonia (Eisenstadt, J. and L.R. Crane. 1994). Also the persistence of Gram-negative bacteria like *P. aeruginosa* and *B. cepacia* in cystic fibrosis patients is an important source of lower respiratory infections. In addition, *P. aeruginosa* is still the most prevalent organism in the airways of CF patients (Pier, 1985). *P. aeruginosa* is perhaps the most important pathogen associated with morbidity and mortality in CF patients. The mean prevalence of *P. aeruginosa* in CF patients in 1990 was 60.7 % (FitzSimmons, 1993). Initial colonization with *P. aeruginosa* quickly progresses towards a chronic infection, which is never eliminated and in most cases eventually results in the death of CF patients. *B. cepacia* also frequently isolated from CF patients. The percentage of patients colonized with *B. cepacia* has been found to be largely dependent on the CF clinic being examined with colonization rates varying from as little as 10% to as much as 40% (Govan and Deretic, 1996,Sajjan et al., 1995).

1.3.1 GRAM-POSITIVE BACTERIA.

Lower respiratory infection can be caused by a variety of streptococcus bacteria such *Streptococcus pneumoniae* is the most common cause of community-acquired bacterial pneumonia throughout the world (Tuomanen et al., 1995), and group B streptococci like *S. agalactiae* is the most common cause of neonatal pneumonia in industrialised countries.

The transmission of GBS rate from colonised women to the new-born is roughly 50%, and is proportional to the density of maternal colonisation (Mandell et al., 2000). Factors associated with increased invasive early-onset perinatal disease include: rupture of amniotic membranes for greater than 18 h prior to delivery; maternal chorio-amnionitis or fever; rupture of membranes under 37 weeks' gestation; and maternal bacteriuria (Schuchat et al., 1994).

Rates of invasive early onset neonatal disease vary between industrialised countries from 1.15 per 1000 live births in the UK, 0.76 per 1000 live births in Finland up to 3 per 1000 births in some US centers (Beardsall et al., 2000,Kalliola et al., 1999). Only 3% of early onset disease presents as pneumonia. Group B streptococci are important causes of sepsis in pregnant women, and a recognised cause of pneumonia in elderly, non-pregnant adults with underlying liver disease, diabetes mellitus or malignancy. Late onset disease, presenting up to 3 months, rarely presents as pneumonia. GroupB streptococcal pneumonia is appearing to be involved in respiratory distress immediately after delivery or within a few hours of birth, with cyanosis, tachypnoea, apnoea and grunting.

The Gram-positive bacterium *Staphylococcus aureus* is a major pathogen responsible for a variety of diseases ranging from minor skin infections to life-threatening conditions such as sepsis. The pathogenicity of *S. aureus* is due to the repertoire of toxins, exoenzymes, adhesins, and immune-modulating proteins that it produces. With the exception of diseases caused by specific toxins, such as enterotoxins and exfoliative or toxic shock syndrome toxins, no single virulence factor has been shown to be sufficient to provoke a staphylococcal infection. Such infection is rather promoted by the coordinated action of various virulence factors, which are cell wall associated and secreted bacterial proteins.

Indeed, both localized infections, such as soft-tissue abscesses, and life-threatening systemic diseases, such as sepsis, result from the ability of this pathogen to attach to cells or tissues; escape the host immune system, i.e., factors that decrease phagocytosis and factors that interact with antistaphylococcal antibodies; and elaborate proteases, exotoxins, and enzymes, factors that specifically cause cell and tissue damage allowing dissemination of *S. aureus* (Projan, 1997). A study of CF patients showed that *S. aureus* could be cultured from the sputum or respiratory tract secretions of 47% of tested CF patients, respectively (Cystic foundation report 1998).

1.3.2 GRAM-NEGATIVE BACTERIA.

Gram-negative is a mixed group of different bacterial species can cause lower respiratory infection. *P. aeruginosa* and *B. cepacia* are example of these bacteria.

1.3.2.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic rod belonging to the bacterial family *Pseudomonadaceae.* Opportunistic pathogen such as *Pseudomonas aeruginosa,* the disease process begins with some alteration or circumvention of normal host defenses. The pathogenesis of *Pseudomonas* infections is multifactorial, as suggested by the number and wide array of virulence determinants possessed by the bacterium. Multiple and diverse determinants of virulence are expected in the wide range of diseases caused, which include septicemia, urinary tract infections, pneumonia, chronic lung infections, endocarditis, dermatitis, and osteochondritis. Respiratory infections caused by *Pseudomonas aeruginosa* occur almost exclusively in individuals with a compromised lower respiratory tract or a compromised systemic defense mechanism. Primary pneumonia occurs in patients with chronic lung disease. Lower respiratory tract colonization of cystic fibrosis patients by mucoid strains of *Pseudomonas aeruginosa* is common and difficult, if not impossible, to treat.

Of the two million nosocomial infections each year, 10% are caused by *P. aeruginosa.* The bacterium is the second most common cause of nosocomial pneumonia and the most common cause of intensive care unit (ICU) pneumonia. Pseudomonas infections can spread within hospitals by health care workers, medical equipments, sinks, disinfectant solutions, and food. These

infections are a very serious problem in hospitals for two reasons. First, patients who are critically ill can die from a Pseudomonas infection, second, many Pseudomonas bacteria are resistant to certain antibiotics, which make them difficult to treat.

The CF lung is particularly susceptible to *P. aeruginosa*, and this organism plays a critical role in the development and progression of pulmonary disease in these patients (Wine, 1999). Chronic airway inflammation with recurrent *P. aeruginosa* infections is the major cause of morbidity and mortality in patients with CF (Khan et al., 1995). In a longitudinal assessment of *P. aeruginosa* in young children with CF, Burns and coworkers (Burns et al., 2001) used bronchoalveolar lavage and showed that 97% of children with CF were colonized with *P. aeruginosa* by the age of 3 years. CFTR has been proposed to function as a receptor that increases clearance of *P. aeruginosa,* therefore, lack of CFTR could directly impair host defense against this organism. Although there are substantial *in vitro* data supporting this hypothesis (Schroeder et al., 2002,Pier et al., 1997), there are no human data that conclusively demonstrate a major role for CFTR in bacterial clearance mechanisms. There is general agreement that the persistent inflammatory response to bacteria infecting CF airways eventually results in lung damage and fibrosis. However, it remains unclear whether excessive inflammation in CF is entirely caused by exogenous bacterial stimulation (Heeckeren et al., 1997,Muhlebach and Noah, 2002) or if CFTR dysfunction leads to endogenous "hyperinflammatory" responses in the CF airway cell. The pathogenesis of *P. aeruginosa* pneumonia is complex, and the outcome of an infection depends on the virulence factors displayed by the bacteria as well as the host response.

The large genome of *P. aeruginosa* (Stover et al., 2000) provides a tremendous amount of flexibility and the metabolic capabilities to thrive in environments that are inhospitable to most other organisms. *P. aeruginosa* expresses a limited number of polar pili, which are involved in attachment to eukaryotic cells. *P. aeruginosa* also produces polar flagella, which are critical for motility. Flagella are involved in the initial stages of pulmonary infection and activate IL-8 production by binding to TLR5 on the apical surface of airway epithelial cells (Adamo et al., 2004). Shortly after colonization of the lung, flagella expression is turned off, coincident with the expression of genes involved in biofilm production. *P. aeruginosa* encodes a type III secretion system that is a major determinant of virulence and allows the bacterium to inject toxins into the host cell.

The type III secretion system is associated with acute invasive infections and requires pilin-mediated bacterial–epithelial contact (Feldman et al., 1998,Hauser et al., 1998). *P. aeruginosa* secretes four known effector proteins via type III secretion system: ExoS, ExoT, ExoU, and ExoY. ExoT is a bifunctional protein possessing an N-terminal GTPase-activating domain and a C-terminal adenosine diphosphate (ADP-) ribosyltransferase domain. ExoS, like ExoT, is a closely related ADP-ribosyltransferase and ExoY is an adenylate cyclase. In a recent study, ExoS was shown to induce tumor necrosis factor α (TNF-α) production via a MyD88-dependent pathway through activation of both TLR2 and TLR4. The ability to activate cells expressing TLR2 was attributed to the C terminus of ExoS, whereas the ability to activate TLR4/MD-2 complex was attributed to the N terminus of Exo S. Exo U is a potent cytotoxin whose host cellular targets and mechanism of action are not completely known (Finck-Barbancon et al., 1997,Finck-Barbancon and Frank, 2001,Sawa et al., 1999). A recent study indicated that ExoU is a member of the phospholipase A family of enzymes, possessing phospholipase A2 activity. In mammalian cells, the direct injection of ExoU has been shown to cause irreversible damage to cellular membranes and rapid necrotic death (Sato and Frank, 2004). ExoY, the most recently discovered protein, has not been yet implicated directly in cellular toxic effects. *P. aeruginosa* produces two major siderophores: pyochelin and pyoverdin (Takase et al., 2000,Meyer et al., 1996,Vasil and Ochsner, 1999). These siderophores bind iron efficiently and are then taken up by the bacteria through specific cell-surface receptors. The siderophores are major virulence factors important not only for providing iron to support bacterial metabolic processes but also for controlling the expression of other *P. aeruginosa* virulence factors, such as exotoxin A, endoprotease, and pyoverdine itself (Lamont et al., 2002).

In addition to the type III secreted proteins and the quorum-sensing system, *P. aeruginosa* expresses many other virulence factors that contribute to its pathogenicity. Some of these factors help colonization, whereas others facilitate bacterial invasion. Bacterial colonization involves multiple factors, including fimbriae or pili, flagella, and surface polysaccharides. Tissue invasion by *P. aeruginosa* is promoted by the production of elastase, alkaline proteases, hemolysins (phospholipase and lecithinase), cytotoxin (leukocidin), siderophores with their uptake systems, and diffusible pyocyanin pigment. *P. aeruginosa* elastases cleave collagen, IgG, IgA, and complement. The elastases disrupt the integrity of the epithelial barrier by disrupting epithelial cell

tight junctions and interfering with mucociliary clearance. *P. aeruginosa* elastase degrades surfactant proteins A and D (SP-A and SP-D), which have an important role in innate immunity (Mariencheck et al., 2003). Alkaline proteases lyse fibrin, interfere with fibrin formation, and inactivate important host defense proteins, such as antibodies, complement, IFN-γ, and cytokines. Leukocidin is a pore-forming protein that has cytotoxic effects on host cells. Phospholipase and lecithinase are hemolysins that act synergistically to break down lipids and lecithin. These proteins promote invasion by causing cytotoxic effects on host cells (Wilson et al., 1987).

LPS is another important component of *P. aeruginosa.* Environmental isolates of *P. aeruginosa* typically express smooth (typable) LPS with long O-side chains, as opposed to the strains that have adapted to CF lung, which are often nontypable and have lost these O-side chains. Ernst and coworkers (Ernst et al., 1999) have demonstrated that LPS from CF isolates has a characteristic penta- or hexa-acylated lipid A structure. This is associated with increased immunogenicity, although it should be noted that *P. aeruginosa* LPS is much less immunogenic and evokes a more modest cytokine response from macrophages other LPS from different bacteria (Ernst et al., 1999).

1.3.2.2 *Burkholderia cepacia*

Burkholderia cepacia was first described by Burkholder in 1950 as a phytopathogen or plant pathogen causing soft rot in onion. *B. cepacia* comprises at least five distinct genotype species that are phenotypically similar species and referred to as genomovar (Govan et al., 1996,Zhao et al., 1995)

The Gram-negative bacterium *B. cepacia* causes serious opportunistic infections in humans and has emerged as an important pulmonary pathogen in patients with cystic fibrosis (CF) (Govan and Deretic, 1996,Govan and Nelson, 1992,Isles et al., 1984). In CF patients the clinical outcome of *B. cepacia* colonization can vary from maintenance of a normal respiratory function to a rapid and ultimately fatal clinical decline (Isles et al., 1984,Rosenstein and Hall, 1980). Although *B. cepacia* is a phytopathogen, it is also recognised as opportunistic pathogen for human. Apart from patients with CF, *B. cepacia* can colonise and cause infections in immunocompromised patients. *B. cepacia* genomovar III (Vandamme et al., 2003) has been linked to most of the cepacia syndrome deaths in CF patients (LiPuma et al., 2001,Clode et al., 2000).

B. cepacia cell wall is like other Grame-negative has two layers, the outer membrane layer which has the LPS and thin Peptidoglycan layer. Under appropriate cultural condition, strains may elaborate extacellular polysaccharides or release material from outer membrane (Allison and Goldsbrough, 1994).

Interestingly, all three *B. cepacia* strains transmissible genomovar III ET12 lineage, predominantly invaded the epithelium by forming biofilms in mucus that ultimately reached epithelial surfaces (Schwab et al., 2002). It showed bacterial clusters in the luminal exudates within the larger bronchi, some of which were associated with the epithelial surface (Sajjan et al., 2001).

B. cepacia is now recognised as a considerable pathogen in nosocomial infection and in patients with chronic pulmonary diseases. *B. cepacia* was first reported as pathogen in cystic fibrosis patients in mid 1970s (Isles et al., 1984). *B. cepacia* has been implicated in various clinical infections in non cystic fibrosis patients. Many cellular and extacellular product of *B. cepacia* have been considered as putative virulence factors such as adhesion, surface hydrophobicity, extacellular polysaccharide, lipopolysaccharide, extacellular enzyme such as proteases and lipases (Mezzetti et al., 2005), hemolysin, siderophores. Study by Gressner and Mortensen 1990 showed that clinical isolates of *B. cepacia* isolated from CF patients has more production of catalase, ornithine decarboxylase, C14 lipase, alginate and trypsin than environmental strain (Gessner and Mortensen, 1990). Among other extracellular products, which considered, as virulent factors are the siderphores. Production of siderophores has been coorelated with the ability of various bacteria to establish and maintain infection (Sokol, 1986) Unlike *P. aeruginosa*, *B. cepacia* do not produce exotoxin A (Vasil et al., 1986). However, the role of these virulent factors in the severity of infection or transmission and mechanism by which *B. cepacia* colonise the lower respiratory tract remain not fully understood.

Adherence and colonisation of *B. cepacia* is the first step in colonisation and bacterial fimbria has been shown to involve in adhesion. After the discovery of the Cable pili (Cbl) and have been identified as one of the adhesion of a specific epidemic *B. cepacia* clone (Goldstein et al., 1995,Sajjan et al., 2000a). Sajjan et al, found that *B. cepacia* adhesion to respiratory mucin (the major glycoproteins of respiratory secretions) is mediated by pilli. However, binding to the epithelial cells also occurred by a mechanism independent of fimbria, involving a disaccharide unit

and glycolipids receptors. Some epidemic strains of *B. cepacia* produce a brownish melanin-like pigment (Govan and Deretic, 1996), however the role of such pigment in virulent is not clear.

1.4 BACTERIAL CELL WALL COMPONENTS

1.4.1 LIPOPOLYSACCHARIDE (LPS)

Lipopolysaccharide (LPS) is a major component of the outermost membrane of Gram-negative bacteria. As such, it plays a central role in the pathogenesis of infections by these bacteria lipopolysaccharide. LPS is found exclusively in the outer membrane of Gram-negative bacteria. LPS forms a hydrophobic barrier, which restricts the entry of many substances such as bile salts, and certain antibiotics, and enable the bacteria to evade many innate immune host defence factors including complement and lysosyme and cationic proteins (Nikaido and Nakae, 1979). LPS is a complex glycolipid composed of two parts, a hydrophilic polysaccharide moiety and a hydrophobic domain known as lipid A (Seydel et al., 2000,Muller-Loennies et al., 1998). LPS is released either in a free form or as a complex with bacterial surface proteins during multiplication of the bacteria or after treatment with antibiotics and after cell death. LPS is a potent activator of the innate immune system and triggers pro-inflammatory responses in many mammalian cells (Heumann and Roger, 2002,Lukasiewicz and Lugowski, 2003,Janeway, Jr. and Medzhitov, 2002).

1.4.1.1 LPS structure

The basic structure of LPS (Fig 1.2A) consists of two main parts: lipid (lipid A) forming the matrix of the outermost membrane leaflet, which is stabilized by divalent cations, and a hydrophilic polysaccharide (PS), extending outward from the bacterium. The PS consists generally of two distinct regions, a core oligosaccharide containing 10–12 sugars, and a polysaccharide chain of repeating units, the O-specific chain. The core is covalently bound through an acidic sugar, usually 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo), to the lipid A.

The proximal "inner core" region also contains heptose residues (Hep), which are often substituted by phosphate (P), pyrophosphate (PP) or diphosphoethanolamine (PPEtN). The "outer core" region usually consists of neutral or amino hexoses such as D-glucose, D-galactose, D-glucosamine, D-

galactosamine or *N*-acetyl derivatives. The presence of O-chains in the LPS structure indicates the smooth morpohological colony appearance termed "smooth" (S), whereas, LPS lacking the O-chain are accordingly termed rough (R) and the bacterial colonies will have rough appearance.

Lipid A have a common Backbone consisting a β-(1-6)-linked glucosamine disaccharide backbone with phosphate monoesters at C-1 and C-4' and β-hydroxyl fatty acyl groups and acyloxyacyl residues at positions 2 and 3, and 2' and 3', respectively (Fig 1.2B). Lipid A is linked to the polysaccharide through the Kdo at position C-6 or the non-reducing glucosamine residue. Lipid A is the primary immunostimulator center of LPS, promoting the activation of the innate immune system via the induction of inflammatory cytokine released by human cells (Medzhitov, 2001,Zahringer et al., 1994). Initial chemical analysis of *B. cepacia* core LPS indicates the absence of detectable 3-deoxy-D-manno-oct-2-ulopyranosonic acid (KDO) (Manniello et al., 1979,Anwar et al., 1983), and with comparison to *P. aeruginosa* LPS, *B. cepacia* LPS has less phosphorous content and more heptose. However it was reported that clinical strains of *B. cepacia* have (KDO) (Straus et al., 1989,Straus et al., 1990).

The O side chain structure consist of repeating units one to eight sugars and the units are polymerized during biosynthesis before addition before addition to the core. The whole chain may contain up to 50 units and the nature and the number of the sugar in within a unit, the nature linkages of the sugars as well as the number of repetitive units produce an enormous structural diversity. The result of such diversity provide the serotypes specificity of each bacterial species or strain (Caroff et al., 2002). Enormous structural diversity exists in O-chains involving the nature and number of sugars within a unit, the nature of the linkages of the sugars as well as the number of repetitive units. The result of such diversity provides the serotype specificity of each bacterial strain.

Nevertheless, there are examples of different bacteria which are serologically the same and share the same O-chain structure and they are, in addition, homopolymeric structures (Caroff et al., 2001). The majority of *B. cepacia* strains isolated from clinical and environmental conditions express smooth LPS (McKevitt and Woods, 1984). There is no evidence so far to confirm that *B. cepacia* strains undergo phenotypic changes from smooth to rough LPS type within the lung as observed with *P. aeruginosa.*

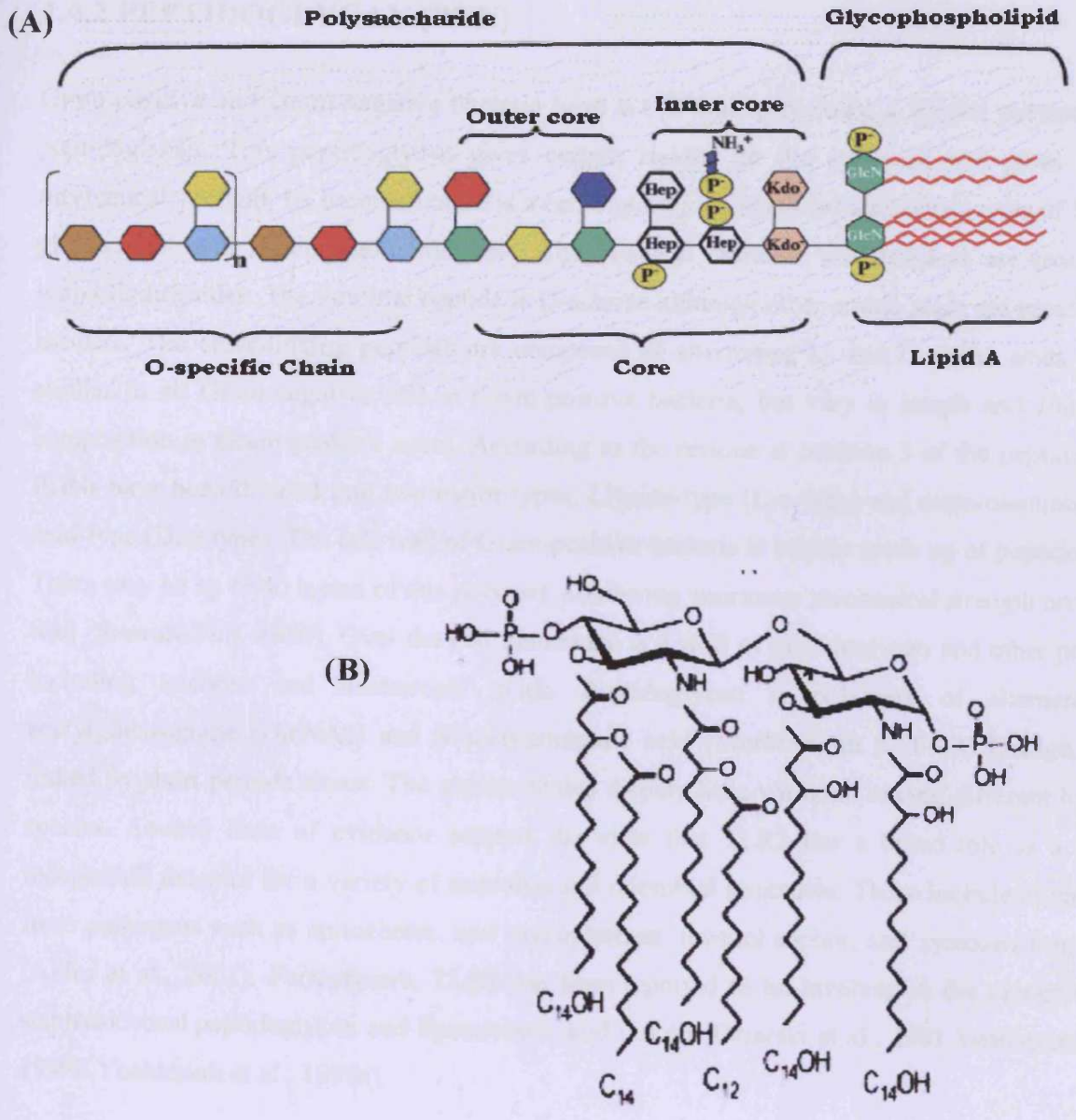


Figure 1.2: Schematic representation of the chemical structure of LPS. Abbreviations: GlcN, glucosamine; Kdo, 2-keto-3-deoxyoctulosonic acid; Hep, L-glycero-D-*manno*-heptose; P, phosphate; EtN, ethanolamine; zig-zag lines, fatty acids (A). Chemical structure of Lipid A (B). Modified from (Caroff et al., 2002).

1.4.2 PEPTIDOGLYCAN (PGN)

Gram-positive and Gram-negative bacteria have a cell wall containing a special polymer called peptidoglycan. This peptidoglycan gives certain rigidity to the cell wall and gives the cell mechanical strength. Its basic structure is a carbohydrate backbone of alternating units of N-acetyl glucosamine and N-acetyl muramic acid. The N-acetyl muramic acid residues are cross-linked with oligopeptides. The terminal peptide is D-alanine although other amino acids are present as D-isomers. The cross-linking peptides are composed of alternating L- and D-amino acids and are similar in all Gram-negative and in Gram-positive bacteria, but vary in length and amino acid composition in Gram-positive cocci. According to the residue at position 3 of the peptide stems, PGNs have been divided into two major types: Llysine-type (Lys-type) and *meso*-diaminopimelic acid-type (Dap-type). The cell wall of Gram-positive bacteria is largely made up of peptidoglycan. There may be up to 40 layers of this polymer, conferring enormous mechanical strength on the cell wall (Stewart-Tull, 1980). Over the cell membrane is a shift of peptidoglycan and other polymers including teichoic and teichuronic acids. Peptidoglycan is polymers of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) in β (1→4) linkage, cross-linked by short peptide stems. The glycan chains display little variation among different bacterial species. Several lines of evidence support the view that TLR2 has a broad role as a pattern recognition receptor for a variety of microbes and microbial structures. These include lipoproteins from pathogens such as spirochetes, and mycoplasmas, inositol anchor, and zymosan from fungi (Akira et al., 2001). Furthermore, TLR2 has been reported to be involved in the recognition of staphylococcal peptidoglycan and lipoteichoic acid (LTA) (Dziarski et al., 2001,Yoshimura et al., 1999b,Yoshimura et al., 1999a).

It has been found that larger amounts of peptidoglycan, in the range of 10 to 100μg/mL range, are necessary to stimulate cellular responses compared to LPS, which induces responses in the ng/ml range (Kusunokiet al., 1995). Since 1×10^6 to 6×10^6 CFU correspond to 1 μg of staphylococcal peptidoglycan (Travassos et al., 2004), whereas 10^7 CFU correspond to 20 ng of LPS, this suggests that whole peptidoglycan is about 100-fold less active than LPS. Studies of *S. aureus* cell walls indicate that only part of peptidoglycan is active. Indeed, insoluble and soluble peptidoglycan chains of high molecular weight are not very inflammatory. Hydrolyzing these chains to sugar and peptide monomers completely abolishes inflammation. In staphylococcal peptidoglycan, three

cross-linked stem peptides appear to be the minimal structural constraint to be inflammatory (Moreillon and Majcherczyk, 2003). Furthermore, treatment of *S. aureus* by lysostaphin, which cleaves the pentaglycine bridge, moderately attenuates release of cytokines, whereas digestion with cellosyl, a muramidase hydrolyzing glycosidic bonds, nearly abrogates the induction of cytokine (Myhre et al., 2004,Yoshimura et al., 2000). This suggests that the glycan strand is crucial for cytokine production, whereas stem peptide structure does not seem to be critical for the inflammatory activities of peptidoglycan

1.4.3 LIPOTEICHOIC ACID (LTA)

Teichoic acids are polymers of glycerol or ribitol linked via phoshodiester bond. These acids can be found in the cell wall of Gram-positive bacteria and appear to extend to the surface of the Peptidoglycan layer. Teichoic acids are not found in the Gram-negative bacteria. They can be either covalently bonded to N-acetylmuramic acid of the peptidoglycan layer or linked to the plasma membrane lipids found in the cytoplasmic membrane. The combined units comprised of teichoic acids and lipids are referred to as Lipoteichoic acids. Teichoic acids are negatively charged and therefore contribute to the negative charge of the Gram-positive cell wall. They may also provide structural support for the cell wall.

The wall of the Gram-positive bacterium constitutes a multifaceted fabric that is essential for survival, shape, and integrity (Weidel and Pelzer, 1964). Macromolecular assemblies of cross-linked peptidoglycan (murein), polyanionic teichoic acids (TAs), and surface proteins function within this envelope. Teichoic acids are composed of wall teichoic acid (WTA) and lipoteichoic acid (LTA) (Wicken and Knox, 1975,Fischer, 1994,Baddiley, 1989). WTA is covalently linked to the peptidoglycan, whereas LTA is a macroamphiphile with its glycolipid anchored in the membrane and its poly (glycerophosphate) chain extending into the wall.

LTA is a member of a class of macromolecules known as modulins that induce a variety of proinflammatory mediators (Fischer, 1994,Keller et al., 1992, Ginsburg, 2002). In synergy with peptidoglycan, LTA causes septic shock and multiple organ failure (De Kimple et al., 1995). The mediators in these host responses include cytokines (IL-1ß, IL-6, IL-8 and TNF-α), nitric oxide, and reactive oxygen intermediate. A clear correlation between the D-alanyl ester content of LTA and virulence has been established for *S. agalactiae*, and *S. aureus* (Collins et al., 2002,Poyart et al., 2003). LTA has been implicated in the virulence of a number of Gram-positive bacteria (Hasty et al., 1992,Sutcliffe and Shaw, 1991). Thus, LTA induces an inflammatory response. However, very large amounts of LTA are necessary to induce responses of cells *in vitro*. Indeed, LTA, in the 1 to 10 μg/mL range is required to trigger cellular responses while LPS in the ng/mL range is sufficient to elicit responses (Kusunoki et al., 1995). It must be taken into consideration that the active concentrations of LTA (1 μg or 10^5 to 10^7 CFU) as well as of LPS (20 ng or 10^7 CFU) are comparable when they are transposed to bacterial cell equivalents (Schroder et al., 2003,von

Aulock et al., 2003), suggesting that LTA and LPS preparations may have similar potency. Comparison of the activity of LPS versus LTA showed that staphylococcal LTA is able to promote the same strong induction of chemoattractants (IL-8, MIP-1α, MCP-1), granulocyte colony-stimulating factor, and anti-inflammatory cytokines (IL-10) as LPS, whereas it is a weaker inducer of TNF-α, IL-1ß and IL-6 (von Aulock et al., 2003,von Aulock et al., 2004). The cytokine pattern produced by LTA is similar to that induced by the whole bacterium (Kusunoki et al., 1995,von Aulock et al., 2003,Wang et al., 2000).

1.5 BACTERIAL ADHERENCE AND INVASION OF LUNG EPITHELIAL CELLS

The epithelial cells in the lung are the first line of contact with bacteria. Adherence is the first important step in initiation of infection, and the initial stage in lower respiratory infection is the bacterial attachment to the epithelial cells, which may lead to bacterial invasion. A confusing plethora of ligand receptor systems has been described for binding bacteria to the epithelial cells. These include pili, outer membrane proteins and even lipopolysaccharide of the bacterium (Saiman and Prince, 1993,Plotkowski et al., 1996) and gangliosides (asialyl-GM-1), fucose residues, heparan sulphate proteoglycan or even mutant CFTR itself on the lung epithelial cells (Imundo et al., 1995,Scanlin and Glick, 1999,Plotkowski et al., 2001).

Many pulmonary pathogens bind to the GalNAc β-4Gal moiety which is available on cells with asialylated glycolipids (Krivan et al., 1988). Examples include lung pathogens such *S. aureus*, and *P. aeruginosa.* While such asialylated receptors are not normally available on the airway surface to any great degree, they are significantly increased in area of cell damage and regeneratin (de Bentzmann et al., 1996). In *P. aeruginosa* and *B. cepacia* there is direct correlation between the adherent and the amount of IL-8 expression in the epithelial cells. The epithelial binding is mediated by pili, which bind directly to GalNAc β-4Gal moiety of asialylated glycolipids.

P. aeruginosa expresses a limited number of polar pili, which are involved in attachment to eukaryotic cells. They bind to the GalNacß1-4 gal moiety exposed on asialylated glycolipids and then activate NF-κB and proinflammatory gene expression through a receptor complex that includes asialoGM1, Toll-like receptor 2 (TLR2), and associated kinases in a lipid raft (Adamo et al., 2004). Because the antigenically dominant epitope of *P. aeruginosa* pili is distinct from the cell-binding domain, strategies to prevent pilin-mediated bacterial adherence have been unsuccessful thus far. *P. aeruginosa* also produces polar flagella, which are critical for motility.

Flagella are involved in the initial stages of pulmonary infection and activate interleukin-8 (IL-8) production by binding to TLR5 on the apical surface of airway epithelial cells (Adamo et al., 2004). Shortly after colonization of the lung, flagella expression is turned off, coincident with the expression of genes involved in biofilm production.

Burkholderia cepacia binds to the epithelial airway in the lung, at least five morphologically different pili have been detected on epidemic and non-epidemic strains including cable, filamentous, spike and mesh form (Goldstein et al., 1995). Of these, cable pili, which are associated with *B. cepacia* ET-12 strain, are the best charaterised (Sajjan et al., 2000b). Strains with this pili bind to cytokeratin 13 (Sajjan et al., 2000a) which is enriched on the hyperplastic epithelia of CF airways (Sajjan et al., 2000b). Some cable-negative *B. cepacia* appear to bind to asailyl GM1 (Sylvester et al., 1996).

1.6 INNATE IMMUNE DETECTION OF BACTERIA

The innate immune response is the first line of defense against microbial pathogens, and plays an important role in activating acquired immunity. When bacteria reach to the lower respiratory tract first interact to the epithelial cells and the start to initiate the binding and invasion. The epithelial cells in the lung express pathogen recognition receptors (PPR) and are therefore, capable of detecting pathogen associated molecular pattern (PAMP) which are conserve product of microbial metabolism (Janeway, Jr., 1989). PAMPs are broadly distributed among pathogens for example, for bacterial LPS (Schulz et al., 2002). The largest and best studied family of PRRs comprises the Toll-like receptors (TLRs), which are membrane associated and have an external leucine-rich repeat recognition domain and an intracellular Toll-interleukin 1 receptor signaling domain (Akira, 2003). Each TLR has been shown to recognize specific components of pathogens, thus demonstrating that the mammalian immune system detects invasion by pathogens via the recognition of microbial components by TLRs. It is now well established that TLRs play an important role in the recognition of microbes during host defences (O'Neill, 2003).

The discovery of Toll, and hence the TLRs, came about as a consequence of a forward genetic screen in the Nüsslein-Volhard laboratory (Anderson et al., 1985), and TLR is the core receptors in the signalling pathway and were identified in insect as a receptors that control dorsal-ventral polarity during embryogenesis (Anderson et al., 1985; Stein et al., 1991). Subsequent studies revealed that it also plays an essential role in the insect immune responses against fungal infection (Lemaitre et al., 1996). Mammalian homologues of TLRs were subsequently identified through expressed sequences tag and genomic sequence database search and named Toll-like receptor (Medzhitov et al., 1997). The TLRs is the member of a bigger family of protein that are characterised by a 150- amino acids domain termed as TIR (Toll/IL-1R/R) domain, which is crucial for signalling, that they share with members of the IL-1R family and plant disease resistance gene (Akira, 2003., Martin et al., 2002). TIR-domain containing proteins are involved in the development and in both animal and plants. There are total of 21 TIR-domain-containing protein so far described in human, 10 of these being TLRs, eight being more like the IL-1R1 and three cytosolic proteins termed myeloid differentiation protein MYD 88 (Janssens et al., 2002), MYD88 adaptor like (Mal) protein (Fitzgerald et al., 2001) and TIR domain-containing adaptor inducing interferon-β (TRIF) (Yamamoto et al., 2003b), which act as adaptors in signalling. The

extracellular domain of TLRs contains 19-25 tandom copies of the leucine-rich repeat (KRR) motif. Each repeat consist of 24-29 amino acids and contains the leucine-rich sequence. The LRR domains of TLRs are thought to be involved directly in the recognition of various pathogens (Akira, 2003). Tow recent papers reveal the distinctive architecture of a TLR sensor domain and hint at how this structural design facilitate the recognition of a wide array of pathogen molecules (Choer et al., 2005; Bell et al., 2005). The cytoplasmic membrane domain-1 receptor (TIR) domain named after the two groups of proteins where it was found initially.

Currently there are at least 11 homologues of Toll designated Toll-like receptors (TLRs) have been found in mammalian (Takeda and Akira, 2005). TLR1-9 are conserved between human and mouse. Of these known TLRs, TLR2, TLR3, TLR4, TLR5, and TLR9 have been extensively characterized (Akira, 2003,Takeda et al., 2003) and showed to recognize distinct PAMPs. TLR1, TLR6, TLR7, and TLR8 have not yet been shown to independently impart signals after recognition of specific microbial products.

In addition to TLRs, a second class PRRs Nod1 and Nod2 that detected PGN. These proteins are located in the cytoplasm and are involved in the detection of bacterial PAMPs that enter into the cell either with an invasive microbe or by translocation by certain pathogenic bacteria through specialized transfer apparatuses (Chamaillard et al., 2003,Girardin et al., 2003c,Girardin et al., 2003a). Like TLRs, Nod proteins have a leucine-rich repeat recognition domain linked to a signaling motif called the caspase activation and recruitment domain. TLRs and Nod1 trigger similar signaling pathways characterized by activation of the transcription factor NF-κB, leading to the production of proinflammatory cytokines. TLRs comprise a family of transmembrane proteins that play an essential role in detection and signaling infections. Individual TLRs recognize distinct structural components of pathogens. TLR domain detect LPS from the outer membrane of Gram-negative bacteria and Peptidoglycan which is found in the cell wall of both Gram-negative and Gram-positive organisms, and Lipoteichoic acid (LTA) (Droemann et al., 2003).

Once detection of the PAMPs has occurred by the host TLRs, the signal is transmitted inside leading to the activation of transcription factors such as NF-κB, which drive the expression of genes, whose protein products play a key role in the defense response against the invading pathogens.

LPS, which part of the cell wall of Gram-negative bacteria is detected through TLR-4 and this will be discussed later. Flagillin, which is part of the bacterial cells of Gram-negative, and also secreted from the bacteria is a potent of inflammatory cytokines. Purification of the culture supernatants containing TLR5-stimulating activity led to the identification of flagellin as the active component (Hayashi et al., 2001). Flagellin is the primary protein component of flagellar, a highly complex structure that extends out from the outer membrane of Gram-negative bacteria. Flagella serve as the propellers that move the bacteria through their aqueous environment. They also aid in the attachment of the bacteria to the host cells, assisting in bacterial invasion and thereby contributing to the virulence of pathogenic bacteria. The flagellin genes from a variety of Gram-negative bacteria share highly conserved regions at their amino- and carboxy-termini, and these regions are responsible for the immunostimulatory activity of flagellin (Eaves-Pyles et al., 2001).

Bacterial DNA is a potent activator of immune cells. The critical involvement of TLR9 in the recognition of bacterial DNA was demonstrated using TLR9-deficient mice (Hemmi et al., 2000). The immunostimulatory activity of bacterial DNA is attributed to the presence of unmethylated CpG motifs, which are relatively infrequent in the vertebrate genome and when they occur are typically methylated on their cytosine residues and lack any immunostimulatory activity.

Recent studies show that bacterial DNA induce cytokine from respiratory epithelial cells and TLR9 is expressed in airway epithelia and is likely involved in this pattern recognition process (Platz et al., 2004).

1.6.1 INNATE IMMUNE DETECTION OF PGN AND LTA

Gram-positive bacterial cell walls are composed of multiple peptidoglycan layers, wall teichoic acids linked to the peptidoglycan and lipoteichoic acid linked to the cytoplasmic membrane. Both wall teichoic acids and lipoteichoic acids are highly charged polymers. They concentrate cations at the cell wall surface and are associated with proteins to form complexes. LTA is the major macroamphiphile molecule of Gram-positive bacteria. The physiochemical properties of LTA are similar to those of LPS in Gram-negative bacteria. Staphylococcal LTA consists of about 25 poly (1-3)-glycerol phosphate linked to a diacylglycerolipid anchor. The hydrophilic polyglycerol phosphate chain is long enough to penetrate the peptidoglycan, and the lipid moiety attaches the polymer to the surface of the cytoplasmic membrane. The glycolipid structure resembles the bacterial membrane composition and usually diverges among Gram-positive bacteria in a genus-specific manner (Fischer, 1988).

Several lines of evidence support the view that TLR2 has a broad role as a pattern recognition receptor for a variety of microbes and microbial structures. These include lipoproteins from pathogens such as mycobacteria, spirochetes, and mycoplasmas, lipoarabinomannan from mycobacteria, *Trypanosoma cruzi* glycosylphosphatidylinositol anchor, and zymosan from fungi (Akira et al., 2001). Furthermore, TLR2 has been reported to be involved in the recognition of staphylococcal peptidoglycan (Schwandner et al., 1999a,Underhill et al., 1999) and lipoteichoic acid (LTA). LTA appears to constitute a broad immunostimulatory factor of Gram-positive bacteria with possibly differing potencies depending on the constituents of the molecule (Ginsburg, 2002,Hermann et al., 2002).

Although crucial for bacterial life, purified wall teichoic acids of *S. aureus* are not very inflammatory (Majcherczyk et al., 2003). However, a number of studies suggest that the bacterial LTA from *S. aureus* has been shown to provoke secretion of cytokines and chemoattractants (TNF-α, IL-1ß, IL-10, IL-12, IL-8, and granulocyte colony-stimulating factor from monocytes or macrophages (Bhakdi et al., 1991,Cleveland et al., 1996a,Keller et al., 1992,Standiford et al., 1994,von Aulock et al., 2003).

TLR2 is expressed by different cells involved in the inflammatory response such as monocytes/macrophages, neutrophils, dendritic cells and mast cells (Esen et al., 2004,McCurdy et al., 2003,Sabroe et al., 2003,Visintin et al., 2001).

Like Gram-negative bacteria, the major components of the Gram-positive bacterial cell wall employ CD14 for immune recognition of both PGN and LTA. Both PGN and LTA have been demonstrated to activate macrophage in a CD14-dependant manner (Gupta et al., 1996,Cleveland et al., 1996b). LTA from *S. aureus* binds to membrane CD14 and acts as an antagonist to other CD14-mediated stimuli (Kusunoki et al., 1995). Recently it has been shown that LTA/TLR2 and its receptors molecules accumulate in the lipid rafts and subsequently targeted rapidly to the golgi apparatus and this process occur in the cell surface (Triantafilou et al., 2004,da Silva et al., 2001).

The expression and activity of TLR signaling is regulated by soluble accessory proteins known as MD-1 and MD-2 (Dziarski and Gupta, 2000). It has been shown that PGN binds to CD14 (Dziarski et al., 1998), and that blockade of this receptor inhibits signaling events induced by both PGN and LTA (Cleveland et al., 1996c,Weidemann et al., 1994) led to the contention that CD14 is involved in signaling of Gram-positive infections. Both bacterial components such as PGN and LTA activate the host defense by engaging TLRs and other pattern recognition receptors of the innate immune system.

Upon recognition of a TLR2 ligand (e.g., PGN or LTA) a range of intracellular signaling events occurs, leading to the activation of signaling kinases of the MAPK family (ERK1/2, JNK, and p38 (Schroder et al., 2001,Vasselon et al., 2002), PKB (Arbibe et al., 2000), and IKKβ (Wang et al., 2001b). Further, several transcription factors, including nuclear factor κB (NF-κB, p50, p65) are activated. Also other factors are activated by the kinase cascade, resulting in the expression of a wide range of proinflammatory mediator genes.

The first event that initiates the signaling cascade is most likely the colocalisation and clustering of receptors and signaling molecules at the plasma membrane. Clustering of TLR2, TLR6, and CD14 in the recognition of secreted microbial products from group B Streptococcus (GBS) have been shown (Henneke et al., 2001). The first intracellular molecule recruited to the complex is the Toll/IL-1 receptor (TIR)-domain containing adapter molecule MyD88 (myeloid differentiation

factor 88 (Muzio et al., 1997). MyD88 then recruits IL-1 receptor associated kinases/IRAKs (Cao et al., 1996). Further on, TAK1 will be activated and released, and may activate IKKβ and MAP kinase (Wang et al., 2001a). The NF-κB-inducing kinase (NIK) is required for the activation of IκB kinase β (Ninomiya-Tsuji et al., 1999), which initiates IκB degradation and NF-κB nuclear translocation (Karin and Delhase, 2000).

Also recent studies have shown that peptidoglycan signaling involve of nucleotide oligomerization domains (NODs), and highlighted the importance of NOD1 and NOD2 in innate immunity. It is thought that they act as cytoplasmic sensors of bacteria and bacterial products. These proteins are part of a larger family of related proteins named CATERPILLER proteins by Ting and co-workers (O'Connor, Jr. et al., 2003), characterized by an LRR and a nucleotide oligomerization domain (NOD) (Girardin et al., 2003c,O'Connor, Jr. et al., 2003).

The ligand binding sites of NOD1 and NOD2 are likely within the leucine-rich repeat domains. NOD1 also contains a single caspase activating and recruitment domain (CARD) while NOD2 contains two CARD domains. The CARD domain provides at least one point for assembly of the NOD signalsome responsible for intracellular signaling triggered by ligand binding. A CARD domain containing kinase known as RICK is required for NOD signaling (Kobayashi et al., 2002). Although NOD2 also detects bacterial peptidoglycan, its role in bacterial sensing is broader than that of NOD1 since it can detect PGN from a wide range of both Gram-positive and Gram-negative organisms. The reason for this is that NOD2 can detect the minimal structure of PGN, which is MDP (Girardin et al., 2003b,Inohara et al., 2003). The precise signaling events triggered via NOD1 and NOD2 are poorly understood.

1.6.2 INNATE IMMUNE DETECTION AND SIGNALLING OF LPS

Gram-negative bacteria cells have different componenents, which comprise the cell wall structure including LPS and PGN in addition to protein like flagella. Moreover, Gram-negative bacteria may excrete proteins that are involved in pathogenicity and induction of inflammatory cytokines. Of these, LPS is the most important extensively studied virulence factor present on Gram-negative negative bacteria.

The pathway that leads to LPS recognition and signalling involves many signalling proteins. Although TLR4 is the principal signalling receptors of LPS (Poltorak et al., 1998), three other accessory proteins are required for LPS-sensing. LPS first bind to LPS binding protein (LBP), which transfer LPS monomer to CD14, which form a ternary complex with LPS and LBP. LBP is a blood-born protein that is produced in the liver (Schumann et al., 1990) and has the properties of an acute phase reactant (Schumann et al., 1999). In addition to the liver, LBP is produced in the lung, where it has numerous proposed important physiological and pathophysiological activities, including a role in ARDS, nosocomial Gram-negative pneumonia, pneumococcal disease and asthma. LBP has opsonic activity, but its importance as a protein involved in LPS response lies with its abilities to accelerate the binding of LPS to CD14 (Yu and Wright. 1996).

CD14 was initially discovered as the receptor for LBP bound LPS, and it is central to mammalian responses to LPS. CD14 is present in two forms: a GPI-linked form and as a soluble proteolytic fragment found in blood (referred to as soluble CD14). Soluble CD14 functions to enhance LPS responses in cells that do not ordinarily express CD14. Over 1400 publications have documented the importance of CD14 in LPS responses (Gangloff et al., 2005), and it has shown that CD14-deficient mice are insensitive to LPS (Moore et al., 2000).

The third protein that TLR4 requires to interact functionally with LPS is MD-2 which was discovered and identified as a molecule that associates with the extracellular portion of TLR4 and enhances LPS responsiveness (Shimazu et al., 1999,Akashi et al., 2000). MD-2 is an 18–25-kDa protein with a cleavable signal sequence that is both bound to TLR4 in the Golgi and is secreted as a soluble molecule from MD-2-expressing cells (Shimazu et al., 1999, Visintn et al., 2001).

It appears to be that CD14 bind to LPS and subsequently presenting it to MD-2 and TLR4 (Poltorak et al., 1998). It was revealed that the presence of MD2 was essential for LPS responses (Akashi et al., 2000). CD14 was found to associate with TLR4 and MD (Pfeiffer 2001) forming a tri-molecular receptor cluster that leads to LPS-induced activation.

Study by Visintin and his colleagues (Visintin et al., 2003) demonstrated that LPS binds to MD2 which is in turn, binds to TLR4 and induces aggregation and signal transduction. Another study demonstrated that MD-2 binds to TLR-4 and this binding was dependent on Cys^{95} and Cys^{105}, which might form an intramolecular disulfide bond that creates a tertiary structure that are required for MD-2-TLR4 interaction (Re and Strominger, 2003).
Despite the evidence that for LPS recognition by TLR4-MD-2 complex, little is known about the binding mode of LPS with regard to the role of TLR4 in this process. Following interaction with LPS and MD-2, TLR4 forms a dimer, which appears to be pivotal in initiation of pr-inflammatory cytokines (Medzhitove et al., 1997). The association of TLR4-MD2 with LPS induces a conformational rearrangement that results in dimerisation of the TLR4 receptor, however, it is not known how for certain (Gangloff et al., 2004) but at any event, LPS-trigger TLR4 dimerisation results in TLR activation that delivers the signal downstream and couples LPS detection with LPS signalling. Some groups have proposed that recognition of LPS by TLR4 involves direct binding, while others have suggested that LPS binds to MD-2 and CD14, and this complex somehow stimulates TLR4 (Lien et al., 2000,Poltorak et al., 2000,Viriyakosol et al., 2001,Akashi et al., 2001). TLRs both bind ligand and transduce signals into the cell following ligand binding. The latter occurs via the TIR domain and formation of a TLR-signals including members of the MyD88 family of adaptor proteins (Cristofaro and Opal, 2003,Beutler et al., 2003). MyD88 also contains a TIR domain, which associates with the TIR domain of the TLRs (Hoebe et al., 2003a).

TLR4 signals by way of four adaptor proteins, which seem to operate in functional pairs: MyD88 with Mal (also known as TIRAP), and TRIF with TRAM (Yamamoto et al., 2003a,Hoebe et al., 2003a,Hoebe et al., 2003b,Yamamoto et al., 2003b). At present, it is believed that the LPS receptor complex uses all of these adaptors when activated and none of them when quiescent.

LPS activates mitogen-activated protein kinases (MAPKs) and the transcription factor nuclear factor (NF)-κB (Sweet and Hume, 1996). The latter is kept sequestered in the cytoplasm by its inhibitor of κB (IκB). Stimulation with LPS sequentially leads to phosphorylation, ubiqutination, and finally, to proteasomal degradation of IκB. This allows NF-κB to be translocated to the nucleus and bind to promoter regions of genes. The discovery of TLR-4 as the crucial receptor for LPS signaling (Poltorak et al., 1998) has boosted a far more detailed investigation into the signaling pathways that mediate this activation. LPS can trigger the so-called Myd88-dependent and independent signaling cascade depending on the nature of TLR-4-associated adaptor proteins (Akira and Takeda, 2004). The adaptor proteins Myd88 and TIRAP mediate signaling via IRAK1/4 to TRAF6, which seem to be important to activate early NF-κB and MAPKs.

Another ligand utilizing MyD88 is IL-1cytokine. The events following the binding of IL-1 to IL-1R binding have been well documented (Dunne and O'Neill 2003). After forming a complex with IL-1R/TLR, MyD88 associates with IL-1R-associated kinase (IRAK)-1 and IRAK-2. This appears to be true also for the TLR4/My D88/Mal complex (Fig 1.3).

In the MyD88-independent pathway the adaptor proteins TRIF and TRAM, conversely, are responsible for initiating IRF-3 activation and thereby cytokine secretion. Additionally, this pathway triggers late NF-κB activation, a process not well understood so far. MyD88-dependent early and MyD88-independent late NF-κB activation is thought to contribute to the initiation of transcription of most proinflammatory cytokines (e.g., IFN-β, IL-1, IL-6, IL-8, and IL-12) (Fig 1.3C).

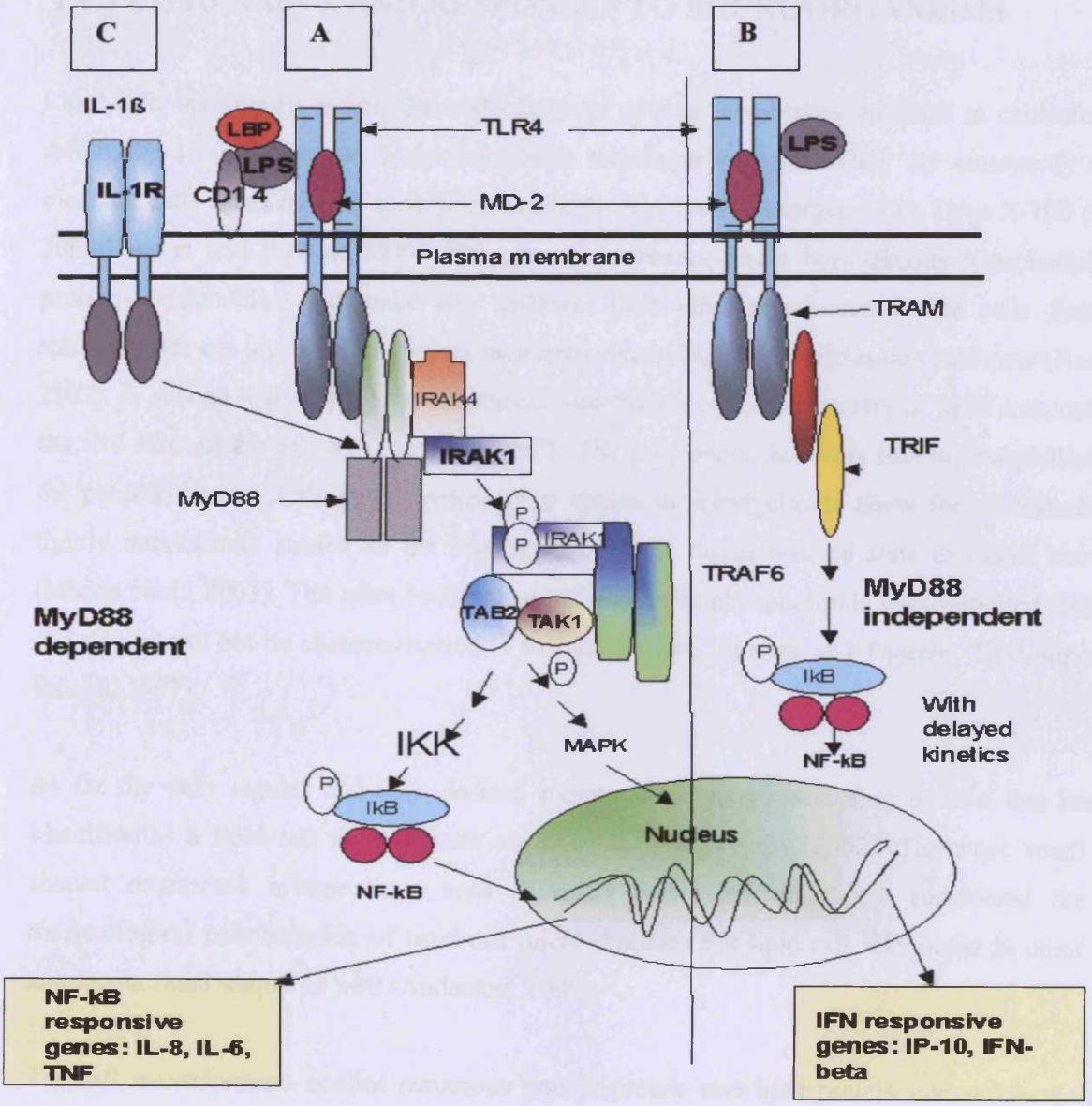


Figure 1.3: Simplified Schematic diagram for LPS-mediated TLR4 and IL-1β mediated IL-1R signalling pathways. Two pathways in the LPS signaling transduction lead to cytokine production: MyD 88 dependant (A) and MyD 88 independent (B) pathways. Also this diagram shows the similarity (MyD88 to down stream) and difference (upstream of MyD88) between LPS MyD 88 dependant (B) and IL-1β pathways (C).

1.6.3 LIPID RAFTS AND RESPONSES TO MICROORGANISMS

Lipid rafts are highly ordered microdomains of plasma membranes enriched in cholesterol and sphingolipids that serve to comparmentalise membrane proteins. They are commonly defined biochemically as membrane complexes insoluble in nonionic detergents like Titon X-100 (Munro, 2003,London and Brown, 2000). The plasma membrane has a homogenous phospholipids and proteins composition that make very different from other membranes of the cells. Sterol and sphingolipids are low level in internal membrane but at high level in plasma membrane (Hao et al., 2002). A second bilayer property of plasma membranes is the asymmetry of lipid composition of the two sides of the bilayer (Bretscher, 1973).The exoplasmic leaflet is rich in phospholipids and the preponderance of saturated hydrocarbon chains in sphingolipids allow for cholesterol to be tightly intercalated, similar to the organization of the liquid ordered state in model membrane (Munro et al., 2003). The inner leaflet is probably rich in phospholipids with saturated fatty acids and cholesterol but its characterisation is still incomplete (Simons and Toomre, 2000,Simons and Ikonen, 1997).

So far the only regular and well defined membrane structure occurring *in vivo* that has been identified as a lipid raft microdomain are caveolae (Kenworthy, 2002). These are small flask-shaped membrane invaginations seen in many cell types and were considered the main morphological manifestation of lipid raft micro domains but lipid raft may occur in other forms and less defined shapes as well (Anderson, 1998).

The raft microdomains control numerous protein-protein and lipid protein interaction at the cell surface. This function is possible on the basis of two important raft properties; their capacity to incorporate or exclude proteins selectively and their ability to coalesce into larger domain. Proteins with raft affinity (eg CD14 or TLR-4), GPI- anchored proteins or proteins that carry hydrophilic modification probably partition into the raft owing to preferential packing of their saturated membrane anchor. It is not yet clear why some transmembrane domains near the exoplasmic leaflet are critical for the partition into the lipid raft (Scheiffele et al., 1997).

It is likely that a given protein can associate with raft at different kinetic. For example, a monomeric transmembrane protein may have a short residency time in rafts, spending most of its time outside the raft. But when the same protein is cross-linked otherwise oligomerised (eg TLR-4 and TLR-2) (Soong et al., 2004), its affinity for the raft increased (Harder et al., 1998). The clustering of separate rafts express proteins to a new membrane environment, enriched in specific enzymes, such as kinase and phosphataes, even a small change in partitioning into a lipid rafts through amplification, initiate signaling cascades (Resh, 1999).

One of most important function of the lipid raft at the cell surface may be their association and role in mediated signal transduction (Scheiffele et al., 1997,Stefanova et al., 1991) as well as microorganism internalisation. In signal transduction, now it is widely appreciated role of the lipid raft is that it serve as a platform in concentration and recruiting for some molecules that are involved in cellular signaling (Pfeiffer et al., 2001,Triantafilou et al., 2002).

Despite the role of lipid raft in activation the immune system, in many cases bacteria and viruses utilising host lipid raft to gain entry to into the cells (Rosenberger et al., 2000), and influencing the fate of phagocytosis (Watarai et al., 2002). The lipid raft in the cells are hijacked by the intracellular organisms at different point in the process of infection and used as a gateway for entry. Recent investigation by Kowalski and his colleague shows that *P. aeruginosa* cause localization of CFTR to the lipid rafts of epithelial cells in cystic fibrosis (Kowalski and Pier, 2004).

Cell stimulation would activate raft clustering, forming specialised domains in which signaling associates could meet and become further activated. These raft-signaling functions would be enhanced by the sequestration of specific elements that are initially found in the raft membrane regions to the raft membrane. Different studies demonstrated the involvement of lipid raft signaling, in human macrophages it has been shown that stimulation with LPS led to TLR4 mobilization to the lipid rafts and MAPK activation (Cuschieri, 2004). Recent studies by (Triantafilou et al., 2004) demonstrated that the receptor molecules that are implicated in LPS activation, such as CD14, heat shock protein (hsp) 70, 90 and TLR4, are present in microdomains following LPS stimulation of MonoMac-6 (MM6) cells

1.7 PULMONARY SURFACTANT

Pulmonary surfactant, a multicomponent complex of several phospholipids, neutral lipids and specific proteins, is synthesized and secreted into alveolar spaces by type II epithelial cells (Creuwels et al., 1997,Griese, 1999). The main functions of pulmonary surfactant are reducing the collapsing force in the alveolus, conferring mechanical stability to the alveoli, and maintaining the alveolar surface relatively free of liquid. Surfactant is primarily composed of phospholipids and proteins. Most of the phospholipids consist of phosphatidylcholine (PC), and one particular PC molecule, dipalmitoylphosphatidylcholine (DPPC), is the most prevalent component (Goerke, 1988) and is composed of 60-70% of phospholipids. The structure of DPPC is suited to form a stable monolayer generating the low surface tension required to prevent alveolar collapse at end-expiration. Phosphatidylglycerol (PG) also contributes to monolayer formation; its synthesis is restricted to type II alveolar cells, and its detection in amniotic fluid is a reliable predictor of lung maturation (Veldhuizen et al., 1998). Phospholipids alone are far from exhibiting all the biophysical properties of pulmonary surfactant. These properties include the ability to generate low minimum surface tension on dynamic compression, to rapidly absorb from the subphase to the interface, to respread when collapse occurs after condensation, and to vary surface tension during expansion and compression at each respiratory cycle. In this respect, the contribution of low-molecular-weight SP-B and SP-C proteins to both structural organization and functional durability is essential (Johansson and Curstedt, 1997,Weaver and Conkright, 2001).

The composition of pulmonary surfactant has both biophysical and immunological function. The biophysical function is related to the ability of surfactant to reduce the surface tension at the air-liquid interface and prevent the lung from collapse (Bastacky et al., 1995, Hills, 1999). In any event, surfactant in the lung play an important role in the lung function by establishing the alveoli and prevent their collapse when the lung undergoes successive cycles of compression during respiration (Bernhard et al., 2004). In addition to its biophysical function, pulmonary surfactant has also properties function that contribute to the pulmonary defence system in the lung. Pulmonary surfactant has been shown to play a protective role against bacterial, viral and fungal pathogens, as well as protection of the lung from injuries by inhaled particles (Coonrod, 1987,Crouch and Wright, 2001,Ofek et al., 2001)

Surfactant is not only found in the alveoli, but also found in bronchioles and small airways (Morgenroth and Bolz, 1985). The presence of surfactant phospolipids and proteins in bronchial secretion has been documented from different studies (Bernhard et al., 1997,Bernhard et al., 2001). Surfactant material forming monolayers and multilayers can be found at the air-liquid interface in the airways. In addition to that the multilamellar vesicles and lattic-like tubular myelin can be found the hypophase of the epithelial lining fluid covering the airways. The mass of these surfactant materials may be originated from the alveoli (Bernhard et al., 1997). The surfactant in the airways, as in the alveoli, has been postulated to have both biophysical and immunological functions. Airways surfactant secure the airway architecture by minimizing the amount of negative pressure in the airways walls (Bernhard et al., 1997,Gehr et al., 1993). It has been described that the phospholipid layer is bound directly to the bronchial and alveolar epithelium (Hills and Chen, 2000),Bernhard et al 1997), and has been demonstrated that the airway mucusa contained less phosphatidylcholine, and higher PE concentration compared to BAL fluid. Moreover, there is an increased concentration of unsaturated fatty acid in the epithelium-bound PC compared with the highly saturated PC composition of tracheal aspirate and BAL.

1.7.1 PULMONARY SURFACTANT COMPOSITION

Pulmonary surfactant is a complex mixture of phospholipids and surfactant specific proteins. Biochemically, pulmonary surfactant is composed of approximately 90% lipids and 10% proteins. The majority of surfactant lipids are phospholipid. The most abundant classes of phospholipids are approximately 85% phosphatidylcholine (PC) compounds, of which 40%-50% are dipalmitoylphosphatidylcholine (DPPC), The other phosphatidylcholine include 9-12% palmitoylmyristoyl-PC (PMPC), 8% palmitoylpalmitoleoyl-PC, 10% palmitoyloleoyl-PC (POPC), 6% palmitoyllineoyl-PC (PLPC), 9% phosphatidylglyserol (PG), 3% phosphatidylethanolamine (PE) and 2% phosphatidyinistol (PI) (Bernhard et al., 2001,Bernhard et al., 1997).

The protein fractions of pulmonary surfactant comprise a high amount of serum proteins and the remaining consists of four surfactant-associated proteins (SPs) (designated SP-A, SP-B, SP-C and SP-D) (Possmayer, 1988). Surfactant proteins SP-A (28-36 kDa) and SP-D (43 kDA) represent the larger of surfactant-associated proteins. They are the members of the member of collagenous carbohydrate binding proteins (collagenous C-type lectins), now commonly known as collectins (Malhotra et al., 1994,Hoppe and Reid, 1994). Recently, It has been reported that SP-D, but not SP-A, increased TNF-α release from human monocytic cells in response to a subset of *P. aeruginosa* and *P. aeruginosa* LPS (Bufler et al., 2004). Recently it has been shown that both SP-A and SP-D increase phagocytosis of *S. pneumonia* and *M. avium* by alveolar macrohage, and SP-A alone interact with TLR2-ligands such as peptidoglycan and inhibit TNF-α expressin (Sano et al., 2006).

Surfactant protein SP-B and SP-C (3.5-3.7 kDa) are extremely hydrophobic in nature and are found in heavy but not light surfactant sub-fractions separated by different centrifugation. SP-B is usually present as a dimeric structure, and SP-C is found as thio-esters of one or more fatty acids (Gustafsson et al., 1997). Whereas, the alveolar epithelial cells are the only cells produce all surfactant components. SP-A, SP-B and SP-D also produce and synthesized by are both secreted and synthesised by both alveolar type II and non-ciliated bronchiolar epithelial cells (Kishore and Reid, 2000,Watford et al., 2000,Kishore et al., 2001).

Because surfactant secretion is a permanent process, there are clearance mechanisms that prevent the alveoli from being filled with this material, thus guaranteeing a relatively stable surfactant concentration. In normal lungs, possible mechanisms for surfactant clearance from the alveoli include its removal via airways, blood, and lymph, its degradation in the alveoli, or its reuptake into cells of the airways, into macrophages, and into cells of the respiratory epithelium (Wright, 1990). All pulmonary surfactant components with the exception of SP-D are synthesized in endoplasmic reticulum of the alveolar type II cells and packaged in the lamellar bodies, which are then translocated into the intraalveolar space. Most of secreted surfactant is taken and metabolized and re-secreted again by the alveolar type II epithelial cells. Re-uptake and surfactant lipids and proteins recycling have been demonstrated (Goerke, 1998,Fehrenbach, 2001).

1.7.1.1 Phospholipids structure and function

Lipids in general are major components of all biological membranes which are crucial to the life of the cells. Despite their differing functions, all biological membrane have a common general structure: each is a very thin film of lipid and protein molecules, held together mainly by noncovalent interactions. Phospolipids are derived from either glycerol or sphingosine. Phospholipids derived from glycerol are called phosphoglycerides, those derived from sphingosine are referred as sphingolipids. Phosphoglycerides consist of glycerol esterified with two fatty acid chains and phosphorylated alcohol group (Figure 1.4). The fatty acids esterified at *sn-1* and *sn-2* positions generally contain an even number of carbon atoms, 16 and 18 carbon fatty acid are the most common, but may contain between 14 and 24 carbon atoms. The grouping at *sn-3* position can take the many forms including serine, choline, inositol or ethanolamine for example (Figure 1.5)

All of lipid molecules in the cell membrane are composed of phospholipids, glycolipids, cholesterol and proteins. In 1972 Singer and Nicolson proposed the fluid mosaic model (Singer and Nicolson 1972). The asymmetrical phospholipid bilayer contains many proteins and in some, the proteins are exposed on the surface of the inner and out leaflet. Theses proteins are often involved to in the transport of molecules across membrane and involve in signalling. The proteins are able to move laterally in the membrane but maintains the integrity of the cell as individual unit,

controls the movement of molecules in and out and provide the sensing and reacting to external environment via various receptors molecules on the cell surface (Stryer, 1988).

Cellular membranes are asymmetrical with regard to the distribution of phospholipids species, proteins and enzyme activities. The asymmetry of the membrane is introduced by positioning of proteins an phospholipids in the membrane during the synthesis by the endoplasmic reticulum and maintained by inability of theses proteins to move from side of the bilayer to the other (Stryer, 1988). Phospholipids on the other hand are able to flip-flop between the inner and the outer leaflet. Within the plasma membrane phosphatidylcholine is predominantly located in the outer leaflet, while PS, PE and PI tend to be located in the inner leaflet (Mathews and Holde, 1990).

The Fluidity of the plasma membrane is dictated by the saturation of the fatty acids within phospholipids and proteins content of the bilayer. Increasing concentration of cholesterol in the membrane reduces fluidity by reducing the proportion of total lipids within the membrane (Mathews and van Holde, 1990). The phospholipids unsaturation introduces link to the fatty acid chains and this may prevent phospholipids from packing tightly, therefore increasing membrane fluidity (Albert et al., 1989).

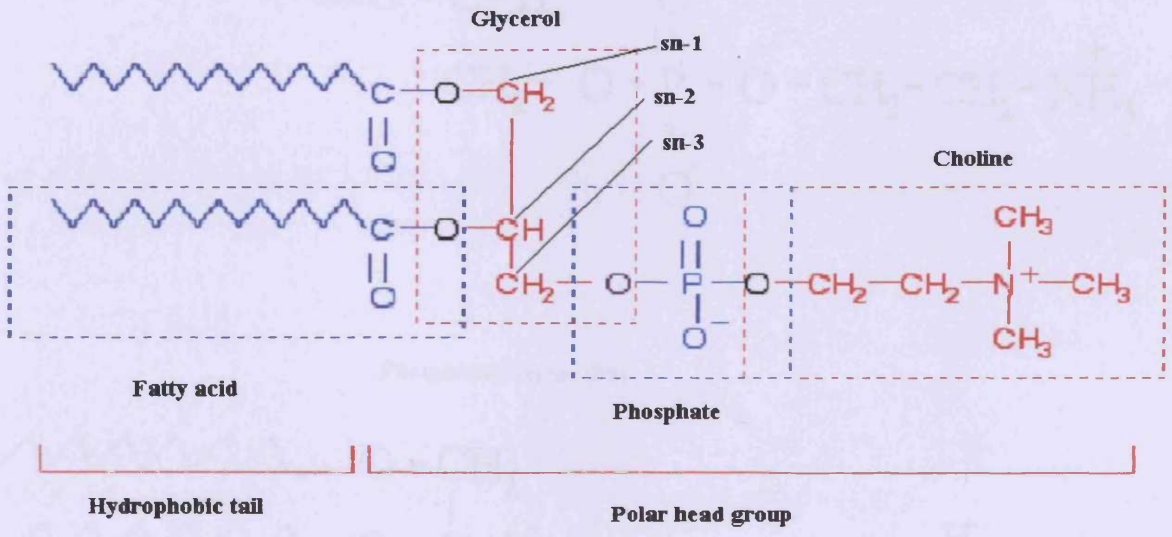
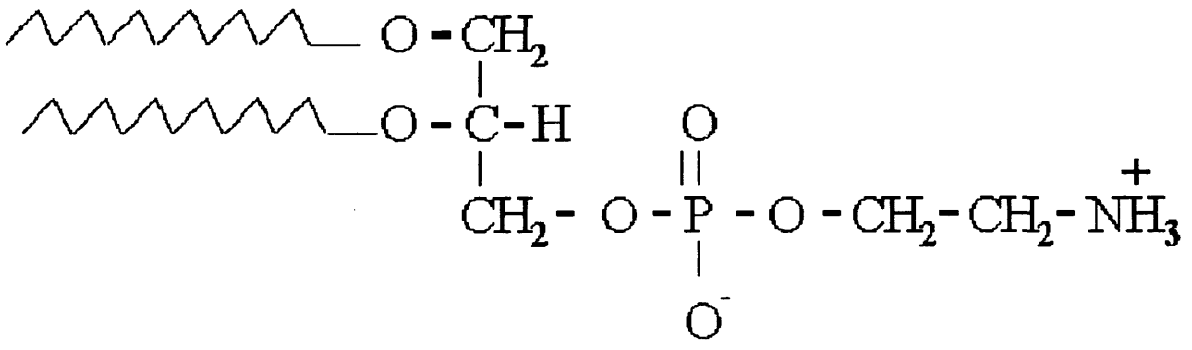


Figure 1.4 Phosphoglycerides structure. General diagram shows the structure of phosphoglyceride indicating the main chemical groups.

Phosphatidylethanolamine (PE)



Phosphatidylserine (PS)

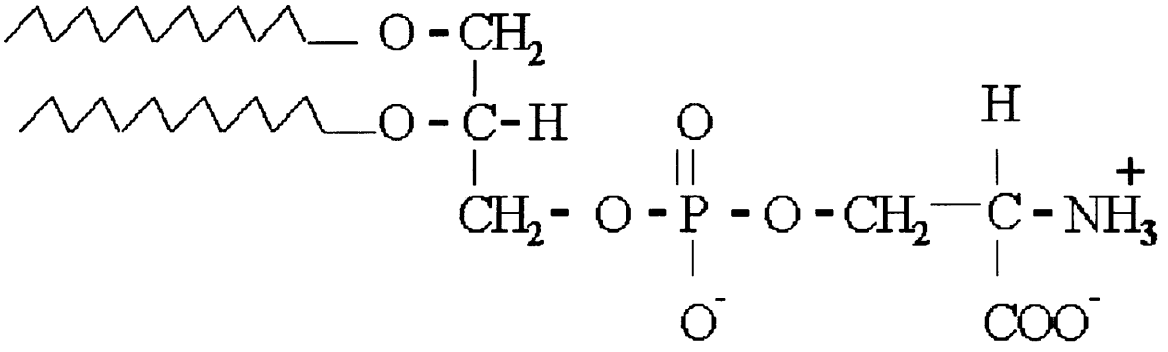


Figure 1.5: Illustrating the head groups of common phospholipids.

1.7.1.2 Surfactant synthesis and alteration

Surfactant is predominantly synthesis by alveolar epithelial type II cells (Wright, 1997). The secretion and reutilization of pulmonary surfactant in the alveolar type II cells and ancillary cells is a dynamic cyclic process. Theses processes involve a number of complex regulatory mechanisms. Surfactant is synthesised in within type II alveolar epithelial cells and stored in intracellular organelles called the lamellar bodies (Mason and Voelker, 1998). The secretion and trafficking of surfactant proteins is a little more complex, with the exception of surfactant proteins SP-A, surfactant proteins are synthesised in polyribosomes, modified in the endoplasmic reticulum, Golgi apparatus and multivesicular bodies and stored in lamellar bodies. Surfactant phospholipids are synthesised in the endoplasmic reticulum transported through Golgi apparatus into multivesicular bodies and packaged into lamellar bodies (Rooney et al., 1994).

Following the formation of the lamellar bodies they are transported to the plasma membrane, where they fuse and are secreted by exocytotic mechanism, aided by annexins and calcium-dependent phospholipid binding proteins (Mason and Volker, 19998; Liu et al, 1996). The intracellular movement of lamellar bodies is believed to involve actin, microtubules and specific attachment proteins (Tsilibary and Williams, 1983). It is widely accepted that upon release, multiple lamellar bodies fuse and form tubular myelin (TM), and with assistant of SP-A, TM is rabidly integrated into surface film at the air water interface (Froh et al., 1990).

It has been suggested that sorting of the TM occurs prior to the integration into the surface active film, with preferential integration of DPPC rich domains, DPPC poor TM being recycled, leading to DPPC poor lipid protein in the subphase (Goerke, 1998). Under physiological condition the film is constantly being compressed and this leads to the collapse of components of the film into the subphase. Subsequently surfactant is taken up by alveolar type II cells and surfactant components retrieved, repackaged and recycled. Also it is documented that alveolar macrophage take part in the catabolization process of surfactant components (Gurel et al., 2001).

The surfactant system can be changed and altered rendered dysfunctional by inflammation that occurs in obstructive or parenchymal lung diseases, which may modify surfactant abundance, structure or function. Also, surfactant deficiency or abnormality may predispose an individual to lung inflammation. The protelytic enzymes, phospholipases and oxidants that are generated during inflammation damage phospholipids and proteins or change their production by the alveolar epithelial cells (Meyer et al., 2002,Zhu et al., 2000). Thus, the anti-inflammatory abrogating may leads to surfactant dysfunction, intensification of inflammatory cascade superimposed on respiratory infection (Mayer et al., 2002).

Deficiency or dysfunction and alteration of pulmonary surfactant play a critical role in the pathogenesis of respiratory diseases. Alterations of the pulmonary surfactant system, including decreased total surfactant levels, have been implicated in the pathophysiology of acute lung injury. Multiple studies of patients with a variety of lung diseases have shown that surfactant levels are decreased in the inflamed, injured, or infected lung (Gregory et al., 1991,Pison et al., 1990,Gunther et al., 1996). In agreement, decreases in alveolar surfactant lipid pools have also been observed in several animal models of lung inflammation induced by both direct insults to the lung, such as bacterial infection(Vanderzwan et al., 1998), oxygen toxicity (Holm et al., 1985), and endotoxin administration (Viviano et al., 1995).

Abnormalities or dysfunction in the phospholipids and apoprotein fractions of surfactant have been described in association with wide variety of pathogens, including bacteria (Baughman et al., 1993, Gunther et al., 1996, Chimote and Banerjee, 2005), viruses and fungi (Hoffman et l., 1992, Phelps et al., 1991).

Because of the chronic lung inflammation seen in COPD and the surface destruction seen in emphysema, the surfactant system may be involved in the underlying pathophysiology. Smokers are likely to have a decreased in phospolipids content in BALF (Hohlfeld et al., 1997), and the level of both SP-A and SP-D are also decreased and theses changes may contribute to the increased incidence of respiratory infections in patients with COPD (Honda et al., 1996). Both functions are dependent on both surfactant-associated proteins and surfactant phospholipids.

It has been shown that *P. aeruginosa* degraded SP-A and SP-D (Mariencheck et al, 2003) and *P. aeruginosa* elastase, was identified as one of the degradative proteins; however, other component can be involved. *P. aeruginosa* protease degrades SP-A and SP-D, resulting in reduction in host defense functions of these SPs, which may contribute to colonisation of the lung. Additionally, protease alters the biophysical function of surfactant by inhibiting the surface tension-reducing property of this material, thus potentially contributing to decreased lung function (Malloy et al., 2005).

Alteration of phospolipids composition in young children with respiratory infections has been described and DPPC was decreased in both infection and CF groups (Mander et al., 2002) and in Asthma (Wright et al 2000). In CF patients, the analysis of surfactant isolated from BALF was reported relatively decreased. Both surfactant proteins especially SP-A and Phospholipids were decreased (Meyer et al., 2000, Griese et al., 2004). It has been reported that surfactant phospholipid levels decreased in lavage from rats (Sheehan et al., 1986) and humans with different pulmonary infections (Escamilla et al., 1993,Hoffman et al., 1992,Rose et al., 1994), whereas SP-A concentrations in lavage are increased (Phelps and Rose, 1991, Phelps and Unstead, 1994). The expression of SP-A and SP-D has also been shown to increase in murine (Atochina et al., 2000), whereas the expression of SP-B can be decreased (Beers et al., 1999) or unchanged (Wright et al 2001) depending on the time of injury.

In pneumonia, bacteria induce changes in pulmonary surfactant. These changes are, mediated by bacteria directly on secreted surfactant or indirectly through pulmonary type II epithelial cells. The bacterial component most likely responsible is LPS since Gram-negative bacteria more often induce these changes than Gram-positive bacteria. Also, endotoxin and Gram-negative bacteria induce similar changes in surfactant. The interaction of bacteria or LPS with secreted surfactant results in changes in the physical (i.e. density and surface tension) properties of surfactant. In addition, gram-negative bacteria or LPS can injure type II epithelial cells causing them to produce abnormal quantities of surfactant, abnormal concentrations of phospholipids in surfactant, and abnormal compositions (i.e. type and saturation of fatty acids) of PC.

1.7.2 THE ROLE OF SURFACTANT PROTEINS IN LUNG IMMUNE DEFENCE AND INFLAMMATORY PROPERTIES

To date there are four surfactant proteins identified. The hydrophobic surfactant protein SP-A was first identified in 1972 by King and colleagues (King and Clements, 1972) and SP-D was originally characterised in 1988 (Persson et al., 1988). SP-A and SP-D are called collectins and are important components in the immune response in the lung. They are oligomeric molecules containing a collagen-like domain and a calcium-dependent lectin domain, known as a carbohydrate recognition domain.

Both SP-A and SP-D have been shown to regulate a variety of immune cell functions *in vitro* and *in vivo*. The lung collectins bind glycoconjugate expressed by a variety of Gram-negative and Gram-positive bacteria including specific strains of such important pulmonary pathogens as *K. pneumoniae*, *P. aeruginosa*, *H. influenzae*, and *E.coli*. SP-A bind specifically to lipid domain rough form of LPS (Van Iwaarden et al., 1994,Kalina et al., 1995), and also bind to lipoteichoic acids LTAs from Gram-positive bacteria (Polotsky et al., 1996). The mechanism by which collectins enhance the immune response is not fully understood, but there is many reports showed that SP-A opsonise and enhance the killing of *H. influenzae* by alveolar macrophages (McNeely and Coonrod, 1993). Although, there is evidence that collectins can increase the internalisation of a variety of microorganisms by modulating the function of phagocytic cells. SP-A and SP-D also increase the uptake of Gram-negative *E.coli* and Gram-positive *S.aureus* and *S.pneumoniae* by neutrophils (Hartshorn et al., 1998). SP-A also has been shown to enhance the phagocytosis of serum opsonised *S.aureus* by alveolar macrophages (van Iwaarden et al., 1990) and group A *streptococcus* (Ohmer-Schrock et al., 1995). SP-D has an important role in the first line defence against lung fungal infections (van Rozendaal et al., 2000). Recently It has been documented that SP-A and SP-D inhibit the growth of Gram-negative bacteria by increasing the permeability of membrane (Wu et al., 2003).

SP-A has also been shown to increase the binding of non-microbial particles to A549 epithelial cells (Wang et al., 1996) and to inhibit production of pro-inflammatory cytokines in response to LPS and to *Candida albicans* in peripheral mononuclear cells and alveolar macrophages respectively (Borron et al., 2000,Rosseau et al., 1999). In contrast, other studies showed that SP-A upregulates the production of TNF-α, IFN-γ, IL-1ß, IL-6 and IL-8 by the THP-1 cell line (Kremlev and Phelps, 1994,Kremlev et al., 1997) and that the addition of bovine surfactant lipids (Survanta®) could inhibit SP-A activity for TNF-α but not IL-8 release.

Surfactant proteins B and C (SP-B and SP-C) are small and extremely hydrophobic. SP-B is essential for the ability of surfactant to reduce surface tension (Nogee, 2004). The hydrophobic surfactant proteins, SP-B and SP-C, promote adsorption of surface-active lipids to the air-liquid interface of the alveoli and are essential for alveolar stability and gas exchange. SP-C deficiency and mutations in the SP-C gene have been associated with interstitial lung disease (Amin et al., 2001). Deletion or mutations in SP-B and SP-C cause an acute and chronic lung disease in neonates and infants. While SP-B plays a critical role in maintaining lung function during infection and/or injury, it is less clear whether loss of SP-B is a cause of pulmonary inflammation. SP-B inhibited endotoxin-induced nitric oxide production by isolated alveolar macrophages, consistent with its potential anti-inflammatory role (Miles et al., 1999b).

SP-B is a 79-amino acid polypeptide, synthesized, processed and secreted from alveolar type II cells. SP-B interacts with surfactant phospholipids, enhancing spreading and stability of surfactant films. Newborn mice and human infants lacking SP-B die of respiratory failure after birth. SP-B deficiency in the newborn is associated with surfactant dysfunction, lack of lamellar bodies, accumulation of incompletely processed pro-SP-C and altered levels of phosphatidylglycerol in surfactant (Beers et al., 2000,Melton et al., 2003,Tokieda et al., 1997) . SP-B expression is reduced in preterm infants and in patients at risk for adult respiratory distress syndrome. SP-C has recently been shown to bind lipopolysaccharide (LPS) (Augusto et al., 2003).

1.7.3 POSSIBLE ROLE OF SURFACTANT LIPIDS IN LUNG IMMUNE DEFENCE

Recent evidence suggests that surfactant lipids may modulate innate immune responses in the lung. The phospholipid components of surfactant inhibited the production of reactive species (ROS) from rabbit, human and guinea pig alveolar macrophages (Hayakawa et al., 1989). In addition Curosurf® was shown to inhibit the release of ROS from human monocytes (Walti et al., 1997). Pulmonary surfactant lipid DPPC has been shown to modulate reactive oxygen intermediate (ROS) released from human monocytes (Tonks et al., 1999). It has been reported that whole surfactant phospholipids or DPPC alone can suppress neutrophil respiratory burst oxidase responses (Ahuja et al., 1996,Suwabe et al., 1998). Also it has been shown that DPPC can modulate LPS induced platelet-activating factor (PAF) in human monocytic cells in dose dependent (Tonks et al., 2003) and TNF-α and PGE_2 (Morris et al., 2000). It has been shown that the whole surfactant phospolipids (Ahuja et al., 1996) or DPPC alone (Suwabw et al., 1995) can suppress neutrophil respiratory burst. Other studies indicated that surfactant could either enhance (Webb et al., 1986) or not modify (Speer et al., 1991) the production of ROS. It has also been shown that DPPC alone inhibits IL-8 release induced by GBS (Doran et al., 2002)

The major surfactant components, PC, PG and PI exert suppressive effect on lymphocytes proliferation, however of the lesser components PE, SM and cholesterol have been shown to augment lymphoproliferative responses (Kremlev et al., 1994; Whilsher et al., 1988), thus the role of surfactant in regulation of immune responses *in vitro* may be determined by the relative proportion of suppressive and stimulatory components, and these may be altered in various pulmonary diseases (Hughes et al., 1989;Lesur et al., 1994).

Pulmonary surfactant preparations like Survanta® is natural/modified surfactant extracted from lung bovine and Curosurf® also is a natural modified surfactant that contains 80 mg/mL of phospholipids from porcine lung, which contains 99% polar lipids. Pulmonary surfactant preparations such as Survanta®, Curosurf® and Exosurf®, which are devoid of surfactant proteins SP-A and SP-D, and also the individual phospholipid component DPPC, have also been reported to modulate the release of inflammatory cytokines and mediators. Survanta® and Curosurf® have

been shown to suppress the LPS induction of TNF-α, IL-1β and IL-6 from human alveolar macrophages (Thomassen et al., 1994,Thomassen et al., 1992). It has been reported that surfactant attenuates both GM-CSF and LPS induced MIP-1 secretion from human alveolar macrophages (Raychaudhuri et al., 2004) suggesting that surfactant may serve an endogenous immunoregulatory role in the normal alveolar space.

Furthermore, pulmonary surfactant or the lipid components alone have been shown to modify the invasion of myeloid and non-myeloid cells by bacteria. Golioto and Wright (2002) noted that treatment of alveolar macrophage with increasing concentration of lipid resulted in the reduction of the uptake of *S. pneumoniae*. Inset et al., 1996 have demonstrated that DPPC inhibits GBS induced injury epithelial cells. Wheras SP-A increased phagocytosis of *S. pneumonia* by alveolar macrophages, this phagocytosis was significantly decreased when exposed to higher concentration of surfactant lipid (Golioto et al., 2002). An earlier study, has demonstrated that the phospholipid DPPC, the major component of human surfactant, inhibits GBS b-h/c–induced injury to A549 lung epithelial cells (Nizet et al., 1996) and, later study showed that DPPC also inhibited the ability of GBS to intracellularly invade these cells and to stimulate their production of IL-8 (Doran et al., 2002). Phospholipid components of human surfactant are known to inhibit GBS beta-hemolysin-associated RBC lysis (Marchlewicz et al., 1980). Nitez et al., 1996 demonstrated that DPPC at 500 μg per mL inhibited 90% of lactate dehydrogenase (LDH) release from lung epithelial cells exposed to beta-hemolysin induced epithelial cells injury. Surfactant prepared from sheep lung showed to inhibit both intracellular killing of bacteria and the intracellular production of H_2O_2 in human monocytes and granulocytes (Geertsma et al., 1993). It has also been reported that surfactant inhibit the killing of *Candida albicans* (Zeligs et al., 1984) and *S. pneumoniae* (Sherman et al., 1988).

There are few studies that have investigated the direct effect of surfactant preparations on the growth of bacteria but with conflicting results. Neumeister and his colleague (Neumeister et al., 1996) show that addition of Survanta® to saline seemed to reduce the viability of *E. coli*, whereas it supported and increased the growth of GBS. Herting and his colleagues (Herting et al., 1994) reported that Curosurf®, consisting of 99% polar lipids (mainly DPPC) and 1% SP-B and SP-C, exerts inhibition of GBS. Another report has shown that surfactant preparation like Curosurf® was

bactericidal in a dose dependant fashion for GBS whereas the Exosurf® only reduced the bacterial number (Rauprich et al., 2000).

Other reports showed that addition of different concentrations of Survanta® to GBS did not alter bacterial survival, whereas Survanta® seemed to protect GBS and *S. aureus* against the bactericidal. Unlike all other tested surfactant preparations, Survanta® significantly promoted the growth of *E. coli*. At phospholipid doses of ≥10 mg/mL, the numbers of CFU per milliliter were increased 4.5 times compared to the bacterial count at the beginning of the experiments (Rauprich et al., 2000b). Furthermore, Pulmonary surfactant or lipid components have been noted to modify and decrease the invasion of *S. pneumoniae* invasion to alveolar macrophages (Golioto and Wright, 2002).

The limited amount of published studies on surfactant lipids, compared to the surfactant proteins, highlights the need to study the possible modulatory role of surfactant lipids. Despite that, there are a few reports indicating that surfactant lipids have an effect on innate immunity responses in the lung (Hayakawa et al., 1989,Morris et al., 2000).

In conclusion, surfactant lipids play an important role in the regulation the immune defence system in the lung. However, the potential role of surfactant lipids on the growth of bacteria and invasion of the epithelial cells by lower respiratory bacterial infectious agents need to be elucidated. In addition to that the role of surfactant lipids on modulating epithelial cells immune response have not been well documented and needed to be studied.

1.8 A549 EPITHELIAL CELLS AS A MODEL FOR BACTERIAL INVASION AND INFLAMMATORY RESPONSES

The complex nature of the lung architecture means that the alveolar epithelium is not readily accessible. Therefore, the use of cultures of alveolar epithelial cells as a reliable *in vitro* experimental model to resemble the lung epithelial cells has gained acceptance among investigators. The most common example of an alveolar epithelial cell line is the human cell A549, initiated in 1972 (Giard et al., 1973) through explant culture of lung carcinomatous tissue from a 58-year-old male. Further studies by M. Lieber et al (Lieber et al., 1976) revealed that A549 cells could synthesize lecithin with a high percentage of desaturated fatty acids using the cytidine diphosphocholine pathway. Multilamellar inclusion bodies were located by transmission electron microscopy.

This cell shows similarities to AETII cells, including the synthesis of phospholipids, the presence of lamellar bodies and microvilli. While A549 cells have been used as a model of the type II cells in a wide range of applications (Asano et al., 1994,Stringer et al., 1996). A549 cell line has characteristic features of Type II cells of the pulmonary epithelium, including lamellar bodies. While this cell line has been extensively referenced in the toxicology literature, many of the typical Type II alveolar epithelial cell biochemical properties and properties on permeable supports have not been reported for A549 cells. Many investigators in the world use A549 cells in scientific research and without doubt the A549 model has provided and contributed knowledge and information to many research fields.

Other cell lines like Calue-3, 16 HBE, bronchial epithelial cell (BEAS-2B) and virus transformed human bronchial epithelial cell line (HBEpC) were used by many investigators (Lee et al., 2005, Schulz et al., 2002, Kunkel et al., 1991; Murphy, 1997) for their studies to represent the lung epithelial cells. These cell lines are either difficult to grow or it takes for long to reach confluence (like Calue-3) or not as physiological representative to normal cell line (like HBEpC). Other sources of epithelial cells from gut and kidney HEK293 are not good example to use in this study since they are originated not from the lung and may express and behave differently. For these reasons A549 epithelial cell line were used in our investigation. In addition to that many

investigators used the A549 epithelial cells as a model to represent airway epithelial cells (Arita et al., 2005) and to study the responses to bacteria and bacterial secretions (Ader et al., 2005).

Also A549 epithelial cells were used to study bacterial invasion to lung epithelial cells (Finlay and Falkow, 1988, Tomich et al., 2002, Bermusez et al., 2002, Cieri et al., 2002) or as a model for inflammatory responses (Palfreyman et al., 1997,Madsen et al., 1999,Doran et al., 2002,Fink et al., 2003,Utaisincharoen et al., 2004,Arita et al., 2004, Rosseau et al., 2005, Carteson et al., 2005).

Despite the similarities and differences to other bronchial cell line, the alveolar A549 epithelial cell line, derived from carcinoma cells of type II pneumocytes behave like bronchial epithelium (Lieber et al., 1976) and has therefore been widely used for studying bronchial epithelial inflammation. In present study the A549 cell line was adopted as a model to study the effect of pulmonary surfactant on bacterial invasion and inflammatory responses.

1.9 AIMS OF THE STUDY

The relationship between the bacterial insults and pulmonary surfactant is one of the important issues to be investigated. Not many studies have investigated the relationship of pulmonary surfactant and growth of bacteria, but a limited number of reports describe contradictory results (Ferrara et al., 2001,Bouhafs and Jarstrand, 1999,Rauprich et al., 2000a). These contradictions may be due to differences in the experimental design or due to differences in the bacterial strains used in the studies.

It is well known that some bacteria like, *B. cepacia,* invade the epithelium and survive in the lung epithelial cells (Keig et al., 2001,Burns et al., 1996,Reddi et al., 2003b) whereas for other bacteria like *P. aeruginosa*, one or two reports showed the invasion to the lung epithelial cells. The relationship between pulmonary surfactant and bacterial invasion of the respiratory epithelial cells is one of the main issues not investigated by researchers and needs to be elucidated in the present study.

Lung epithelial cells release cytokines such as CXC chemokines and other neutrophil chemotaxis upon stimulation with bacteria (Mikamo et al., 2004a,Reddi et al., 2003a), but it is not known if this stimulation is related to the invasion or is independent of invasion by bacteria. Also, bacterial products such as LPS, PGN, LTA and other secretary products may be involved in the pathogenesis but it is not clear whether the lung epithelial cells respond directly to these bacteria and bacterial products, and what role surfactant lipids play in these responses.

The aims of this project are: to investigate the effect of pulmonary surfactant on bacterial growth and invasion of alveolar epithelial cells. Subsequently it aims to elucidate the bacteria and bacterial product induced IL-8 release from lung epithelial cells and the possible regulatory role of pulmonary surfactant and surfactant phospholipids on the production and release of IL-8.

The specific objectives of the present study, therefore, are:

1- To determine the Effect of pulmonary surfactant and surfactant lipids on the growth of bacteria involved in lower respiratory infections.

2- To investigate the uptake of bacteria by A549 lung epithelial cells, and the possible effect of pulmonary surfactant and surfactant lipids on this uptake.

3- To characterise the IL-8 release from lung epithelial cells in response to bacteria and bacterial products.

4- To investigate the possible immunoregulatory role of pulmonary surfactant and surfactant phospholipids on the IL-8 released from lung epithelial cells and the mechanism involved this modulation.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 MATERIALS

All chemicals and reagents used for experiments in this thesis were purchased from Sigma-Aldrich company, Ltd (Poole, Dorset, UK) unless otherwise stated.

2.1.1 CELL LINE, MEDIA AND SUPPLEMENTS

Human lung epithelial cells A549 (CCL-185) (American Type Culture Collection, Maryland, USA)

Roswell Park Memorial Institute (RPMI) 1640 medium without L-glutamine (Gibco™ Cell culture, Paisley, UK)

Foetal calf serum (Gibco™ Cell culture, Paisley, UK)

L-glutamine (Gibco™ Cell culture, Paisley, UK)

Sodium pyruvate solution (Gibco™ Cell culture, Paisley, UK)

Tryptone soy broth (Oxoid Limited, Hampshire, UK)

2.1.2 SURFACTANT

2.1.2.1 Survanta®

Commercial pulmonary surfactant preparation Survanta® (Abbott Laboratories Ltd, Kent, UK) is a natural/modified sterile, and non-pyrogeninc bovine lung surfactant extract. Survanta® contains phospholipids (25mg/mL), neutral lipids, fatty acids (1.4-3.5 mg/mL) and surfactant protein. Each millilitre of Survanta® contains 25mg of phospholipids, <1mg of proteins (SP-B, SP-C) and 0.5-1.75 mg of triglycerides. The primary lipid is phosphatidylcholine; is 11-15.5 mg/mL. Of the PC species, DPPC constitute 75%.

2.1.2.2 Curosurf®

Curosurf® (Chiesi Farmaceutici, Parma, Italy) is a natural modified surfactant that contains 80 mg/mL of phospholipids from porcine lung which contains 99% polar lipids with more than 40% DPPC and 1% hydrophobic associated proteins (SP-B, SP-C).

2.1.2.3 Dipalmitoylphosphatidylcholine (DPPC)

1,2-Dipalmitoyl-sn-Glycero-3-phosphatidylcholine (DPPC) was purchased from (Avanti® Polar-Lipid inc, Albaster, USA) in powder or in chloroform solution. Results showed no difference between DPPC in powder and chlloroform solution.

2.1.3 BACTERIAL STRAINS

All bacterial strains used in this study were purchased from Oxoid (Oxoid Limited, Hampshire, U.K) unless otherwise stated. The bacteria used were:

Pseudomonas aeruginosa NCTC 10662 (Oxoid Limited, Hampshire, U.K)

Pseudomonas aeruginosa (clinical strain isolated from patient with lung infection), a gift from Dr. H. Ryley Dept. Medical Microbiology, School of Medicine, Cardiff University, UK)

Burkholderia cepacia NCTC 10661 (Oxoid Limited, Hampshire, U.K)

Burkholderia cepacia ET-12 (clinical strain isolated from patient with Cystic fibrosis), a gift from Dr. H. Ryley Dept. Medical Microbiology, School of Medicine, Cardiff University, UK)

2.1.4 ANTIBODIES

2.1.4.1 For ELISA assay

All antibodies for ELISA were purchased from (R&D, Oxon, UK):

Monoclonal mouse anti-human IL8 antibody (capture antibody)

Biotinylated goat anti-human IL-8 antibody

Monoclonal mouse anti-human IL-1 β antibody (capture antibody)

Biotinylated goat anti-human IL-1 β (detection antibody)

IL-8 and IL-1 β standard proteins were purchased from (R&D, Oxon, UK)

2.1.4.2 For Neutralising assay

Anti-human TLR4 antibody (clone HTA125), (e-bioscience, San Francisco, USA)

Anti-human CD14 antibody (clone: 134620) (R & D, Oxon, UK)

Anti-human TLR2 antibody (clone TL2.1), (Santa Cruz Biotechnology)

Isotype control anti-human antibody IgG2a (clone Ebm2a) (e-bioscience, San Francisco, USA)

2.1.4.3 For Western blot

Rabbit polyclonal anti-human TLR4 (clone HT80), (Cell Signalling Technology, Hertfordshire, UK)

Rabbit anti-human horse radish (HRP) conjugated antibody (Cell Signalling Technology, Hertfordshire, UK)

2.1.4.4 For flow cytometry

Anti-human TLR4-phycoerthrin (PE) conjugated antibody (e-bioscience, San Francisco, USA)

IgG_{2a}-PE, isotype antibody for TLR4 (e-bioscience, San Francisco, USA)

2.1.5 ANTIBIOTICS & CHEMICALS

Penicillin/Streptomycin (Gibco™ Cell culture, Paisley, UK)

Gentamicine (50 mg/mL IN deionized water)

Ceftazidime (500 mg in powder form)

Polymyxin B sulfate salt

Methyl-β-Cyclodextrin

Cytochalasin D

2.1.6 RECOMBINANT CYTOKINES AND RELATED MATERIALS

Recombinant Human IL-1β (R & D, Oxon, UK)

Streptavidin conjugated horseradish-peroxidase (kirkegaard and Perry Laboratories, Wembley Middlesex, UK).

Tetramethylbenzidine (TMB) microwell peroxidase (Kirkegaard and Perry Laboratories, Wembley Middlesex, UK)

2.1.7 RT-PCR REAGENTS

Deoxyriboneucleotides (dNTP) mix: dATP, dCTP, dGTP, dTTP (Ambion, Cambridgeshire, UK)

DNA ladder (Invitrogen Life Technologies, Paisley, UK)

$MgCl_2$ (Promega, Southampton, UK)

PCR buffer without $MgCl_2$ (Promega, Southampton, UK)

RNA isolation Kit (Ambion, Cambridgeshire, UK)

RT-PCR RETROscript® kit (Ambion, Cambridgeshire, UK)

Taq DNA polymerase (Promega, Southampton, UK)

RNA isolation Kit (Ambion, Cambridgeshire, USA)

PRIMERS: (Sigma-Genosys, Cambridgeshire, UK)

IL-8 primer: The sequence of IL-8 primer (Gilmour et al., 2001):

sense: **CGA TGT CAG TGC ATA AAG ACA**

antisense: **TGA ATT CTC AGC CCT CTT CAA AAA**

(melting temperature $T_m = 65^0C$)

GAPDH primer: The sequence of GAPDH primer (Borchers et al., 1999):

sense: **TGC TGG GGC TGG TGG TC**

antisense: **TCA AGT GGG GCG ATG CTG**

(melting temperature $T_m = 58.2^0C$)

2.1.8 LIPOPOLYSACCHARIDE (LPS) AND BACTERIAL CELL WALL COMPONENTS

LPS from *Pseudomonas aeruginosa* serotype 10 (Sigma-Aldrich, Dorset, UK)

LPS from *Pseudomonas aeruginosa* clinical strain LPS (isolated in Dept. Medical Microbiology, School of medicine, Cardiff University, UK)

LPS from *Burkholderia cepacia* NCTC 10661 (isolated in Dept. Medical (isolated in Dep. Medical Microbiology, School of Medicine, Cardiff University, UK)

LPS from *Burkholderia cepacia* ET-12 LPS (isolated in Dept. Medical Microbiology, School of Medicine, University, UK)

Staphylococcus aureus Peptidoglycan (PGN) (Sigma-Aldrich, Dorset, UK)

Staphylococcus aureus Lipoteichoic acid (LTA) (Sigma-Aldrich, Dorset, UK)

2.1.9 PREPARATION OF LPS (LPS ISOLATON)

2.1.9.1 Growing and source of bacteria

LPS was extracted and purified from 2 isolates of *B. cepacia* (*B. cepacia* NCTC 10661, *B. cepacia* ET-12), and one isolate of *P. aeruginosa* clinical strain. Each bacterial isolate was inoculated onto blood agar plate and incubated for overnight at 37°C. The bacterial colonies were scraped by sterile loops and inoculated into 4 sterile flasks with 250 mL of TSB and incubated overnight at 37°C with shaking. The liquid cultures were harvested by centrifugation at 3600 xg for 25 minutes and washed twice in sterile PBS. The bacterial pellet was frozen, lyophilised and stored at 4°C until used.

2.1.9.2 LPS extraction using phenol-water method

Many methods have been developed for the extraction of LPS from gram- negative bacteria. One of the methods, which are widely used, is the phenol-water procedure (Westphal *et al.* 1952) because it is applicable to many different groups of bacteria. LPS isolation was based on the method of Westphal *et al.*, 1952 and adopted by Qureshi *et al.*, 1982. The lyophilised

bacteria were weighed and suspended into phenol-chloroform petroleum ether reagent (5mL per 500 μg of dried bacteria) and homogenised for a minimum of 5 minutes using a tissue grinder with PTFE Pestle (KarteII) immersed in an ice bath. The homogenised mixture was then transferred into a 15 mL polypropylene (organic solvent resistant) tube and centrifuged for 30 minutes at 3600 xg at 4°C. The tube was removed and the supernatant was filtered through a Whitman No 1 filter paper into 250 mL round bottom flask. The chloroform and petroleum ether was removed from the filtered supernatant by using a rotary evaporator at 60°C for at least 15 minutes. The remaining solution was removed with a glass pipette and transferred to a 15 mL polypropylene tube and 6 volumes of diethyl ether/acetone (diethyl ether 17% acetone 83%) was added and left to stand at RT for one hour. The precipitated LPS was collected by centrifugation at 3600 xg for 30 minutes.

The supernatant was discarded and the pellet was washed three times with 5 mL diethyl ether /acetone. The LPS pellet was frozen and dried in a vacuum dessicator for at least 60 minutes before 3mL of pyrogen free water was added by heating to 37°C in a water bath and by suction/displacement of the solution using a 5 mL syringe with 23 gauge needle. The LPS suspension was centrifuged at 100,000 rpm for one hour using a Beckman Optima ultracentrifuge with a TLA-100 rotor. The supernatant was discarded and the final resultant was a clear and transparent sediment, which was dispensed into pyrogen-free water.

For further purification, the LPS was treated with 1μg of DNase/mL (from bovine pancreas; Sigma Chemical Co) plus 1μg RNase/mL (from bovine pancreas; BDH, Poole, UK) at 30°C for 90 minutes at pH 7.5, followed by the addition of 1μg/mL of trypsin (Sigma Chemical Co) and incubation at 30°C for 90 minutes. Finally 1μg of Proteinase K/mL (Sigma) was added and incubated at 30°C for 90 minutes.

Following these enzymatic treatments the solution was dialysed against distilled water overnight and lyophilised. This preparation was then treated with 45% phenol containing tiethylamine and Sodium DOC and the LPS, extracted in the aqueous phase, was precipitated with ethanol by the DOC-phenol-water extraction method (Manthey et al., 1994) to obtain the LPS.

The supernatant was discarded and the final resultant was a clear/transparent sediment which was dispensed into pyrogen-free water before being transferred to a glass sterile bijoux, frozen and lyophilised overnight. The dried LPS was weighed and stock solution was made up at a concentration of 1mg/mL in pyrogen-free water then was quantified using LAL assay before was kept at –80°C.

2.1.10 REAGENTS AND BUFFERS FOR WESTERN BLOTTING

ECL advance western blotting detection kit (Pharmacia Biotech, Ltd., Buckinghamshire, UK)
NuPAGE® antioxidant (Invitrogen Life Technologies, Paisley, UK)
NuPAGE® LDS (Invitrogen Life Technologies, Paisley, UK)
NuPAGE®reducing agent (Invitrogen Life Technologies, Paisley, UK)
10x Blocking Buffer (Sigma-Aldrich, Dorset, UK)
NuPAGE® SDS MOPS running buffer (Invitrogen Life Technologies, Paisley, UK)
NuPAGE® transfer buffer (Invitrogen Life Technologies, Paisley, UK)
Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) Pre-cast gel (Invitrogen Life Technologies, Paisley, UK)
Ultra pure water (Pharmacia Biotech, Ltd., Buckinghamshire, UK)

2.2 METHODS

2.2.1 CELL CULTURE

2.2.1.1 A549 epithelial cells

The human lung epithelial cell line A549 (CCL-185) was maintained in complete medium consisting of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% foetal calf serum, 200mM L-glutamine and 1% Penicillin/Streptomycin. Cells were seeded initially at a density of 10000/mL in 20 mL medium, and grown to confluence at 37°C in 5% humidified atmosphere. The medium was changed every two days. When the cells became confluent, they were sub-cultured (1:10 splitting ratio) after being detached with 0.25% (w/v) trypsin-0.5 mM Ethylaminediaminetetraacetic acid (EDTA) solution. Cells were used after the third passage, and only up to 20 passages for all experiments that were done in this project.

2.2.1.2 Freezing and storage of A549 cell line

A549 epithelial cells were grown to confluent before they were trypsinized and washed twice with complete medium. The cells were harvested by low speed centrifugation (200 xg) for 5 minutes and were then re-suspended in fresh growth medium. Cells were counted after viability was assessed with trypan blue dye and adjusted to 2-3x10^6 /mL. The preservative, dimethyl sulfoxide, DMSO, (10%, v/v) was added to the suspension, the cells were aliquoted into 1mL cryovials for freezing at -80°C overnight and then transferred to liquid nitrogen for long-term preservation.

2.2.1.3 Thawing and growing frozen cells

Cells were removed from liquid nitrogen and thawed rapidly at 37°C for a few minutes and the cell suspension was centrifuged for 5 minutes at 500xg after being transferred to a universal container that contained 10 mL of complete medium to remove dimethyl sulfoxide. The cells were washed twice and re-suspended in 10 mL of fresh complete medium, transferred to 15cm^2 tissue culture flask and incubated at 37°C. Cells were transferred to 25cm^2 tissue culture flask when they reached confluence, and were sub cultured as described in sections (2.2.1.1).

2.2.1.4 Growing A549 cells for cytokine induction and for other experiments

The epithelial A549 cells were seeded in complete RPMI 1640 medium onto 12-well plate at desired density, and incubated at 37°C. The cells were fed for two days with the complete medium until establishing confluence. When the cells become confluent, the monolayers were washed three times with PBS and the cells were incubated with medium with L-glutamine overnight (A549 epithelial cells were prepared as described here for all experiments unless otherwise stated).

2.2.2 CELL VIABILITY ASSESSMENT

The effect of surfactants, bacteria or bacterial products on the A549 cell viability were analysed by two methods:

2.2.2.1 Trypan blue method

Counting and cell viability was determined by mixing an equal volume of filtered trypan blue dye solution (0.4% w/v in HEPES solution) and cell suspension. The mixture was incubated for 5-10 minutes at room temperature and 10 μL of the suspension was placed between the coverslip of the haemocytometer counting chamber (Arnold R Horwell limited, London, UK). The cells were then examined by Inverted phase contrast microscopy, and viable cells were distinguished from dead (blue) cells by dye exclusion.

2.2.2.2 Cell titer 96® aqueous one-solution cell proliferation assay

The CellTiter 96® Aqueous one solution cell proliferation assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assay. All different conditions were tested for cell viability using this method by adding 20 μL of CellTiter 96® A_{queous} one solution reagent to each well of a 96 well assay plate containing samples from different conditions in 100 μL of culture medium. Two controls were used; untreated cells (positive control) and cells treated with DMSO overnight (negative control)

The plate was incubated for 4 hours at 37°C in humidified atmosphere. Optical densities were measured at 490 nm using photospectrometer. The optical densities are proportional to the formazan product and this represents the proportional number of living cells. Results were expressed as a mean percentage of viable when compared with untreated cells, and compared with the negative control to ensure the validity of this assay in this situation.

2.2.3 BACTERIAL CULTURES

2.2.3.1 Growing bacteria from –80° C

Preserved bacteria in microbeads (PRO-LAB Diagnostics, Ontario, Canada) stored at –80°C were taken out and one bead was transferred to 5 mL of tryptic soy broth (TSB) and incubated at 37°C overnight. A blood agar plate was inoculated from the TSB culture and bacteria were streaked to give isolated colonies before incubated aerobically at 37°C overnight.

2.2.3.2 Growing bacteria in liquid medium

5-10 bacterial colonies were picked from fresh blood agar cultures and 10 mL of TSB was inoculated and incubated at 37°C in a shaking incubator. After 18 hours incubation TSB culture was adjusted spectrophotometrically to the desired optical density (OD) and kept at –80°C for use. Bacteria were kept for a maximum of 2 weeks for use.

2.2.3.3 Bacterial cell quantification

Bacterial cells were quantified using turbidity or optical density, which is a rapid method of estimating the number of bacteria in solution. Bacterial cells were inoculated into TSB and incubated at 37°C in a shaking incubator overnight. Serial dilutions from the broth cultures were made in TSB and the optical density (OD) for all dilutions were measured at a wavelength of 600 nm ($O.D_{600}$). The optical densities were recorded after readings were adjusted to zero with a blank TSB. Using a fresh pipette tip and starting with the highest dilution, 0.1 mL of each serial dilution were transferred to the surface of blood agar plates and spread out using sterile spreader over the whole surface of the plate. Plates were left to dry before incubated at 37°C overnight. Appropriate plates containing between 100-200 CFU countable numbers of bacterial colonies were selected and the colonies were counted and recorded. From this count, the colony forming units (CFU) of bacteria in the original samples were calculated using this equation:

CFU= number of bacteria x 10x dilution factor.

The optical density experiment and colony-forming unit (CFU) were repeated three times and the average for each experiment was taken. Standard curves for each bacterial strain were established relating the average number of colony-forming unit/mL (CFU) to the average of the optical density (OD) measurement (determined by spectrophotometer) and plotted using the EXCEL computer program. The number of colony forming unit (CFU) or the optical density (OD) was calculated using the equation in each standard curve in EXCEL for all bacteria used in this project.

2.2.3.4 Preparing heat killed bacteria (HK)

Bacteria were cultured and quantified (section 2.2.3.1-2.2.3.3) before centrifuging at (10,000xg) for 20 minutes, then washed twice in sterile PBS and heated at 95°C in a water-bath for 30 minutes. The suspension of heated-killed (HK) bacteria was resuspended in the same volume of RPMI media with L-glutamine and aliquoted before kept at -80°C. Heat killed bacteria were thawed at room temperature and vortex-mixed before use.

Sterility was confirmed by inoculating a loopfull from the prepared heat killed and streaked onto blood agar plate before was incubated aerobically at 37°C. The blood agar plate was checked for the presence or absence of colonial appearance.

2.2.3.5 Preparing conditioned media (CM)

Conditioned media was prepared from cultured and quantified bacteria in TSB (section 2.2.3.1-2.2.3.3). The bacterial suspension was passed through a 0.22 micron filter and the supernatant was collected. The supernatant was cooled down to room temperature and checked for sterility (as explained earlier) before being aliquoted and kept at -80°C. Conditioned media was thawed at room temperature and vortex-mixed before use.

2.2.3.6 Preparing heat conditioned media (hCM)

Heat- conditioned media was prepared by heating conditioned media (section 2.2.3.5) at 95°C in a water-bath for 30 minutes. Heat-conditioned media was cooled down at room temperature and checked for sterility before aliquoted and kept at -80°C. Heat conditioned media from bacteria (hCM) was thawed at room temperature and vortexed before use.

2.2.4 SURFACTANT PHOSPHOLIPID PREPARATION.

2.2.4.1 Preparation of silanised bijoux vials

Dipalmitoylphosphatidylcholine and related phospholipids adhere to glass. To lessen the adherence of DPPC and facilitate removal, darkened glass bijoux vials used were silicone coated. The glass Bijoux vials were soaked overnight in 1M HCl solution. Following acid washing, the vials were rinsed and soaked in de-ionized water overnight. The vials were then inverted and left to dry at 37°C overnight before silanising with RepelCoat (BDH, Dorset, UK) and autoclaved at 121°C at 15 PSI for 20 minutes.

2.2.4.2 DPPC preparation

The DPPC, which was provided by the supplier either in powder (to which chloroform was added to dissolve) or ready dissolved in chloroform, was aliquoted at 5mg/mL in silianized bijoux vials and kept at –20°C until needed. Desired amounts of the DPPC were taken into silianized bijoux vials and dried under a very low stream of nitrogen gas. The DPPC which was a very thin shiny/waxy layer was re-suspended in desired RPMI medium, and was incubated at 37°C for 20 minutes before vortex-mixing intermittently and sonicated (under ice to minimize heat) three times for 20 seconds each time at amplitude of 10 μm using an MSE Soniprep 150 sonicator (Sanyo Gallenkamp PLC, Leicestershire, UK). DPPC was prepared freshly just before use.

2.2.5 INHIBITORY EFFECT OF SURFACTANT

The effect of different surfactants on the growth of bacteria was measured used two methods:

2.2.5.1 Optical density method

The inhibitory concentrations of surfactants were determined using a microdilution method in a 96 well round bottom plate in a (trypticase soy broth (TSB), 0.9% saline or saline supplemented with 5% human serum). Surfactant; Survanta®, Curosurf® or DPPC lipid were prepared in the same media and 200 μL was added to wells in triplicate at the desired concentration (500μg/mL for the DPPC, and 250μg/mL for Survanta® and Curosurf®). Bacteria at a density of 10^5 CFU/mL were added to all wells.

The plates were read using the Labsystem multiscan reader at 600 nm optical density (OD), and the reading was subtracted from the wells with bacteria only. OD results were recorded at (0 hour) before incubated at 37°C. The plate was read again and the optical density (OD) was recorded at 6 hours and 24 hours incubation before subtractions were made from controls without surfactant.

2.2.5.2 Colony forming unit (CFU) method

The inhibitory effect of the surfactants and DPPC lipid were determined using macrodilution method in 5 mL glass tubes. DPPC was prepared and sonicated (2.2.4.2), Survanta® and Curosurf® were prepared in 1% TSB, 0.9% saline or saline supplemented with 5% human serum at concentration (500μg/mL for the DPPC, and 250μg/mL for Survanta® and Curosurf®) and 2mL were dispensed to all. Bacteria at a density of 10^5 CFU/mL were added to all tubes and incubated at 37°C.

The number of bacteria was checked (0,12, 24 hours) by making serial dilutions of 100 μL from each tube and culturing 100 μL from each dilution onto blood agar plate. The plates were checked after 18 hours incubation at 37°C and the colonies were counted. Final calculations were made by multiplying the dilution factors to get the colony forming units per mL (CFU/mL) in the original tubes.

2.2.6 BACTERIAL INTERNALISATION BY A549 EPITHELIAL CELLS AND EFFECT OF PULMONARY SURFACTANT

The study of internalisation of bacteria to A549 epithelial cells was investigated using three different methods: fluorescent microscopy and antibiotic protection assay. In addition electron microscopy was used to confirm the location of the bacteria inside the A549 cells.

2.2.6.1 Bacterial internalisation by A549 epithelial cells measured by fluorescent microscopy

To study the uptake of bacteria by the A549 epithelial cells, the bacteria were labeled with fluorescent dye. Tryptone soya broth (TSB) was inoculated from an over night bacterial blood agar plate by touching 5-10 colonies and incubated at 37°C aerobically with shaking overnight until it reached 10^9-$5x10^9$ CFU/mL as determined by the optical density (OD_{600}) (section 2.2.3.3). Bacteria were conjugated with FITC as described by (Hazenbos et al., 1994) with minor changes and optimisation to suit our experiment. Bacteria were pelleted in 10 mL of (0.2 M $NaCO_3$ in 50 mM NaCl) buffer, pH 9.2 containing 0.05 mg/mL of (FITC) and left for 15 minutes in the dark. The bacteria were washed five times in PBS (3000xg for 30 minutes), and then were resuspended in 10 mL of PBS. Aliquots were made before storage at -80°C.

2.2.6.1.1 Coating bacteria with surfactants

Bacteria alone or FITC-conjugated bacteria were washed and resuspended in 1 mL of RPMI media without antibiotic in 1.5 mL eppendorf tubes. 500μg/mL DPPC or 250μg/mL of Survanta® or Curosurf® were added and incubated at 37°C in a wheel rotor for 1 hour.

2.2.6.1.2 Assessment of bacterial internalisation by fluorescent microscopy

Bacteria conjugated to FITC with or without surfactant coating were prepared and coated with different surfactant or DPPC (section 2.2.6.1.1) before was added to A549 epithelial cells. A549 epithelial cells ($5x10^5$ cell/mL) in RPMI medium without antibiotics were grown on 18 mm round coverslips in 12 well tissue culture plates for 6 hours. The coverslips were then washed twice with PBS before being exposed to the surfactant coated bacteria at multiplicity of infection (moi) of (10 bacteria: A549 cells) and incubated for 2 hours. The coverslips were washed three times with PBS and fixed with 3.7% paraformaldehyde in PBS containing 15 mM sucrose pH 7.4 for 30 minutes at room temperature. After neutralising with 50 mM HCl, the coveslips were washed with PBS pH. 7.4 and mounted with 5μg/mL of trypan blue to quench FITC stained bacteria outside the cells. Coverslips were examined using fluorescent microscopy and 4000 A549 cells were counted and the number of cells containing fluorescent bacteria was calculated using the Image-Pro® Plus software (Media Cybernetics, MD, USA).

2.2.6.2 Bacterial internalisation by A549 cells measured by antibiotic protection assay

This experiment was performed as described by (Burns et al., 1996) with modification. A549 cells (10^6 cell/mL) were dispensed in 12 well tissue culture plates with complete media without antibiotic. Surfactant coated bacteria (section 2.2.6.1.1) or bacteria alone were added at 10:1 moi (number of bacteria: number of A549 cells) and incubated at 37° C with.
Following 4 hour incubation at 37°C, the A549 epithelial cells were washed three times with sterile PBS to remove non adherent bacteria, and 1mL of RPMI medium containing 500μg/mL of gentamicin and 1mg/mL of ceftazidime were added and left for one hour at room temperature to kill extracellular bacteria. The wells were washed three times with sterile PBS and lysed with 1mL of 0.25% (v/v) Triton X-100 in PBS.

In some circumstances the step of incubation with antibiotics was omitted to calculate the number of adherent and internalised bacteria. The number of the bacteria (CFU) in the cell lysis suspension was determined by culturing serial dilutions on blood agar plates and incubation at 37°C for 24 hours. Colonies were counted for viability and the result was calculated as:
Number of internalised bacteria = the number of bacteria counted on the blood agar plate (after treatment with antibiotics and lysis) X dilution factors.

2.2.6.3 Bacterial internalisation by A549 cells measured by electron microscopy

A549 epithelial cells were seeded into 6 tissue culture plates at concentration of 10^6 cells in 3 mL complete media and incubated at 37°C. The monolayers were fed every day till confluent. The media was changed to without antibiotics and 1 mL was added to all wells just to cover the monolayers. Bacteria were prepared from logarithmic phase and were added to some wells at moi (500 bacteria: 1 A549 cell) before were incubated at 4°C for 60 minutes for attachment. Control cells without bacteria were included and pulmonary surfactant Survanta® or DPPC lipid also were added to A549 to study the uptake of surfactant by the cells. The plates were warmed at RT for 30 minutes before washed with warm PBS were incubated at 37°C. The monolayers were washed x3 with sterile PBS before 3ml of media without antibiotics were added and incubated at 37°C. The cells were washed with PBS (x3) at different time (1, 2, 4, and 24 hours) and processed for electron microscopy for dehydration and fixation (Glauert, 1975).

2.2.6.3.1 Fixation

The monolayers were rinsed twice with warm cacodylate buffer before 1 mL of cold cacodylate was added. The cells were fixed with a mixture of osmium tetroxide/glutaraldehyde (concentrations usually 1% / 2.5% in cacodylate buffer and were left for at least 6 hours in a sealed box at 4°C protected from light. The fixative solution was removed and the cells were washed x3 with 1 mL of 0.9% cold saline. The fixed cells were scraped by adding cold saline and using a rubber policeman (the plunger from 1 mL syringe) before were removed using a glass Pasteur and transferred to Eppendorf tubes. This was repeated twice to gather up all remaining cells. The cells were centrifuged at low speed at 300g for 10 minutes in a sealed rotor microcentrifuge and the tube were filled with saline before stored at 4°C for dehydration.

2.2.6.3.2 Embedding, cutting and double-staining

The fixed cells were dehydrated in a series of graded ethanolic solutions 30%, 50%, 70% and 90% for 10 minutes then in 100% for 15 min and repeated twice ending with pure acetone, and finally embedded in Epon 812. Ultrathin sections were made and placed onto 200-mesh standard square copper grids and double-stained with 2% methanolic uranyl acetate followed by 1.8% aqueous lead citrate. The sections were evaluated with a Phillips TEM410 transmission electron microscope. (This part of work (2.2.6.3.2) was done by Dr. M Rittig, University of Nottingham)

2.2.7 INCUBATION OF A549 CELLS WITH CYTOCHALASIN D FOR INVASION ASSAY

A549 cells were seeded into 12 well culture plates and allowed to adhere for 6 hours in complete media without antibiotics. The monolayers were then pre-incubated with 5μM of cytochalasin D for 30 minute in fresh complete medium. After pre-incubation with cytochalasin D, the monolayers were then stimulated with bacteria at moi (10:1) number of bacteria to the number of the cells, and the tissue culture plate was incubated at 37°C with humidified atmosphere for 3 hours. The wells were washed with sterile PBS and the antibiotic protection assay was performed (section 2.2.6.2).

2.2.8 STIMULATION OF A549 EPITHELIAL CELLS AND EFFECT OF SURFACTANTS.

2.2.8.1 Time course of cytokine production from A549 cells stimulated with live bacteria

A549 epithelial cells were prepared as described earlier (2.2.1.4) and the media was changed before stimulation with live bacteria at moi 10:1 (bacteria: A549 cells) in the presence or absence of 1% human serum. The plate was incubated at 37°C. Supernatants were collected at different times for time course experiment and kept at –80°C before cytokines (IL-8, IL-1β) were assayed (section 2.2.10). For the effect of surfactant on IL-8 production, the A549 cells were incubated with Survanta®, Curosurf® or DPPC at 250 μg/mL for 2 hours before removing the surfactant and washing the cells with sterile PBS prior adding bacteria and then incubated for 18 hours.

2.2.8.2 Incubation of A549 epithelial cells with bacterial Lipopolysaccharide

The A549 epithelial cells were seeded (2.2.1.4) in complete RPMI 1640 medium into 12-well tissue culture plates. The cells were fed with complete medium everyday until establishing confluence. When the cells become confluent, the monolayers were washed three times with PBS and the cells were incubated with RPMI 1640 medium with only L-glutamine for 18-24 hours. For a dose-response, the cells were stimulated with different concentrations of LPS (100 ng-1000 ng) from *P. aeruginosa* serotype 10, *P. aeruginosa* clinical strain, *Burkholderia cepacia* ET-12 and *Burkholderia cepacia* NCTC 10661in the presence or absence of 1% human serum (HS). One concentration point of LPS (100 ng /mL) was chosen for time course experiment for IL8 cytokine, the cells were challenged in the presence or absence of 1% human and incubated at 37°C with or without of surfactant for (0, 6, 12, 24, 48 hours). Human serum was used in our experiment as a source of (CD14 and LBP) and at 1% showed that no effect on the growth of bacteria (chapter 3).

2.2.8.3 Incubation of A549 cells with recombinant IL-1β for IL-8 induction and effect of surfactant

The A549 epithelial cells grown to confluence as described earlier (2.2.1.4) before stimulation with 5ng/mL of recombinant IL-1β in the presence of 1% human serum. For the effect of surfactant the A549 cells were incubated with Survanta®, Curosurf® or DPPC at 250 μg/mL for 2 hours before adding stimulation with IL-1β. The tissue culture plate was incubated at 37°C incubator and supernatants were collected after 18 hours and IL-8 was determination by ELISA (2.2.10)

2.2.8.4 Stimulation A549 epithelial cells with heat killed and conditioned media for IL-8 and the effect of surfactant

The human A549 cells were grown as described (2.2.1.4) and stimulated with different amount of HK bacteria or CM in RPMI with only L-glutamine in presence or absence of 1% human serum. For the effect surfactant, the A549 cells were incubated with Survanta®, Curosurf® or DPPC at 250μg/mL for 2 hours before stimulation with the desired amount of HK or CM. The tissue culture plates were incubated at 37°C with humidity before supernatants were collected after18 hours.

2.2.9 DOSE RESPONSE OF PEPTIDOGLYCAN (PGN) AND LIPOTEICHOIC ACID (LTA) INDUCED IL-8 & IL-1β FROM A549 EPITHELIAL CELLS

The confluent human A549 epithelial cells grown as described (2.2.1.4) and incubated with different concentrations of peptidoglycan or lipoteichoic acid (0, 50,100, 250,500,1000 ng/mL) in the presence or absence of 1% human serum in RPMI 1640 with L-glutamine. The tissue culture plates were incubated for 18 hours at 37°C with humidity, and the supernatants were collected for IL-8 and IL-1β was determination by ELISA. For surfactant effect, one concentration was chosen and the A549 cells were incubated with 250μg/mL of Survanta®, Curosurf® or DPPC before for 2 hours prior adding PGN or LTA and incubated for 18 hours for IL-8 protein determination by ELISA assay.

2.2.10. ELISA ASSAY

Enzyme-linked Immunosorbent Assay (ELISA) is combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily assayed enzyme that possesses a high turnover number. ELISA can provide a useful measurement of antigen or antibody concentration.

Sandwich ELISA Assays is one of the most useful of the immunoassays is the two antibody "sandwich" ELISA. This assay is used to determine the antigen concentration in unknown samples. This ELISA is fast and accurate, and if a purified antigen standard is available, the assay can determine the absolute amount of antigen in an unknown sample. The sandwich ELISA requires two antibodies that bind to epitopes that do not overlap on the antigen. This can be accomplished with either two monoclonal antibodies that recognize discrete sites or one batch of affinity-purified polyclonal antibodies (Perlmann and Perlmann, 1994)

To utilize this assay, one antibody (the "capture" antibody) is purified and bound to a solid phase typically attached to the bottom of a plate well. Antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash, and a labeled second antibody (the "detection" antibody) is allowed to bind to the antigen, thus completing the "sandwich". The assay is then quantitated by measuring the amount of labeled second antibody bound to the matrix, through the use of a colorimetric substrate. Major advantages of this technique are that the antigen does not need to be purified prior to use, and that these assays are very specific. However, one disadvantage is that not all antibodies can be used. Monoclonal antibody combinations must be qualified as "matched pairs", meaning that they can recognize separate epitopes on the antigen so they do not hinder each other's binding (Goding, 1996).

The cytokines released from A549 epithelial cells were assayed by ELISA using paired antibody as follows: mouse monoclonal anti-human IL-8 or IL-1β capture antibody in PBS (4μg/mL) was added to a flat-bottom 96 well microtiter plate (Nunc^{TM} Maxisorb) and incubated at room temperature. Then the coated plate was aspirated and washed three times with washing buffer (PBS/0.05% Tween 20). 300μL blocking buffer (PBS/1%BSA/5% Tween 20) was added to each well and the plate was incubated for 1 hour. After washing the plate three times, IL-8 or IL-1β standards were prepared in doubling dilutions (from 1000-16.125 pg/mL) and 100μL of standards and samples were added and the plate was incubated for 2 hours (diluent was included as blank). The ELISA plate was washed and 100μL of 10μg/mL of anti IL-8 or IL-1β biotinylated detection antibody were added to each well and the reaction mixtures were incubated for a further 2 hours at room temperature. After the plate was washed three times and dried, 100μL of 1:20,000 diluted Streptavidin-HRP conjugate was added to each well and incubated for 20 minutes. At the end of 20 minutes at room temperature, the plate was washed three times and 100μL of TMB substrate was added and the plate was incubated for 20-30 minutes at room temperature in the dark after which 50μL of 0.5M sulfuric acid was added to stop the reaction mixture. Then, the absorbance was read on a microplate reader at 450 nm and the reading was subtracted automatically by readings at 540 nm to correct for optical imperfection in the plate.

2.2.11 BLOCKING STUDIES

The effect of neutralization receptors TLR2, TLR4 and CD14 on IL-8 induction was investigated. A549 cells ($5x10^5$) were seeded into 12-well plates and grown to confluence and media changed everyday. When cells become confluent, they were washed three times with PBS and the medium was changed to serum free and incubated overnight. The cells were washed again and serum free media was added prior adding 20 μg/mL of anti-TLR4 antibody (HTA125), anti-TLR2 (clone TL2.1), anti-CD14 or isotype control antibody and the tissue culture plate was incubated at room temperature. After 45 minutes (for anti TLR2 and anti TLR4) and 30 minutes (for anti CD-14), 100 ng/mL LPS or live bacteria (moi 10 bacteria: 1 A549) were added to the A549 epithelial cells in presence of 1% of human serum and incubated at 37°C. The supernatants were collected after the cells were further incubated for 18 h. and IL-8 was assayed as described in section (2.2.10).

2.2.12 POLYMYXIN NEUTRALIZATION ASSAY

A549 cells ($5x10^5$) were seeded onto 12-well tissue plates. The growth medium was changed everyday until the cells establish confluence. When the cells become confluent, the monolayers were washed and RPMI medium with L-glutamine was added and the cells were incubated at 37°C overnight. LPS from different sources at concentration of 100 ng/mL, heat killed bacteria (HK), live bacteria, or conditioned media from bacteria (CM) or heated conditioned media from bacteria (hCM) were incubated with PMB at 10 μg/mL at 37°C for 30 minutes before the LAL assay was performed or before were added to the A549 cells in presence of 1% of human serum. Supernatants were collected after 18 hours incubation for determination of IL-8 by ELISA assay (2.2.10).

2.2.13 LIMULUS AMOEBOCYTE LYSATE (LAL) ASSAY

The Limulus Amebocyte Lysate (LAL) assay is used to detect endotoxin (LPS) from Gram-negative bacteria. The endotoxin activity was analysed by the kinetic LAL assay (kinetic-QCL) purchased from Biowhittaker Ltd (Wokingham, UK). The test was performed according to the manufacturer's instruction with extra care taken at all stages to avoid contamination with endotoxin and by using pyrogen-free tubes, tips and pyrogen-free water (PFW). In brief, endotoxin standard was prepared in PFW to give a concentration of 50 endotoxin unit (EU)/mL and were vortexed for 5 minutes prior to use. The standard was diluted to give 5.0, 0.5, 0.05 and 0.005EU /mL (EU approximately equals 100 picogram of endotoxin). The sample tests (LPS, HK and CM) were prepared in pyrogen-free glass tubes and appropriate dilutions were made in PFW water. Each standard and sample (50μL) were added in duplicate to a 96 flat-bottom microtiter plate. The plate was placed for 10 minutes in the holder of the KQCL reader, which had been warmed up to 37°C. The LAL reagent was reconstituted and prepared by adding 2.6 mL of PFW and swirled gently. 50 μL of LAL reagent was added to each well and the reading plate was taken for 100 minutes using the computer software program (Win 1.1 KQCL).

2.2.14 INCUBATION OF A549 CELLS WITH LIVE BACTERIA FOR INDUCTION OF IL-8 mRNA EXPRESSION

A549 cells were seeded as described (section 2.2.1.4) and the media was changed again before Survanta® or DPPC were added at final concentration of 250 μg/mL and incubated for 2 hours. The cells were challenged with different types of bacteria at 10 moi (bacteria: A549) that was prepared (centrifuged at 500 xg) for 20 minutes and resuspended in RPMI media with L-glutamine. After appropriate time of incubation, the cells were lysed and the total RNA was isolated as described in section 2.2.17.1. Following quantification of the total RNA, RT and PCR were done as described (section 2.2.17.2- 2.2.17.4).

2.2.15 INCUBATION OF A549 CELLS WITH LPS FOR INDUCTION IL-8 mRNA

A549 cells were prepared (section 2.2.1.4) and the media were changed again before Survanta® or DPPC were added at final concentration of μg/mL and incubated for 2 hours. The cells were then stimulated in presence of 1% human serum with different types of LPS 100ng/mL from *P. aeruginosa* serotype 10, *P. aeruginosa* clinical strain, *B.cepacia* NCTC 10661 and *B.cepacia* ET-12 strain. After appropriate time of incubation, the cells were lysed and the total RNA was isolated as described (section 2.2.17.1). Following quantification of the total RNA, RT and PCR were done as described (section 2.2.17.2- 2.2.17.4).

2.2.16. INCUBATION A549 CELLS WITH HEAT KILLED AND HEATED CONDITIONED MEDIA (hCM) FOR INDUCTION IL-8 mRNA

A549 cells were prepared as described (section 2.2.1.4) and the media were changed again before Survanta® or DPPC were added at final concentration of 250μg/mL and incubated for 2 hours. The cells were challenged with 10 μL (represent 10^6 CFU) of heated heat killed bacteria or heated conditioned media (hCM) of *P.aeruginosa* clinical strain. After appropriate time of incubation, the cells were lysed and the total RNA was isolated as described (section 2.2.17.1). Following quantification of the total RNA, RT and PCR were done as described (section 2.2.17.2- 2.2.17-4).

2.2.17 RT AND PCR

PCR is a method for in vitro amplification of DNA. It has substantially accelerated the pace of research in many fields of biology, both by reducing the time required to perform routine manipulations of DNA and by making new manipulations possible. In essence, PCR is multiple rounds of primer extension reactions in which, complementary strands of defined region of the DNA molecule, are simultaneously synthesized by a thermostable DNA polymerase. During repeated rounds of these reactions, the number of newly synthesized DNA strands increases exponentially so that after 20 to 30 reaction cycles, the initial template DNA will have been replicated several million–fold. This power to faithfully, amplify along with the low cost and simplicity of the method have made PCR an indispensable tool.

PCR utilizes template DNA - the starting DNA of interest, two primers - short, single-stranded, synthesized pieces of DNA that complement sequences on each side of the region of the template DNA that is being amplified, thermostable DNA polymerase - typically *Taq* (*Thermus aquaticus)*, a heat stable enzyme capable of adding nucleotides to a growing DNA strand, dNTPs - a supply of the 4 nucleotides needed to make the new DNA strands, magnesium - a cofactor for the polymerase and a buffer solution - to maintain the pH and salt concentrations appropriate for the polymerase. Once these components are combined they go through a series of temperature changes (cycles), repeatedly, in a machine called a thermocycler. This process will generate exponential copies of the DNA segment of interest. Each cycle consists of three parts: denaturation, annealing, and elongation or extension.

In denaturation the reaction is heated to greater than 90°C. at this temperature the double helix is destabilized and DNA molecules separate into the single strand capable of being copied by the DNA polymerase. The reaction is cooled to a temperature that allows binding of the primers to the single stranded DNA without permitting double helix to reform between the template strands. This process is called annealing. The temperature used varies (typically 40-60°c) and is determined by the sequence and number of bases in the primers. Extension step is carried out at the temperature at which the DNA polymerase is most active. The DNA polymerase, directed by the position of the primers, copies the intervening target sequence

using the single stranded DNA as a template. A total of 20-40 PCR cycles is carried out depending on the abundance of the target sequence in the template DNA. Sequences up to several thousands of base pairs can be amplified. The PCR products can be evaluated using an agarose gel when run alongside a DNA size standard, or marker, with DNA bands of known sizes.

2.2.17.1 Total RNA isolation

RNA was isolated from the cell cultures by trypsinising the cells after washing 3 times with sterile PBS and then neutralizing the trypsin with complete media. The cells were collected and centrifuged at 200 xg and washed three times with PBS. The total RNA was isolated from the cells using Ambion RNAqueous® kit (Cambridgeshire, UK). This method is a phenol-free, rapid and filter-based RNA isolation method, which is based on the ability of glass fibers to bind nucleic acid in concentrated chaotropic salt solutions (Boom, et al. 1990, Marko, et al.1982).

Precautions were taken during working with RNA. These include: wearing gloves throughout experiments to prevent contamination from RNases found in human sweat; regularly changing gloves; using a dedicated set of pipettes that are used solely for RNA work; using tips and tubes that are tested and guaranteed to be RNase-free and using RNase-free chemicals and reagents. The isolation was done by following the manufacturer's instructions. Briefly 350µL of lysis/binding solution was added to the cells and vortexed for 1 minute before the same volume of 64% ethanol was added and vortexed vigorously for another minute. The lysate/ethanol mixture was pipetted onto the RNAqueous filter cartridge and centrifuged for 45 second at 10,000 xg. The flow-through was discarded and 700µL of solution #1 was added onto the filter cartridge before centrifuged for 15 seconds at 10,000 xg for 45 seconds. The flow-through was discarded and 500µL of solution #2/3 was added and washed through the filter as in the previous step. The filter cartridge was transferred into a fresh collection tube and the 40µL of elution solution (preheated to 80°C) was added to the center of the filter and centrifuged at 10,000 rpm for 30 seconds. This elution step was repeated and the RNA collected into the same tube as the first elution.

2.2.17.2 RNA quantification and purity assessments

The concentration and purity of total RNA were determined by dilution an aliquots of the RNA preparation 1:20 in Nuclease-free water, and reading the absorbance spectrophotometrically using Genequant (Amersham Biosciences UK Limited, Bucks, UK) at 260nm and 280 nm after the Genequant was zero with DNA/RNA free water. RNA sample (10 μL) was diluted 1/20 with DNA/RNA free water (80 μL). Concentration of RNA was calculated as following:
RNA concentration = Spectrophotemetric conversion x A_{260} x dilution factor.
The ratio of A_{260} to A_{280} value is measure of RNA purity, and it should fall in the range of 1.8 to 2.1.

2.2.17.3 Reverse transcription

The isolated total RNA (2 μg) was added to 2 μL of Random Decamers and Nuclease free water was added and mad up to 12 μL. The mixture was centrifuged briefly before heated for 3 minutes at 85^0C. The mixture was removed and put on ice before 8 μL of the master mix of reverse transcriptase components (2 μL of 10X RT buffer, 4 μL of dNTP mix, 1 μL of RNase inhibitor, 1 μL of Reverse transcriptase) was added to all tubes. The mixtures were mixed gently and spun briefly before subjected to the reverse transcription using thermal cycler (PTC-100 Peltier thermal cycler "MJ Research™ Inc, Massachusetts"). The reaction mixtures in the tubes were incubated at 42°C for 1 hour and then heated at 92°C for 10 minute to inactivate the reverse transcriptase. The RT products then subjected to PCR or kept at –20°C.

2.2.17.4 Polymerase chain reaction (PCR)

The synthesised cDNA (RT reactions) were amplified by Polymerase chain reaction (PCR) in 50 µL PCR tube with 1 units of thermostable DNA polymerase (Taq polymerase) in Taq buffer, 3 mM $MgCl_2$ and 5 µM of oligonucleotide primers specific for IL-8 or GAPDH genes (2.1.7). The cycle profile for PCR were 30s at 94°C, 45s at the annealing temperature (Ta) specific for each primer pair (Ta= for IL-8, 60°C, for GAPDH, 52°C), and 60s at 72°C for 'n' number of cycles (n= 28 for IL-8, for GAPDH n= 35. The durations of the denaturation at the first cycle and extension at the last cycle were made for 5 minute. Subjecting 2µg of RNA to PCR without reverstransciptition has been made for negative controls.

2.2.17.5 Agarose gel electrophoresis for PCR product

Agarose (2%, w/v) was added to the Tris-Borate-EDTA (TBE) running buffer and the mixture was heated in a microwave oven to allow the agarose to dissolve. The mixture was then cooled to 50-60°C and poured into the gel-forming apparatus containing a comb. The comb was removed after gel was solidified and the electrophoresis tank was filled with TBE running buffer until the surface of the gel was just covered. 10µL of each of PCR product was mixed with 2.5 µL high performance gel loading dye and 10 µL of the mix was then loaded to the gel and electrophoresed at 100v for 1 hour. Gel was stained with ethidium bromide in TBE buffer (50 µg/100mL TBE buffer) for one hour and visualied under UV light using Geldoc (Bio-Rad Laboratories, California, USA). Densitometry analysis was performed on the PCR product using UVIDoc software (UVITec Limited, Cambridge, UK).

2.2.18 FLOW CYTOMETRY

The basic flow cytometer consists of a laser beam intersecting of a stream of fluid containing single cells passing sequentially. As the cells flow past a focused laser beam of appropriate wavelength, the probes fluorescence and the emitted light is collected and directed to appropriate detectors. These detectors, in turn, translate these light signals into electronic signals proportional to the amount of light collected. When the laser beam hits the edge of an individual cell, some of the photons of the light are deflected slightly and the amount of this light, called "forward angle" light scatter is an indication of the cell size. Other photons may hit internal structures (granules, etc.) of the cell, which causes them to deflect on a wide angle. This light scatter is often called "side scatter" or "90 degree light scatter". All of the fluorescence light (up to 8-12 colours in the state of the art flow cytometer) and scattered light signals are measured, digitized and correlated particle by particle. These signals can then be converted into histograms for immediate results and/or stored as raw data for experimental analysis later.

Most of flow cytometer are equipped with an argon laser with one or more different wavelengths, which are used with different fluorochrome, fluorescein isothiocyante (FITC) or phycoerythrin (PE). Many of the signals of most interest to users of flow cytometers are of very low amplitude. Flow cytometers come in two different verities: analyzers and analyzer/cell sorters. The flow stream is agitated and droplets are formed. If the desired cell is known to be in a particular droplet, it is charged negatively or positively when the droplet passing the charging collar. There are two deflection plates just beyond the charging collar, one of which is positively charged to deflect the negatively charged droplet while the other is negatively charged to deflect the positively charged droplets. The deflection is controlled in such a way the cells land in a tube filled with medium. The advantage of the cell sorters is that you can sort two different types of cells simultaneously and, if desired, collected unsorted cells (Ormerod, 1999,Shapiro, 1995).

Routine immunofluorescence measurements often require detection of only a few thousand fluorescently labeled antibody molecules bound to a cell surface. In such cases, the signal from the fluorescence detectors may be only slightly above background levels, and its use as a

trigger signal is likely to result in an unacceptably high level of false triggering by light and electrical noise fluctuations, resulting in accumulation of spurious data values.

This is prevented if a signal with a higher signal-to-noise ratio, e.g., a light scattering signal, is used for triggering and used as a gating signal for the fluorescence measurement, meaning that a fluorescence value will only be recorded when the light scattering signal indicates that a cell is present in the observation region. Most, if not all, of the artifactual data generated by false triggering are eliminated when such gated analysis are done (Shapiro, 1995)

Most modern instruments are capable of analysing at least five parameters. Clearly, all the parameters can be displayed in a correlated fashion. To make full use of the information collected, 'gating' is employed. Data from one or two parameters are displayed, regions of interest are defined to select certain populations of cells for display of further parameters (Ormerod, 1999).

2.2.18.1 Expression of TLR4

To investigate the surface expression of TLR4, A549 epithelial cells were grown to confluence prior to incubation with Survanta® or DPPC (250μg/mL) for 2 hours. The cells were washed 3 times with sterile PBS before were scraped using rubber policeman and then washed once with complete medium. The cells were harvested and centrifuged at 200 xg for 5 minutes in fresh growth medium. 10^6 cells were counted and resuspended in 150μL of staining buffer (PBS-1% bovine serum albumin BSA) and transferred to 96 wells plate (15μL of cells suspension into all wells (10^5cells per well). To determine the expression of TLR4, isotype control and anti-TLR4 monoclonal antibody coupled to phycoerythrin (PE) were diluted 1:4, and 10 μL of the diluted antibody or the isotype control were added to the wells at final concentration of (20μg/mL). The cells were incubated at 4°C in the dark for 30 minutes and 150 μL of flow cytometric flow (optimized sheath fluid from Becton Dickinson) were added to each well. The cells were harvested by spinning them at 200 rpm for 5 minutes and resuspended in 25 μL of flow cytometry. 20,000 cells per sample were analysed by flow cytometric calibur and the data acquired in acquisition to analysis mode processed using CellQuest (BD, UK). The A549 cells

were analysed for the expression of TLR4 after gating on side and forward scatters (Fig 7.2a) and the isotype background control. Cells Debris were excluded from all analyses on the basis of light scatter. Acquired flow cytometric data were analyzed using WinMDI (Joe Trotter, Pharmingen, San Diego, CA). The expression of TLR4 was determined from the autofluorescence of identically treated and untreated (Survanta® or DPPC) A549 cells and the fluorescence was recorded in the FL2 emission channel. Results of the expression of TLR4 in Survanta/DPPC treated cells was compared to untreated A549 cells and expressed as the mean fluorescence intensity (MFI) measured after subtracting the MFI of the cells with isotype controls.

2.2.19 EFFECT OF METHYL β-CYCLO DEXTRIN (MβCD) ON LIPID RAFT AND IL-8 INDUCTION BY LPS OR LIVE BACTERIA

The confluent A549 epithelial cells were incubated with incomplete media with L-glutamine overnight before MβCD (20mM) was added and incubated for 30 minutes. The cells were challenged with LPS (100 ng/mL) or live bacteria (moi: 10 bacteria: 1 A549) in the presence of 1% human serum and the plates were incubated at 37°C with humidity. Supernatants were collected after 18 hours for IL-8 ELISA assay (2.2.10).

2.2.20 LIPID RAFTS

2.2.20.1 Isolation of lipid rafts

Experiment was prepared by incubating the 2x 10^{10}A549 cells with or without Survanta® or DPPC (250μg/mL) for 2 hours before washed 3 times with sterile PBS and incubated with 100 ng/mL of LPS or live bacteria at moi (10 bacteria: 1 A549) in presence of 1%human serum. After 50 minutes the cells were washed with PBS and trypsinized and washed 3 times with cold PBS. Lipid rafts were isolated from A549 cells as described in Triantafilou *et al*, 2002 with slight modifications. Briefly, A549 cells from the previous step were lysed in 250μL lysis buffer (50mm Tris, 100mM NaCl, 5mM EDTA) containing 5% Triton X-100 and protease inhibitor cocktails for 45 minutes on ice. The lysates were then homogenised by sonicating (Soniprep 150, MSE, Sanyo) on ice three times for 10 second intervals and mixed with an equal volume of 90% sucrose in Tris NaCl EDTA buffer (TNE) before were carefully placed at the bottom of centrifuge tubes. The sample was overlaid with 1.6 mL of 30% sucrose in TNE buffer and 200μL of 5% sucrose in TNE buffer and centrifuged at 100, 000xg for 24 hours. Fractions (270μL) were gently removed from the top to the bottom of the gradient and n-octylglucoside was added to each fraction at 60μM final concentration to solubilize the lipid rafts. The lysate fractions were stored at –80° C.

To determine the efficiency of the isolation of lipid rafts, 2.5μL of each fraction was dot blotted onto Nitrocellulose transfer membrane. After drying (30min), the membrane was blocked with a casein-based blocking buffer (Sigma) for 30 min at room temperature and washed twice in PBS with 0.1% Tween 20. Cholera toxin β (CTB) binds specifically to GM1 gangliosides which reside exclusively in lipid raft domains of the cell membrane (Harder et al., 1998,Janes et al., 1999). The lipid rafts was detected using HRP-linked CTB. The HRB-CTB at dilution 1:2000 to 0.21μg/mL was used to overlay the membrane for 1 hour at room temperature. The membrane was washed four times with PBS-Tween 20 followed by two 2-minute washes with water. The signal was detected using chemiluminescet substrate.

2.2.21 WESTERN BLOTTING

2.2.21.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE electrophoresis)

Electrophoresis was performed using the NuPAGE® electrophoresis system using XCell SureLock™ Mini-Cell was obtained from Invitrogen (Invitrogen Life Technologies, Paisley, UK). This system is based upon a Bis-Tris-HCL buffered (pH 6.4) 10% polyacrylamide10 mm gel run under reduced conditions with 3-(N-morpholino) propane sulphonic acid (MOPS) running buffer. Samples of protein lysate with equal of protein concentrations were mixed with, 12.5µL of 4X NuPAGE® LDS (lithium dodecyl sulphate) sample buffer and 5µL NuPAGE® reducing agents (0.5M DTT in stabilized liquid form) to a final volume of 50µL. The mixture was vortexed and then heated at 70°C for 10 minutes and centrifuged briefly before placed on ice. The appropriate volumes of samples (20µL) were loaded to each well of the gel 10% SDS PAGE gel using extra fine tips. Electrophoresis was carried out and the proteins separated at 200V for 50 minutes using NuPAGE® SDS MOPS running buffer at room temperature. Also molecular weight (magic mark) and pre-stained standard (see Blue® Plus2) were included.

2.2.21.2 Electro blotting

Once the electrophoresis was finished, the gel cassette was carefully opened and the gel left to rest on the larger plate. A pre-soaked filter paper was placed on top of the gel and the plate was turned over the gel was separated from the plates. The surface of the gel was wetted and a pre-soaked nitrocellulose membrane was put on the gel. Another pre-soaked filter paper was placed on top of the transfer membrane and air bubbles were removed using a glass rode. The gel membrane assembly was then placed on two soaked pads that were placed on the blot module. Another two pre-soaked pads were placed on top so that the gel-membrane assembly was sandwiched between the pads.

The blotting assembly module was placed in XCell SureLock™ Mini-Cell tank and filled with NuPAGE™ transfer buffer until the gel-membrane sandwich is just covered with the transfer buffer. The outer chamber was also filled with the rest of transfer buffer with approximately 650mL, and the gels were electroblotted at 35V for 75 minutes.

2.2.21.3 Blocking the membrane and antibody probing

Nitrocellulose membranes were washed twice with 20 mL of ultra pure water on a rotary shaker before incubation in 5 X blocking solution (Sigma) for 60 minutes. The membranes were then washed twice and incubated with primary antibodies (TLR4) diluted 1:500 in 1 X blocking solution in PBS for 1 h at room temperature. Thereafter, the membranes were washed four times for five minutes each in antibody washing buffer before being incubated for 30 minutes at room temperature with secondary antibodies (anti rabbit-HRP) diluted 1:2000 in 1X blocking solution. The membranes were washed four times for five minutes each in antibody wash buffer before washing twice with ultra pure water. The excess water was taken from the membranes using tissue paper before the membranes were developed using ECL advance detection kit according to the protocol supplied by Amersham (Amersham, Buckinghamshire, UK). Finally, the chemiluminescence's signal was detected with KODAK X-ray photographic films (Amersham, Buckinghamshire, UK) that were exposed for 30 seconds to 5 minutes. The films were developed with 20% (v/v) developer and were fixed using fixing agents (Sigma-Aldrich Company Ltd, Dorset, UK). The films were dried and the bands for TLR4 protein was identified by comparison with pre-stained molecular weight markers. The translocation/mobilization and the concentration of the bands in lipid rafts and none lipid raft were also identified and compared in all fractions and different condition treatment.

2.2.22 DATA ANALYSIS

All data were analysed using one-way analysis of variance (ANOVA). Differences between groups were determined using Tukey's test for pairwise differences. Between group comparisons were analysed by Paired T-test. P value of <0.05 was considered significant. All data were expressed as mean ± SD. Minitab software version 14.0 was used for all results analysis.

CHAPTER 3. RESULTS

EFFECT OF PULMONARY SURFACTANT LIPIDS ON BACTERIAL GROWTH

The lower respiratory tract is lined with epithelial cells that are the primary sites for the control of immune responses in the bronchi compartment. The alveoli are lined with type 1 and type 2 alveolar epithelial cells. Type II alveolar epithelial cells (AEII) are responsible for production and secretion of surfactant.

The defense system of the respiratory tract is complex and consists of various well-described components (Pison et al, 1996). The epithelium of the lung is lined with extracellular pulmonary surfactant. This is the surface that invading bacteria first come into contact with when they reach the alveoli. A major cause of lower respiratory infections is Gram-negative bacteria including *Pseudomonas aeruginosa* and *Burkholderia cepacia.* Although pulmonary surfactant lipid components have been shown to regulate cellular inflammatory responses to bacterial molecules, a direct effect of the lipids on bacterial growth has not been investigated. Therefore before investigating specific mechanisms and possible modulation of bacterial-induced innate immune responses by epithelial cells, the direct effect of pulmonary surfactant lipids on bacterial growth was investigated.

3.1 EFFECT OF PULMONARY SURFACTANT ON BACTERIAL GROWTH IN TRYPTONE SOY BROTH (TSB)

The effect of the different surfactants (Survanta®, Curosurf®) and DPPC lipid on the growth of bacteria was investigated using tryptone soy broth (TSB) as nutritional growth media. The concentration of surfactants used here represent the levels that may be found within human pulmonary surfactant (Hayakawa et al. 1992;Speer et al. 1991;Thomassen et al. 1992). The quantified number of bacteria was inoculated into the broth media in microtiter wells in presence or absence of Survanta®, Curosurf® and DPPC lipid before incubation at 37°C. The optical density was read at different times (0h, 12h and 24), and the results showed Survanta®, Curosurf® at 250 μg/mL and DPPC at 500 μg/mL did not have any significant effect on the growth of either environmental or clinical strains of *B. cepacia* or *P. aeruginosa* (Fig 3.1).

The effect of pulmonary surfactant lipids on bacteria numbers in terms of colony forming units (CFU) was investigated and the results shown (Fig 3.2) that Survanta®, Curosurf® (250 μg/mL 250 and DPPC 500 μg/mL do not significantly effect the number of colony forming units (CFU).

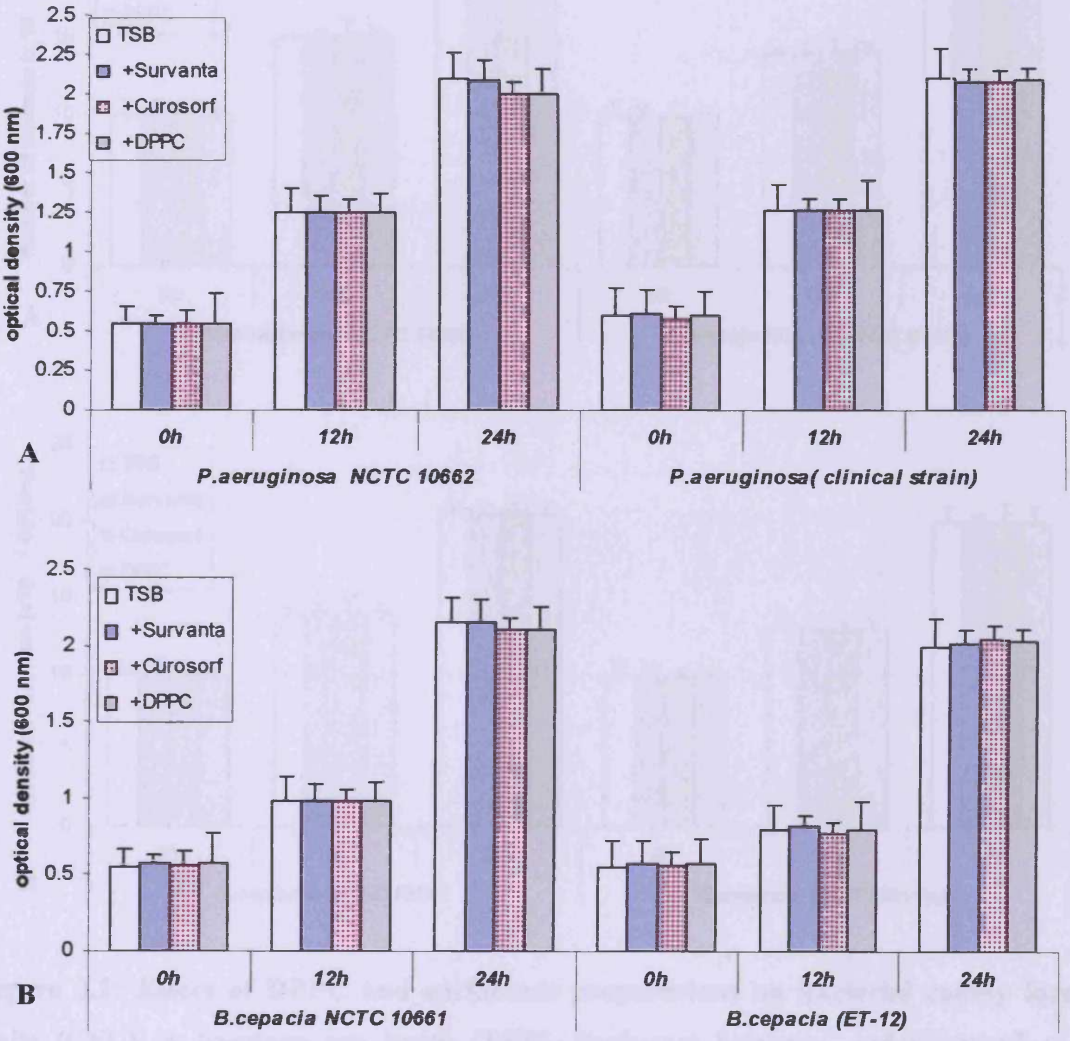


Figure 3.1: Effect of DPPC and surfactant on bacterial growth in tryptone soy broth (TSB). Surfactant Survanta® and Curosurf® at 250 μg/mL or DPPC lipid (500 μg/mL) did not show significant effects on the growth of *P. aeruginosa* NCTC 10662 or *P. aeruginosa* clinical strain (A) and *B. cepacia* NCTC 10661 or *B. cepacia* ET-12 strain (B). Results are expressed as mean ± SD of three independent experiments.

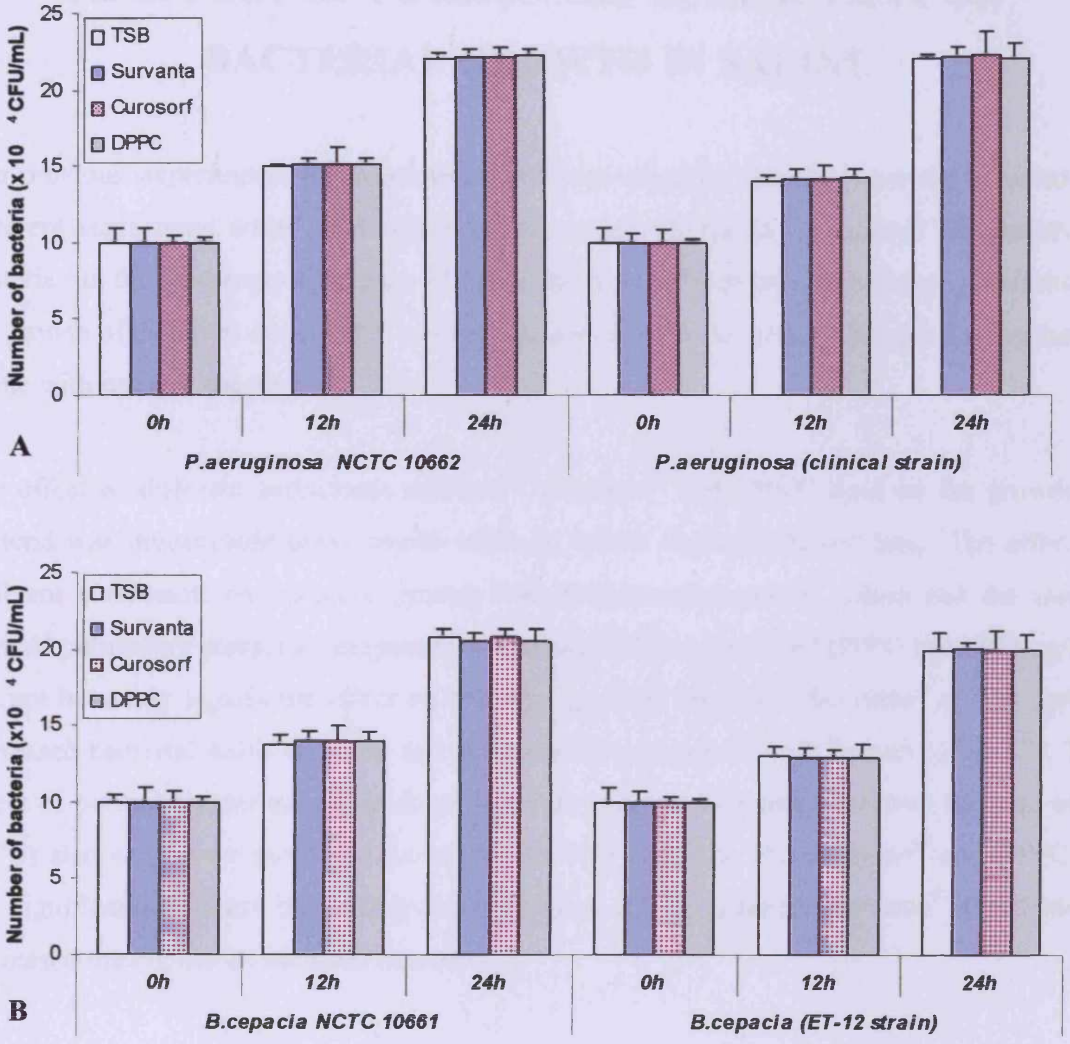


Figure 3.2: Effect of DPPC and surfactant preparations on bacterial colony forming units (CFU) in tryptone soy broth (TSB). Surfactant Survanta® and Curosurf® at 250 μg/mL or DPPC lipid (500 μg/mL) did not have a significant effect on the CFU number of *P. aeruginosa* NCTC 10662 or *P. aeruginosa* clinical strain (A) and *B. cepacia* NCTC 10661 or *B. cepacia* ET-12 strain, (B). Results are expressed as mean ± SD of three independent experiments.

3.2 EFFECT OF PULMONARY SURFACTANT ON BACTERIAL GROWTH IN SALINE

The previous experiments demonstrated that Gram-negative bacteria were not affected by different surfactants when TSB was used as nutritional media to support the growth of bacteria. In the next experiments the effect of different pulmonary surfactant preparations on the growth of different strains of *P. aeruginosa* and *B. cepacia* were investigated using sterile saline with out any supplement.

The effect of different surfactants survanta®, Curosurf® and DPPC lipid on the growth of bacteria was investigated using sterile saline to mimic the un-inflamed lung. The effect of different surfactants on bacterial growth was determined in sterile saline and the results showed pulmonary surfactant preparations Curosurf® 250 μg/mL and DPPC lipid 500 μg/mL did not have any significant effect on bacterial growth. Moreover Survanta® at 250 μg/mL decreased bacterial death in saline and supported the growth of both bacteria (Fig 3.3). The effect of pulmonary surfactant lipids on bacteria numbers in terms of colony forming units (CFU) also was investigated and the results showed (Fig 3.4) that Curosurf® and DPPC do not significantly effects the colony forming units (CFU) whereas Survanta® significantly decreased the decline of bacterial number.

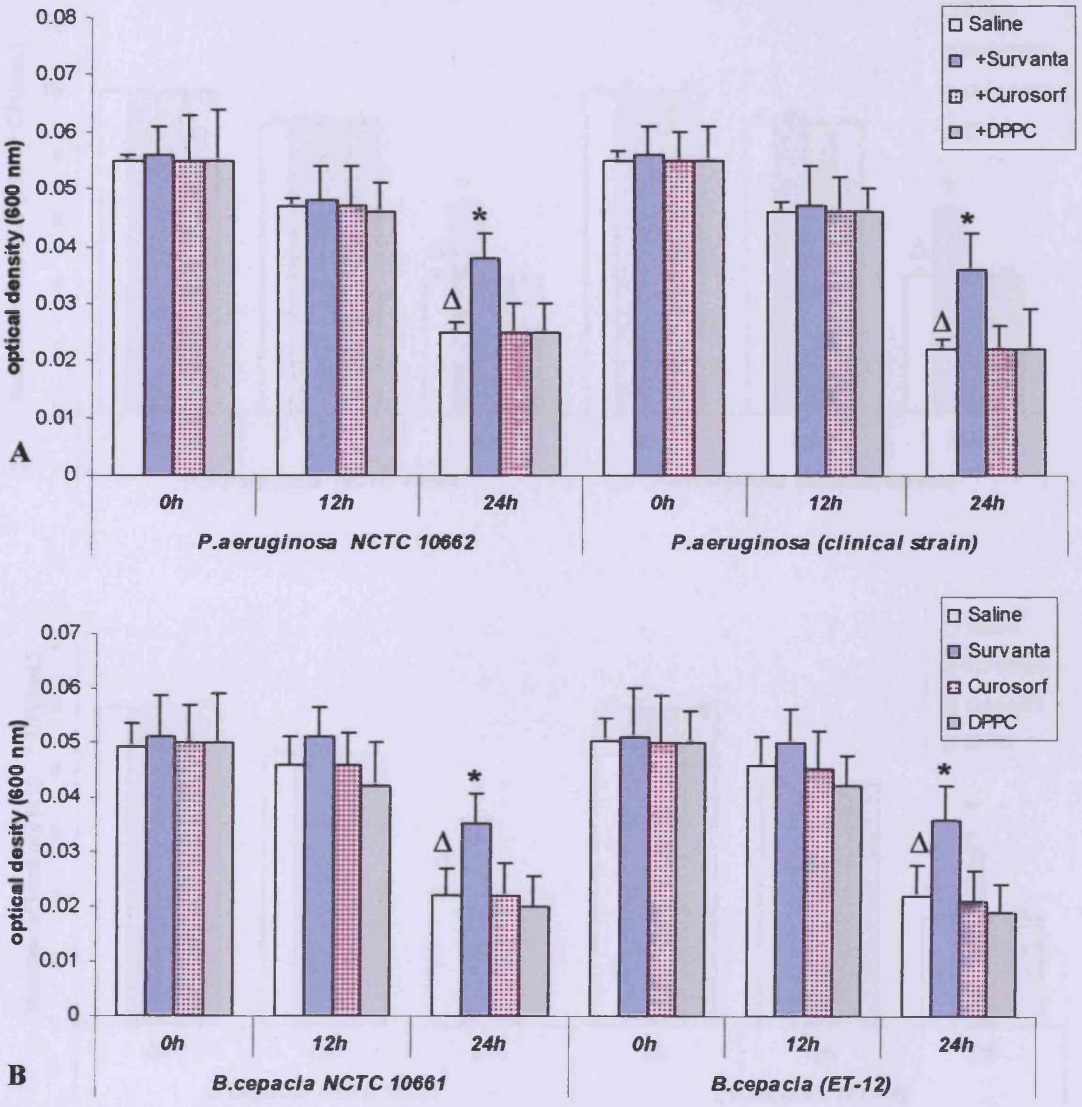


Figure 3.3: Effect of DPPC and surfactant on bacterial growth in saline. Surfactant Curosurf® and DPPC lipid did not show significant effect on the growth of *B. cepacia* and *P. aeruginosa*, however Survanta® significantly decreased the decline of bacterial growth of both strains of *P. aeruginosa* (A) and *B. cepacia* (B). Results are expressed as mean ± SD of three independent experiments. ΔP<0.01 analysed by ANOVA and *P<0.05 by Tukey's.

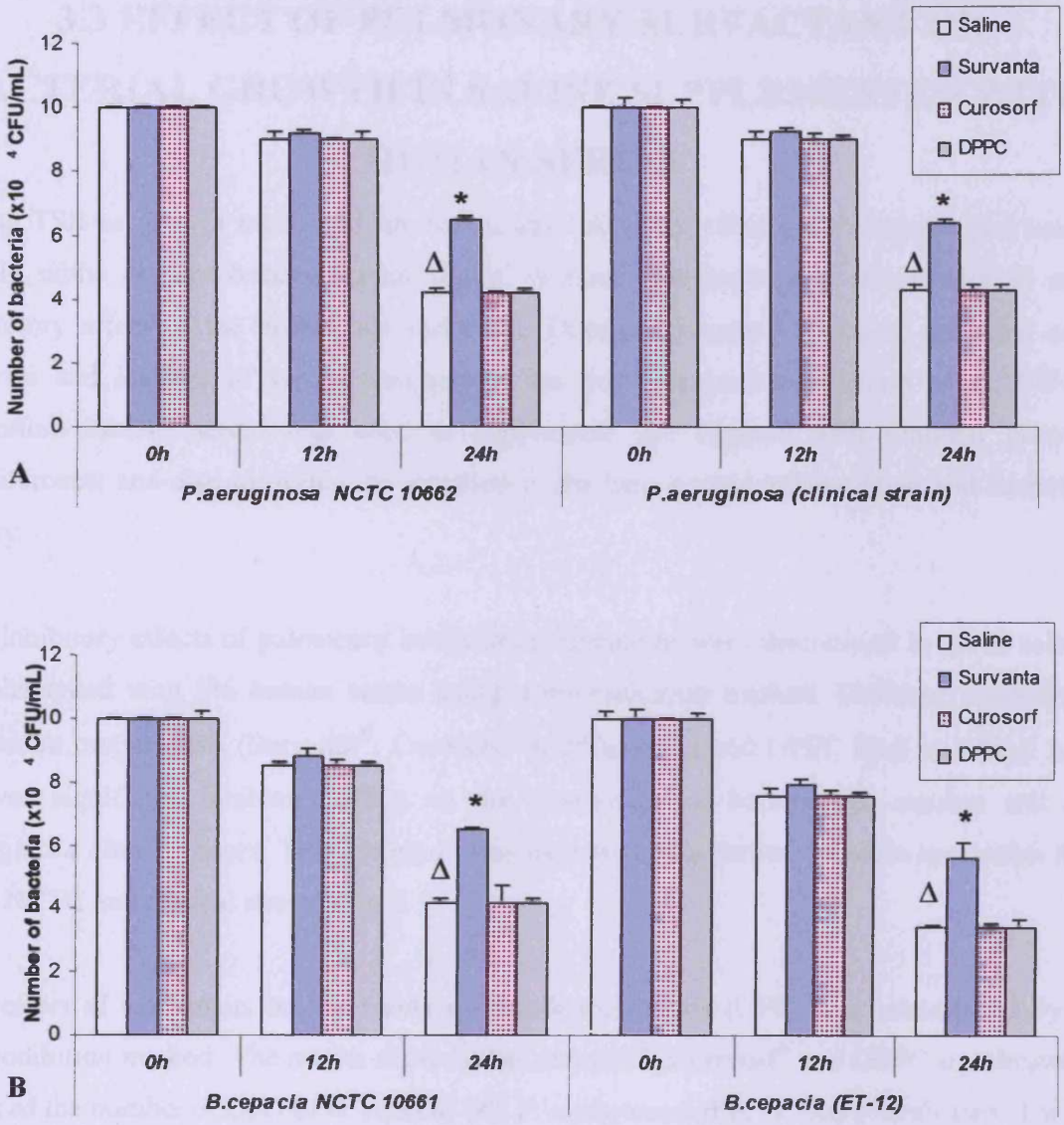


Figure 3.4: Effect of surfactant preparations on bacterial colony forming unit (CFU) in saline. Surfactant Survanta® significantly decreased the decline of bacterial numbers of both strains of *P. aeruginosa* (A) and *B. cepacia* (B) whereas Curosurf® and DPPC did not show significant effect on *P. aeruginosa* NCTC 10662 and *P. aeruginosa* clinical strain (A), and *B. cepacia* NCTC 10661 and *B. cepacia* ET-12 strain (B). Results are expressed as mean ± SD of three independent experiments. ΔP<0.01 analysed by ANOVA and *P<0.05 by Tukey's.

3.3 EFFECT OF PULMONARY SURFACTANT ON BACTERIAL GROWTH IN SALINE SUPPLEMENTED WITH HUMAN SERUM

Using TSB as growth media did not reveal any inhibitory effect of surfactants, and using sterile saline as 'non-bacteriological media' without supplement also did not reveal any inhibitory effect of the pulmonary surfactant. During pulmonary infection, epithelial cell injuries and leakage of serum components has been documented (Elborn et al. 1994). Therefore human serum was used to supplement the bacteria with minimal growth requirements and also to mimic the situation in the lung during inflammation and bacterial injury.

The inhibitory effects of pulmonary surfactant preparations were determined in sterile saline supplemented with 5% human serum using a microdilution method. Different pulmonary surfactant preparations (Survanta®, Curosurf® at 250μg/mL) and DPPC lipid at 500μg/ mL showed significant inhibitory effect on the Gram-negative bacteria; *B. cepacia* and *P. aeruginosa* after 12 hours. This inhibition was increased after further 24 hours incubation for both NCTC and clinical strains (Fig 3.5).

The effect of surfactants on the viable colony forming units (CFU) was investigated by a macrodilution method. The results showed that survanta®, Curosurf® and DPPC significantly reduced the number of CFU of *B. cepacia* and *P. aeruginosa* after 12 hours incubation. These significant reductions were increased after 24 hours incubation (Fig 3.6).

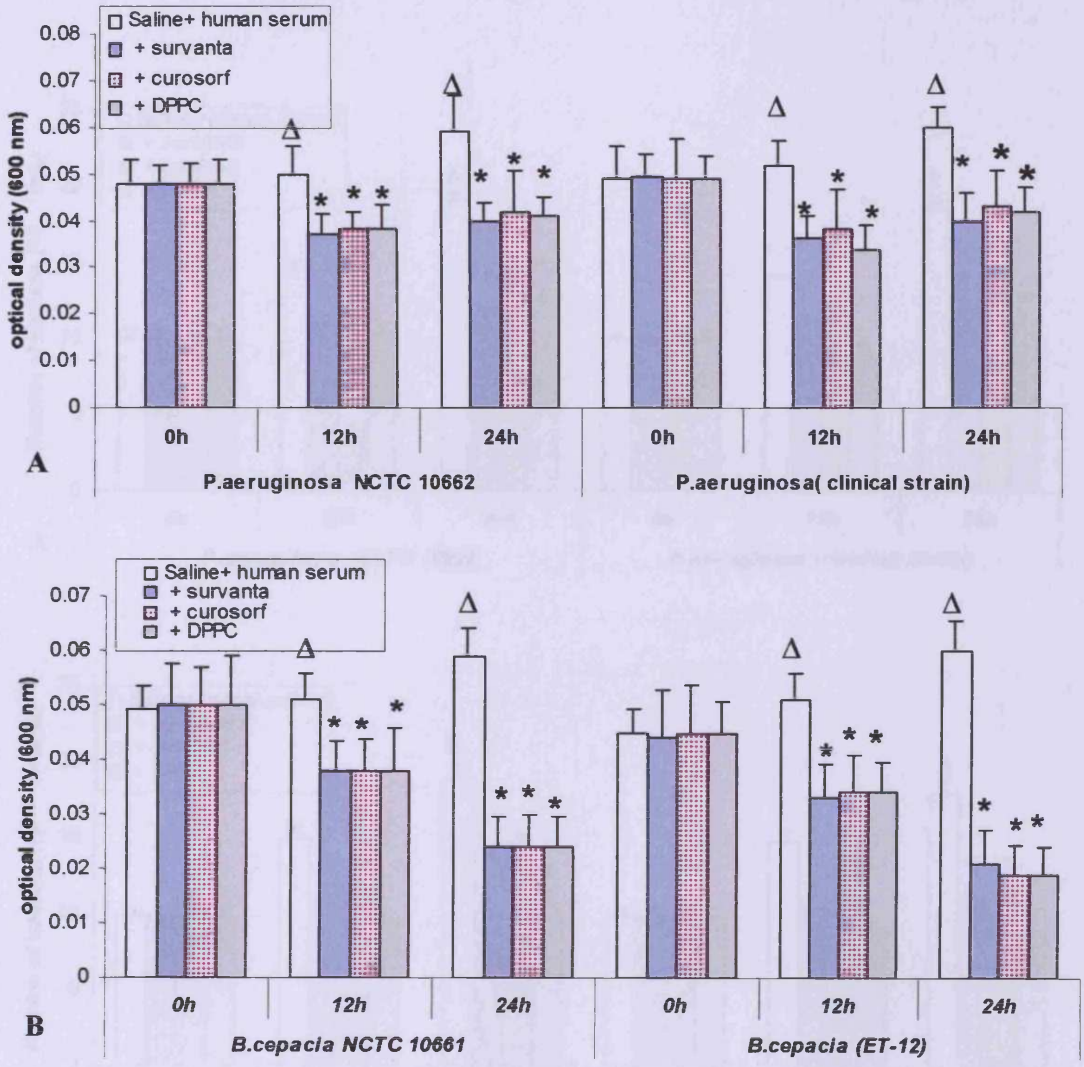


Figure 3.5: Effect of surfactant on bacterial growth in saline supplemented with 5% human serum. Surfactant®, Curosurf® and DPPC lipid significantly inhibited the growth of both NCTC and clinical strain of *P. aeruginosa* (A) and *B. cepacia* (B) at 12 and 24 hours. Results are expressed as mean ± SD of three independent experiments. ΔP<0.01 analysed by ANOVA and *P<0.05 by Tukey's.

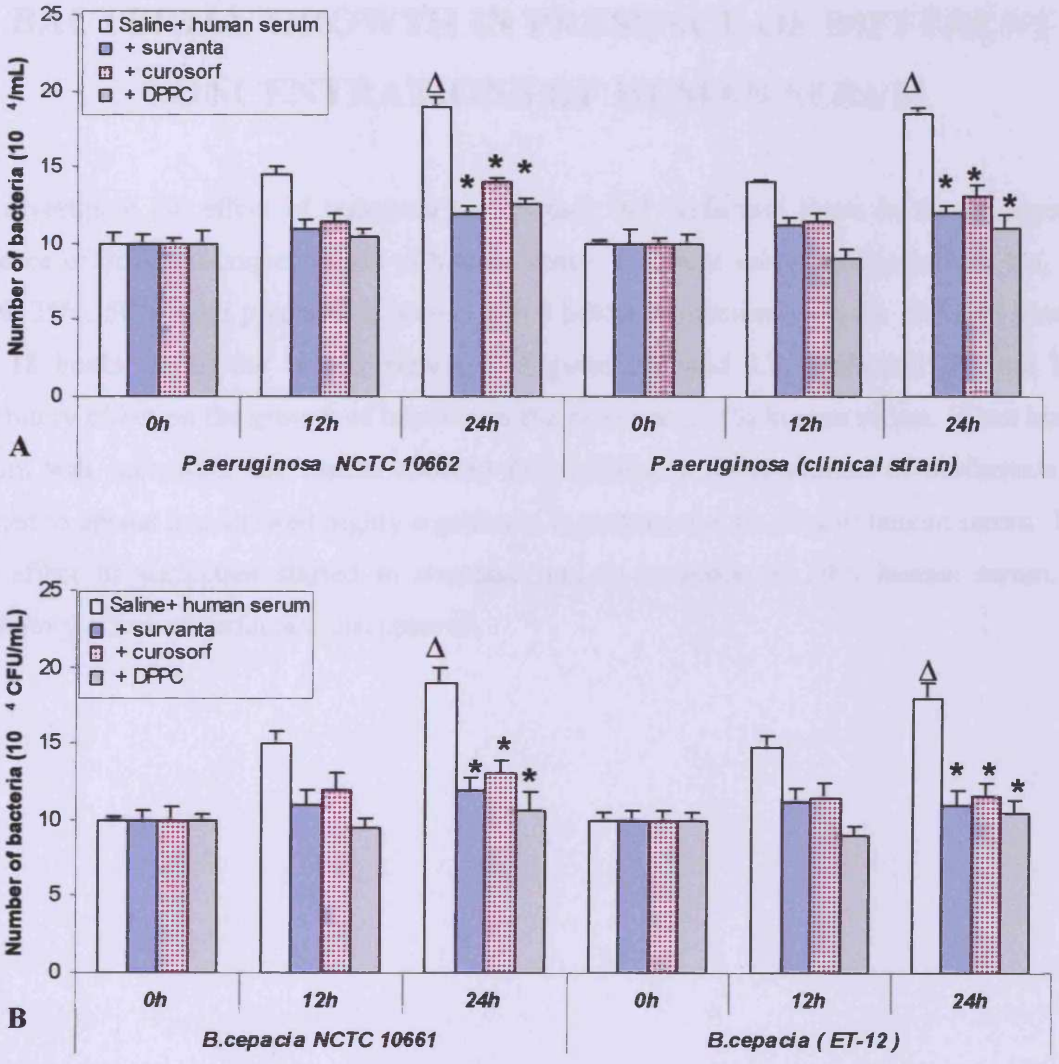


Figure 3.6: Effect of surfactant preparations on bacterial colony forming unit (CFU) in saline supplemented with 5% human serum. Surfactant Survanta®, Curosurf® and DPPC lipid significantly decreased the number of CFU for both strains of *P. aeruginosa* (A) and *B. cepacia* (B) after 24 hours incubation. Results are expressed as mean ± SD of three independent experiments. ΔP<0.01 analysed by ANOVA and *P<0.05 by Tukey's.

3.4 THE EFECT OF PULMONARY SURFACTANT ON BACTERIAL GROWTH IN PRESENCE OF DIFFERENT CONCENTRATIONS OF HUMAN SERUM

To investigate the effect of pulmonary surfactant and surfactant lipids in the presence or absence of different concentration of human serum, different serum concentrations 1%, 5%, 10%, 25%, 50% were prepared in sterile saline before incubation with the different bacteria for 18 hours. From the results shown in Figures 3.7 and 3.8, surfactant did not have inhibitory effect on the growth of bacteria in the presence of 1% human serum. When human serum was increased, the results showed that inhibitory concentration of surfactants are started to appear and showed highly significant in presence of 10-25% of human serum. Then the effect of surfactant started to decrease and in presence of 50% human serum, the inhibitory effect of surfactant disappeared.

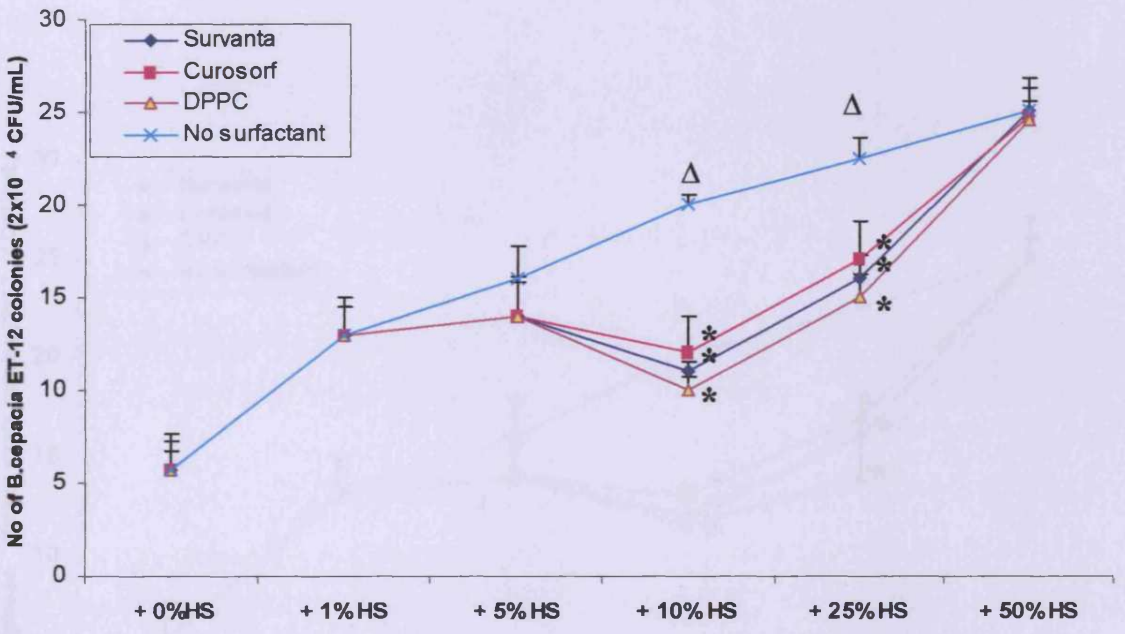


Figure 3.7: Effect of pulmonary surfactant on the growth of *B. cepacia* in the presence of different concentrations of human serum. Survanta®, Curosurf® and DPPC lipid significantly decreased the number of CFU of *B. cepacia* ET-12 in presence of 5-25% of human serum after 18 hours incubation. The results were expressed as mean ± SD of three independent experiments. $\Delta P<0.01$ analysed by ANOVA and Tukey's ($^*P<0.05$) when comparing surfactant treatment to controls.

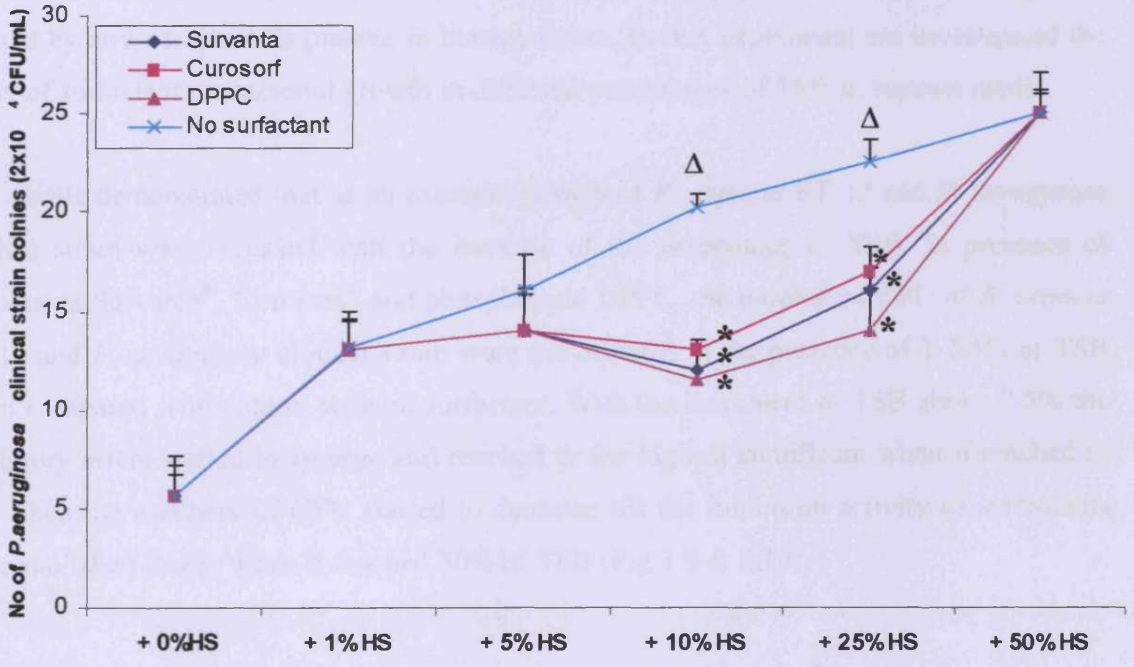


Figure 3.8: Effect of pulmonary surfactant on the growth of *P. aeruginosa* in the presence of different concentrations of human serum. Surfactant Survanta®, Curosurf® and DPPC lipid significantly decreased the number of CFU of *P. aeruginosa* clinical strain in presence of 5-25% of human serum after 18 hours incubation. The results were expressed as mean ± SD of three independent experiments. $\Delta P<0.01$ analysed by ANOVA and Tukey's (*$P<0.05$) when comparing surfactant treatment to controls.

3.5 THE EFECT OF PULMONARY SURFACTANT ON BACTERIA IN PRESENCE OF DIFFERENT CONCENTRATIONS OF TSB

Our previous result showed that surfactants have highest significant inhibitory effect on bacterial growth in the presence of 10-25 % of human serum. One speculation is that the human serum may harbor specific factors in association with the inhibitory activity of surfactants. The aim of this experiment was to exclude any possible effect that might be exerted by any components present in human serum. In this experiment we investigated the effect of surfactant on bacterial growth in different percentages of TSB as support media.

The results demonstrated that as an example growth of *B. cepacia* ET-12 and *P. aeruginosa* clinical strain was increased with the increase of the percentage of TSB. In presence of surfactants Survanta®, Curosurf® and phospholipid DPPC, the number of CFU of *B. cepacia* ET-12 and *P. aeruginosa* clinical strain were not affected in the presence of 1-2.5% of TSB when compared with control without surfactant. With the increment of TSB above 2.5% the inhibitory effect started to emerge and reached to the highest significant when it reached to 5%. Then the numbers of CFU started to decrease till the inhibition activity of surfactants were abolished totally when it reached 50% of TSB (Fig 3.9 & 3.10).

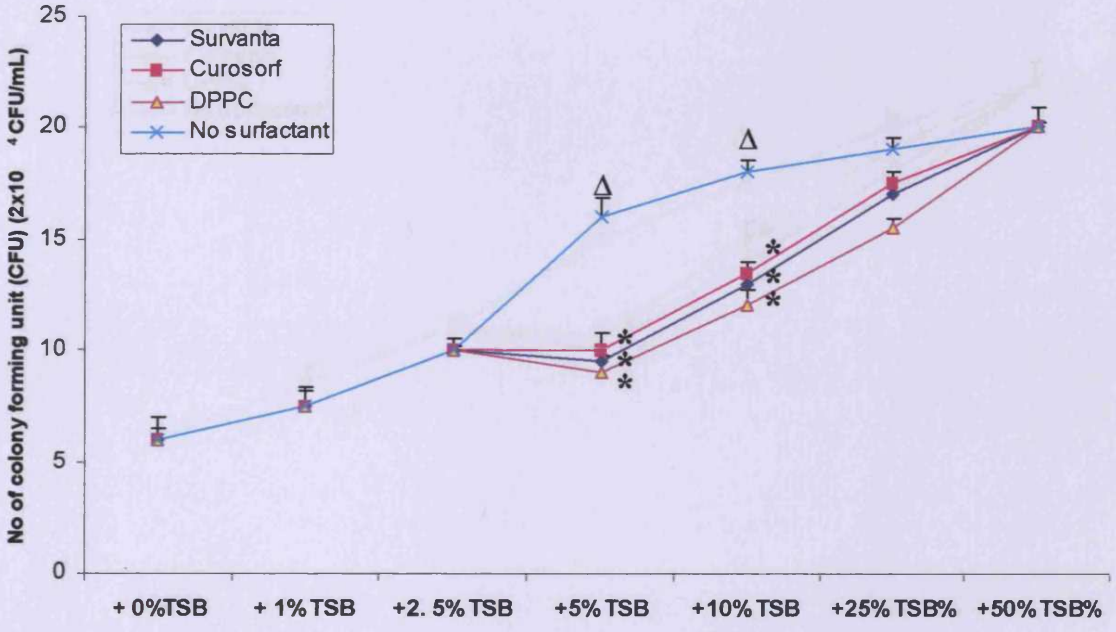


Figure 3.9: Effect of pulmonary surfactant on the growth of *B. cepacia* in presence of different concentrations of TSB. Surfactant Survanta®, Curosurf® and DPPC lipid significantly decreased the number of CFU of *B. cepacia* ET-12 strain when the TSB reached to percentage of 5-25%. The results were expressed as mean ± SD of three independent experiments. ΔP<0.01analysed by ANOVA and Tukey's (*P<0.05) when comparing surfactant treatment to controls.

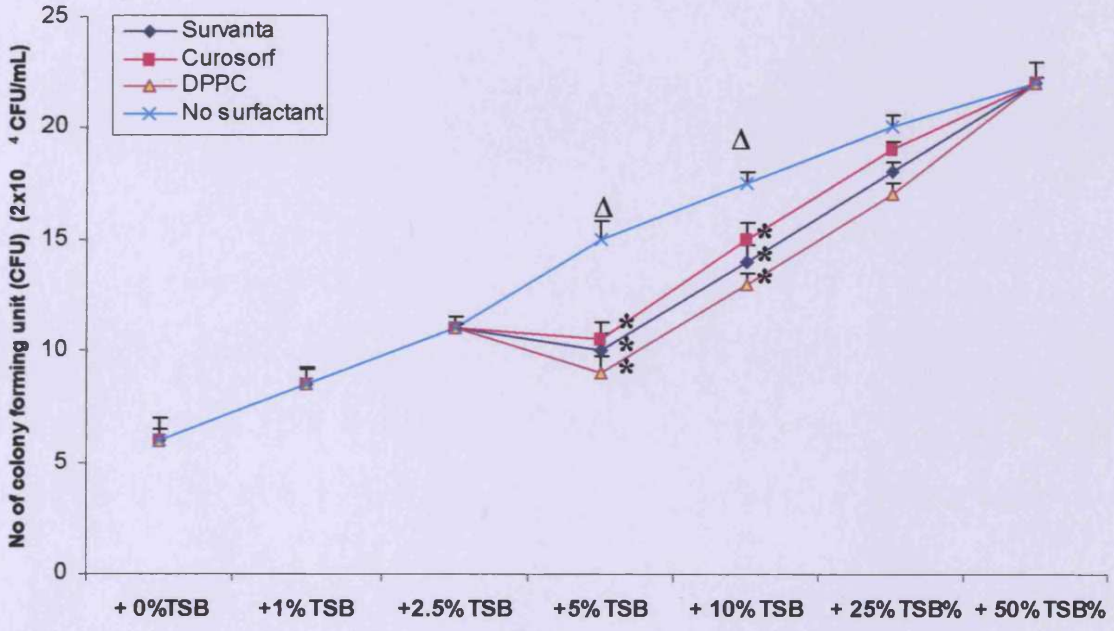


Figure 3.10: Effect of pulmonary surfactant on the growth of *P. aeruginosa* in presence of different concentrations of TSB. Surfactant Survanta®, Curosurf® and DPPC lipid significantly decreased the number of CFU of *P. aeruginosa* clinical strain when the TSB reached to percentage of 5-25%. The results were expressed as mean ± SD of three independent experiments. $\Delta P<0.01$ analysed by ANOVA and Tukey's ($^*P<0.05$) when comparing surfactant treatment to controls.

3.6 SUMMARY OF RESULTS

The results from this chapter show that surfactants used at their physiological concentration can inhibit the growth of bacteria. This inhibition was only significant in the presence of 5-25% of human serum or tryptone soya broth.

CHAPTER 4. RESULTS

EFFECT OF PULMONARY SURFACTANT LIPIDS ON BACTERIAL UPTAKE BY A549 EPITHELIAL CELLS

The effect of pulmonary surfactant on the internalisation of clinical and NCTC strains by A549 epithelial cells was investigated using two different methods; fluorescent microscopy, antibiotic protection assay. The results of internalisation were confirmed by electron microscopy. In the previous chapter this study investigated the effect of pulmonary surfactant on the growth of bacteria. Since the major objective of the study was to determine the effect of pulmonary surfactant on the inflammatory responses induced by bacteria and their products, the study did not concentrate further on the mechanism of surfactant on bacterial growth. In order to draw relation between the inflammatory responses such as IL-8 and bacteria, the internalisation of bacteria to the A549 cells will be studied and the effect of pulmonary surfactant on this internalisation was examined. Furthermore the relation of internalisation with IL-8 induction needs to be investigated in order to make sure and exclude any possible effect on the bacterial invasion may interfere with the next study of IL-8 regulation.

4.1 THE EFFECT OF PULMONARY SURFACTANT LIPIDS ON BACTERIAL INTERNALISATION BY A549 EPITHELIAL CELLS USING FLUORESENT MICROSCOPY

Epithelial cells have long been considered to act primarily as a physical barrier to protect the integrity of epidermal or mucosal surfaces. The lung epithelial cells are continuously exposed to bacteria. Many studies have shown that bacterial internalisation by A549 epithelial cells depends on the bacterial species (Keig et al., 2002). In this study we sought to investigate the internalisation of environmental and clinical strains of *B. cepacia* and *P. aeruginosa* by A549 epithelial cells, and the effect of surfactant lipids on such internalisation.

The internalisation of bacteria by A549 epithelial cells and the effect of pulmonary surfactant lipids on this internalisation were investigated using fluorescein isothiocyanate (FITC) conjugated bacteria. The internalisation of FITC-conjugated bacteria was determined by fluorescent microscopy after quenching external fluorescence with trypan blue dye.

Examination of the internalised bacteria after three hours incubation showed that (*B. cepacia* NCTC 10661and *B. cepacia* ET-12, *P. aeruginosa* NCTC 10662 and *P. aeruginosa* clinical strain) were internalised by the epithelial A549 cells. A typical example of fluorescent micrograph is shown in Fig 4.1.

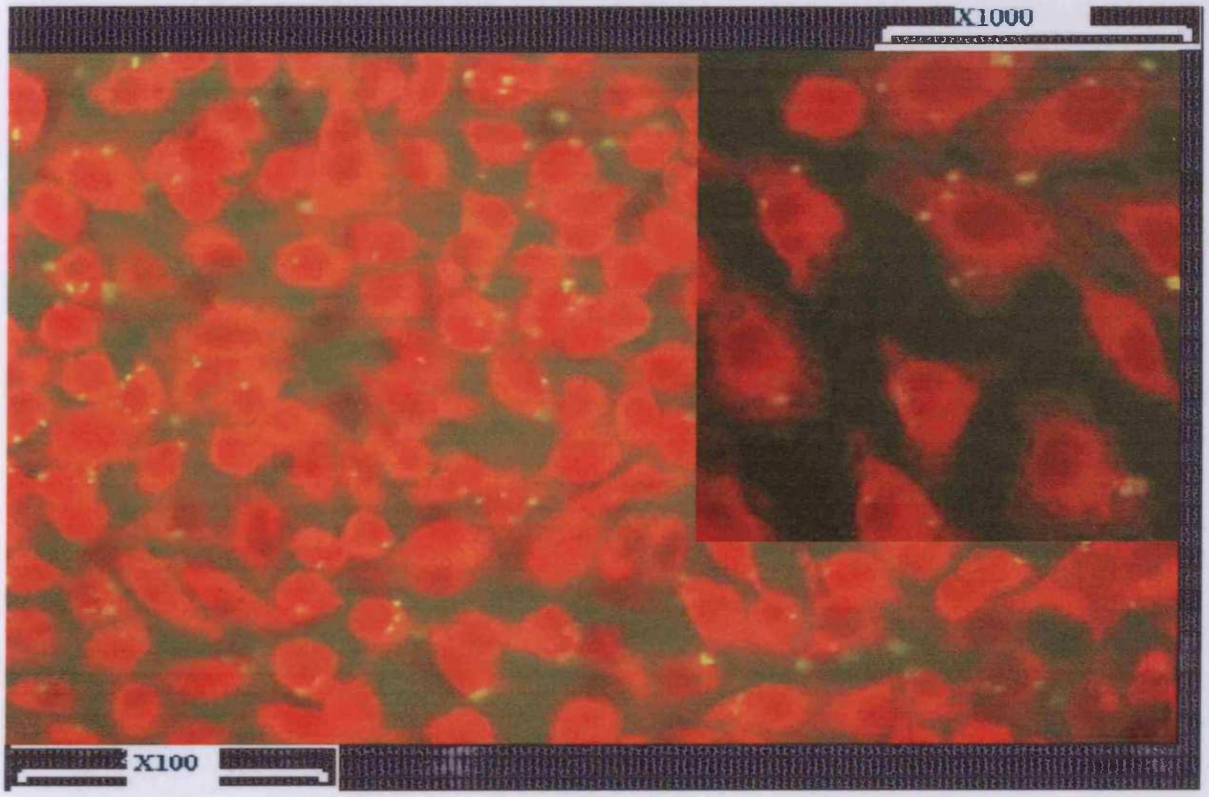


Figure 4.1: Fluorescent micrograph showing internalisation of FITC-conjugated *P. aeruginosa* clinical strain by A549 cells. FITC-conjugated bacteria were incubated with A549 epithelial cells at moi 10 bacteria: 1 A549 cell. The extracellular bacteria were quenched with trypan blue. The insert shows internalised bacteria at higher magnification (x1000) using Image-Pro® Plus program (Media Cybernetics, Georgia, USA).

The effect of pulmonary surfactant on the internalisation of different NCTC and clinical strains bacteria by A549 epithelial cells was investigated. Whereas Curosurf® and DPPC increased the internalisation of both NCTC and clinical strain of *P. aeruginosa,* the study found that Survanta® significantly decrease of this internalisation (Fig 4.2). However, with *B. cepacia* the presence of Survanta®, Curosurf® or DPPC significantly increased the internalisation by A549 epithelial cells (Fig 4.3).

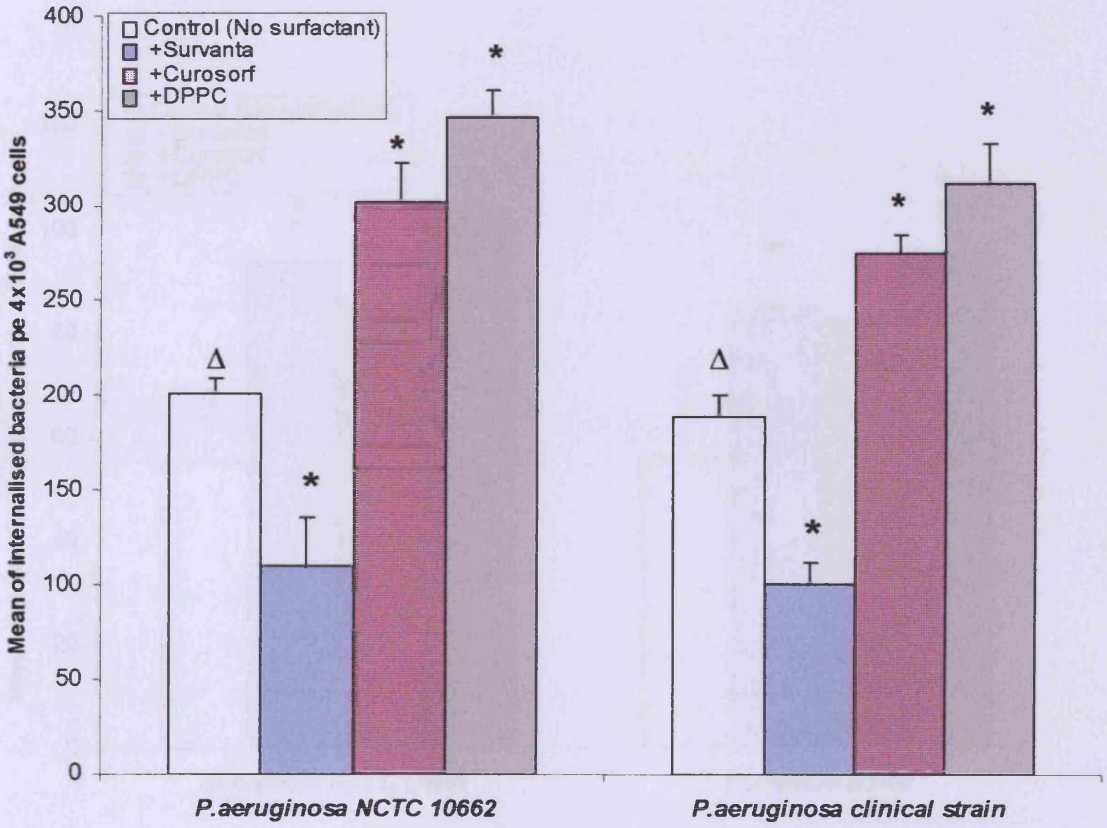


Figure 4.2: The effect of surfactant on internalisation of *P. aeruginosa* by A549 epithelial cells. Both Curosurf® and DPPC increased the internalisation of *P. aeruginosa* NCTC 10662 and *P. aeruginosa* clinical strain in A549 epithelial cells. Survanta® significantly decreased the internalisation. The numbers of internalised FITC-conjugated bacteria were counted using Image-Pro® Plus program (Media Cybernetics, Georgia, USA) and the results are expressed as the mean ± SD of three independent experiments. $\Delta P<0.01$ analysed by ANOVA and $*P<0.05$ by Tukey's.

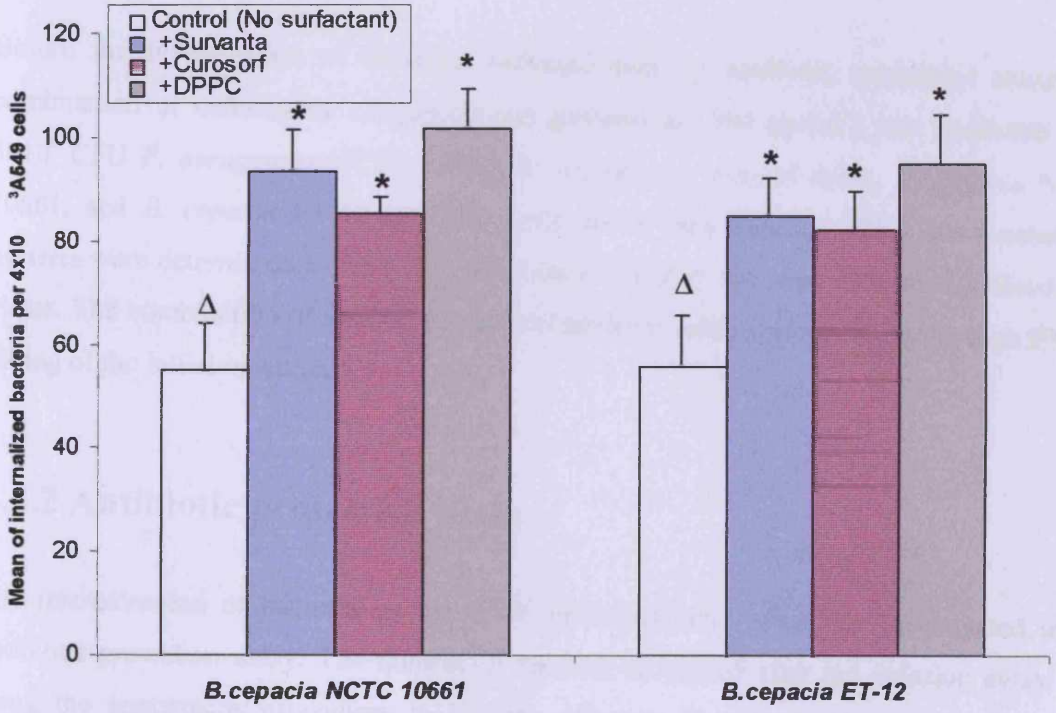


Figure 4.3: The effect of surfactant on internalisation of *B. cepacia* by A549 epithelial cells. All surfactants Survanta®, Curosurf® and DPPC lipid increased the internalisation of *B. cepacia* 10661 and *B. cepacia* ET-12 clinical strain in A549 epithelial cells. The numbers of internalised FITC-conjugated bacteria were counted using Image-Pro® Plus program (Media Cybernetics, Georgia, USA) and the results are expressed as the mean ± SD of three independent experiments. ΔP<0.01 analysed by ANOVA and *P<0.05 by Tukey's.

4.2 THE EFFECT OF PULMONARY SURFACTANT LIPIDS ON BACTERIAL INTERNALISATION BY A549 EPITHELIAL CELLS USING AN ANTIBIOTIC PROTECTION ASSAY

4.2.1Gentamicin/ceftazidime killing assay

Before the investigation of bacterial internalisation by antibiotic protection assay, the combination of ceftazidime (1mg/mL) and gentamicin (500 μg/mL) was incubated with $5x10^6$ CFU *P. aeruginosa* NCTC 10662, *P. aeruginosa* clinical strain, *B. cepacia* NCTC 10661, and *B. cepacia* ET-12 in TSB. After 1hour incubation at 37°C, the numbers of bacteria were determined by culturing serial dilutions from the broth cultures on Blood agar plates. The combination of gentamicin and ceftazidime used resulted in greater than 99.9 % killing of the initial inoculums.

4.2.2 Antibiotic protection assay

The internalisation of bacteria by the A549 epithelial cells were also investigated using antibiotic protection assay. The number of bacteria recovered after the invasion assay and using the gentamicin/ceftazidime to kill the adherent bacteria to the A549 cells were determined by lysing the cells with 0.25% Triton-X-100 and incubating serial dilutions on blood agar plates.

The invasion of *P. aeruginosa* NCTC 10662 and *P. aeruginosa* clinical strain was analysed and the results showed that both strains of *P. aeruginosa* used in our study invade the A549 epithelial cells. The effect of surfactant on internalisation revealed that Survanta® significantly decreased the invasion of both strains of *P. aeruginosa*, whereas Curosurf® and DPPC increased this internalisation when compared with the controls without surfactant (Fig 4.4).

The internalisation of *B. cepacia* NCTC 10661 and *B. cepacia* ET-12 strain by the A549 epithelial cells shows that these bacteria can be internalised by the A549 epithelial cells. Survanta®, Curosurf® and DPPC show significant increase of internalisation of both *B. cepacia* NCT 10661 and *B. cepacia* ET-12 clinical strains (Fig 4.4). Whereas Survanta® significantly decreased the internalisation of *P. aeruginosa*, Curosurf® and DPPC significantly increased the internalisation to A549 cells (Fig 4.5).

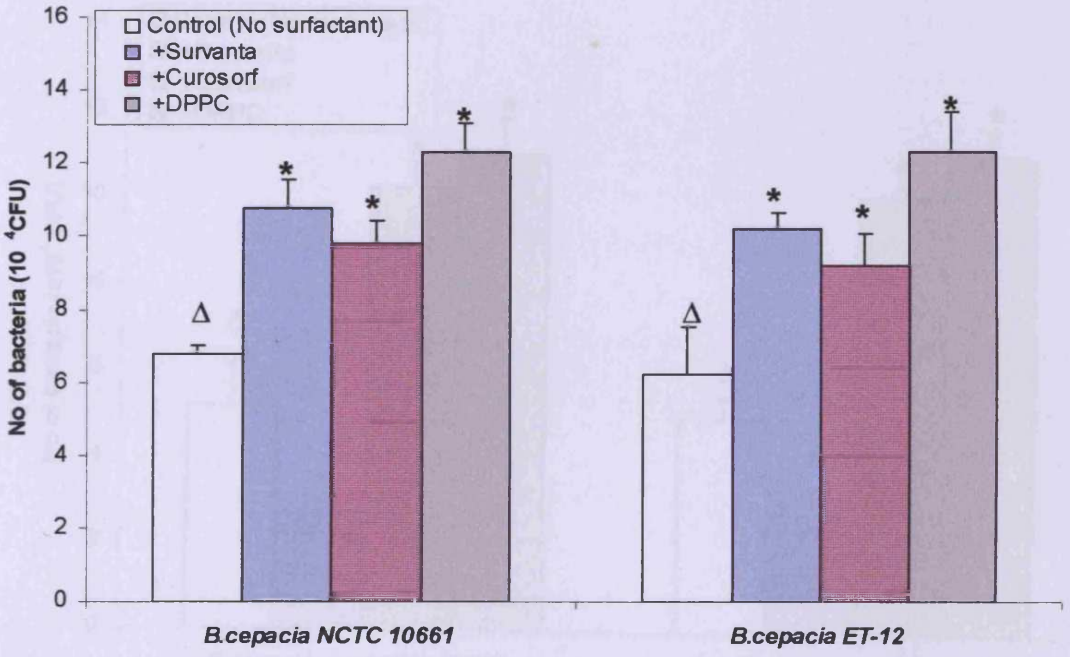


Figure 4.4: Antibiotic protection assay. All surfactants Survanta®, Curosurf® and DPPC lipid increased the internalisation of *B. cepacia* 10661 and *B. cepacia* clinical strain to A549 epithelial cells. The numbers of internalised bacteria recovered was counted and expressed as CFU and the results are expressed as the mean ± SD of three independent experiments. ΔP< 0.01 analysed by ANOVA and *P<0.05 by Tukey's.

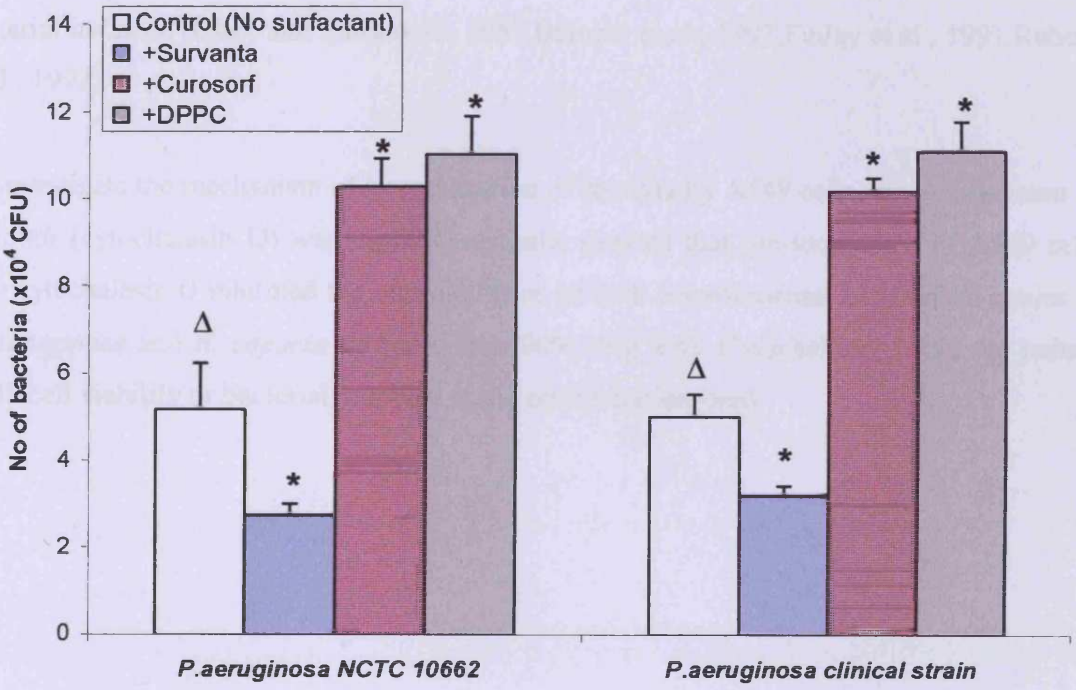


Figure 4.5: Antibiotic protection assay. Survanta® significantly decreased the internalisation, and Curosurf® and DPPC increased the internalisation of *P. aeruginosa* NCTC 10662 and *P. aeruginosa* clinical strain to A549 epithelial cells in significant manner. The numbers of internalised bacteria recovered was counted and expressed as CFU and the results are expressed as the mean ± SD of three independent experiments. ΔP<0.01 analysed by ANOVA and *P<0.05 by Tukey's.

4.3 EFFECT OF CYTOCHALASIN D ON BACTERIAL INTERNALISATION BY A549 EPITHELIAL CELLS

A common strategy employed by bacteria during cell invasion is to trigger epithelial cells to undergo cytoskeletal rearrangements mediated by actin polymerization (Finlay and Cossart, 1997). Cytochalasin D, is an actin polymerization inhibitor which has been shown to inhibit bacterial invasion (Clerc and Sansonetti, 1987,Bianchi et al., 1993,Finlay et al., 1991,Rubens et al., 1992).

To investigate the mechanism of internalisation of bacteria by A549 cells, the cytoskeleton inhibitor (cytochalasin D) was used. The results showed that pre-incubation of A549 cells with cytochalasin D inhibited the internalisation of both environmental and clinical strains of *P. aeruginosa* and *B. cepacia* by more than 90% (Fig 4.6). Cytochalasin D did not reduce A549 cell viability or bacterial numbers at the concentration used.

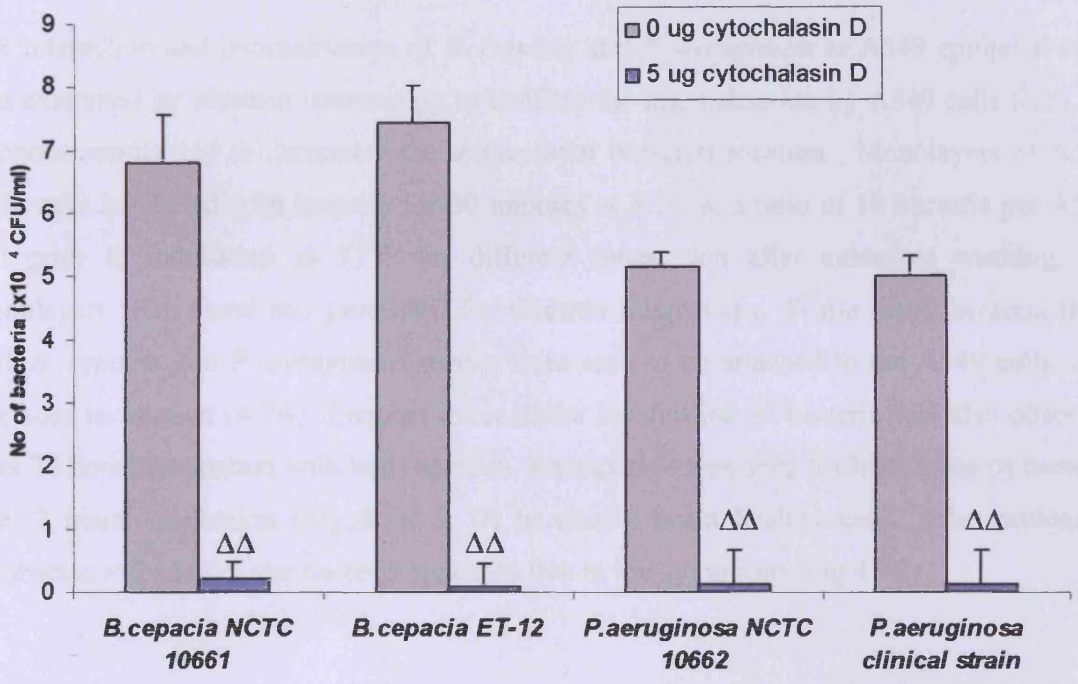


Figure 4.6: Effect of cytochalasin D on bacterial internalisation by A549 epithelial cells. The number of bacteria recovered after treatment of A549 with 5 μg/mL Cytochalasin D for 1 hour and postinfection for 3 hours with 10 moi (10 bacteria: A549 cells). Antibiotic protection assay was carried out by incubating with gentamicin (500μg/mL)/ceftazidime (1μg/mL) for 30 minutes to kill the extacellular bacteria before lysis with 0.25% Trinton-X-100 to quantify the number of internalised bacteria. Control wells were included with bacteria without Cytochalasin D. Results are expressed as the mean ± SD of bacteria in A549 cells for three independent experiments. $\Delta\Delta P<0.001$ vs. controls analysed by paired T-test.

4.4 INVESTIGATION OF BACTERIAL INTERNALISATION BY A549 EPITHELIAL CELLS BY ELECTRON MICROSCOPY

The interaction and internalisation of *B. cepacia* and *P. aeruginosa* to A549 epithelial cells was examined by electron microscopy to confirm the internalisation by A549 cells from the previous results and to determine the intracellular bacterial location. Monolayers of A549 cells were incubated with bacteria for 30 minutes at 37°C at a ratio of 10 bacteria per A549 cell prior to incubation at 37°C for different times, and after extensive washing, the monolayers were fixed and processed for electron microscopy. While many bacteria from both *B. cepacia* and *P. aeruginosa* strains were seen to be attached to the A549 cells after one hour incubation (4.7A), frequent intracellular localisation of bacteria was also observed after 24 hours incubation with both bacteria. Endocytosis was seen for both types of bacteria after 2 hours incubation (Fig 4.7B & D) in clearly intact healthy cells. After prolonged incubation to 24 hours, the bacteria appeared free in the cytoplasm (Fig 4.7C).

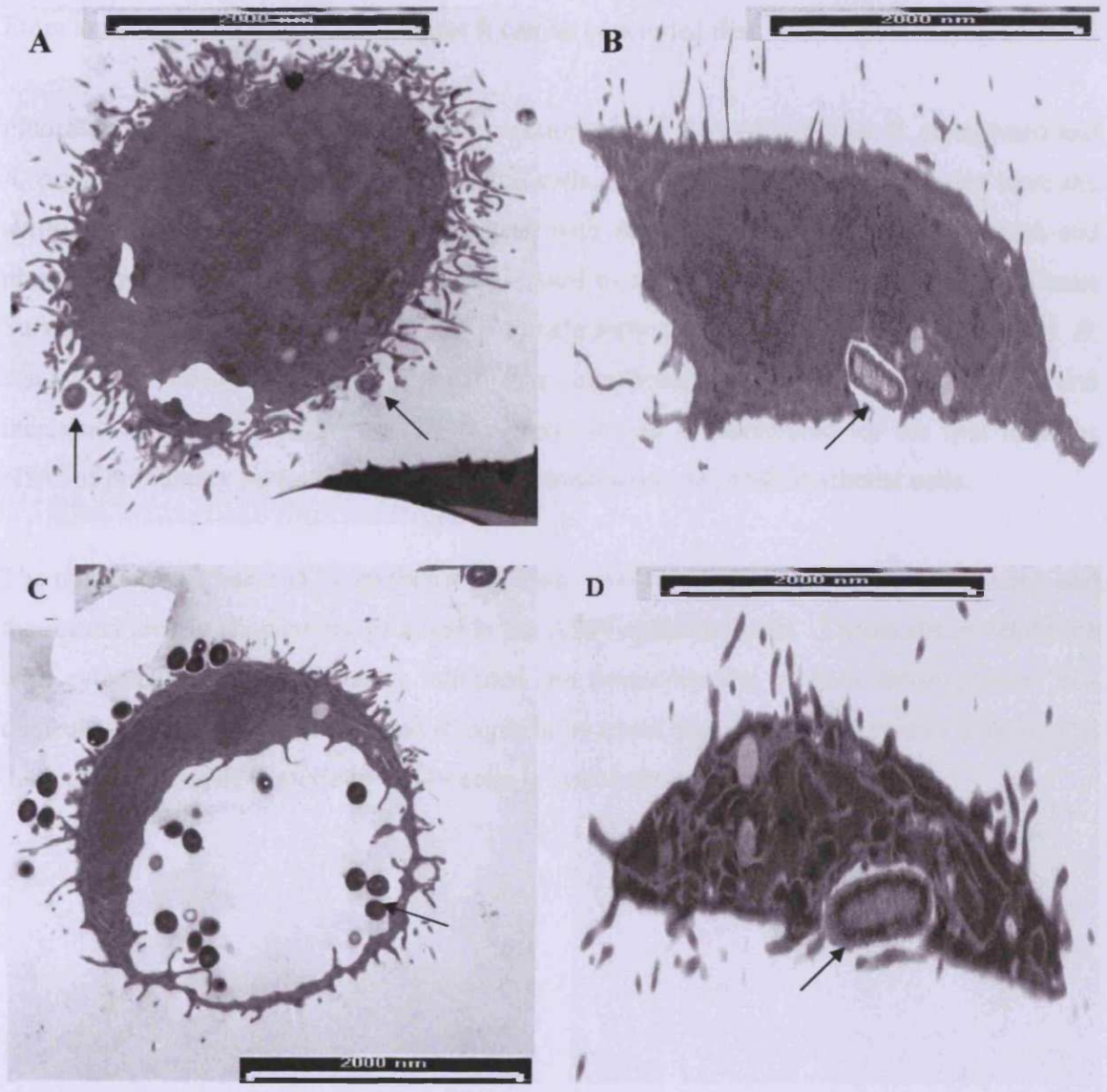


Figure 4.7: Electron micrographs showing the internalisation of bacteria to A549 epithelial cells. Attachment of *B. cepacia* to A549 cells (A), engulfment of *B. cepacia* (B) and *P. aeruginosa* (D) by A549 cells, and internalisation of *B. cepacia* by the A549 cells (C). Arrows indicate the bacteria (*B. cepacia* ET-12 strain tends to be rounded like).

4.5 SUMMARY OF RESULTS

From the work described in this chapter it can be concluded that:

Fluorescent microscopy and antibiotic protection assays showed that both *P. aeruginosa* and *B. cepacia* are internalised by the epithelial cells. Both NCTC and clinical strains have the ability to invade after 3 hours of incubation with A549 cells. The effect of surfactant and phospholipids on this internalisation was assessed by this method and showed that surfactants Survanta®, Curosurf® and DPPC lipid significantly increased the internalisation of *B. cepacia*. *P. aeruginosa* internalisation was significantly decreased by Survanta® and increased by both Curosurf® and DPPC. These results demonstrated for the first time the effect of pulmonary surfactant on bacterial internalisation by A549 epithelial cells.

The uptake of the bacteria by epithelial cells was confirmed using electron microscopy and the results clearly showed bacteria inside the A549 epithelial cells. Cytoskeleton Inhibition with cytochalasin D significantly inhibited the internalisation of both environmental and clinical strains of *P. aeruginosa* and *B. cepacia* by more than 90%. These results indicate that the internalisation of bacteria by A549 cells is cytoskeleton dependant.

CHAPTER 5. RESULTS

INDUCTION OF IL-8 FROM A549 EPITHELIAL CELLS BY BACTERIA AND THE EFFECT OF PULMONARY SURFACTANT LIPIDS

5.1 BACTERIAL INDUCTION OF IL-8 IN A549 EPITHELIAL CELLS

Until recently, the epithelial lining in the lower respiratory tract was considered to be merely a mechanical barrier to antigen or microorganism penetration. However, more recently attention has been turned to its metabolic activity and the way in which the cells interact with other elements of host defence. A growing body of evidence now suggests a more active role of the epithelial cells in host defence (Diamond et al 2000; Nelson et al 1998; Zhang et al 2000). The epithelial cells in the lung are continuously exposed to Gram-positive and Gram-negative bacteria and their products such LPS, PGN, LTA or other secreted components during infection. However the role of the epithelial cells in response to bacterial infection is not well elucidated. The cytokine IL-8 plays a role in recruiting neutrophils to the site of infection, however overproduction of this cytokine may lead to progressive inflammation in the lung, therefore it has been chosen for investigation and the possible effect of pulmonary surfactants on IL-8 release will be elucidated.

5.1.1 Time course of IL-8 induction in absence and presence of human serum

This study characterised the A549 epithelial cell responses to different bacteria for release of the cytokines IL-8 and IL-1β in the presence and absence of human serum. A549 cells released IL-8 in response to different bacterial stimuli at moi 10:1 (bacteria: A549). IL-8 was estimated at different times of incubation (0, 2, 4, 6, 12, 24, 48 hours) in the absence and presence of human serum by ELISA assay and compared with controls without bacteria.

Stimulation of A549 epithelial cells with Gram-negative bacteria *B. cepacia* NCTC 10661, *B. cepacia* ET-12, *P. aeruginosa* NCTC 10662 and *P. aeruginosa* clinical strain without human serum showed that IL-8 was induced after 4 hours and increased gradually until it reached a maximum after 48 hours incubation. The clinical strain of *P. aeruginosa* induced higher amount of IL-8 than the other bacterial strains after 24 hours of incubation in absence or presence of human serum (Fig 5.1 and Fig 5.2).

In the presence of human serum, the A549 cells were more responsive and produced almost twice as much IL-8 (Fig 5.2) when compared with the same induction in the absence of human serum (Fig 5.1). Comparing IL-8 induced by the environmental and clinical strain, the clinical strain of *P. aeruginosa* induced more than the environmental, whereas *B. cepacia* ET-12 induced less than the environmental strain in the presence and absence of human serum (Fig 5.1 and Fig 5.2).

When IL-1β was investigated, live bacteria did not induce IL-1β from A549 epithelial cells up to 48 hours of incubation. Increasing the incubation to 72 hours and the moi to (50 bacteria: 1 A549 cells) did not show any effect on the IL-1β induction in the presence and absence of human serum (data not shown).

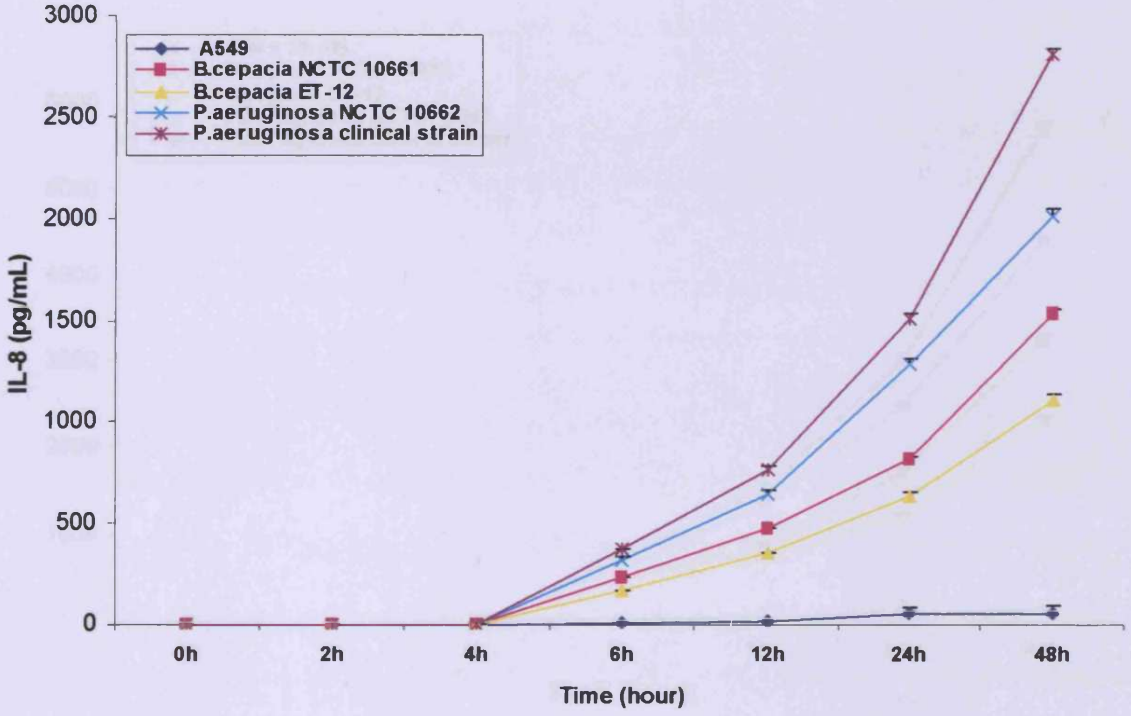


Figure 5.1: Time course of IL-8 induction from A549 epithelial cells by *B. cepacia* NCTC 10661, *B. cepacia* ET-12, *P. aeruginosa* NCTC 10662 and *P. aeruginosa* clinical strain in the absence of human serum. Confluent A549 cells were stimulated with the different Gram-negative bacteria at moi 10 bacteria: 1A549 in the absence of 1% human serum. Supernatants were collected at different times and IL-8 was measured by ELISA. IL-8 concentrations are expressed as mean ± SD of three independent experiments.

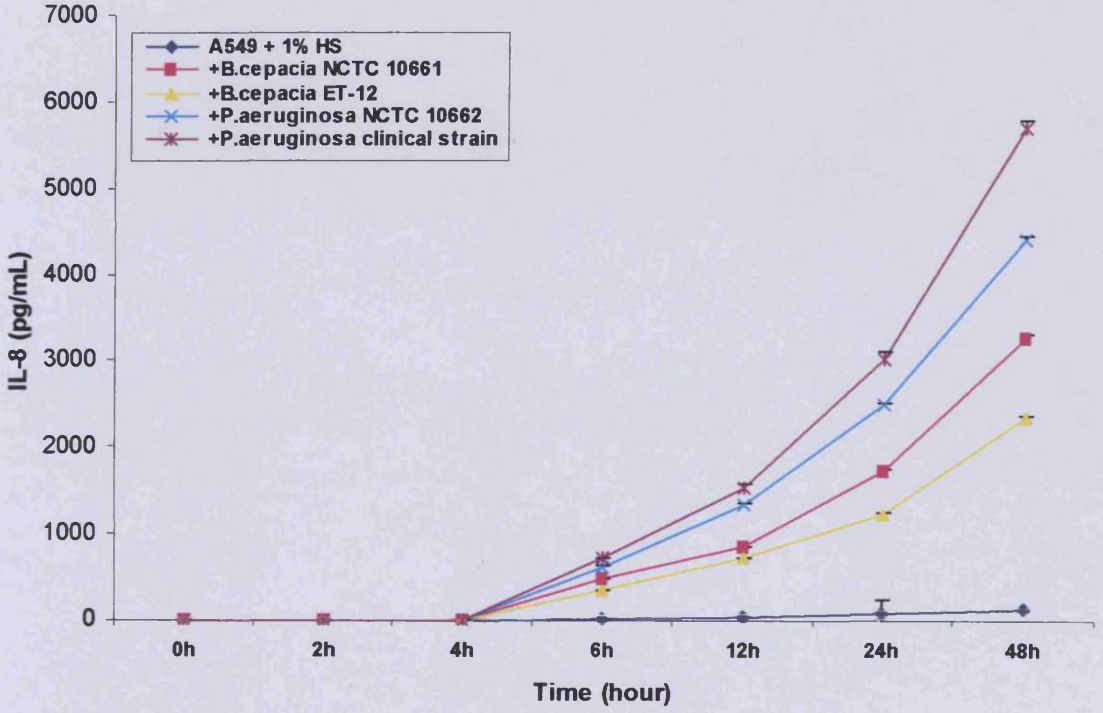


Figure 5.2: Time course of IL-8 induction from A549 epithelial cells by *B. cepacia* NCTC 10661, *B. cepacia* ET-12, *P. aeruginosa* NCTC 10662 and *P. aeruginosa* clinical strain in the presence of human serum. Confluent A549 cells were stimulated with the different Gram-negative bacteria at moi 10 bacteria: 1A549. Supernatants were collected at different times and IL-8 was measured by ELISA. IL-8 concentrations are expressed as mean ± SD of three independent experiments.

5.2 EFFECT OF SURFACTANT LIPIDS ON BACTERIAL INDUCTION OF IL-8 FROM A549 EPITHELIAL CELLS

During lower pulmonary tract infections, bacteria come into contact with the epithelial cells and may colonize the microenvironment. As a result of this, epithelial and endothelial injury may be established and capillary leakage of serum and inflammatory mediators may occur. Vascular permeability increases and polymorphonuclear leukocytes (PMNs) arrive at the area of infection. IL-8 is chemoattractant for neutrophils to the site of infection therefore here we investigated the response of the A549 epithelial cells to live bacteria in IL-8 release in the absence of human serum to study the early response to bacteria and in the presence of serum to mimic the microenvironment in the alveolar space after the leakage of serum components. The effect of surfactant lipids also was investigated to study the possible modulation on this induction.

5.2.1 Effect of surfactant lipids on IL-8 induced from A549 cells by live bacteria in the absence and the presence of human serum

To investigate the effect of different surfactants on bacterial induction of IL-8 cytokine from A549 epithelial cells, Survanta®, Curosurf® or DPPC were incubated with confluent, adherent A549 epithelial cells ($5x10^5$ cells/mL) for 2 hours prior to stimulation with different bacteria at moi (10 bacteria: 1 A549). Surfactants did not affect bacterial growth at the concentration used in this study (chapter 3). Pre-incubation of the cells with the pulmonary surfactants Survanta®, Curosurf® or DPPC lipid significantly inhibited the release of IL-8 induced by either NCTC or clinical strains of *P. aeruginosa* and *B. cepacia* (Fig 5.3- 5.6).

As shown in figures 5.3-5-6, surfactant Survanta® was the most active surfactant in the inhibition of IL-8 from A549 cells, producing over 50% inhibition in presence and absence of human serum. There were no significant differences in the induction of IL-8 between the different bacterial strains used to stimulate this cytokine. Generally this study found that there were no critical differences between clinical and environmental NCTC strains in regards to the inhibitory effect of pulmonary surfactants for the induction of IL-8.

Since pulmonary infection with bacteria is frequently accompanied by leakage of human serum into the interstitial spaces, the inhibition of IL-8 cytokine by the surfactant was reported in this study in the presence of human serum by live bacteria. It was noted that IL-8 induced in the presence of human serum almost twice as much as induced by live bacteria without human serum (Fig 5.1 & Fig 5.2) and moreover the higher amounts of IL-8 were still down regulated by the surfactants to a similar extent as in the absence of human serum. Thus, in the presence of human serum, Survanta®, Curosurf® and DPPC all at (250μg/mL) markedly inhibited IL-8 induced by *B. cepacia* NCTC 10661 (Fig 5.3), the clinical strain *B. cepacia* ET-12 (Fig 5.4) and both the NCTC and clinical strain by *P. aeruginosa* (Fig 5.5 & 5.6).

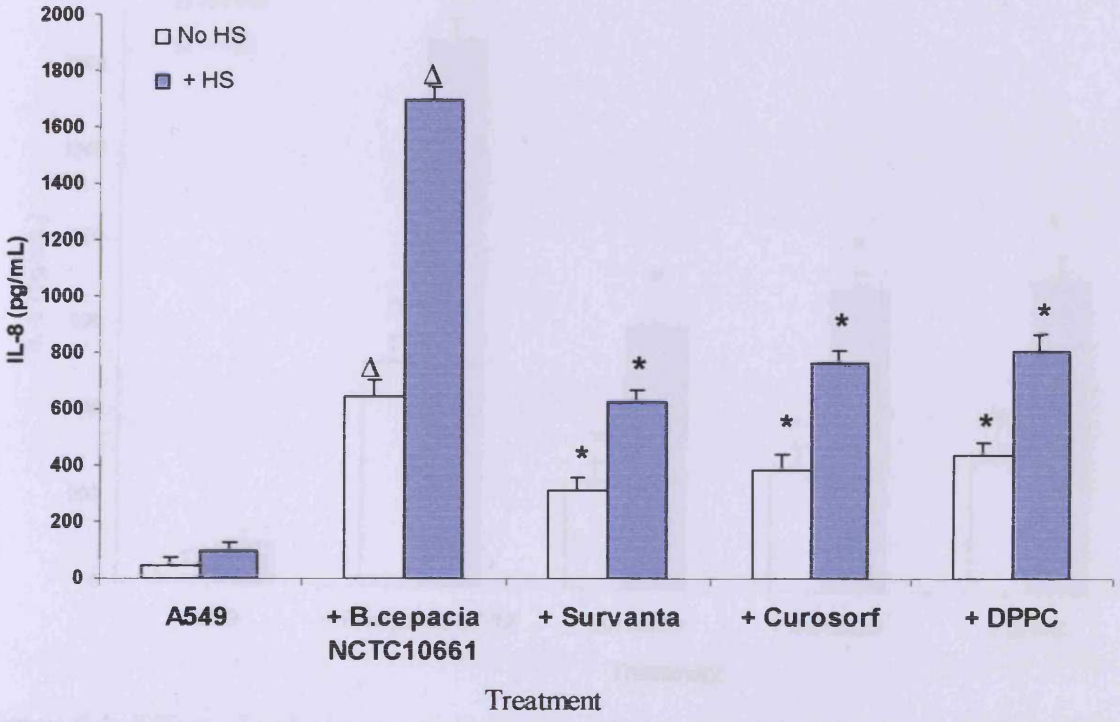


Figure 5.3: Effect of pulmonary surfactant on IL-8 induced by *B. cepacia* NCTC 10661. A549 cells were grown to confluence and incubated with 250 µg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior to stimulation with bacteria at moi (10 bacteria: 1A549 cell) in the absence or presence of human serum. Supernatants were collected after 18 hours and were assayed by ELISA. Results are expressed as the mean ± SD of three independent experiments. ΔP<0.01 analysed by ANOVA and Tukey's (*P<0.05) when comparing surfactant treatment to controls.

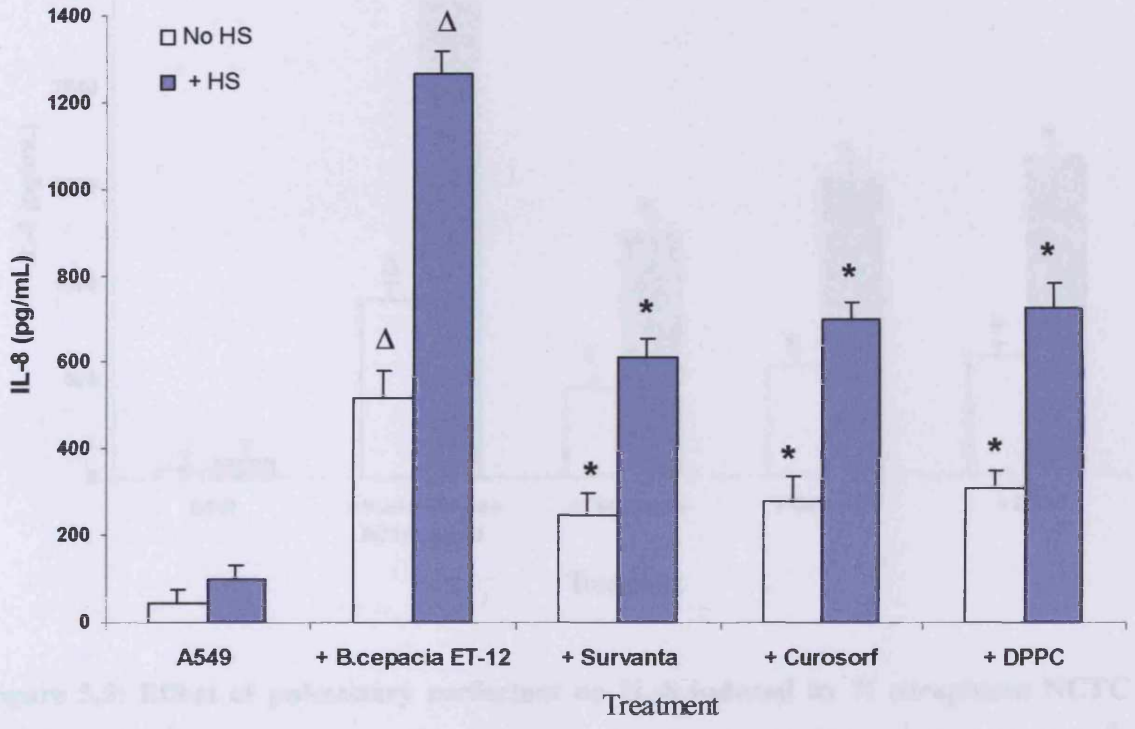


Figure 5.4: Effect of pulmonary surfactant on IL-8 induced by *B. cepacia* ET-12. A549 cells were grown to confluence and incubated with 250 μg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior stimulation with bacteria at moi (10 bacteria: 1A549 cell) in the absence or presence of human serum. Supernatants were collected after 18 hours and were assayed by ELISA. Results are expressed as the mean ± SD of three independent experiments. Δ$P<0.01$ analysed by ANOVA and Tukey's (*$P<0.05$) when comparing surfactant treatment to controls.

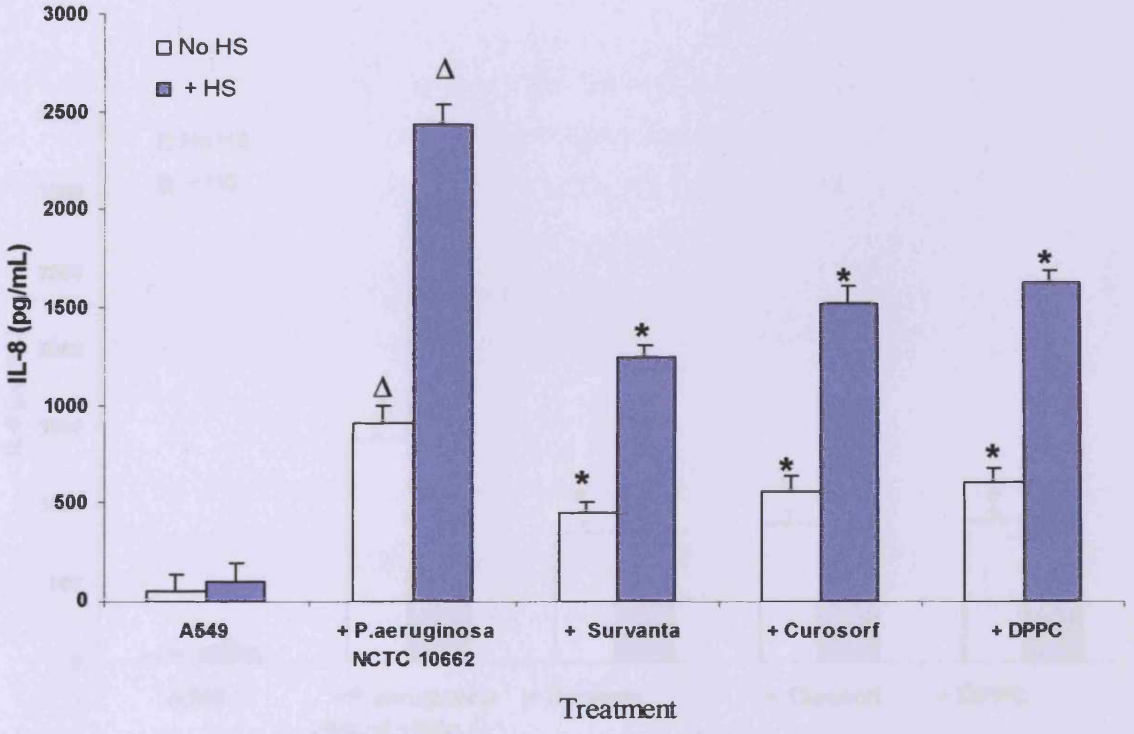


Figure 5.5: Effect of pulmonary surfactant on IL-8 induced by *P. aeruginosa* NCTC 10662. A549 cells were grown to confluence and incubated with 250 μg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior stimulation with bacteria at moi (10 bacteria: 1A549 cell) in the absence or presence of human serum. Supernatants were collected after 18 hours and were assayed by ELISA. Results are expressed as the mean ± SD of three independent experiments. Δ$P<0.01$ analysed by ANOVA and Tukey's (*$P<0.05$) when comparing surfactant to controls.

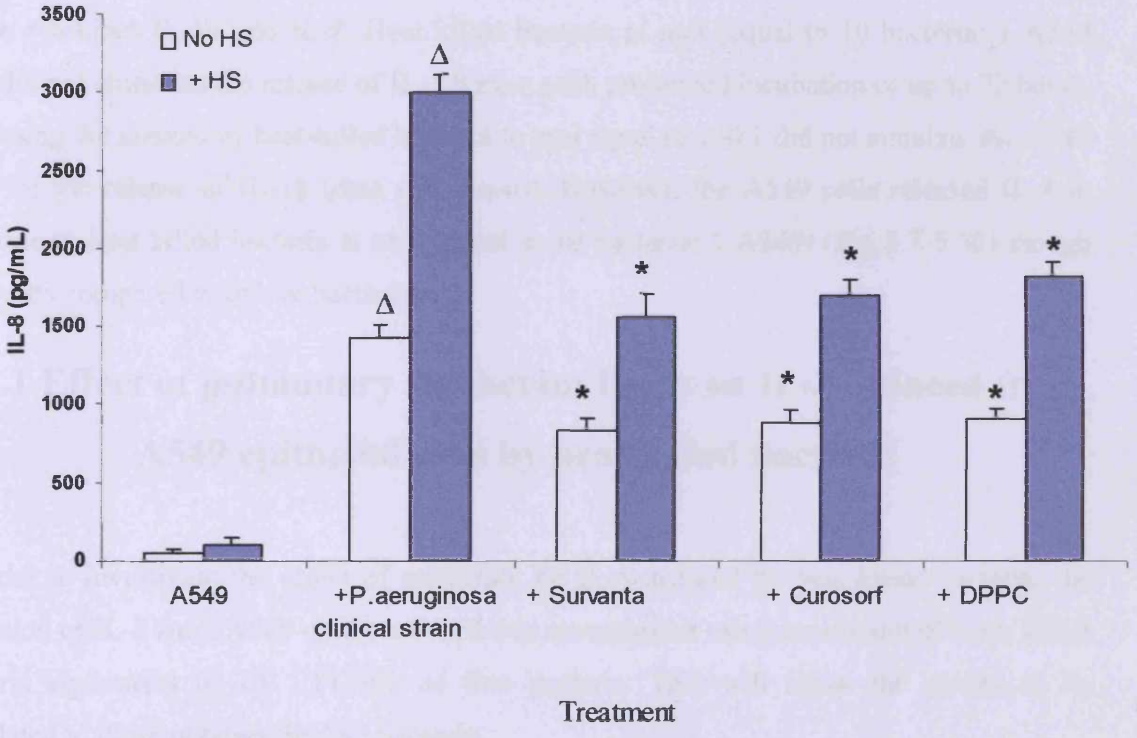


Figure 5.6: Effect of pulmonary surfactant on IL-8 induced by *P. aeruginosa* clinical strain. A549 cells were grown to confluence and incubated with 250 µg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior stimulation with bacteria at moi (10 bacteria: 1A549 cell) in the absence or presence of human serum. Supernatants were collected after 18 hours and were assayed by ELISA. Results are expressed as the mean ± SD of three independent experiments. ΔP<0.01 analysed by ANOVA and Tukey's (*P<0.05) when comparing surfactant treatment to controls.

5.3 A549 EPITHELIAL CELL CYTOKINE RESPONSES TO HEAT KILLED BACTERIA

The response of A549 epithelial cells to heat killed bacteria was characterised for the release of the cytokines IL-1β and IL-8. Heat killed bacteria at moi (equal to 10 bacteria: 1 A549 cell) did not stimulate the release of IL-1 β even with prolonged incubation of up to 72 hours. Increasing the amount of heat-killed bacteria to moi equal to 100:1 did not simulate the A549 cells for the release of IL-1β (data not shown). However, the A549 cells released IL-8 in response to heat killed bacteria at moi (equal to 10 bacteria: 1 A549) (Fig 5.7-5.10) though less when compared with live bacteria.

5.3.1 Effect of pulmonary surfactant lipids on IL-8 induced from A549 epithelial cells by heat-killed bacteria

In order to investigate the effect of surfactant on IL-8 induced by heat-killed bacteria, the induction of IL-8 from A549 epithelial cells was investigated using an amount of heat- killed bacteria equivalent to 10^6 CFU/mL of live bacteria. This will allow the results to be correlated to those obtained by live bacteria.

When A549 epithelial cells were challenged with heat killed preparation of all bacteria tested in the study in the absence of human serum, they produced significant amounts of IL-8 in a time dependent manner (Fig 5.1). The effect of pulmonary surfactants on IL-8 induction by heat killed bacteria was studied and the results revealed that heat killed (HK) bacteria from environmental and clinical strains of *P. aeruginosa* and *B. cepacia* was not significantly modulated by Survanta®, Curosurf® and DPPC in the absence of human serum (Fig 5.7-5.10).

Previous experiments with live bacteria demonstrated that some bacterial components that utilize surfactant modulation were serum dependant components (Section 5.2.1). Such components require serum-derived factors to be present to induce responses in the A549 cells (eg sCD14) and serum was included in these experiments to provide these components.

When A549 epithelial cells were challenged with heat-killed bacteria in the presence of human serum, all bacteria used in this study induced significant production of IL-8. In this study we have shown the secretion of IL-8 from A549 epithelial cells induced by heat-killed bacteria from both environmental and clinical strains of *P. aeruginosa* and *B. cepacia* was significantly increased by more than 40% in the presence of human serum. These IL-8 inductions were significantly suppressed when incubated with Survanta®, Curosurf® and DPPC. This down regulation was demonstrated with heat-killed bacteria from both environmental and clinical strains of *P. aeruginosa* (Figure 5.7 and 5.8) and *B. cepacia* (5.9 and 5.10).

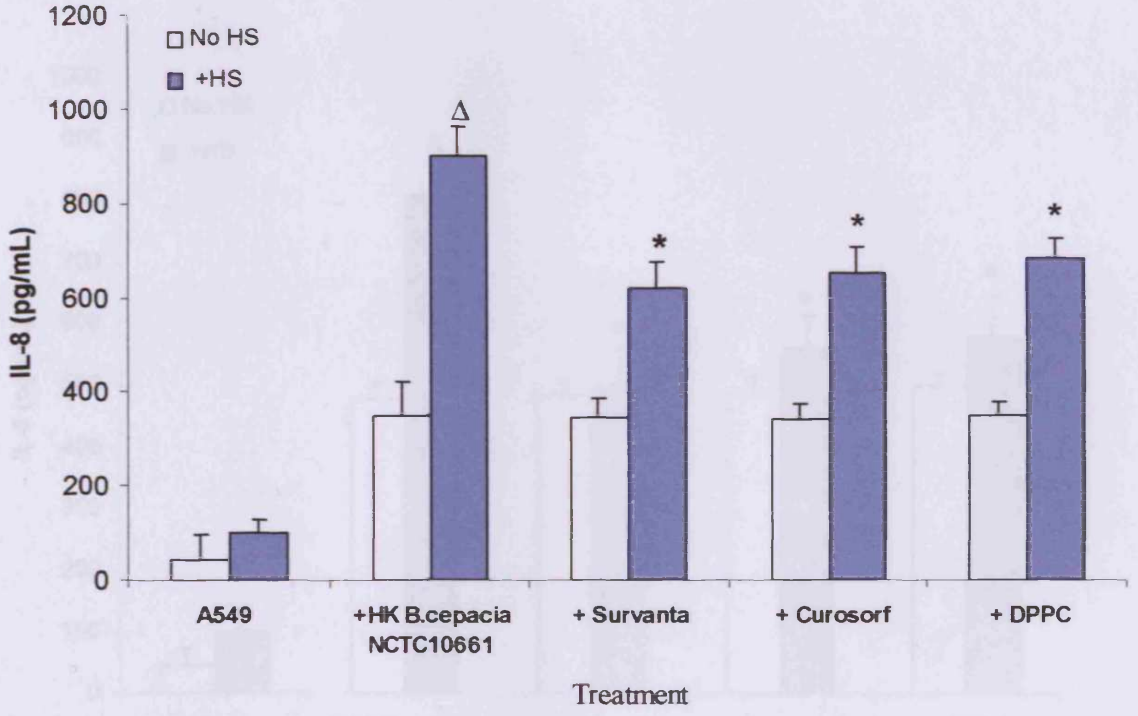


Figure 5.7: Effect of pulmonary surfactant on IL-8 induced by heat killed of *B. cepacia* NCTC 10661. A549 cells were grown to confluence and incubated with 250 μg/mL of Survanta®, Curosurf ® or DPPC for 2 hours prior to stimulation with heat killed *B. cepacia* NCTC 10661 in presence or absence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD of three independent experiments. $\Delta P<0.01$ analysed by ANOVA and Tukey's ($^*P<0.05$) when comparing surfactant treatment to controls.

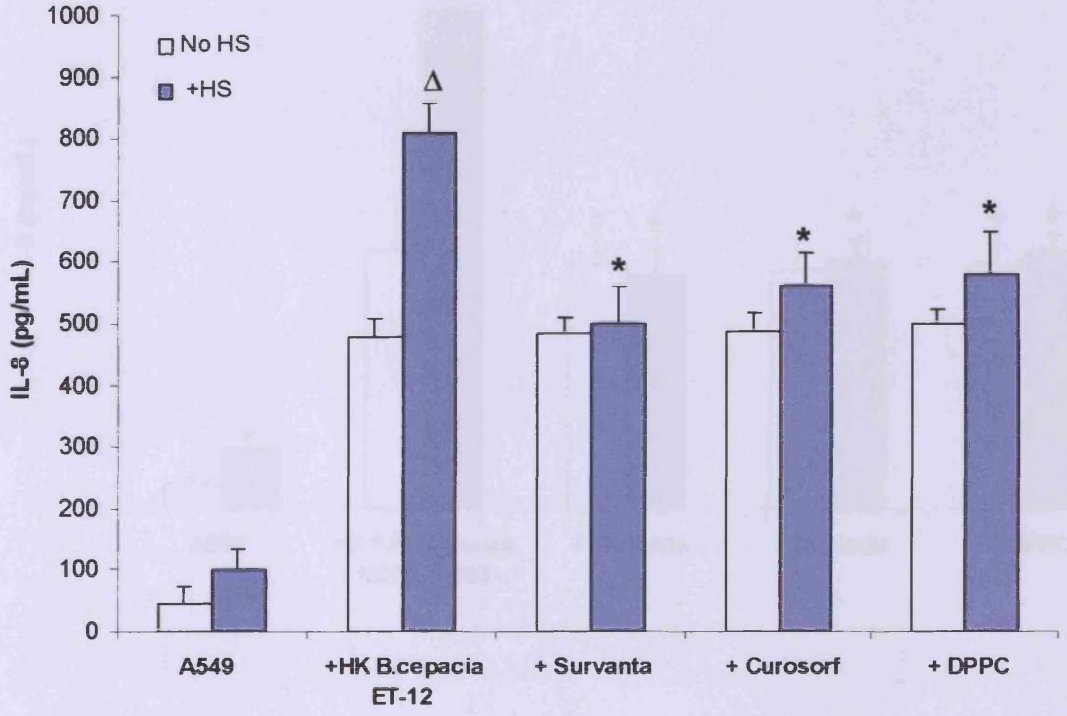


Figure 5.8: Effect of pulmonary surfactant on IL-8 induced by heat killed of *B. cepacia* ET-12. A549 cells were grown to confluence and incubated with 250 µg/mL of Survanta®, Curosurf ® or DPPC for 2 hours prior to stimulation with heat killed *B. cepacia* ET-12 in presence or absence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD of three independent experiments. $\Delta P < 0.01$ analysed by ANOVA and Tukey's ($^*P<0.05$) when comparing surfactant treatment to controls.

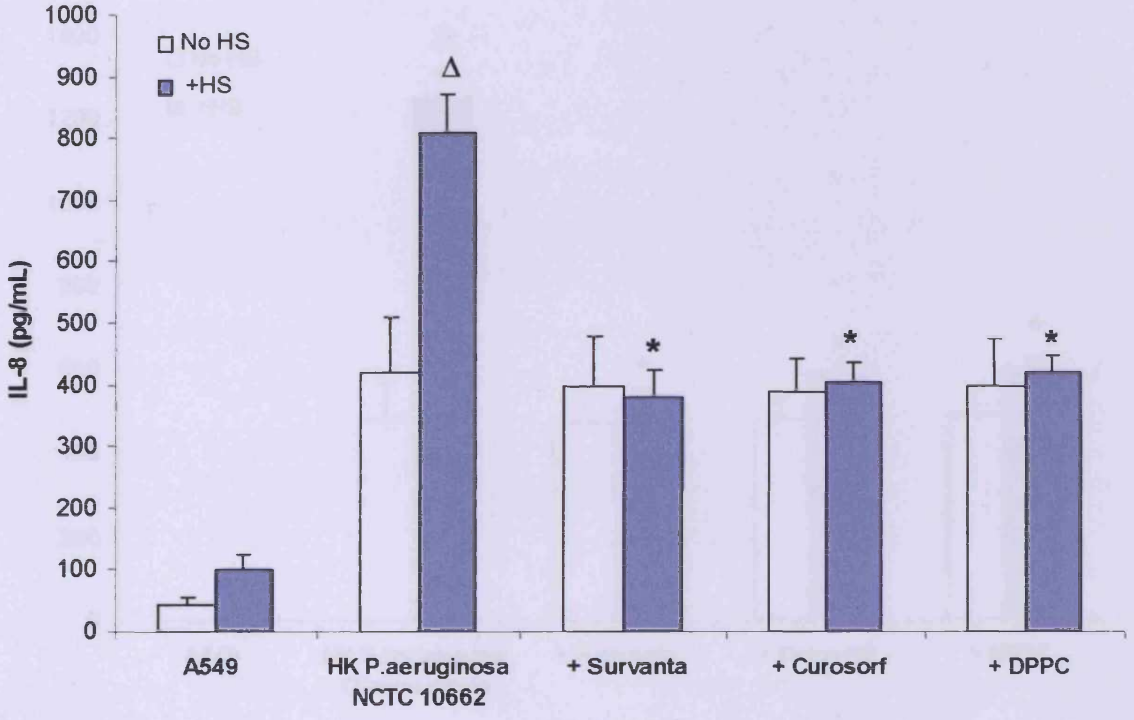


Figure 5.9: Effect of pulmonary surfactant on IL-8 induced by heat killed of *P. aeruginosa* NCTC 10662. A549 cells were grown to confluence and incubated with 250 μg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior to stimulation with heat killed *P. aeruginosa* NCTC 10662 in presence or absence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD of three independent experiments. Δ$P<0.01$ analysed by ANOVA and Tukey's (*$P<0.05$) when comparing surfactant treatment to controls.

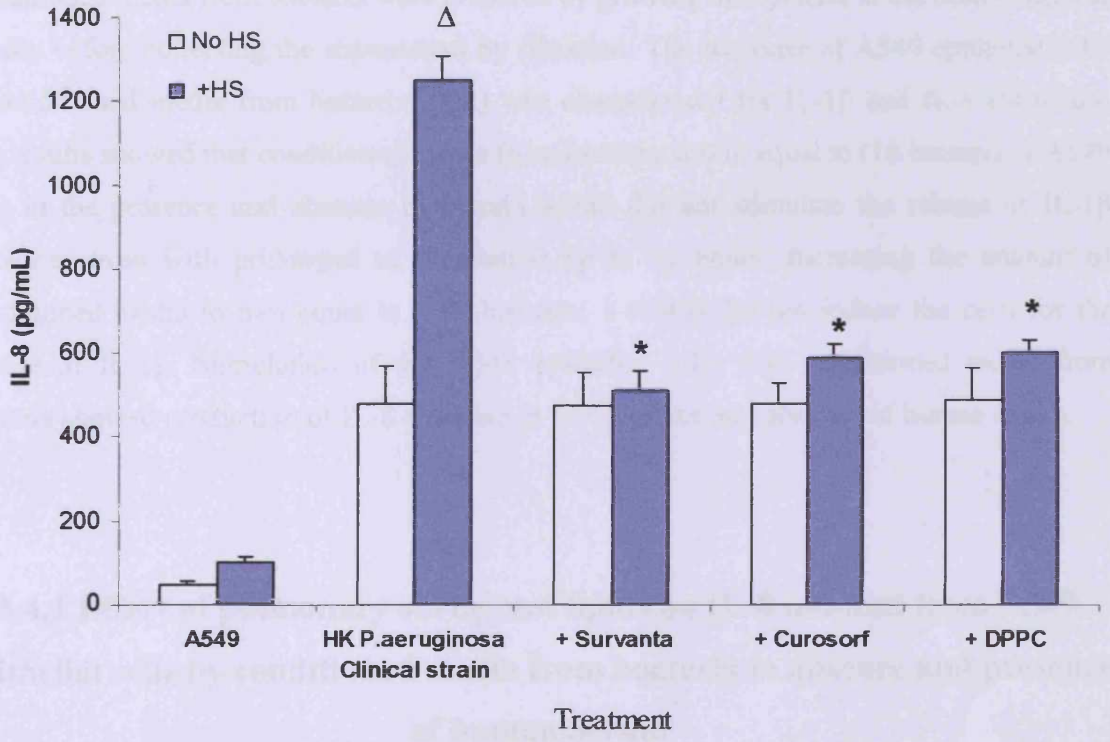


Figure 5.10: Effect of pulmonary surfactant and surfactant phospholipid DPPC on IL-8 cytokine from A549 epithelial cells induced by heat killed *P. aeruginosa* clinical strain. A549 cells were grown to confluence and incubated with 250 µg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior to stimulation with heat killed *P. aeruginosa* with clinical strain in presence or absence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD of three independent experiments. Δ$P<0.01$ analysed by ANOVA and Tukey's (*$P<0.05$) when comparing surfactant treatment to controls.

5.4 A549 EPITHELIAL CELLS RESPONSE TO CONDITIONED MEDIA FROM BACTERIA ON IL-8 CTOKINE RELEASE

Conditioned media from bacteria were prepared by growing the bacteria at the desired optical density before collecting the supernatant by filtration. The response of A549 epithelial cells to conditioned media from bacteria (CM) was characterised for IL-1β and IL-8 cytokines. The results showed that conditioned media from bacteria at moi equal to (10 bacteria: 1 A549 cell) in the presence and absence of human serum did not stimulate the release of IL-1β cytokines even with prolonged of incubation up to 72 hours. Increasing the amount of conditioned media to moi equal to (100 bacteria: 1 A549) did not induce the cells for the release of IL-1β. Stimulation of the A549 epithelial cells with conditioned media from bacteria showed production of IL-8 cytokine in the presence and absence of human serum.

5.4.1 Effect of pulmonary surfactant lipids on IL-8 induced from A549 epithelial cells by conditioned media from bacteria in absence and presence of human Serum

Conditioned media (CM) from bacteria were prepared from different bacteria and have been tested for sterility before use in the study. In order to investigate the effect of surfactant lipids on IL-8 cytokine induction, the induction of IL-8 from A549 epithelial cells using conditioned media equal to moi (10 bacteria: 1 A549) was investigated.

When A549 epithelial cells were challenged with conditioned media (CM) from different bacteria tested in this study, they showed considerable and significant amounts of IL-8 induction. Pulmonary surfactant Survanta®, Curosurf® and DPPC lipid did not inhibit IL-8 cytokine induced by conditioned media from both strains of *B. cepacia* (5.11, 5.12) and both of *P. aeruginosa* (5.13, 5.14) strains.

When serum was added prior to incubation with conditioned media from bacteria, IL-8 induction was increased and when pulmonary surfactant was investigated, Survanta®, Curosurf® and DPPC did not inhibit IL-8 induction.

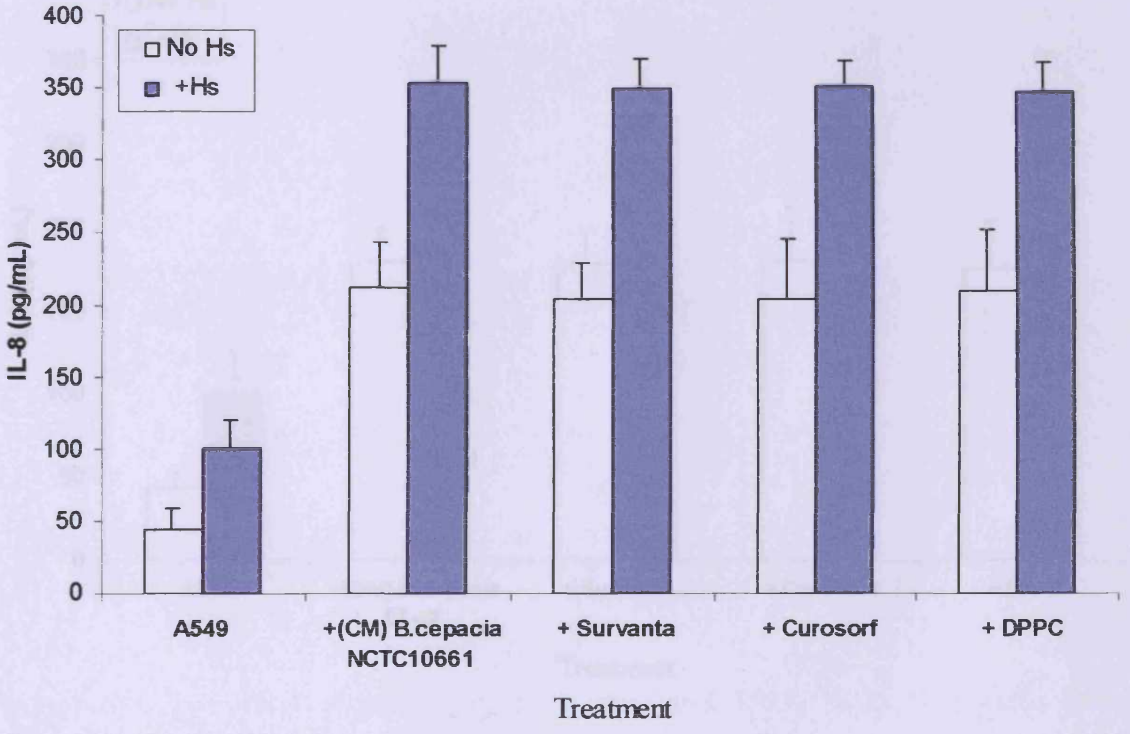


Figure 5.11: The effect of pulmonary surfactant and DPPC on IL-8 cytokine from A549 epithelial cells induced by conditioned media (CM) from *B. cepacia* NCTC 10661. A549 cells were grown to confluence and incubated with 250 μg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior to stimulation with conditioned media from *B. cepacia* NCTC 10661. Supernatants were collected after 18 hours and were assayed for IL-8 by ELISA. Pre-treatment of A549 cells with surfactants did not show any significant effect on IL-8 release. Results are expressed as the mean ± SD of three representative experiments.

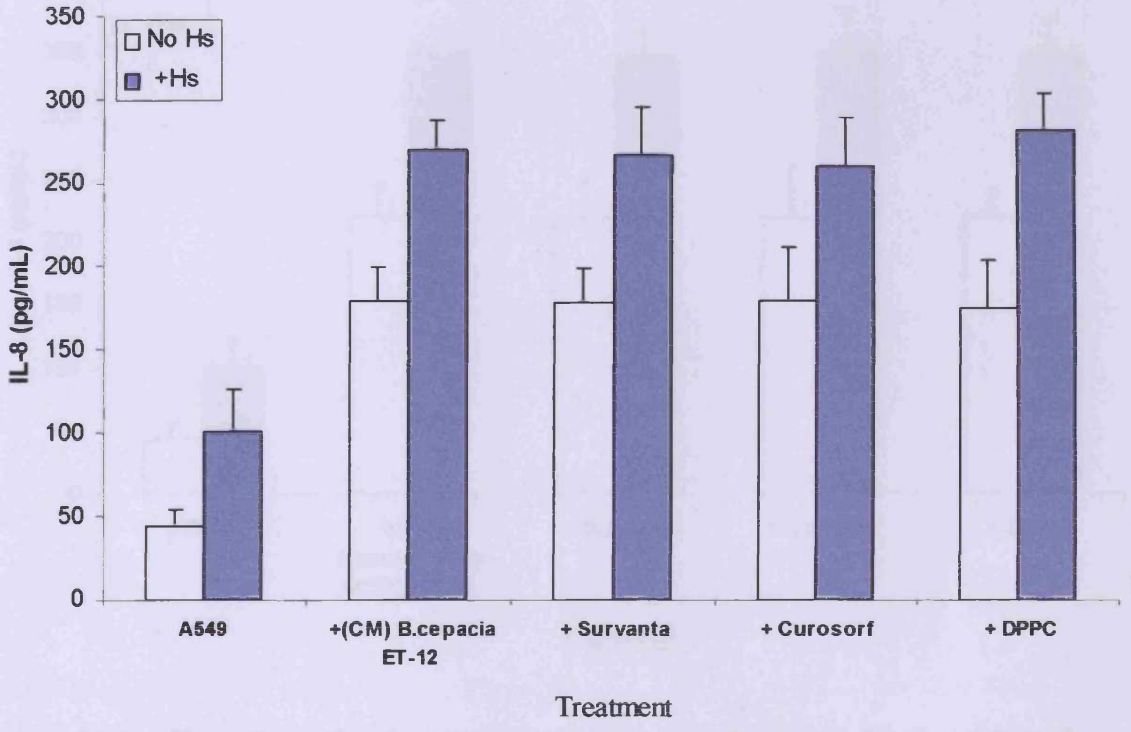


Figure 5.12: The effect of pulmonary surfactant and DPPC on IL-8 cytokine from A549 epithelial cells induced by conditioned media (CM) from *B. cepacia* ET-12. A549 cells were grown to confluence and incubated with 250 µg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior to stimulation with conditioned media from *B. cepacia* ET-12. Supernatants were collected after 18 hours and were assayed by for IL-8 by ELISA. Pre-treatment of A549 cells with surfactants did not show any significant effect on IL-8 release. Results are expressed as the mean ± SD of three representative experiments.

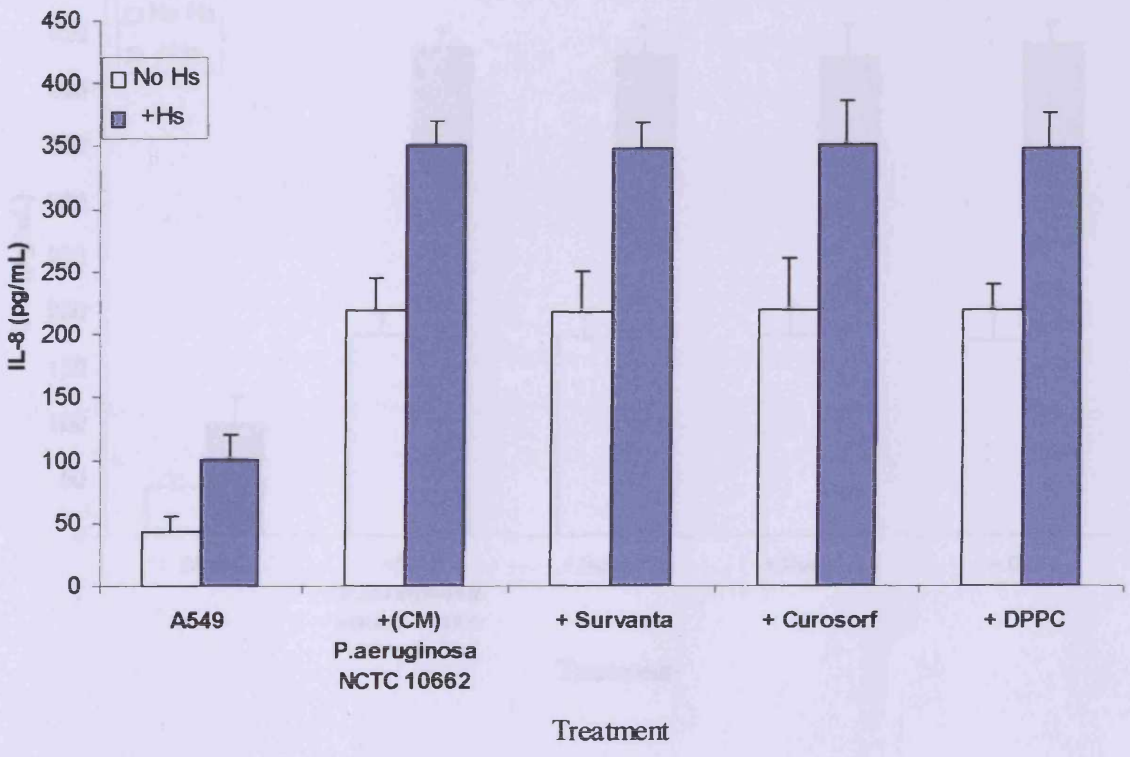


Figure 5.13: The effect of pulmonary surfactant and DPPC on IL-8 cytokine from A549 epithelial cells induced by conditioned media (CM) from *P. aeruginosa clinical strain*. A549 cells were grown to confluence and incubated with 250 µg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior to stimulation with conditioned media from *P. aeruginosa clinical strain*. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Pre-treatment of A549 cells with surfactants did not show any significant effect on IL-8 release. Results are expressed as the mean ± SD of three representative experiments.

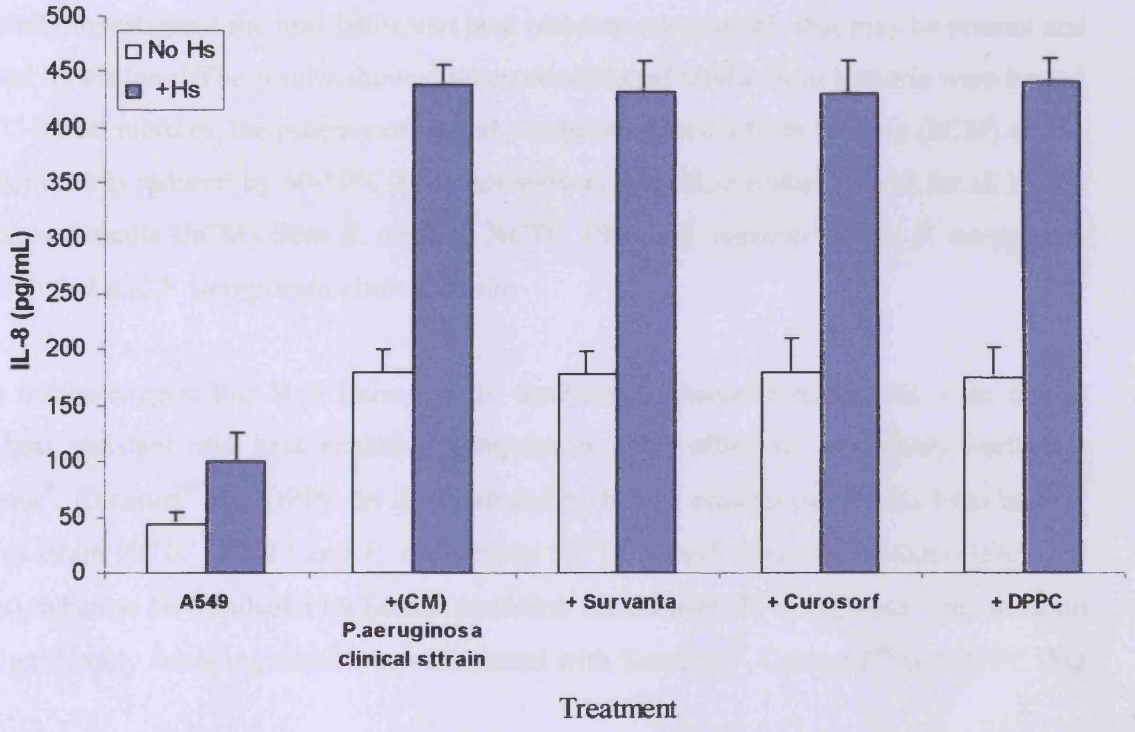


Figure 5.14: The effect of pulmonary surfactant and DPPC on IL-8 cytokine from A549 epithelial cells induced by conditioned media (CM) from *P. aeruginosa* NCTC 10662. A549 cells were grown to confluence and incubated with 250 µg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior to stimulation with conditioned media from *P. aeruginosa* NCTC 10662. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Pre-treatment of A549 cells with surfactants did not show any significant effect on IL-8 release. Results are expressed as the mean ± SD of three representative experiments.

5.4.2 Effect of pulmonary surfactant lipids on IL-8 induced from A549 epithelial cells by heated conditioned media from bacteria in the presence of human serum

This study investigated the heat labile and heat resistant components that may be present and secreted by bacteria. The results showed when conditioned media from bacteria were heated at 95°C for 30 minutes, the potency of heated conditioned media from bacteria (hCM) on IL-8 induction was reduced by 40-50% (Data not shown). This IL-8 reduction was for all heated conditioned media (hCM) from *B. cepacia* NCTC 10661, *B. cepacia* ET-12, *P. aeruginosa* NCTC 10662 and *P. aeruginosa* clinical strain.

These results suggest that IL-8 induction by conditioned media from bacteria were due to both heat resistant and heat sensitive components. The effect of pulmonary surfactant Survanta®, Curosurf® and DPPC on IL-8 induced by heated conditioned media from both *B. cepacia* strain NCTC, ET-12 and *P. aeruginosa* NCTC 10661 were not inhibited (Data not shown), whereas IL-8 induced by heated condition media from *P. aeruginosa* clinical strain was significantly down regulated when incubated with Survanta®, Curosurf ® and DPPC (Fig 5.15).

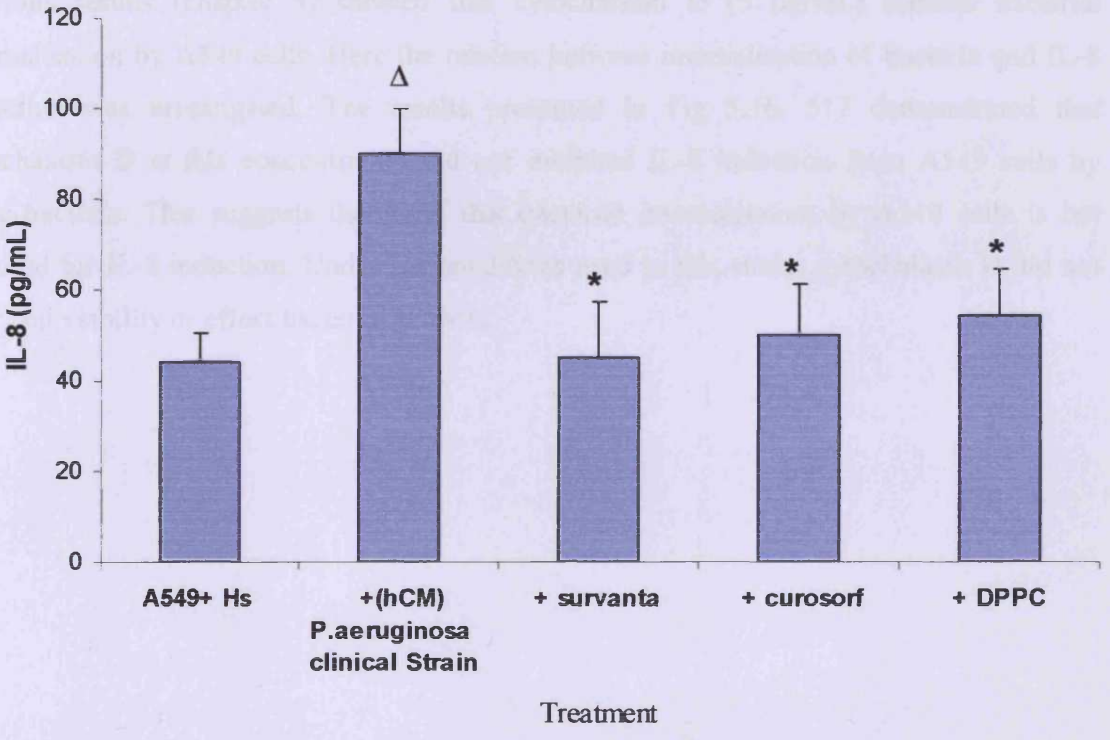


Figure 5.15: Effect of pulmonary surfactant and DPPC on IL-8 cytokine induced by heated conditioned media (hCM) from *P. aeruginosa* clinical strain. A549 cells were grown to confluence and incubated with 250 µg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior to stimulation with heated conditioned media (hCM) from *P. aeruginosa* clinical strain in presence of 1% human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Pre-treatment of A549 cells with surfactants significantly suppressed the IL-8 release. Results are expressed as the mean ± SD of three independent experiments. $\Delta P<0.01$ analysed by ANOVA and Tukey's ($^*P<0.05$) when comparing surfactant treatment to control.

5.5 EFFECT OF CYTOCHALASIN D ON IL-8 INDUCED FROM A549 EPITHELIAL CELLS BY LIVE BACTERIA

Previous results (chapter 3) showed that cytochalasin D (5 μg/mL) inhibits bacterial internalisation by A549 cells. Here the relation between internalisation of bacteria and IL-8 induction was investigated. The results presented in Fig 5.16, 517 demonstrated that cytochalasin D at this concentration did not inhibited IL-8 induction from A549 cells by these bacteria. This suggests therefore, that bacterial internalisation by A549 cells is not required for IL-8 induction. Under the conditions used in this study, cytochalasin D did not alter cell viability or effect bacterial growth.

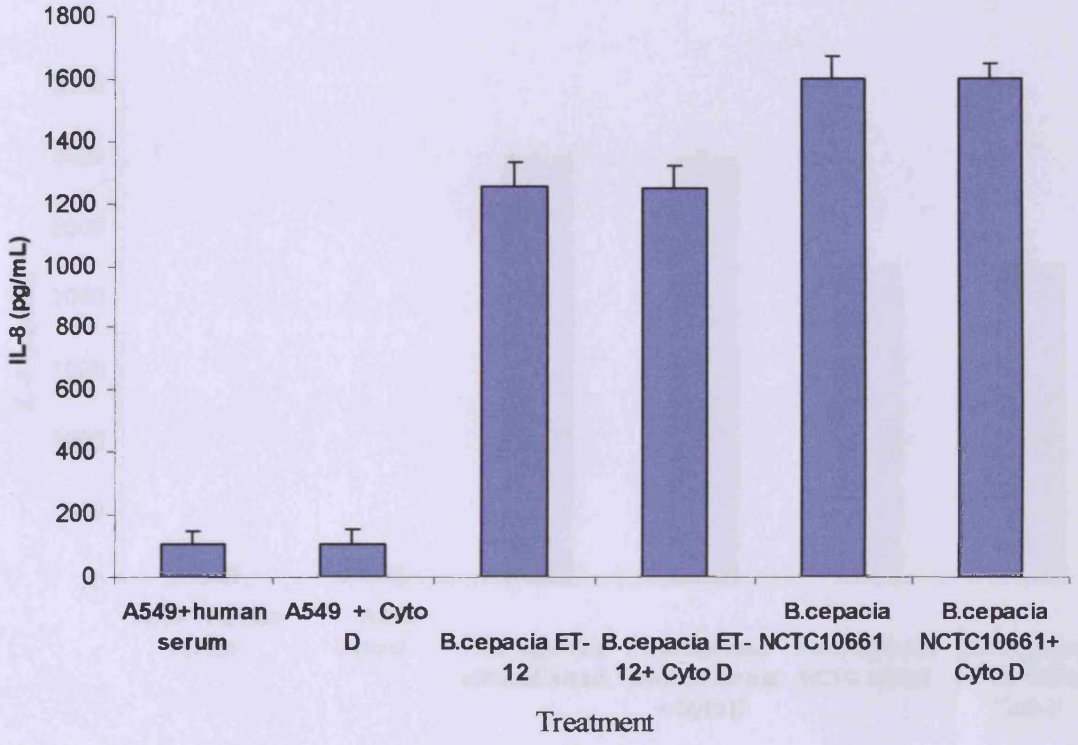


Fig 5.16: Effect of cytochalasin D on IL-8 induction by *B. cepacia* in A549 cells. A549 cells were grown to confluence and pre-incubated with 5μg/mL cytochalasin D (cyto D) for 2 hours before stimulation with *B. cepacia* ET-12 or *B. cepacia* NCTC 10661 in presence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD from three separate experiments.

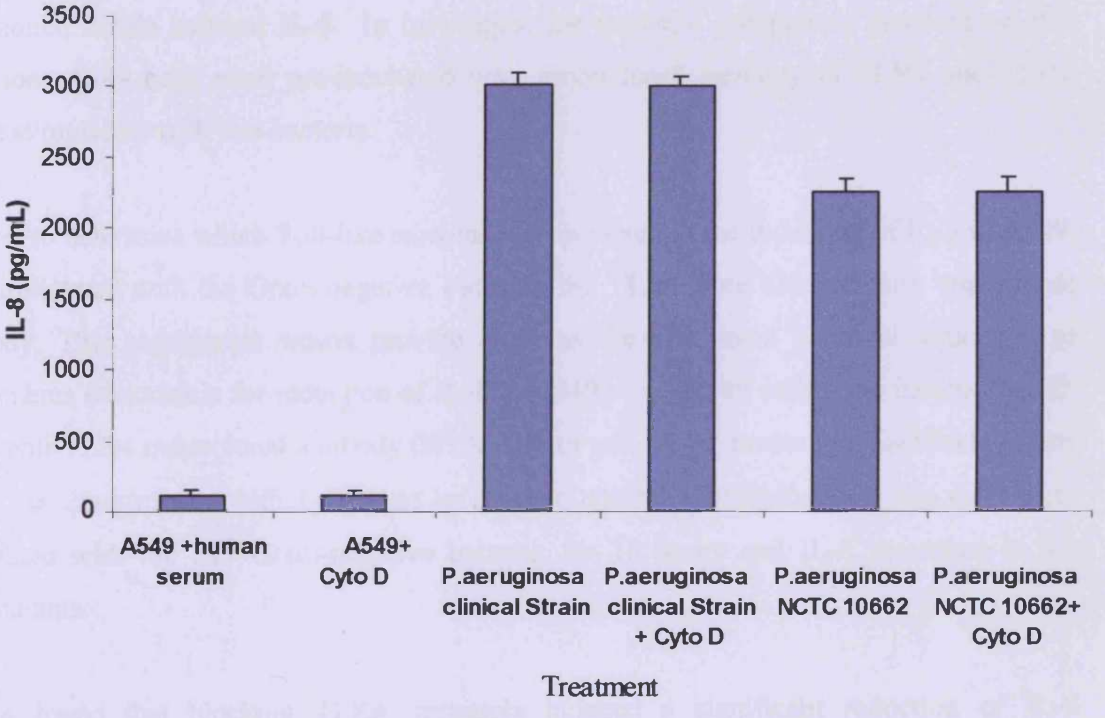


Figure: 5. 17: Effect of cytochalasin D on IL-8 induction by *P. aeruginosa* in A549 cells. A549 cells were grown to confluence and pre-incubated with 5μg/mL cytochalasin D (cyto D) for 2 hours before stimulation with *P. aeruginosa* NCTC 10662 or *P. aeruginosa* clinical strain in presence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD from three separate experiments.

5.6 EFFECT OF TLR4 AND TLR2 BLOCKADE ON IL-8 INDUCTION FROM A549 CELLS BY LIVE BACTERIA

Toll like receptors (TLRs) play an essential role in the recognition of bacteria and bacterial products like LPS and PGN (Gon et al., 2004,Takeda and Akira, 2005). Previous results demonstrated that live bacteria, heat killed bacteria, conditioned media and heated conditioned media induced IL-8. To investigate the bacterial component involved on this induction, A549 cells were pre-incubated with monoclonal antibody to TLR4 and TLR2 before stimulation with live bacteria.

In order to determine which Toll-like receptors are involved in the induction of IL-8 in A549, cells stimulated with the Gram-negative bacteria, the TLRs were blocked with appropriate antibody. This experiment would provide clues as the prominent bacterial structures or components responsible for induction of IL-8 in A549 cells. A549 cells were incubated with either anti-TLR4 monoclonal antibody (HTA 125) or anti-TLR2 monoclonal antibody (clone TL2.1) at 20μg/mL or with IgG2A as an isotype control. Following this, the cells were stimulated with the live Gram-negative bacteria for 18 hours and IL-8 measured in the supernatants.

It was found that blocking TLR4, receptors induced a significant reduction of IL-8 production after 18 hours incubation with *P. aeruginosa* and *B. cepacia* suggesting a prominent role for TLR4 ligands (Fig 5.18, 5.19). Blocking TLR2 receptors did not show significant affect the release of IL-8 induced by *P. aeruginosa* and *B. cepacia* and suggests that TLR2 is not significantly involved in the induction of IL-8 by these bacteria (5.20-5.21), however when A549 cells incubated with anti-TLR2 antibody prior stimulation with *P. aeruginosa* or *B. cepacia,* did not significantly inhibited IL-8 production (5.20, 5.22) when compared with peptidoglycan control (5.22).

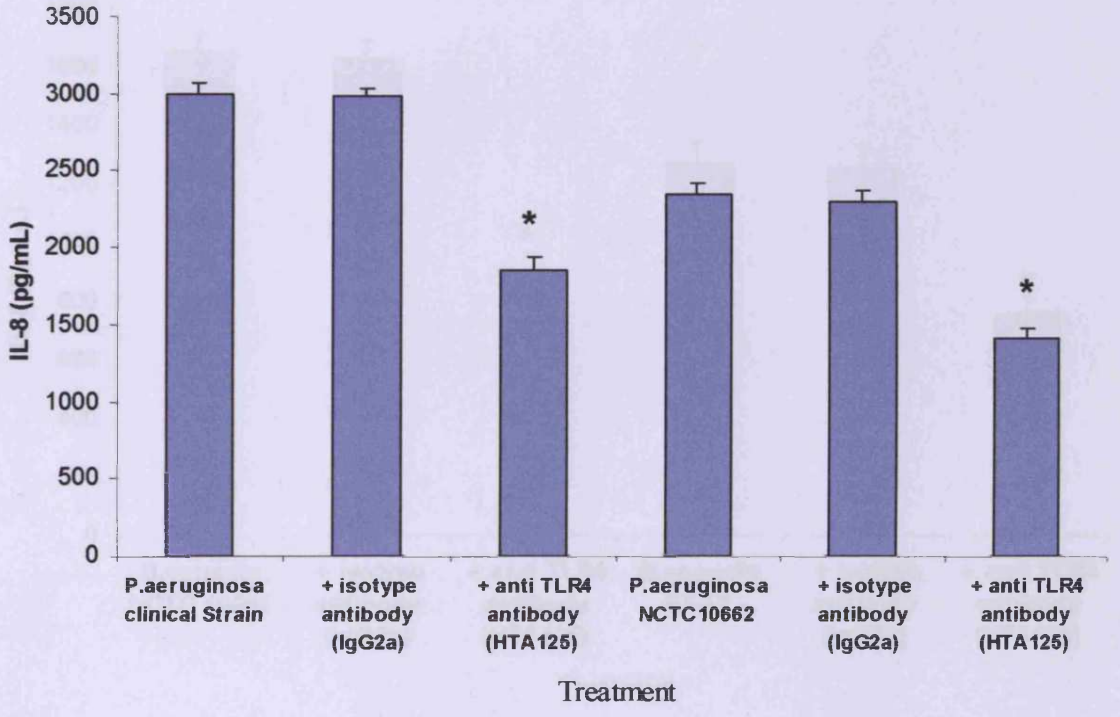


Figure 5.18: The role of TLR4 in *P. aeruginosa* induction of IL-8 from A549 cells. A549 cells were pre-incubated with or without 20μg/mL of anti-TLR4 (HTA125) or isotype antibody (IgG2a) for 2 hours before being stimulated with *P. aeruginosa* NCTC 10662 or *P. aeruginosa* clinical strain in presence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD of three independent experiments. *P<0.05 compared with cells stimulated with bacteria alone (analysed by Paired T-test).

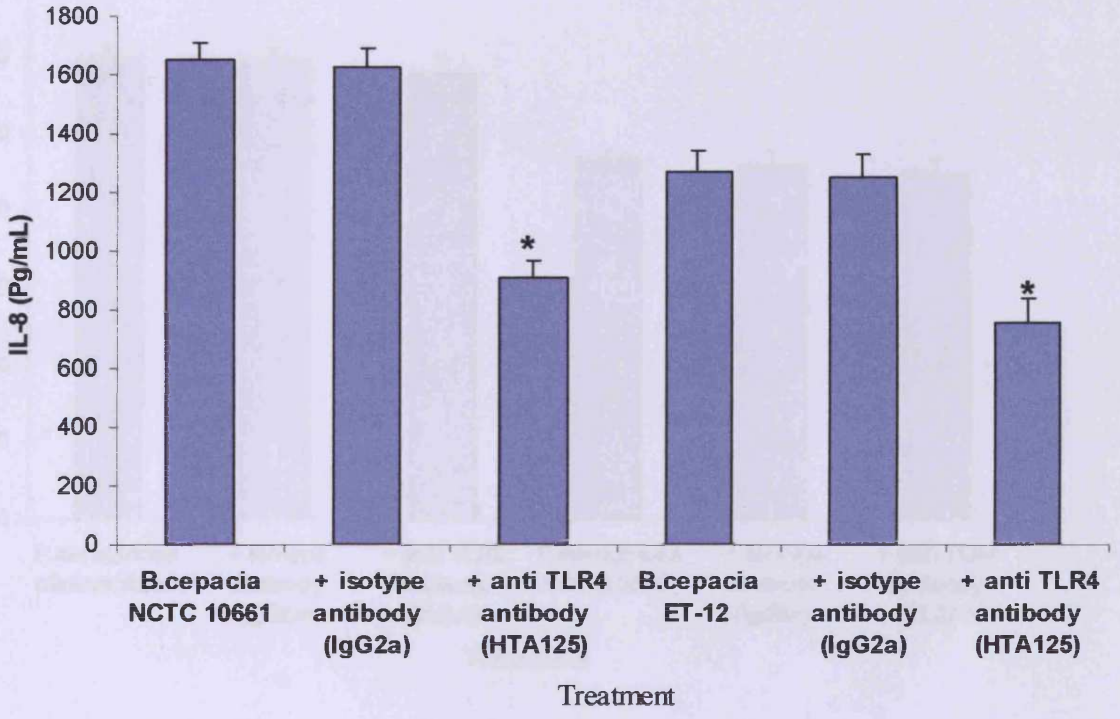


Figure 5.19: The role of TLR4 in *B. cepacia* induction of IL-8 from A549 cells. A549 cells were pre-incubated with or without 20μg/mL of anti-TLR4 (HTA125) or isotype antibody (IgG_{2a}) for 2 hours before being stimulated with *B. cepacia* NCTC 10661 or *B. cepacia* ET-12 in presence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD of three independent experiments. *$P<0.05$ compared with cells stimulated with bacteria alone (analysed by Paired T-test).

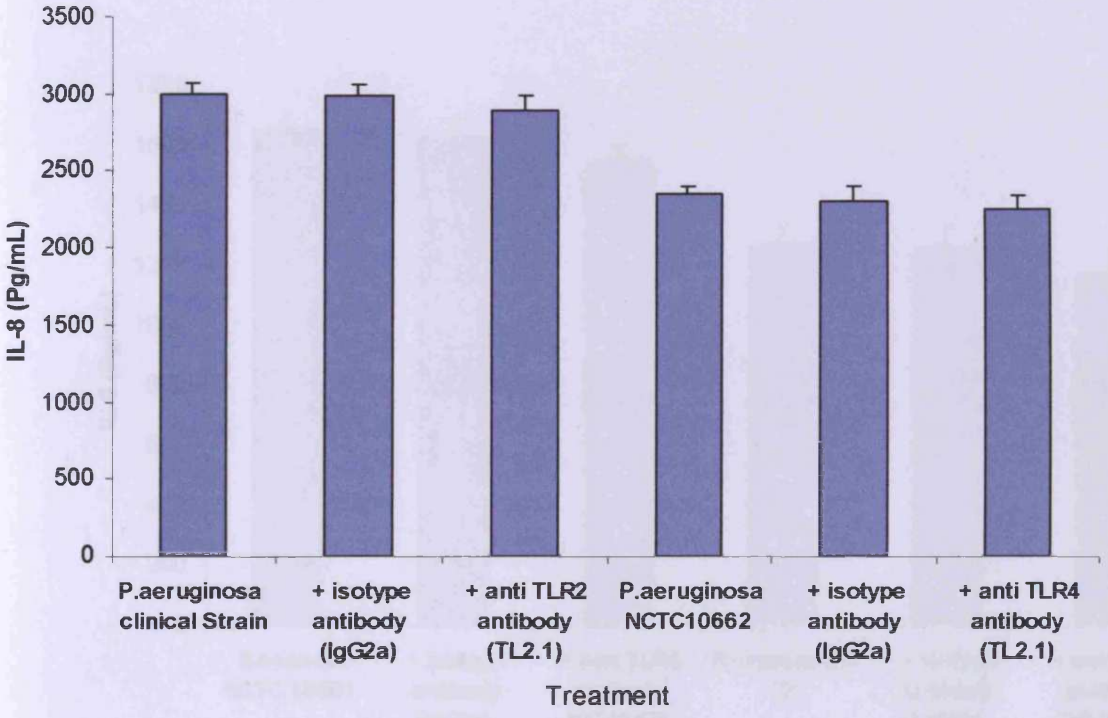


Figure 5.20: Blocking TLR2 does not show significant reduction on *P. aeruginosa* induction of IL-8 from A549 cells. A549 cells were pre-incubated with or without 20μg/mL of anti-TLR2 (TL2.1) or isotype antibody (IgG_{2a}) for 2 hours before being stimulated with *P. aeruginosa* NCTC 10662 or *P. aeruginosa* clinical strain in presence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD of three independent experiments.

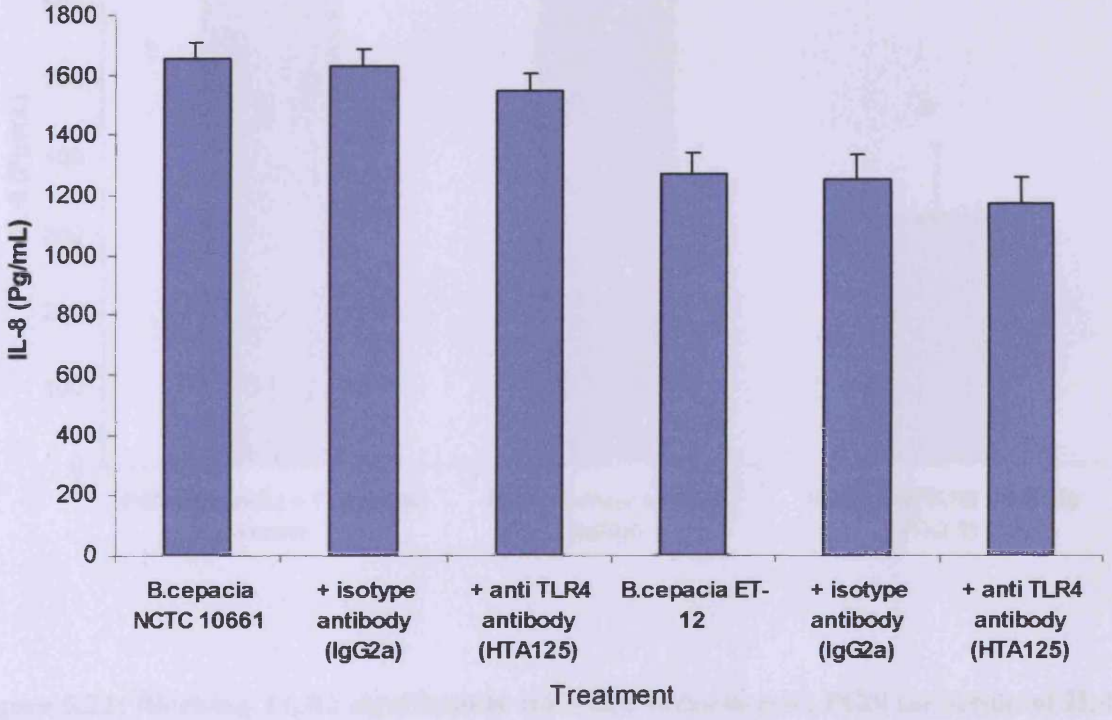


Figure 5.21: Blocking TLR2 does not show significant reduction on *B.cepacia* induction of IL-8 from A549 cells. A549 cells were pre-incubated with or without 20μg/mL of anti-TLR2 (TL2.1) or isotype antibody (IgG_{2a}) for 2 hours before being stimulated with *B. cepacia* NCTC 10661 or *B. cepacia* ET-12 strain in presence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD of three independent experiments.

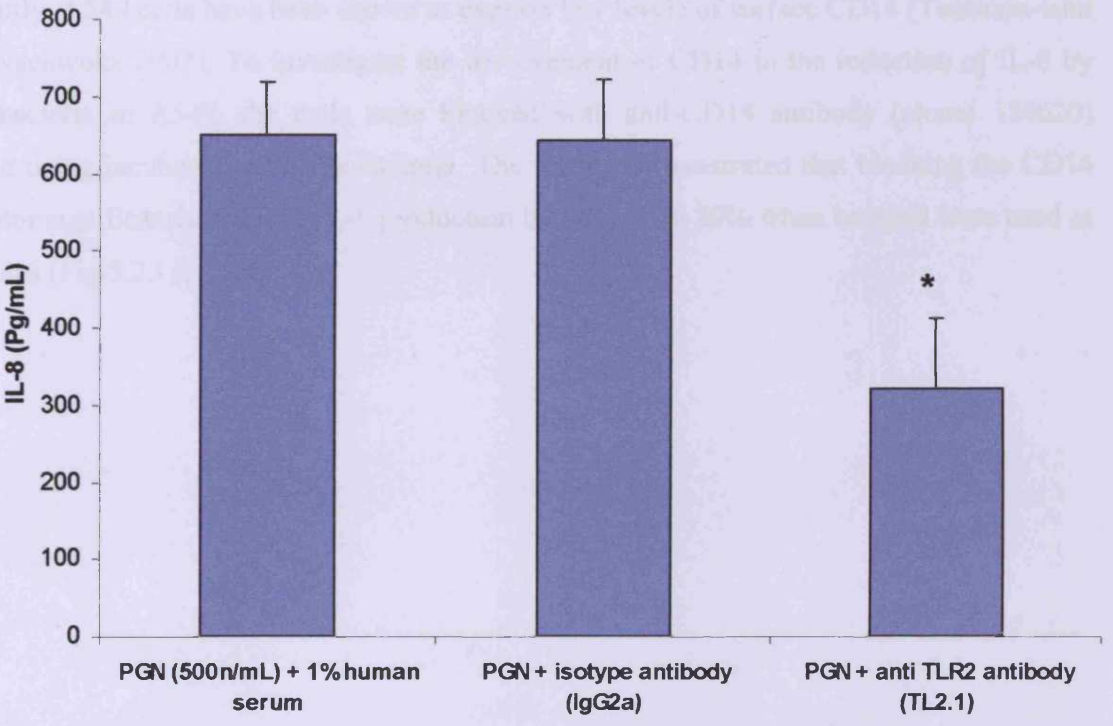


Figure 5.22: Blocking TLR2 significantly inhibited reduction on PGN induction of IL-8 from A549 cells. A549 cells were pre-incubated with or without 20μg/mL of anti-TLR2 (TL2.1) or isotype antibody (IgG_{2a}) for 2 hours before being stimulated with peptidoglycan presence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD of three independent experiments. *P< 0.05 compared with cells stimulated with PGN in the presence of human serum (analysed by Paired T-test).

5.7 EFFECT OF CD14 BLOCKADE ON IL-8 INDUCTION FROM A549 EPITHELIAL CELLS BY LIVE BACTERIA

CD14 has been shown to be an important component of the receptor complex for LPS. Recently, A549 cells have been shown to express low levels of surface CD14 (Tsutsumi-ishii and Nagawoka 2003). To investigate the involvement of CD14 in the induction of IL-8 by live bacteria in A549, the cells were blocked with anti-CD14 antibody (clone: 134620) before being incubated with live bacteria. The results demonstrated that blocking the CD14 receptor significantly reduced IL-8 production by more than 30% when bacteria were used as stimulus (Fig 5.23 & 5.24).

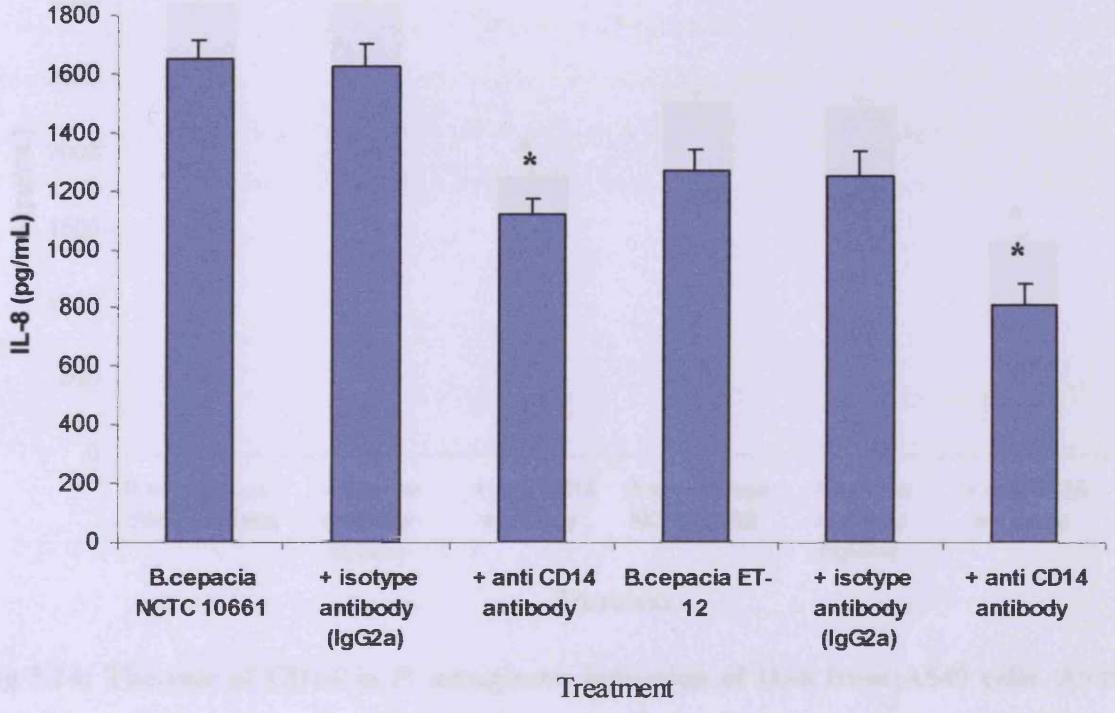


Fig 5.23: The role of CD14 in *B. cepacia* induction of IL-8 from A549 cells. A549 cells were pre-incubated with or without 20μg/mL of anti-CD14 or isotype antibody (IgG2a) for 2 hours before being stimulated with *B. cepacia* NCTC 10661 or *B. cepacia* ET-12 in presence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD of three independent experiments. *P<0.05 compared with cells stimulated with bacteria alone (analysed by Paired T-test).

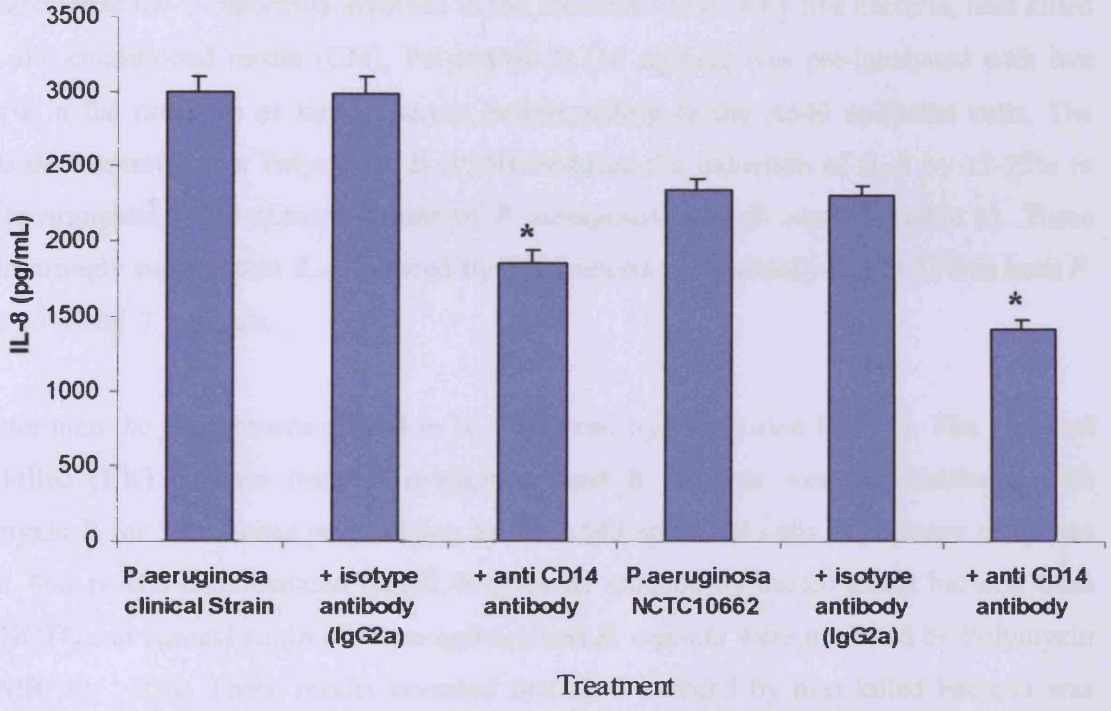


Fig 5.24: The role of CD14 in *P. aeruginosa* induction of IL-8 from A549 cells. A549 cells were pre-incubated with or without 20μg/mL of anti-CD14 or isotype antibody (IgG2a) for 2 hours before being stimulated with *P. aeruginosa* in presence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results are expressed as the mean ± SD of three independent experiments. *P<0.05 compared with cells stimulated with bacteria alone (analysed by Paired T-test).

5.8 EFFECT OF POLYMYXIN B ON IL-8 INDUCTION FROM A549 EPITHELIAL CELLS

Polymyxin B has been shown to bind to LPS and inhibit its activity (Uehara et al., 2002). To characterise the components involved in the induction of IL-8 by live bacteria, heat killed (HK) and conditioned media (CM), Polymyxin B (10 μg/mL) was pre-incubated with live bacteria in the presence of human serum before adding to the A549 epithelial cells. The results demonstrated that Polymyxin B (PMB) reduced the induction of IL-8 by 12-15% in both environmental and clinical strains of *P. aeruginosa* and *B. cepacia* (table 1). These results strongly suggest that IL-8 induced by live bacteria was partially due to LPS in both *P. aeruginosa* and *B. cepacia.*

To determine the components shared in IL-8 induced by heat killed bacteria. The prepared heat killed (HK) bacteria from *P. aeruginosa* and *B. cepacia* were pre-incubated with Polymyxin B for 30 minutes prior adding to the A549 epithelial cells in presence of human serum. Our results demonstrated that IL-8 cytokine induced by heated killed bacteria from both NCTC and clinical strain of *P. aeruginosa* and *B. cepacia* were inhibited by Polymyxin B (PMB) by >30%. These results revealed that IL-8 induced by heat-killed bacteria was partially due to the presence of LPS components (table 1).

The results demonstrate that Polymyxin B clearly inhibited IL-8 released by more than 10% when (CM) from *P. aeruginosa* clinical strain was used. These results revealed that (CM) from *P. aeruginosa* clinical strain secreted more LPS than other strains used in this study (table 1).

	IL-8 Concentration (Pg/mL)		
	Without Polymyxin B	With Polymyxin B	% Reduction
***B. cepacia* NCTC 10661**	1660 ±101	1040 ± 37	37.3 ±15
***B. cepacia* ET-12**	1300 ±89	806 ± 42	38 ±9
***P. aeruginosa* NCTC 10662**	2500 ±122	1720 ± 64	31.2 ±17
***P. aeruginosa* clinical strain**	3050 ±129	2195 ± 84	28 ±19
***B. cepacia* NCTC 10661 (HK)**	905 ± 64	520 ± 39	42.5 ±12
***B. cepacia* ET-12 (HK)**	824 ± 55	505 ± 30	38.7 ±10
***P. aeruginosa* NCTC 0662(HK)**	892 ± 48	650 ± 35	27.1±21
***P. aeruginosa* clinical (HK)**	1108 ± 69	708 ± 48	36.3±14
***B. cepacia* NCTC 10661 (CM)**	490 ± 35	484 ± 28	1.2 ±13
***B. cepacia* ET-12 (CM)**	350 ± 29	344 ± 21	1.7 ±8
***P. aeruginosa* NCTC 0662(CM)**	354 ± 38	350 ± 27	1.12± 11
***P. aeruginosa* clinical (CM)**	640 ± 41	570 ± 25	10.9±12

Table 1: Effect of Polymyxin B on IL-8 induced by live bacteria, heat killed (HK) and conditioned media (CM) by A549 cells. Confluent A549 cells were incubated without or with pretreated Polymyxin B (10 μg/mL) before stimulation with live bacteria heat killed (HK) or conditioned media (CM) from bacteria in the presence of human serum. Supernatants were collected and IL-8 concentrations were determined by ELISA. Polymyxin B inhibited IL-8 released when compared with controls. Results are expressed as the mean ± SD of three independent experiments.

5.9 DETERMINATION OF LPS ENDOTOXIC ACTIVITY OF HEAT KILLED BACTERIA AND CONDITIONED MEDIA BY LAL ASSAY

Lipopolysaccharide is a major potent component of the cell wall in Gram-negative bacteria. The endotoxin activity of LPS that may be present in heat killed and conditioned media from different strains of *B. cepacia* and *P. aeruginosa* were investigated and measured using a kinetic LAL assay. The results showed that conditioned media from different bacteria tested here have very low endotoxic activity when assayed.

The endotoxin activity in HK and CM used in our IL-8 stimulation experiments were determined using the LAL assay and based on the amount of CM used in our experiment (10 mL) which originated from 10^6 bacteria, we concluded that condition CM from *B. cepacia* ET-10 and *B. cepacia* NCTC 10661 is equivalent to 3 ng and 2 ng of endotoxic LPS activity respectively, *P. aeruginosa* clinical strain and *P. aeruginosa* NCTC 10662 have 10ng and 4ng of endotoxic LPS activity respectively. Polymyxin B abolished completely the endotoxin activity in all CM tested in our study (see table 2).

Endotoxin activity of LPS may be present in heat killed bacteria (HK) of different strains of *B. cepacia* and *P. aeruginosa* were measured using LAL assay. The effects of Polymyxin B on this activity were measure and compared to the activity without treatment with Polymyxin B. The result showed that heat killed bacteria tested here harbor endotoxic activities when compared with the same LPS.

The results show that Polymyxin B significantly decreased LPS activity for all LPSs and heat killed bacteria tested here. Polymyxin B (10 μg/mL) also neutralised over 50% of the endotoxic activities of LPS tested here. Polymyxin B also neutralised the endotoxic activity of heat killed *P. aeruginosa and B. cepacia* by more than 90% (Table 2).

The amounts of endotoxin in heat killed bacteria used in our stimulation experiments were determined based on the result of the LAL assay and based on the amount of HK used (10 mL) which have 10^6 bacteria, we conclude that heat killed bacteria of *B. cepacia* ET-12 and *B. cepacia* NCTC 10661 harbor 32 ng, 43 ng of endotoxic activity respectively, *P. aeruginosa* clinical strain and *P. aeruginosa* NCTC 10662 harbor 56ng and 42ng of endotoxic activity respectively.

	Heat killed Bacteria (HK)	**Heat killed bacteria + Polymyxin B**	**Conditioned media (CM)**	**Conditioned media CM + Polymyxin B**
***B. cepacia* ET-12**	322	9	3	*
***B. cepacia* NCTC10661**	344	5	2	*
***P. aeruginosa* clinical strain**	425	21	10	*
***P. aeruginosa* NCTC 10662**	561	17	4	*

Table 2: Endotoxin activity of heat killed bacteria and conditioned media from different bacteria was measured by LAL assay. LPS, heat killed or conditioned media from bacteria was incubated with and without Polymyxin B (PMB) for 30 minutes prior to LAL. The results of endotoxin activity was calculated and expressed in ng/mL. * below detection.

5.10 EFFECT OF SURFACTANT LIPIDS ON IL-8 mRNA INDUCTION

The previous results showed that IL-8 protein is induced by; live bacteria, heat killed bacteria and bacterial conditioned media. At the same time this study demonstrated that surfactant Survanta® Curosurf® and DPPC lipid down regulated this induction. To investigate whether these infectious agents activated gene transcription and if the surfactants could inhibit this transcription or merely blocked protein secretion, IL-8 mRNA was isolated and measured by semi-mutative RT-PCR.

5.10.1 Effect of surfactant lipids on IL-8 mRNA induced by live bacteria in the presence and absence of human serum

In the previous results we showed that surfactants Survanta®, Curosurf® and DPPC lipid down regulate secreted IL-8 protein induced by live bacteria from A549 epithelial cells. To investigate this effect of surfactant at the IL-8 mRNA level, the conflunt A549 cells were weaned with RPMI media has L-glutamine only and incubated for 4 hours with different surfactants at 250 μg/mL for 2 hours before being challenged with the different bacteria in the absence and presence of 1% human serum. mRNA was then isolated, quantified and transcribed. The resultant cDNA was subjected to PCR using IL-8 or GAPDH primers and separated and assessed by gel electrophoresis (2.2.20.5).

The results are shown in Figures 5.27-5.28. Results of PCR analysis showed that both live NCTC and clinical strain of *B. cepacia* and *P. aeruginosa* significantly ($P<0.05$, n= 3) induced IL-8 mRNA from A549 epithelial cells. 4 hours after stimulation with live bacteria, the expression of IL-8 mRNA was increased 6-7 fold, in comparison to untreated cells. Survanta® and DPPC significantly down-regulated the IL-8 mRNA expression induced by *B. cepacia* NCTC 10661 (Fig 5.25), *B. cepacia* ET-12 (5.28), *P. aeruginosa* NCTC 10662 (Fig 5.29) and *P. aeruginosa* clinical strain (Fig5.25) in the presence of 1% human serum.

The results demonstrated that pulmonary surfactant Survanta® and DPPC phospholipid at 250 μg/mL significantly down regulated IL-8 gene expression induced by both NCTC and clinical strains of *B. cepacia* and *P. aeruginosa*.

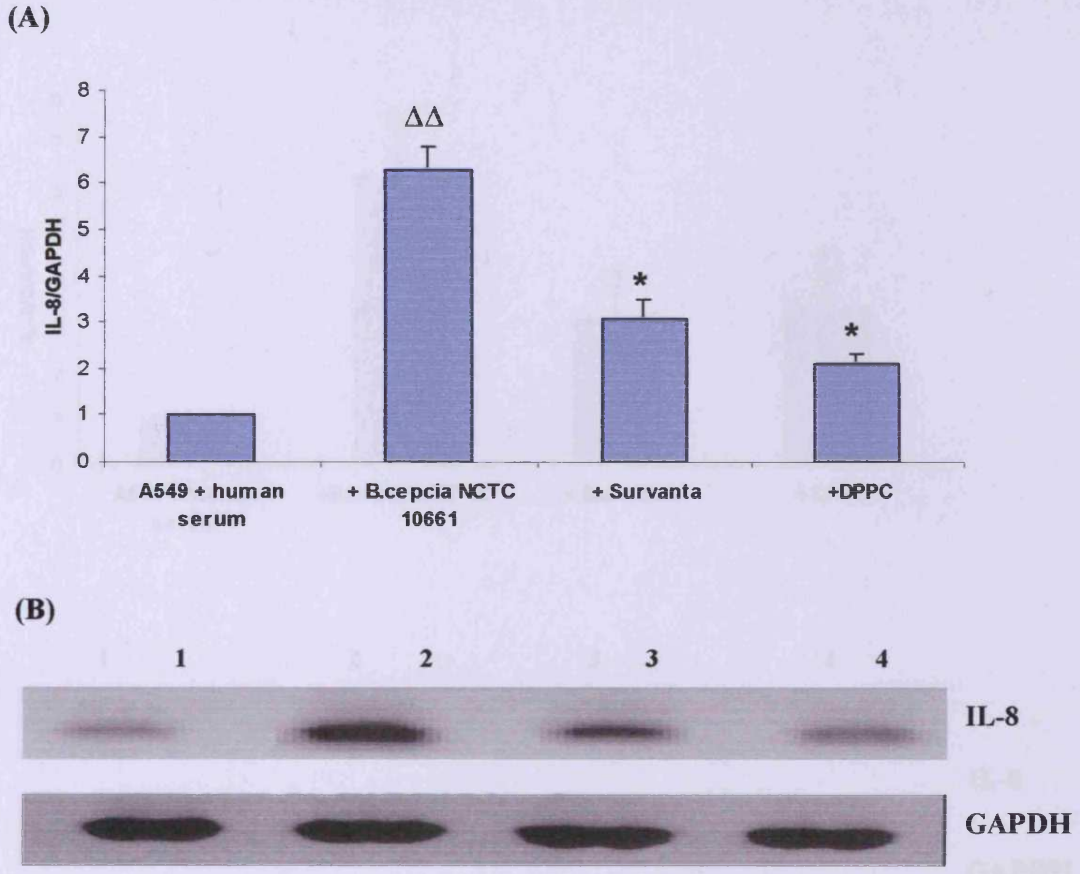
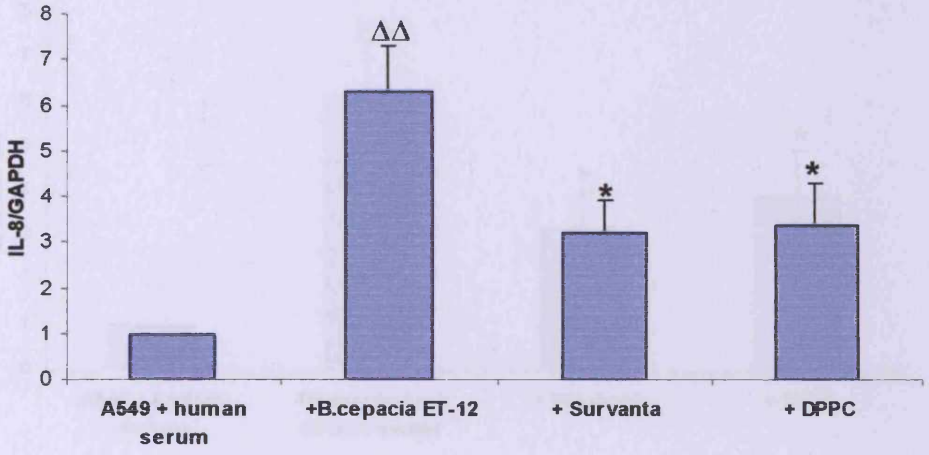


Figure 5.25: PCR analysis of IL-8 mRNA induced by *B. cepacia* NCTC 10661 with and without surfactant treatment. Monolayers of A549 cells were incubated with Survanta® or DPPC (250μg/mL) for 2h prior to stimulation with *B. cepacia* NCTC 10661 at moi (10 bacteria: A549) for 4 hours in presence of human serum. Total RNA was isolated and RT-PCR was performed. (A): Densitometric analysis was performed using (UVIDoc software) for all bands and the volumetric means for each test group were divided by the mean of the control (cells without stimulation). The expression were plotted as a ratio of IL-8 mRNA to GAPDH mRNA and the results shown as the mean ± SD of 3 independent experiment ($\Delta\Delta P<0.001$ analysed by ANOVA and $*P<0.05$ by Tukey's). (B): Representative gel photo for IL-8 and GAPDH mRNA. Lane 1: A549 with human serum, 2: A549 with bacteria, 3 & 4: As lane 2 but treated with Survanta® or DPPC respectively before stimulation.

(A)



(B)

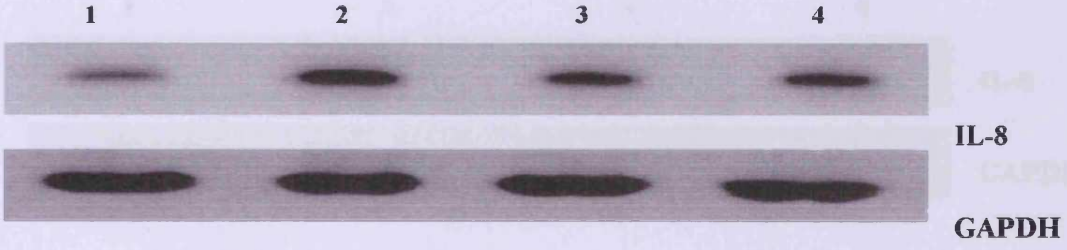
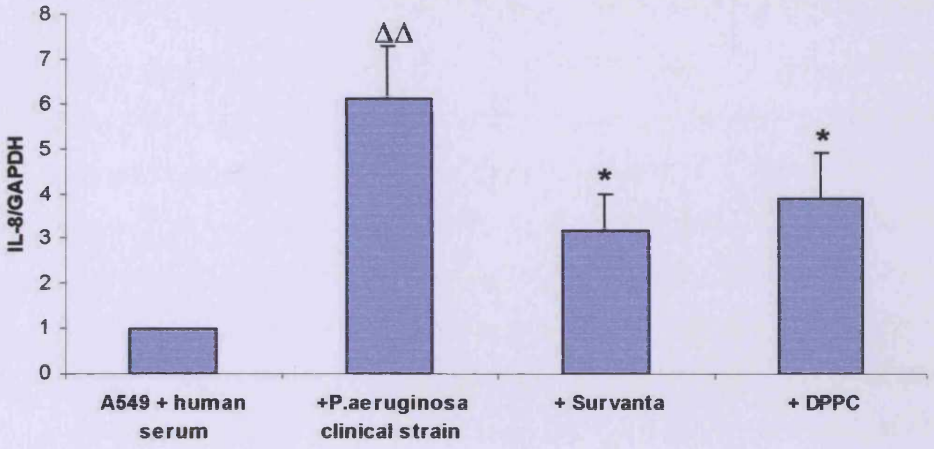


Figure 5.26: PCR analysis of IL-8 mRNA induced by *B. cepacia ET-12* with and without surfactant treatment. Monolayers of A549 cells were incubated with Survanta® or DPPC (250μg/mL) for 2h prior to stimulation with *B. cepacia* ET-12 at moi 10 bacteria: 1 A549 for 4 hours in presence human serum. Total RNA was isolated and RT-PCR was performed. (A): Densitometric analysis was performed using (UVIDoc software) for all bands and the volumetric means for each test group were divided by the mean of the control (cells without stimulation). The expression were plotted as a ratio of IL-8 mRNA to GAPDH mRNA and the results shown as the mean ± SD of 3 independent experiment ($\Delta\Delta P<0.001$ analysed by ANOVA and $*P<0.05$ by Tukey's). (B): Representative gel photo for IL-8 and GAPDH mRNA. Lane 1: A549 with human serum, 2: A549 with bacteria, 3 & 4: As lane 2 but treated with Survanta® or DPPC respectively before stimulation.

(A)



(B)

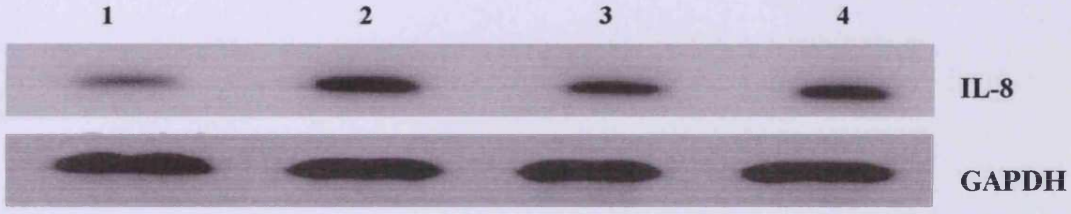


Figure 5.28: PCR analysis of IL-8 mRNA induced by *P. aeruginosa* clinical strain with and without surfactant treatment. Monolayers of A549 cells were incubated with Survanta® or DPPC (250µg/mL) for 2h prior to stimulation with *P. aeruginosa* clinical strain at moi 10 bacteria: 1 A549 for 4 hours in presence of human serum. Total RNA was isolated and RT-PCR was performed. (A): Densitometric analysis was performed using (UVIDoc software) for all bands and the volumetric means for each test group were divided by the mean of the control (cells without stimulation). The expression were plotted as a ratio of IL-8 mRNA to GAPDH mRNA and the results shown as the mean ± SD of 3 independent experiment ($\Delta\Delta P<0.001$ analysed by ANOVA and $*P<0.05$ by Tukey's). (B): Representative gel photo for IL-8 and GAPDH mRNA. Lane 1: A549 with human serum, 2: A549 with bacteria, 3 & 4: As lane 2 but treated with Survanta® or DPPC respectively before stimulation.

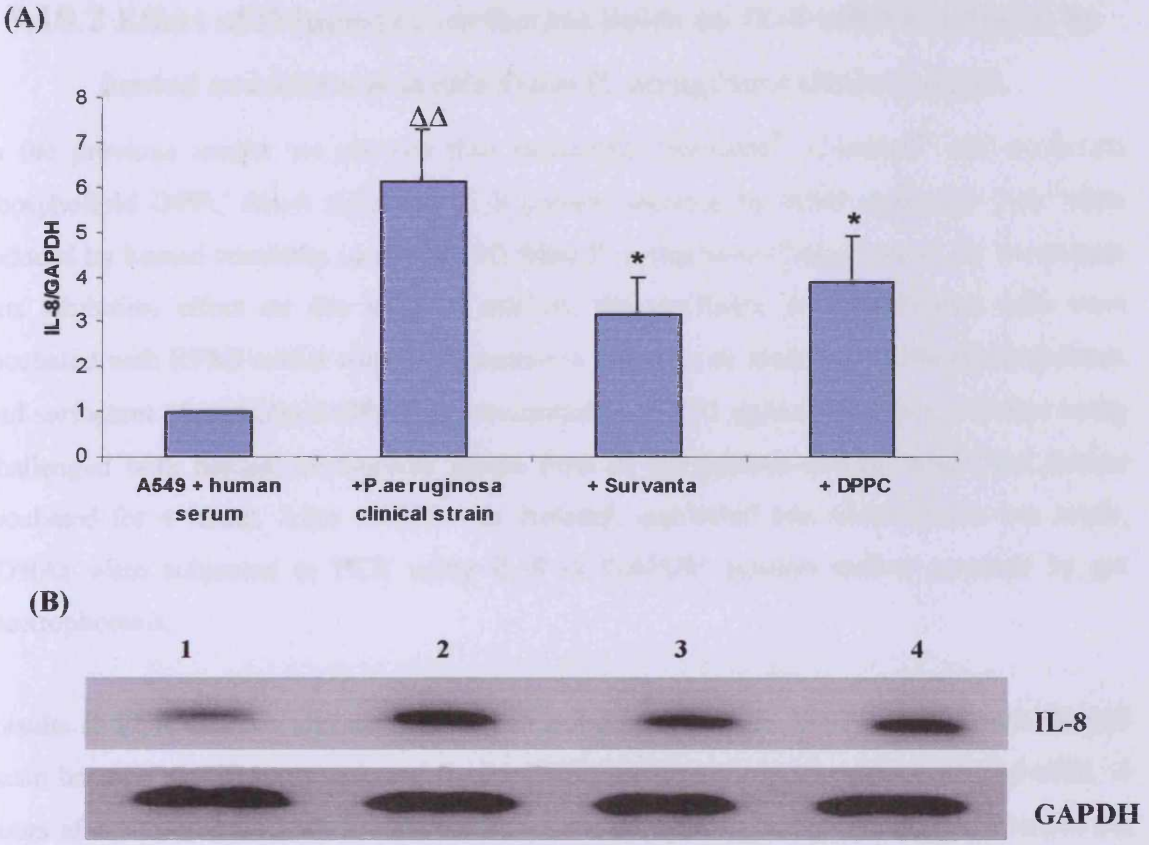


Figure 5.28: PCR analysis of IL-8 mRNA induced by *P. aeruginosa* clinical strain with and without surfactant treatment. Monolayers of A549 cells were incubated with Survanta® or DPPC (250μg/mL) for 2h prior to stimulation with *P. aeruginosa* clinical strain at moi 10 bacteria: 1 A549 for 4 hours in presence of human serum. Total RNA was isolated and RT-PCR was performed. (A): Densitometric analysis was performed using (UVIDoc software) for all bands and the volumetric means for each test group were divided by the mean of the control (cells without stimulation). The expression were plotted as a ratio of IL-8 mRNA to GAPDH mRNA and the results shown as the mean ± SD of 3 independent experiment ($\Delta\Delta P<0.001$ analysed by ANOVA and $*P<0.05$ by Tukey's). (B): Representative gel photo for IL-8 and GAPDH mRNA. Lane 1: A549 with human serum, 2: A549 with bacteria, 3 & 4: As lane 2 but treated with Survanta® or DPPC respectively before stimulation.

5.10.2 Effect of Pulmonary surfactant lipids on IL-8 mRNA induced by heated conditioned media from *P. aeruginosa* clinical strain

In the previous results we showed that surfactants Survanta®, Curosurf® and surfactant phospholipid DPPC down regulated IL-8 protein secreted by A549 epithelial cells when induced by heated condition media (hCM) from *P. aeruginosa* clinical strain. To investigate this inhibition effect on the level of mRNA, the confluent A549 epithelial cells were incubated with RPMI media with L-glutamine in presence or absence of different surfactants and surfactant phospholipid DPPC at concentration of 250 μg/mL for 2 hours before being challenged with heated conditioned media from *P. aeruginosa* clinical strain and further incubated for 4 hours. After mRNA was isolated, quantified and transcription was made, cDNAs were subjected to PCR using IL-8 or GAPDH primers before assessed by gel electrophoresis.

Results of PCR analysis showed that heated conditioned media from *P. aeruginosa* clinical strain bacteria significantly induced IL-8 mRNA ($P<0.05$, $n= 3$) by A549 epithelial cells. 4 hours after stimulation with heated condition media from *P. aeruginosa* clinical strain, the expression of IL-8 mRNA was more than 3 times higher when compared to the matched untreated cells. The effect of surfactants Survanta® and phospholipid DPPC on mRNA expression were clearly and significantly inhibited when compared with matched control cells (Fig 5.29).

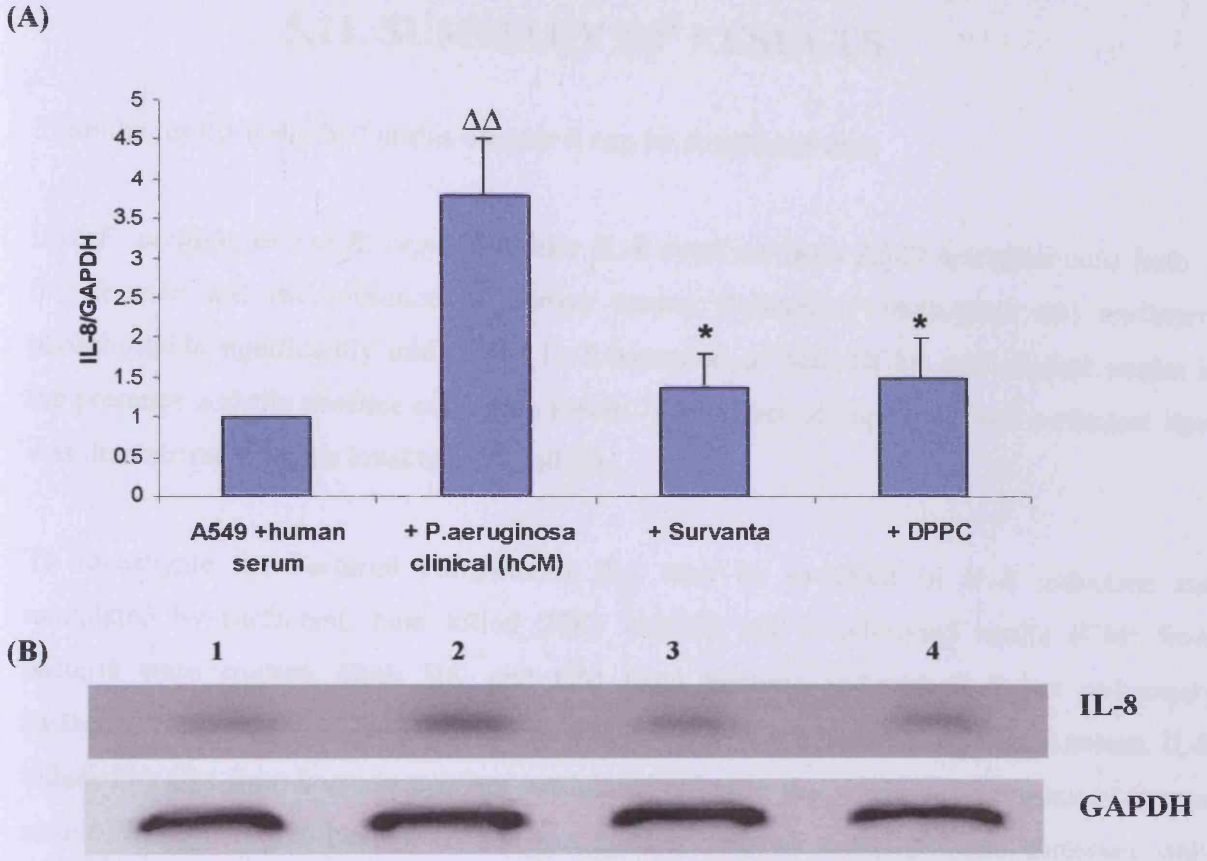


Figure 5.29: PCR analysis of IL-8 mRNA induced by heated conditioned media from *P. aeruginosa* clinical strain with and without surfactant treatment. Monolayers of A549 cells were incubated with Survanta® or DPPC (250μg/mL) for 2h prior to stimulation with heated conditioned media from *P. aeruginosa* clinical strain at moi equal to 10 bacteria: 1 A549 for 4 hours in presence of human serum. The total RNA was isolated and RT-PCR was performed. (A): Densitometric analysis was performed using (UVIDoc software) for all bands and the volumetric means for each test group were divided by the mean of the control (cells without stimulation). The expression were plotted as a ratio of IL-8 mRNA to GAPDH mRNA and the results shown as the mean ± SD of 3 independent experiment ($\Delta\Delta P<0.001$ analysed by ANOVA and $*P<0.05$ by Tukey's). (B): Representative gel photo for IL-8 and GAPDH mRNA. Lane 1: A549 with human serum, 2: A549 with bacteria, 3 & 4: As lane 2 but treated with Survanta® or DPPC respectively before stimulation.

5.11. SUMMARY OF RESULTS

From the results described in this chapter it can be concluded that:

Live *P. aeruginosa* and *B. cepacia* induce IL-8 cytokine from A549 epithelial cells both in the absence and the presence of human serum. Pulmonary surfactants and surfactant phospholipids significantly inhibit this IL-8 induction of both NCTC and clinical strains in the presence and the absence of human serum. This effect of surfactant and surfactant lipid was demonstrated on the level of IL-8 mRNA.

To investigate the bacterial components that may be involved in IL-8 induction and modulated by surfactant, heat killed (HK) bacteria and conditioned media (CM) from bacteria were studied. Both HK and CM from bacteria induced IL-8 but pulmonary surfactants only inhibited IL-8 induced by HK bacteria in the presence of human serum. IL-8 induced by CM from bacteria was not modulated either in the absence or presence of human serum. When CM was heated, IL-8 induction was reduced and pulmonary surfactant only modulated IL-8 induced by CM from *P. aeruginosa* clinical strain when serum was included. This effect of pulmonary surfactants was shown at the level of IL-8 gene transcription.

The involvement of TLR4, TLR2 and CD14 in IL-8 induction by live bacteria was investigated by using blocking antibodies. The results demonstrated that TLR4 components are involved in IL-8 induced by live bacteria. Further identification of the bacterial component utilized the down regulation effect by surfactant, neutralization with Polymyxin B and LAL assay was performed, and the results demonstrated that LPS was involved in the induction of IL-8 and as well part of the modulated components by surfactant when IL-8 induced by bacteria. Further characterisation of these components will be investigated in the next chapter.

CHAPTER 6. RESULTS

A549 CELL RESPONSES TO BACTERIAL CELL WALL COMPONENTS AND THE EFFECT OF PULMONARY SURFACTANT LIPIDS

6.1 LIPOPOLYSACCHARIDE (LPS) ISOLATION AND BIOACTIVITY

Lipopolysaccharide (LPS) was isolated from *P. aeruginosa* clinical strain, *B. cepacia* ET-12 and *B. cepacia* NCTC 10661 using the phenol-water procedure (2.2.7.2). The purified LPS were dissolved in pyrogen-free water at a stock concentration of 1mg/mL. To confirm the LPS isolation, the endotoxic activity of purified LPS and commercially available LPS from *P. aeruginosa* serotype 10, were measured using the kinetic Limulus endotoxin (LAL) assay. The effects of Polymyxin B on this activity also were determined and compared with the activity without treatment with Polymyxin B.

The results showed that LPS isolated from *P. aeruginosa* clinical strain has endotoxin activity > *P. aeruginosa* serotype 10 LPS> *B. cepacia* NCTC 10661> *B. cepacia* ET-12. The results also demonstrated that incubating LPS (100ng/mL) with Polymyxin B 10 μg/mL for 30 minutes inhibited the endotoxic activity by more than 50% as determined using the LAL assay.

6.2 A549 CELLS RESPONSE TO (LPS)

The A549 response to different types of LPS on the release of IL-1β and IL-8 cytokines was investigated in the absence and presence of human serum. LPS, PGN or LTA at the concentration used in our study in the presence or absence of Survanta®, Curosurf® or DPPC were not toxic to the A549 epithelial cells.

6.2.1 Dose response of LPS on IL-β and IL-8 induction in the absence and presence of human serum

A549 epithelial cell monolayers in 12 well tissue culture plates were grown in complete RPMI media with antibiotics for 2 days before the cells were weaned with RPMI medium with L-glutamine only overnight before being incubated overnight with or without different concentrations of LPS (10, 100, 250, 500, 1000 ng/mL) in absence or presence of 1% human serum.

A549 cells did not release IL-1β cytokine upon incubation with different concentrations of LPS from *P. aeruginosa* and *B. cepacia*. All isolated LPS from *P. aeruginosa, B. cepacia* as well as the commercial LPS from *P. aeruginosa* serotype 10 did not induce IL-1β in absence or presence of human serum even with 1000 ng/mL LPS and prolonged incubation to 72 hours (result not shown).

However the results showed that LPS induces IL-8 from A549 cells poorly in the absence of human serum (Fig 6.1) and strongly in a dose-dependant manner in the presence of human serum (Fig 6.2). In the absence of human serum, at least 500ng/ mL LPS was required to elicit IL-8 production but in the presence of human serum, 10ng/mL of LPS induced IL-8. Potency to stimulate IL-8 by LPS isolated from different bacteria was as follows: *P. aeruginosa* clinical strain> *P. aeruginosa* serotype 10> *B. cepacia* NCTC 10661> *B. cepacia* ET-12.

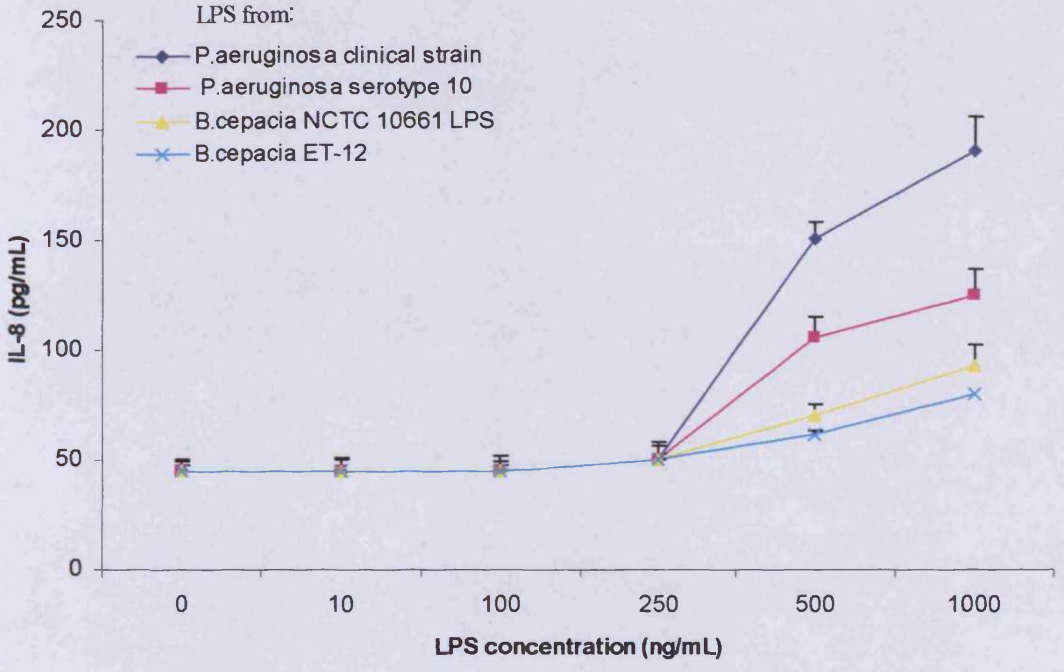


Fig 6.1: Induction of IL-8 responses with different LPS in A549 cells in the absence of human serum. A549 cells were incubated in complete RPMI media with antibiotics for two days. Wells were washed and media were changed to RPMI with L-glutamine only prior stimulation with different concentration of various LPS. Supernatants were collected after 18 hours and IL-8 was measured by ELISA. Results are expressed as mean ± SD of three independent experiments.

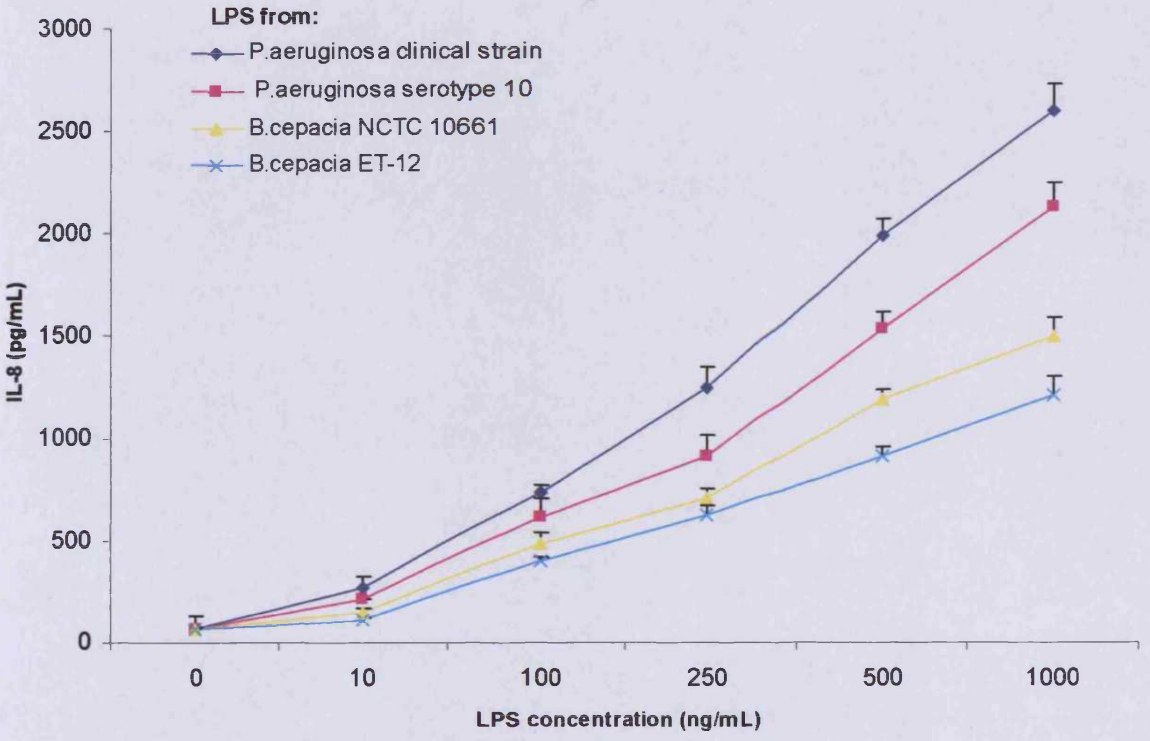


Fig 6.2: Induction of IL-8 responses with different LPS in A549 epithelial cells in the presence of human serum. A549 cells were incubated in complete RPMI media with antibiotics for two days. Wells were washed and media were changed RPMI with L-glutamine only prior stimulation with different concentration of various LPS in presence of 1% human serum. Supernatants were collected after 18 hours, aliquoted and were diluted with the same media when needed before IL-8 was measured by ELISA. Results were expressed as mean ± SD of three independent experiments.

6.3 EFFECT OF PULMONARY SURFACTANT LIPIDS ON LPS INDUCED IL-8 FROM A549 CELLS

To investigate the effect of different surfactants and surfactant lipid DPPC on the induction of IL-8 cytokine, A549 cells were grown for 2 days in complete media and the cells were weaned with media with L-glutamine only. The cells were then incubated with or without 250 μg/mL of Survanta®, Curosurf® or DPPC and incubated for 2 hours before challenged with 100ng/mL LPS for 18 hours.

The results show that each surfactant Survanta®, Curosurf® and DPPC lipid at a concentration of 250μg/mL markedly inhibited IL-8 production from A549 epithelial cells after 18 hours incubation with LPS from both *P. aeruginosa* clinical strain (Fig 6.3) and serotype 10 (Fig 6.4), *B. cepacia* NCTC 10661 and *B. cepacia* ET-12 (Fig 6.5, 6.6). Surfactant lipids inhibited 40-50% of IL-8 induced by LPS, and Survanta® inhibited more than Curosurf® more than DPPC.

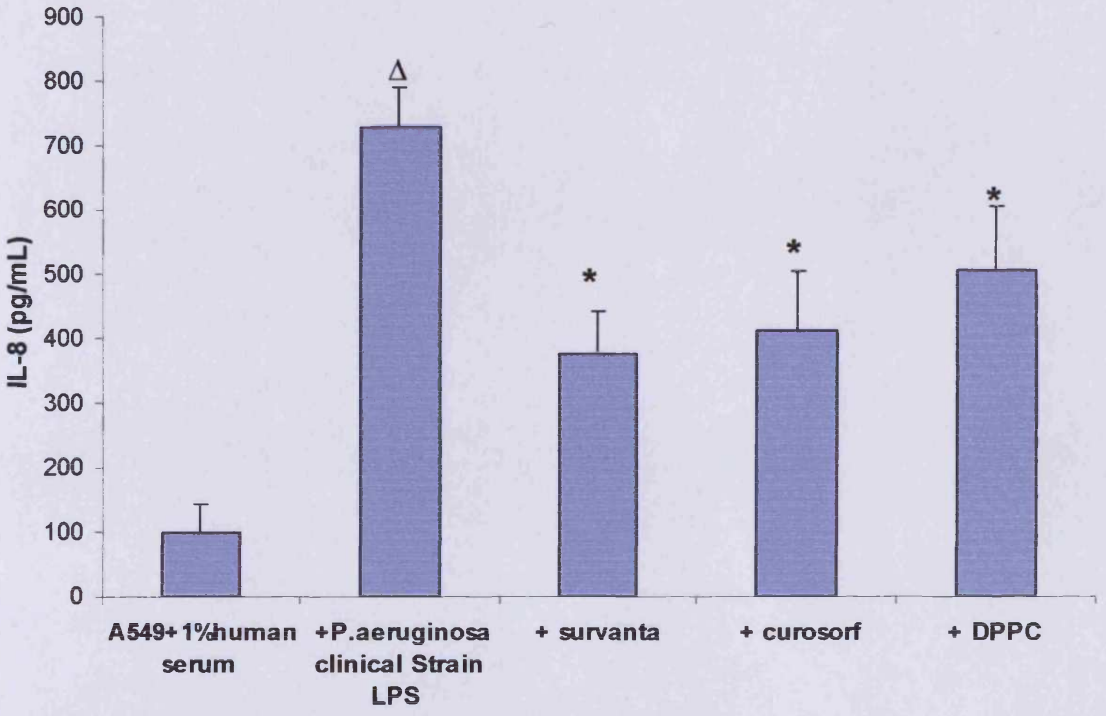


Fig 6.3: Effect of pulmonary surfactants on IL-8 induced by LPS from *P. aeruginosa* clinical strain. A549 cells were grown to confluence and incubated with 250 μg/mL of Survanta® Curosurf® or DPPC for 2 hours prior stimulation with LPS (100 ng/mL) in presence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Pre-treatment of A549 cells with surfactants significantly suppressed the IL-8 release. Results are expressed as the mean ± SD of three independent experiments. $\Delta P<0.01$ analysed by ANOVA and Tukey's ($^*P<0.05$) when comparing surfactant treatment to controls.

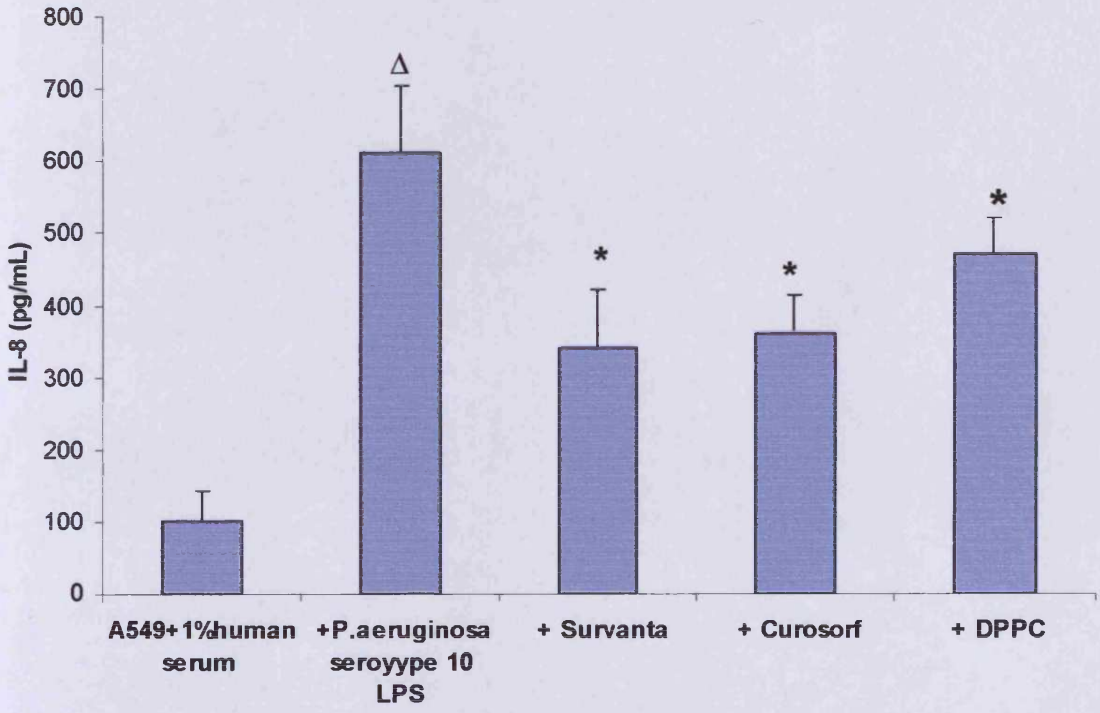


Fig 6.4: Effect of pulmonary surfactants on IL-8 induced by LPS from *P. aeruginosa* serotype 10. A549 cells were grown to confluence and incubated with 250 μg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior stimulation with LPS (100 ng/mL) in presence of human serum. Supernatants were collected after 18 hours and were by ELISA. Pre-treatment of A549 cells with surfactants significantly suppressed the IL-8 release. Results are expressed as the mean ± SD of three independent experiments. Δ$P<0.01$ analysed by ANOVA and Tukey's (*$P<0.05$) when comparing surfactant treatment to controls.

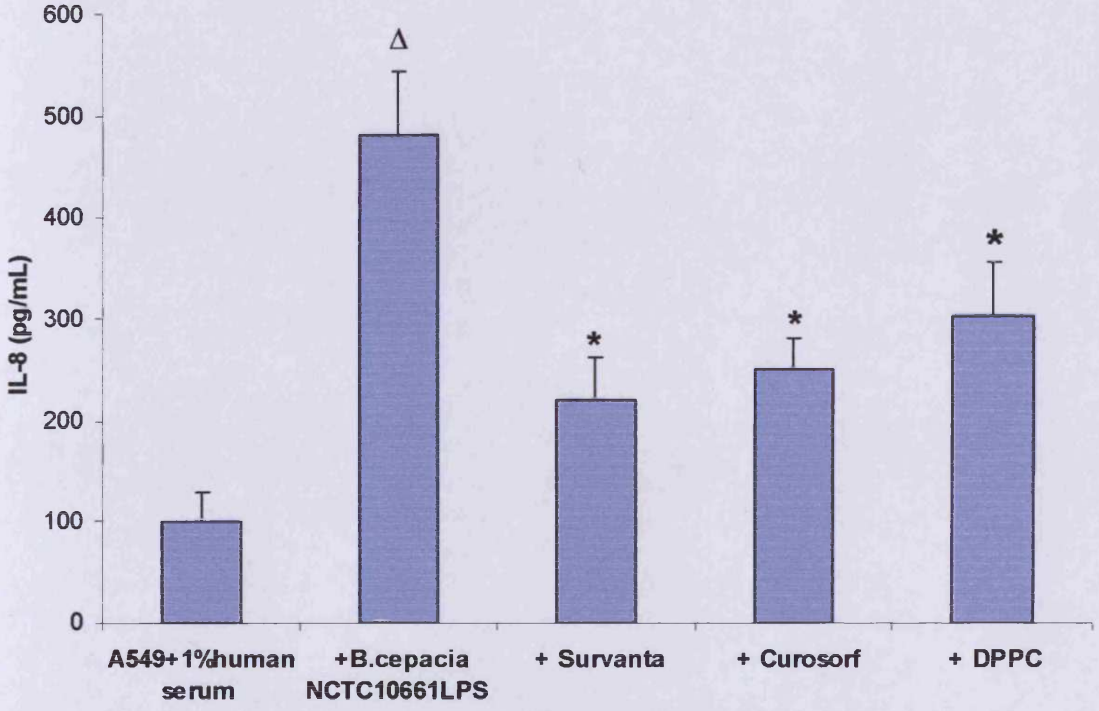


Fig 6.5: Effect of pulmonary surfactants on IL-8 induced by LPS from *B. cepacia* NCTC 1066. A549 cells were grown to confluence and incubated with 250 μg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior stimulation with LPS (100 ng/mL) in presence of human serum. Supernatants were collected after 18 hours and assayed by ELISA. Pre-treatment of A549 cells with surfactants significantly suppressed the IL-8 release. Results are expressed as the mean ± SD of three representative experiments. Δ$P<0.01$ analysed by ANOVA and Tukey's (*$P<0.05$) when comparing surfactant treatment to controls.

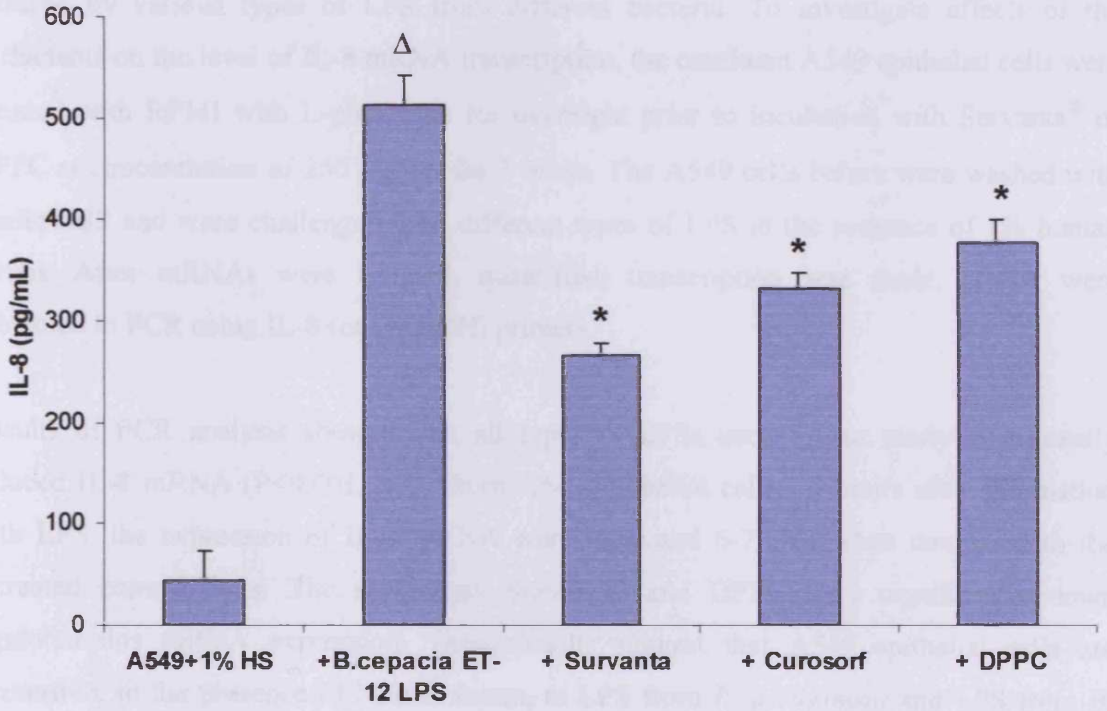


Fig 6.6: Effect of pulmonary surfactants on IL-8 induced by LPS from *B. cepacia* ET-12. A549 cells were grown to confluence and incubated with 250 µg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior stimulation with LPS (100 ng/mL) in presence of human serum. Supernatants were collected after 18 hours and assayed by ELISA. Pre-treatment of A549 cells with surfactants significantly suppressed the IL-8 release. Results are expressed as the mean ± SD of three independent experiments. Δ$P<0.01$ analysed by ANOVA and Tukey's (*$P<0.05$) when comparing surfactant treatment to controls.

6.4 EFFEECT OF PULMONARY SURFACTNTS LIPIDS ON LPS INDUCED IL-8 mRNA FROM A549 EPITHELIAL CELLS

In the previous ELISA results we showed that surfactants Survanta®, Curosurf® and surfactant phospholipid DPPC inhibited IL-8 protein from A549 epithelial cells that were induced by various types of LPS from different bacteria. To investigate effects of the surfactants on the level of IL-8 mRNA transcription, the confluent A549 epithelial cells were weaned with RPMI with L-glutamine for overnight prior to incubation with Survanta® or DPPC at concentration of 250 μg/mL for 2 hours. The A549 cells before were washed with sterile PBS and were challenged with different types of LPS in the presence of 1% human serum. After mRNAs were isolated, quantified, transcription was made, cDNA were subjected to PCR using IL-8 (or GAPDH) primers.

Results of PCR analysis showed that all types of LPSs used in our study significantly induced IL-8 mRNA ($P<0.001$, $n=3$) from A549 epithelial cells. 4 hours after stimulation with LPS, the expression of IL-8 mRNA were increased 6-7folds when compared to the untreated control cells. The surfactants Survanta® and DPPC lipid significantly down regulated this mRNA expression. These results suggest that A549 epithelial cells are responsive, in the presence of human serum, to LPS from *P. aeruginosa* and LPS from *B. cepacia* and that the surfactants Survanta® and DPPC modulated IL-8 mRNA induction.

In detail, the results show that pulmonary surfactant Survanta® and DPPC lipid at 250μg/mL inhibited IL-8 gene expression induced by LPSs from both *P. aeruginosa* and *B. cepacia* in a significant manner for *P. aeruginosa* clinical (Fig 6.7), *P. aeruginosa* serotype 10 (Fig 6.8), *B. cepacia* NCTC 10661 (Fig 6.9) and *B. cepacia* ET-12 (Fig 6.10).

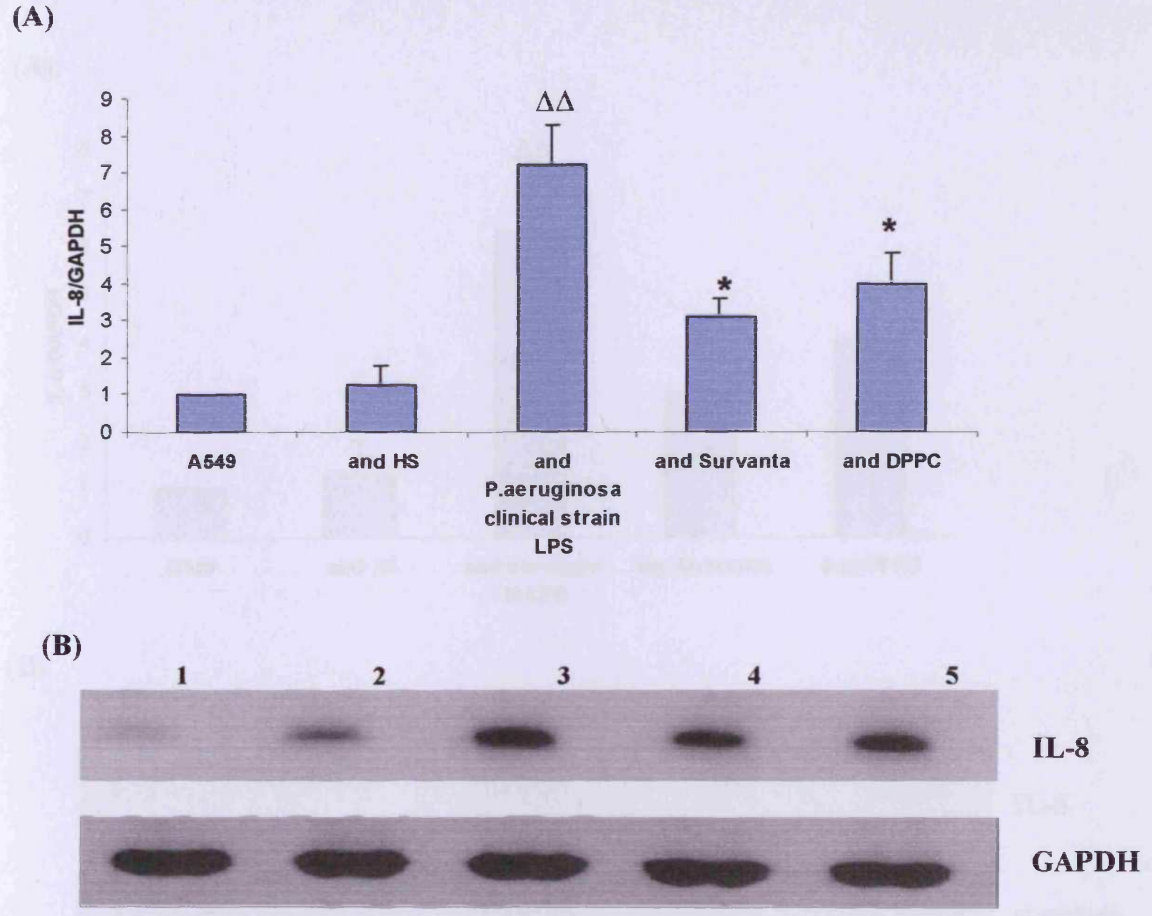


Fig 6.7: PCR analysis of IL-8 mRNA induced by LPS from *P. aeruginosa* clinical strain with and without surfactant treatment. The monolayers were incubated with Survanta® or DPPC (250μg/mL) for 2 hours prior to stimulation with LPS (100 ng/mL) in presence of 1% human serum. Total RNA was isolated and RT-PCR was performed. (A): Densitometric analysis was performed using (UVIDoc software) for all bands and the volumetric means for each test group were divided by the mean of the control (cells without stimulation). The expression were plotted as a ratio of IL-8 mRNA to GAPDH mRNA and the results shown as the mean ± SD of 3 independent experiment ($\Delta\Delta P<0.001$ analysed by ANOVA and $*P<0.05$ by Tukey's). (B): Representative gel photo for IL-8 and GAPDH mRNA. Lane 1: A549 with human serum, 2: A549 with bacteria, 3 & 4: As lane 2 but treated with Survanta® or DPPC respectively before stimulation.

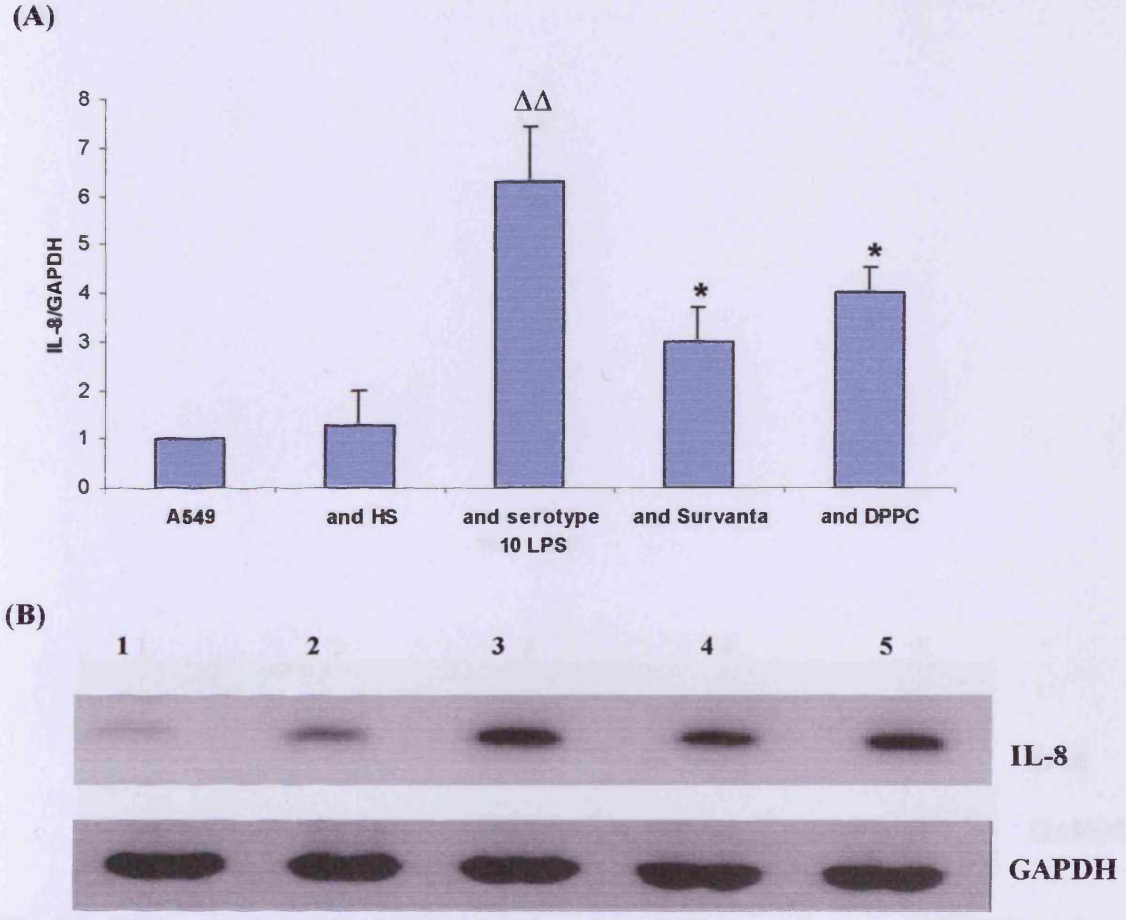


Fig 6.8: PCR analysis of IL-8 mRNA induced by LPS from *P. aeruginosa* serotype 10 with and without surfactant treatment. The monolayers were incubated with Survanta® or DPPC (250μg/mL) for 2 hours prior to stimulation with LPS (100 ng/mL) in the presence of 1% human serum. Total RNA was isolated and RT-PCR was performed. (A): Densitometric analysis was performed using (UVIDoc software) for all bands and the volumetric means for each test group were divided by the mean of the control (cells without stimulation). The expression were plotted as a ratio of IL-8 mRNA to GAPDH mRNA and the results shown as the mean ± SD of 3 independent experiment ($\Delta\Delta P<0.001$ analysed by ANOVA and $*P<0.05$ by Tukey's). (B): Representative gel photo for IL-8 and GAPDH mRNA. Lane 1: A549 with human serum, 2: A549 with bacteria, 3 & 4: As lane 2 but treated with Survanta® or DPPC respectively before stimulation.

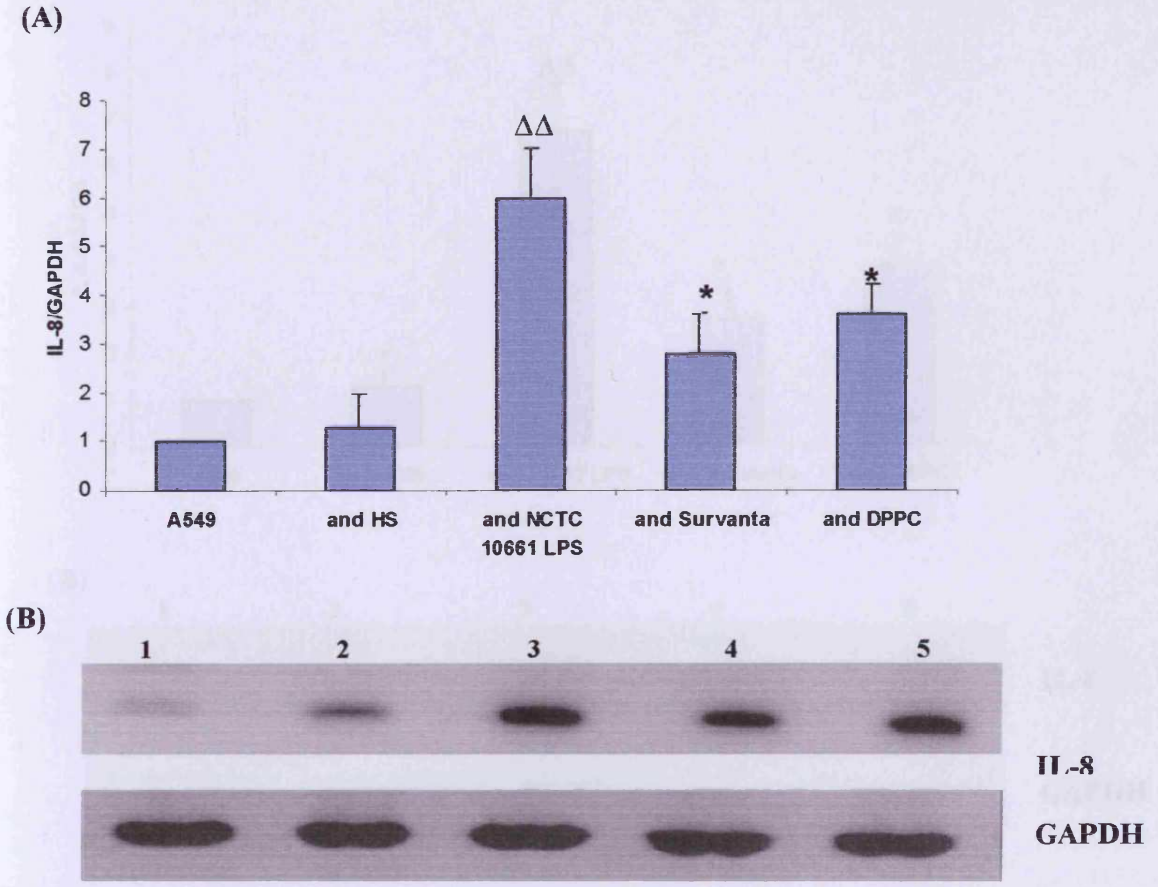


Fig 6.9: PCR analysis of IL-8 mRNA induced by LPS from *B. cepacia* NCTC with and without surfactant treatment. The monolayers were incubated with Survanta® or DPPC (250μg/mL) for 2 hours prior to stimulation with LPS (100 ng/mL) in the presence of 1% human serum. Total RNA was isolated and RT-PCR was performed. (A): Densitometric analysis was performed using (UVIDoc software) for all bands and the volumetric means for each test group were divided by the mean of the control (cells without stimulation). The expression were plotted as a ratio of IL-8 mRNA to GAPDH mRNA and the results shown as the mean ± SD of 3 independent experiment ($\Delta\Delta P<0.001$ analysed by ANOVA and $*P<0.05$ by Tukey's). (B): Representative gel photo for IL-8 and GAPDH mRNA. Lane 1: A549 with human serum, 2: A549 with bacteria, 3 & 4: As lane 2 but treated with Survanta® or DPPC respectively before stimulation.

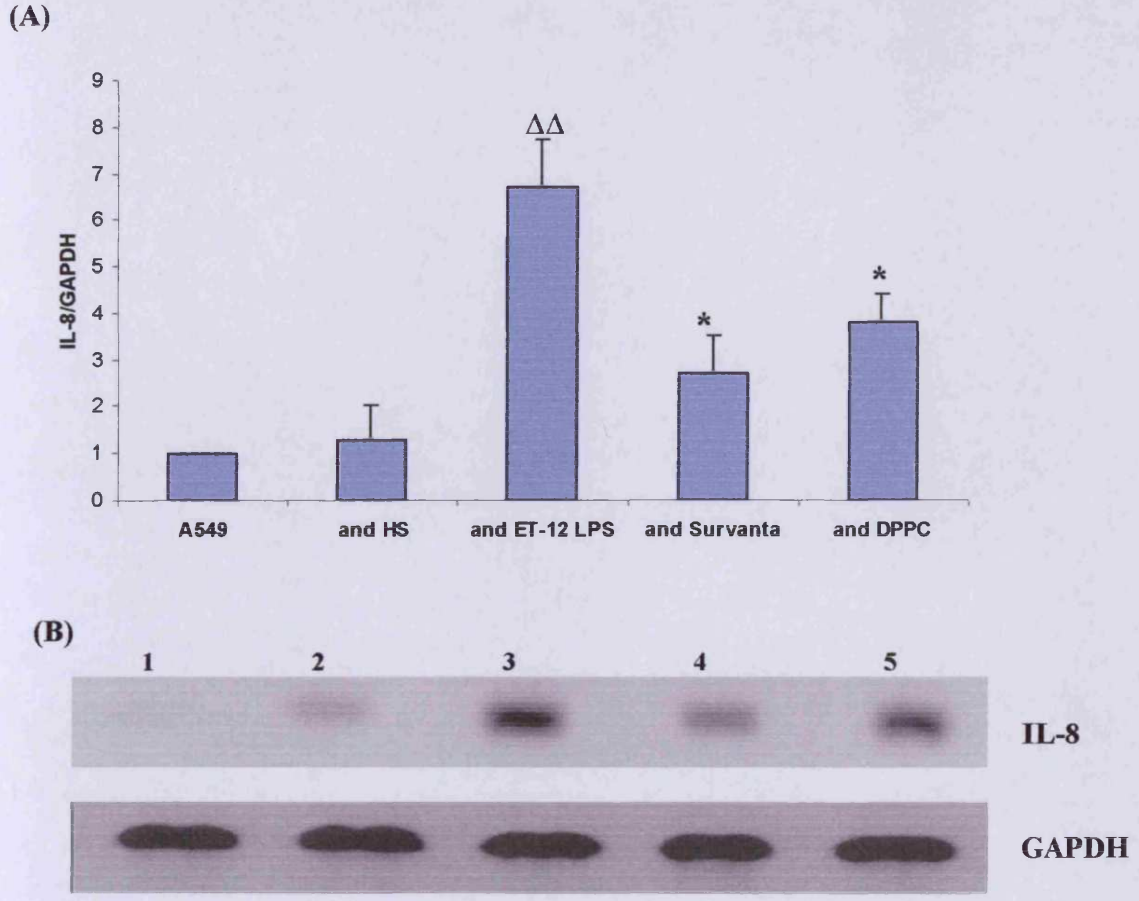


Fig 6.10: PCR analysis of IL-8 mRNA induced by LPS from *B. cepacia* ET-12 with and without surfactant treatment. The monolayers were incubated with Survanta® or DPPC (250μg/mL) for 2 hours prior to stimulation with LPS (100 ng/mL) in the presence of 1% human serum. Total RNA was isolated after 4 hours incubation and RT-PCR was performed. (A): Densitometric analysis was performed using (UVIDoc software) for all bands and the volumetric means for each test group were divided by the mean of the control (cells without stimulation). The expression were plotted as a ratio of IL-8 mRNA to GAPDH mRNA and the results shown as the mean ± SD of 3 independent experiment ($\Delta\Delta P<0.001$ analysed by ANOVA and $*P<0.05$ by Tukey's). (B): Representative gel photo for IL-8 and GAPDH mRNA. Lane 1: A549 with human serum, 2: A549 with bacteria, 3 & 4: As lane 2 but treated with Survanta® or DPPC respectively before stimulation.

6.5 EFFECT OF ANTI-TLR4 ON LIPOPOLYSACCHARIDE INDUCED IL-8 FROM A549 EPITHELIAL CELLS

Toll like receptor 4 (TLR4) is an essential element in the signal transduction of LPS. In this study the involvement of TLR4 receptor in IL-8 induction from A549 epithelial cells by LPS was investigated using anti-TLR4 antibody to block this receptor. A549 epithelial were incubated with or without anti-TLR4 antibody (HTA125) and isotype control antibody IgG2a at (20 μg/mL) for 1 hour at 37°C before adding the LPS (100ng/mL) and incubation for 18 hours in the presence of 1% human serum. The results show that pre-incubation of the A549 cells with anti-TLR4 antibody significantly reduced LPS-induced IL-8 from *P. aeruginosa* and *B. cepacia* when compared with untreated controls (Fig 6.11 and Fig 6.12).

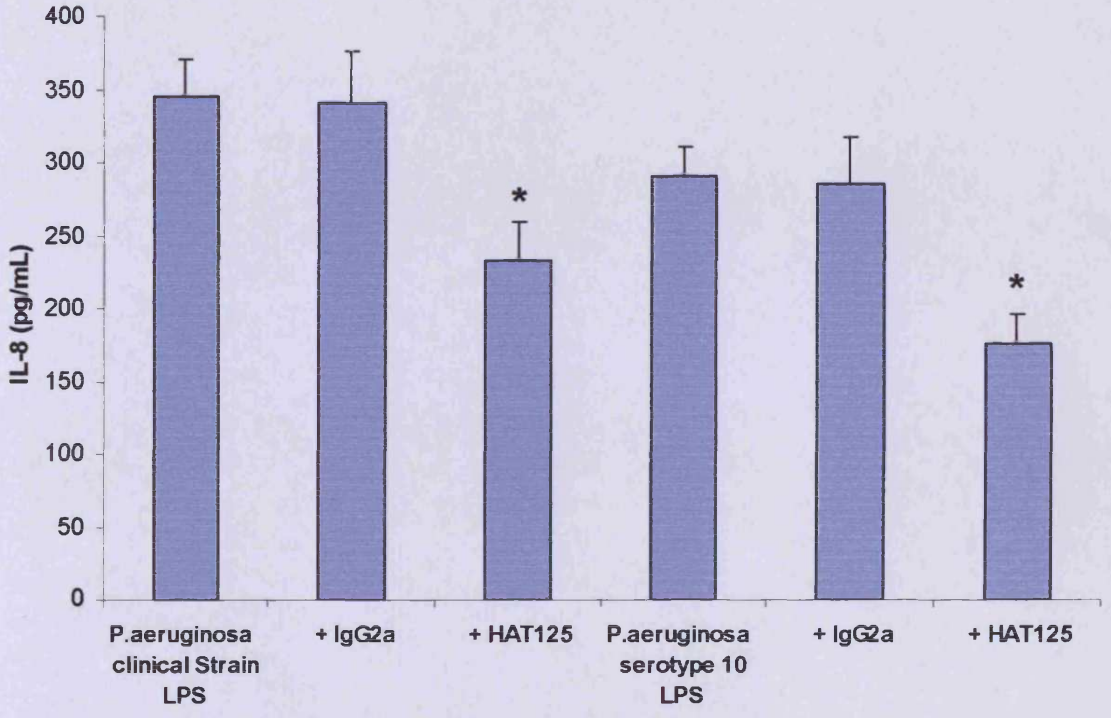


Fig 6.11 Effect of anti-TLR4 on IL-8 induced by LPS from *P. aeruginosa*. A549 epithelial monolayers were grown in tissue culture plates and weaned and media was changed to RPMI with L-glutamine only. Anti-TLR-4 antibody HTA125 or isotype control antibody IgG2a was added (20 μg/mL) and incubated for 1 hour prior to stimulation with LPS in the presence of 1% of human serum. Supernatants were collected after 18 hours incubation and IL-8 was measured by ELISA. Values are mean ± SD, n=3. *P<0.05 vs LPS only (analysed by Paired T-test).

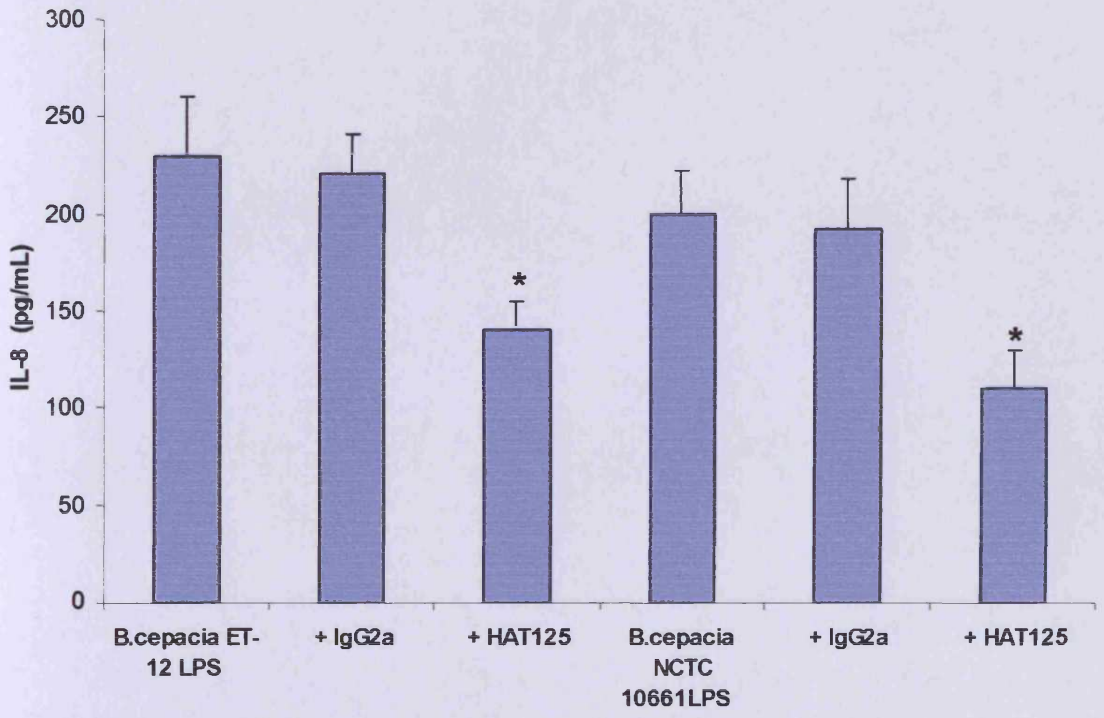


Fig 6.12 Effect of anti-TLR4 on IL-8 induced by LPS from *B. cepacia*. A549 epithelial monolayers were grown in tissue culture plates and were weaned and media was changed to RPMI with L-glutamine only. Anti-TLR-4 antibody HTA125 and isotype control antibody IgG2a was added (20 μg/mL) and incubated for 1hour prior to stimulation with LPS in the presence of 1% of human serum. Supernatants were collected after 18 hours and IL-8 was measured by ELISA. Values are mean ± SD, n=3. *P<0.05 vs. LPS alone (analysed by Paired T-test).

6.6 EFFECT OF ANTI-CD14 ON LIPOPOLYSACCHARIDE INDUCED IL-8 FROM A549 CELLS

The involvement of soluble CD14 (present in human serum) on IL-8 inducted by different types of LPS was investigated. The human serum was incubated with or without anti-CD14 antibody (clone: 134620) and isotype control antibody $IgG2_a$ at (20 μg/mL) for 1 hour prior to adding to the A549 cells in the presence 100 ng/mL of LPS. The results show that anti-CD14 antibody significantly inhibited IL-8 production when compared with control cells without blocking.

Blocking soluble CD14 significantly reduced the secretions of IL-8 cytokine by >45% when induced with LPS from *P. aeruginosa* LPS, serotype 10, *B. cepacia* ET-12 and *B. cepacia* NCTC 10661 respectively (Fig 6.13 and Fig 6.14). These results demonstrate the involvement of CD14 in human serum on LPS induced IL-8 from A549 epithelial cells

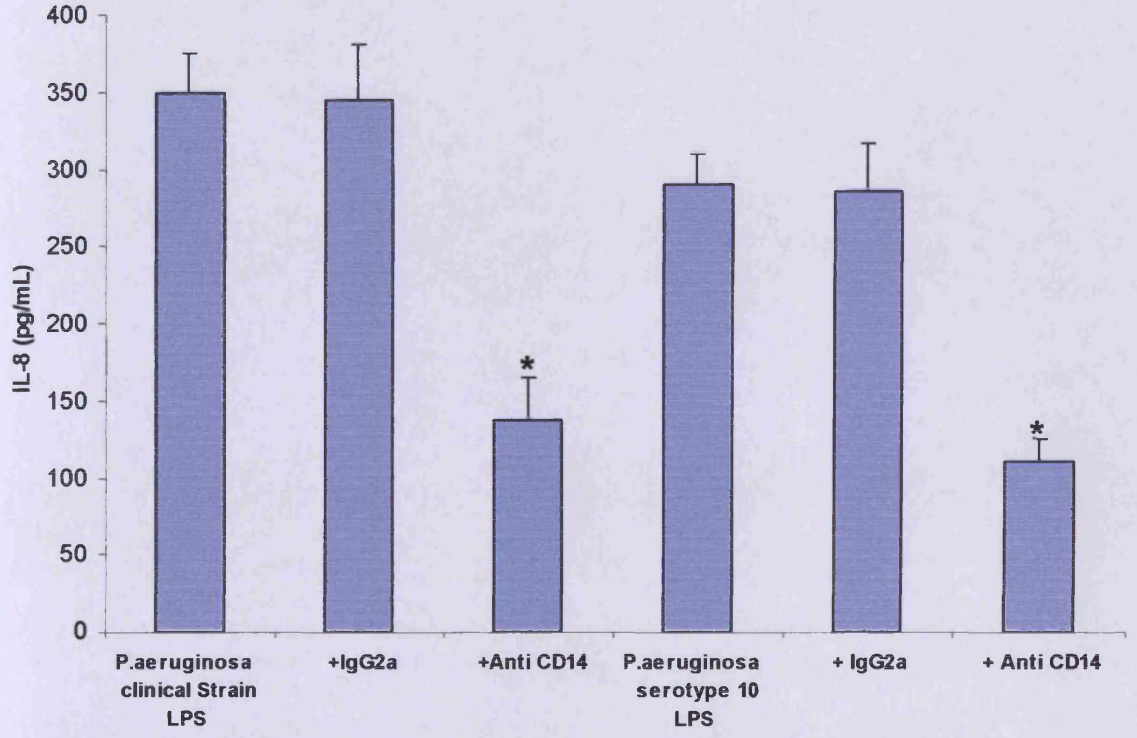


Fig 6.13 Effect of anti-CD14 on IL-8 induced by LPS from *P. aeruginosa*. A549 epithelial monolayers were grown in tissue culture plates and were weaned and media was changed to RPMI with L-glutamine only. Anti-CD14 antibody (clone: 134620) or isotype control antibody IgG2a (20 μg/mL) was incubated with human serum for 1 hour before adding to A549 cells in the presence of 100 ng/mL LPS. Supernatants were collected after 18 hours and IL-8 assayed by ELISA. Values are mean ± SD, n=3. *P<0.05 vs LPS alone (analysed by Paired T-test).

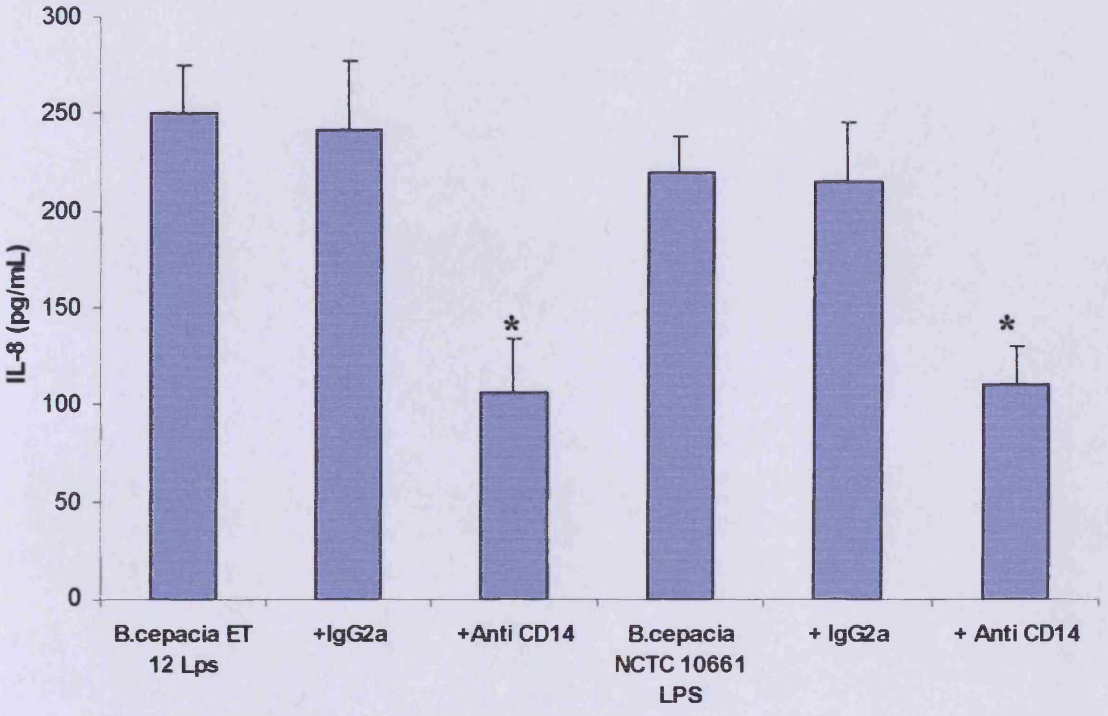


Fig 6.14: Effect of anti-CD14 on IL-8 induced by LPS from *B. cepacia*. A549 epithelial monolayers were grown in tissue culture plates and were weaned and media was changed to RPMI with L-glutamine only. Anti-CD14 antibody (clone: 134620) or isotype control antibody IgG2a (20 μg/mL) was incubated with human serum for 1 hour before adding to A549 cells in the presence of 100 ng/mL LPS. Supernatants were collected after 18 hours and IL-8 assayed by ELISA. Values are mean ± SD, n=3. *P<0.05 vs. LPS alone (analysed by Paired T-test).

6.7 A549 CELL RESPONSES TO PEPTIDOGLYCAN (PGN)

Previous results demonstrated that pulmonary surfactants down-regulated IL-8 induced by live and heat killed bacteria. Peptidoglycan is one of the cell wall components that may be involve in IL-8 induction, and surfactants may possibly modulate their active actions. Before evaluating the effects of the surfactants Survanta®, Curosurf® and DPPC lipid on IL-1β & IL-8 cytokines, the dose response of peptidoglycan (PGN) on induction of IL-1β and IL-8 from A549 epithelial cells was investigated.

6.7.1 Dose responses of PGN and IL-8 induction in absence and presence of human Serum

A549 epithelial cells monolayers at density of ($5x10^5$ cell /mL) in 12 well tissue culture plates were grown in complete RPMI media with antibiotic for 2 days before the cells were weaned with RPMI medium containing L-glutamine only overnight and incubated with different concentrations of PGN (10, 100, 250, 500, 1000 ng/mL) in the absence or in the presence of 1% human serum.

The results demonstrated that A549 epithelial cells did not release IL-1β cytokine upon stimulation with different concentrations of peptidoglycan (PGN) up to 1000 ng/mL in the presence or absence of human serum (data not shown).

A549 cells show insignificant responses to low doses of PGN (10-250 ng/mL) for IL-8 induction in the absence of human serum. This amount of IL-8 was increased and became significant with the increase of PGN concentration ≥500 ng/mL or more (Fig 6.15). In presence of human serum, the A549 cells showed more response to PGN and released IL-8 cytokine to a lower dose (250ng/mL) when compared with induction without human serum (Fig 6.15).

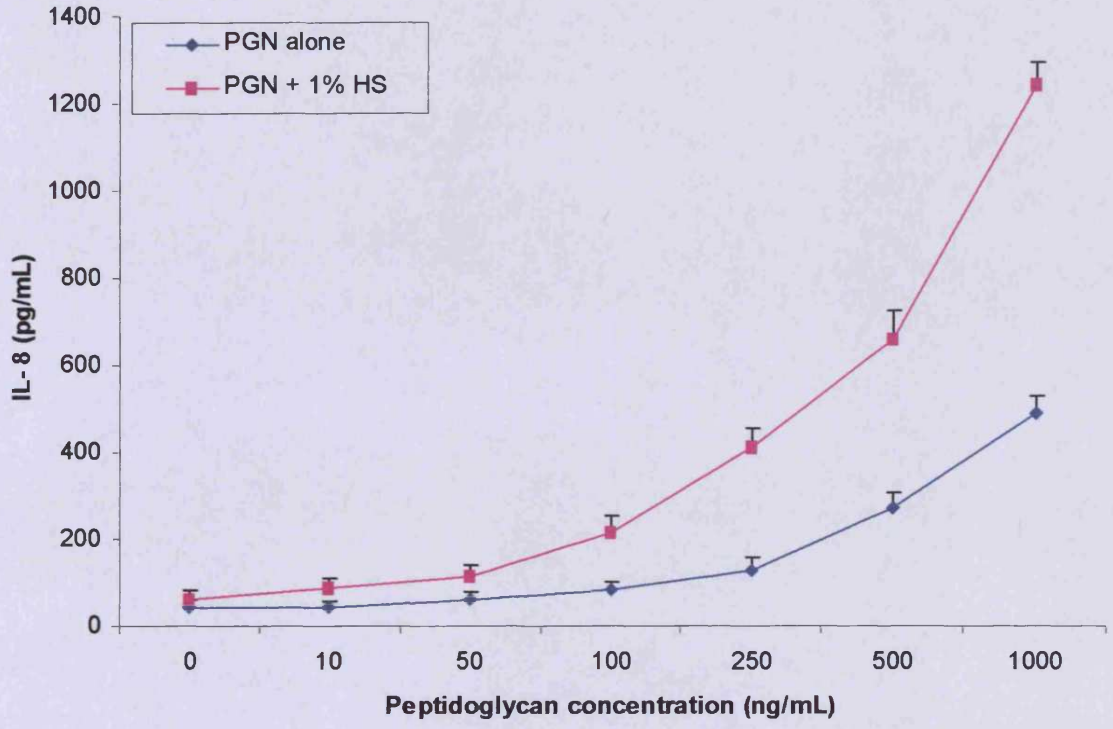


Fig 6.15: Dose responses of Peptidoglycan and IL-8 induction from A549 epithelial cells. A549 cells were incubated in complete RPMI media with antibiotics for two days. Wells were washed and media were changed to RPMI with L-glutamine prior stimulation with different concentration of PGN in the presence or absence of human serum. Supernatants were collected after 18 hours and IL-8 cytokines was measured by ELISA assay. Results are expressed as mean ± SD of three independent experiments

6.7.2 Effect of pulmonary surfactant lipids on peptidoglycan induced IL-8 from A549 epithelial cells in absence and presence of human serum

The induction of IL-8 by peptidoglycan from A549 epithelial cells was investigated in the absence of human serum to characterise the possible effect of pulmonary surfactant on the early response of the epithelial cells. At the same time the effect of pulmonary surfactant and surfactant lipid was investigated in the presence of human serum to mimic the early injury of the lung and the leakage of serum.

The effects of different surfactants and surfactant lipid DPPC on the induction of IL-8 was investigated by pre-incubation the A549 epithelial cells with 250 μg/mL of Survanta®, Curosurf® and DPPC at 250μg/mL for 2 hours prior to addition of PGN (500ng/mL) in the absence and presence of human serum. The result show that in absence (data not shown) and presence of human serum, IL8 induced by PGN was not modulated by surfactant Survanta®, Curosurf® or DPPC lipid (Fig 6.16 showed the effect of surfactants in presence of human serum). Increasing the concentration of surfactant to 500μg/mL did not show any different change on PGN induced IL-8 (Data not shown).

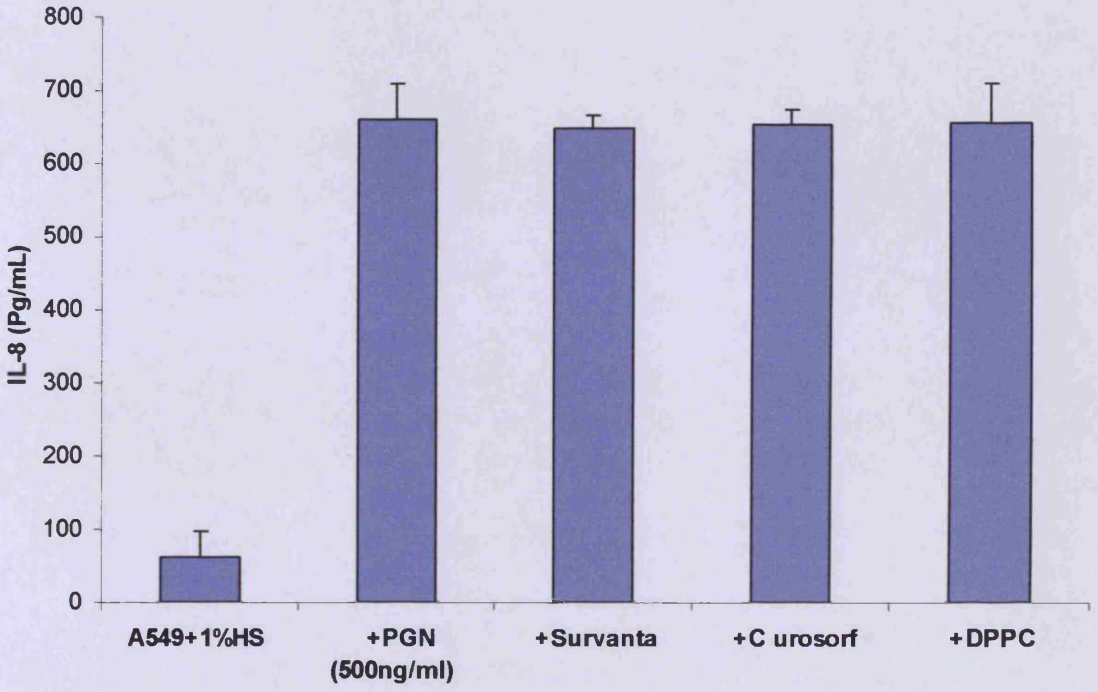


Fig 6.16: Effect of pulmonary surfactants on IL-8 induced by PGN. A549 cells were grown to confluence and incubated with 250 μg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior stimulation with PGN (500 ng/mL) in presence of human serum. Supernatants were collected after 18 hours and assayed by ELISA. Pre-treatment of A549 cells with surfactants did not have any significant affect on PGN induce IL-8. Results are expressed as the mean ± SD of three independent experiments.

6.8 A549 CELL RESPONSES TO LIPOTEICHOIC ACID (LTA)

Lipoteichoic acid (LTA) is an important virulent factor in Gram-positive bacteria. The aim of using LTA in this study was to compare with LPS, which is the most important virulent factor in Gram-negative bacteria, and to investigate the possible modulatory effect by surfactants. Commercially prepared LTA from *S. aureus* was used and the dose response was examined for IL-1β and IL-8 release from A549 epithelial cells.

6.8.1 Dose responses of LTA on IL-1β AND IL-8 induction from A549 in presence and absence of human serum

A549 epithelial cells monolayers at density of ($5x10^5$ cell /mL) in 12 wells tissue culture plate were grown in complete RPM media with antibiotic for 2 days before the cells were weaned with RPMI medium contains L-glutamine only for overnight and incubated overnight with and without different concentrations of LTA (10, 100, 250, 500, 1000 ng/mL) in absence or presence of 1% human serum.

The results show that incubation of A549 cells with different concentrations of LTA did not induce IL-1β in the presence or absence of human serum (Data not shown). However A549 epithelial cells responded poorly to LTA and release IL-8 in absence of human serum. When human serum was added, the cells responded and released more IL-8 in dose dependent manner. The A549 epithelial cells secrete more IL-8 with higher doses of LTA (500ng/mL or more) in the absence of human serum, and the dose of LTA response went down to ≤ 300ng/mL when 1% human serum was added (Fig 6.17).

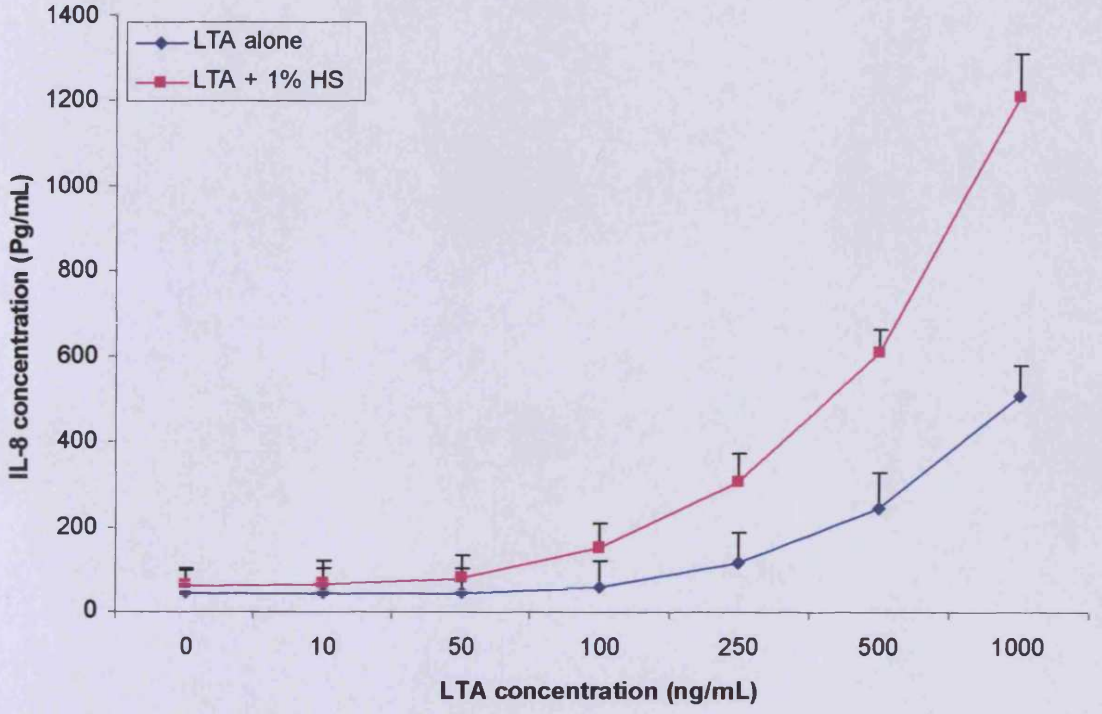


Fig 6.17: Dose responses of LTA and IL-8 induction from A549 epithelial cells. A549 cells were incubated in complete RPMI media with antibiotics for two days. Wells were washed and media were changed to RPMI with L-glutamine prior to stimulation with different concentration of LTA in the presence or absence of human serum. Supernatants were collected after 18 hours and IL-8 assayed by ELISA. Results are expressed as mean ± SD of three independent experiments.

6.8.2 Effect of pulmonary surfactant lipids on LTA induced IL-8 from A549 epithelial cells in presence or absence of human serum

The effects of different surfactant preparations and surfactant phospholipid DPPC on the induction of LTA induced IL-8 was investigated after adding 250 μg/mL of Survanta®, Curosurf® or 500μg/mL of DPPC and incubated for 2 hours with A549 epithelial cells before LTA (750ng/mL) was added in presence and absence of human serum.

The results showed that the A549 epithelial cells are more responsive to LTA in IL-8 production when human serum was included during the stimulation. The effect of surfactant Survanta®, Curosurf® and DPPC lipid at a concentration of 250μg/mL did not significantly effect of IL-8 production from A549 epithelial cells in presence and absence of human serum after 18 hours incubation (Fig 6.18 showed the effect of surfactant in presence of human serum).

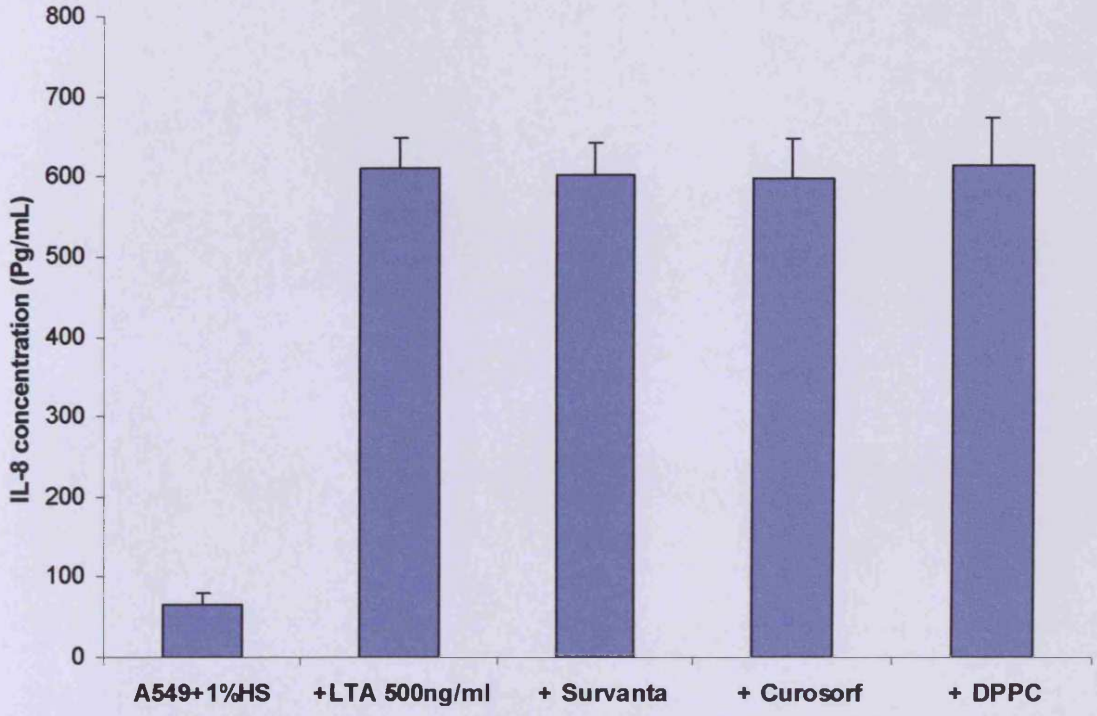


Fig 6.18: Effect of pulmonary surfactants on IL-8 induced by LTA. A549 cells were grown to confluence and incubated with 250 μg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior stimulation with LTA (500 ng/mL) in presence of human serum. Supernatants were collected after 18 hours and assayed by ELISA. Pre-treatment of A549 cells with surfactants did not have any significant affect on PGN induce IL-8. Results are expressed as the mean ± SD of three independent experiments.

6.9 SUMMARY OF RESULTS

From the work described in this chapter it can be concluded that:

A549 cells respond poorly to LPS in the absence of serum factors. In the presence of such factors, these cells respond to <100 ng/mL LPS with production of significant amount of IL-8 protein and mRNA. Antibody blocking experiments showed that CD14 from serum is important in the LPS-induced IL-8 response of A549 cells and these cells produce IL-8 via LPS activation of TLR4 in the classical LPS response.

The surfactants Survanta®, Curosurf® and DPPC lipid significantly inhibited the LPS-induced IL-8 release in A549 cells. The surfactant did not inhibit the IL-8 production in response to PGN and LTA. These results suggest that the down-regulation of IL-8 responses in A549 cells by the surfactant is specific to LPS.

CHAPTER 7. RESULTS

INVESTIGATION OF THE POSSIBLE MECHANISMS BY WHICH PULMONARY SURFACTANT LIPIDS INHIBIT IL-8 PRODUCTION BY A549 CELLS

7.1 INDUCTION OF IL-8 CYTOKINE BY RECOMBINANT IL-1β AND THE EFFECT OF PULMONARY SURFACTANTS

Our previous results showed that pulmonary surfactants Survanta® and phospholipid DPPC inhibited IL-8 cytokine induced from A549 cells when stimulated with LPS and live bacteria. The mechanisms of how surfactants modulate IL-8 induction were thus investigated. It is known that IL-1β shares signal transduction pathways with LPS down stream of MyD88 and also share common pathways down stream of TRAF6 (Martin and Wesche, 2002,Maschera et al., 1999,Zhang and Ghosh, 2001).

Since previous results (chapter 6) suggested the effect of the surfactants were specific for LPS-induced response in A549 cells, investigation of IL-8 produced in the response to LPS and IL-1 would clarify where in the LPS signaling pathway the surfactant were acting. The induction of IL-8 by rIL-1β and the effect of surfactants on this were first investigated. A549 epithelial cells were stimulated with 5 ng/mL of recombinant IL-1β (rIL-1β) and the effect of different surfactants were investigated by pre-treatment of the epithelial cells with Survanta®, Curosurf® or DPPC before stimulation with rIL-1 β.

The results in Figure 7.1 show that A549 epithelial cells produce high concentration of IL-8 upon stimulations with rIL-1β. When A549 epithelial cells were pre-incubated with different surfactants for 2 hours before stimulation with rIL-1β, the IL-8 release was not significantly modulated when compared to unstimulated control cells (Fig 7.1). This result suggests that the surfactants are acting at a point upstream of MyD88 or TRAF6.

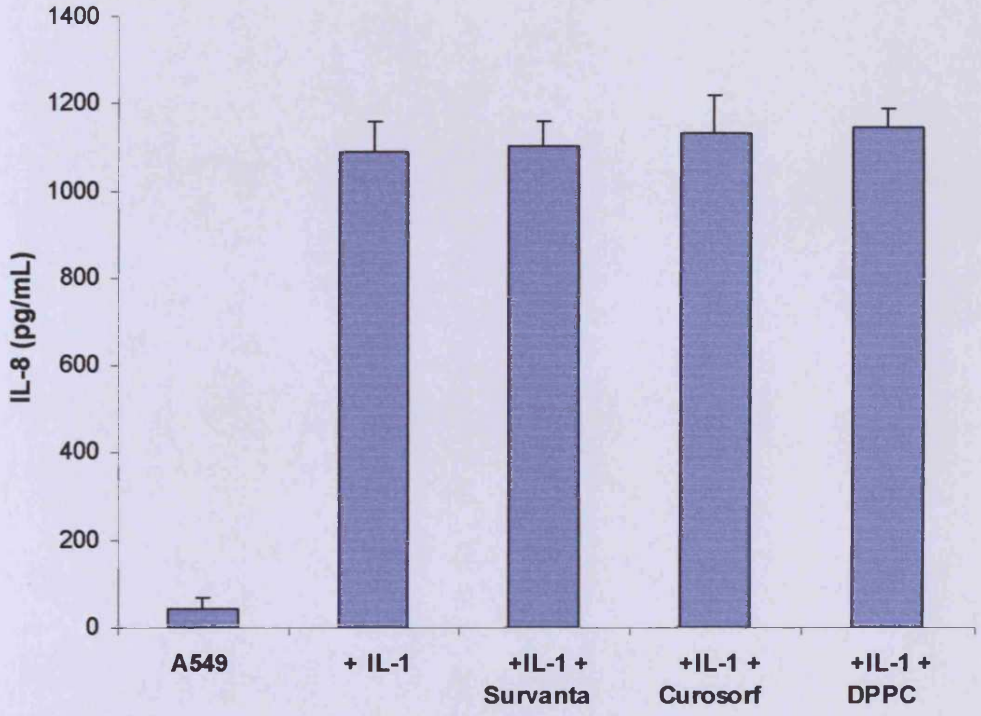


Fig 7.1: Induction of IL-8 by recombinant IL-1β and effect of pulmonary surfactant. A549 cells were grown to confluence and incubated with 250 μg/mL of Survanta®, Curosurf® or DPPC for 2 hours prior stimulation with IL-1β (10ng/mL). Supernatants were collected after 18 hours and IL-8 was assayed by ELISA. Pre-treatment of A549 cells with surfactants did not show any significant inhibition effect on IL-8 release. Results are expressed as the mean ± SD of three independent experiments.

7.2 EFFECT OF SURFACTANT LIPIDS ON TLR4 RECEPTOR IN A549 CELLS

TLR4 is the main transmembrane receptor component involved in LPS signaling. To investigate the possible mechanism of action of pulmonary surfactant lipids on IL-8 induced by *P. aeruginosa*, *B. cepacia* or their LPS products, the direct effect of surfactants on TLR4 was first examined using flow cytometry (see methods 2.2.18) and the TLR4 expression was quantified by flow cytometry. The distribution of A549 cells was displayed according there forward (FSC) and side scatter (SSC) properties before gated (Figure 7.2A) and analysed. Typical flow cytometry profiles analysis is shown in (Fig 7.2B & C).

In the analysis of the cell surface expression of TLR4, different fluorescent pattern were acquired and displayed and the histogram of PE (red) fluorescence in FL2 due to the bound of the TLR receptors on A549 cells PE-conjugated with anti-TLR4 antibody. The results showed that pre-incubating A549 with Survanta® or DPPC has no effect on expression of TLR4 (Fig 7.2A & B) and the results were of suggests that surfactants do not down regulate expression of TLR4 receptors.

Previous work in our laboratory has shown that pulmonary surfactants do not inhibit binding of LPS to cell surface receptors (Abate, 2005). Therefore, the surfactants might affect the formation of a signaling receptors complex in the membrane. It has been shown that upon LPS stimulation, TLR4 and other components of LPS receptors complex transfer into membrane lipid domains known as lipid rafts (Triantafilou et al., 2002). Thus, the involvement of lipid rafts in LPS-induced IL-8 responses in A549 cells was investigated to determine possible mechanisms for surfactant action.

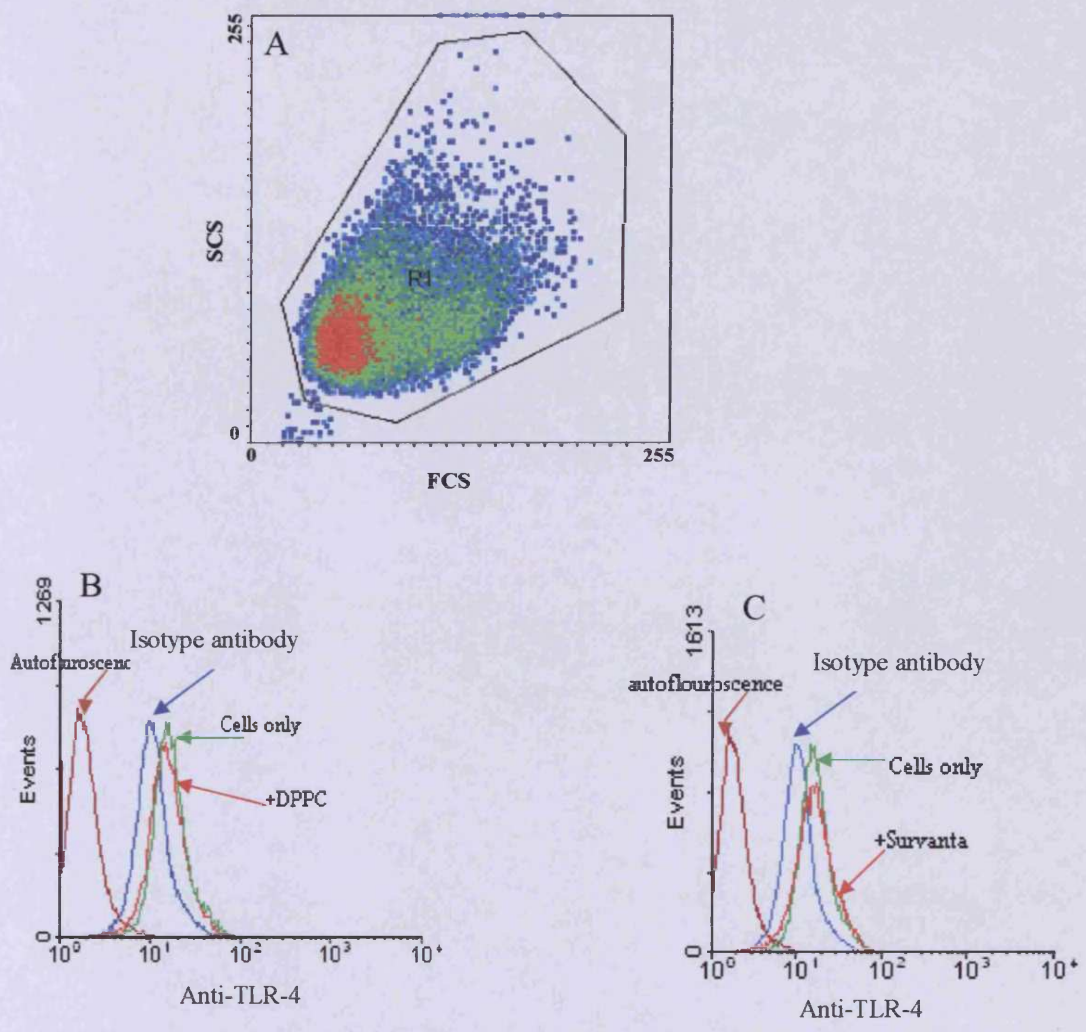


Figure 7.2: Typical flow cytometry profiles of surface expression of TLR4 receptors on A549 cells. A549 cells was incubated with isotype or anti-TLR4 PE conjugated antibodies to cell surface TLR4 antigen for 1 hour. Cells were washed and 20,000 cells were analysed. An example of typical flow cytometry analysis of expression of TLR-4 are shown. Forward side (FSC) side scatter (SSC) plots of gating of the cells population (A), and histograms of the gated region only (B, C): pre-incubated with or without DPPC (250µg/mL) (B) or Survanta® (250µg/mL) (C) for 2 hours (red line). Results showed that Survanta® and DPPC did not significantly affect the expression of TLR4

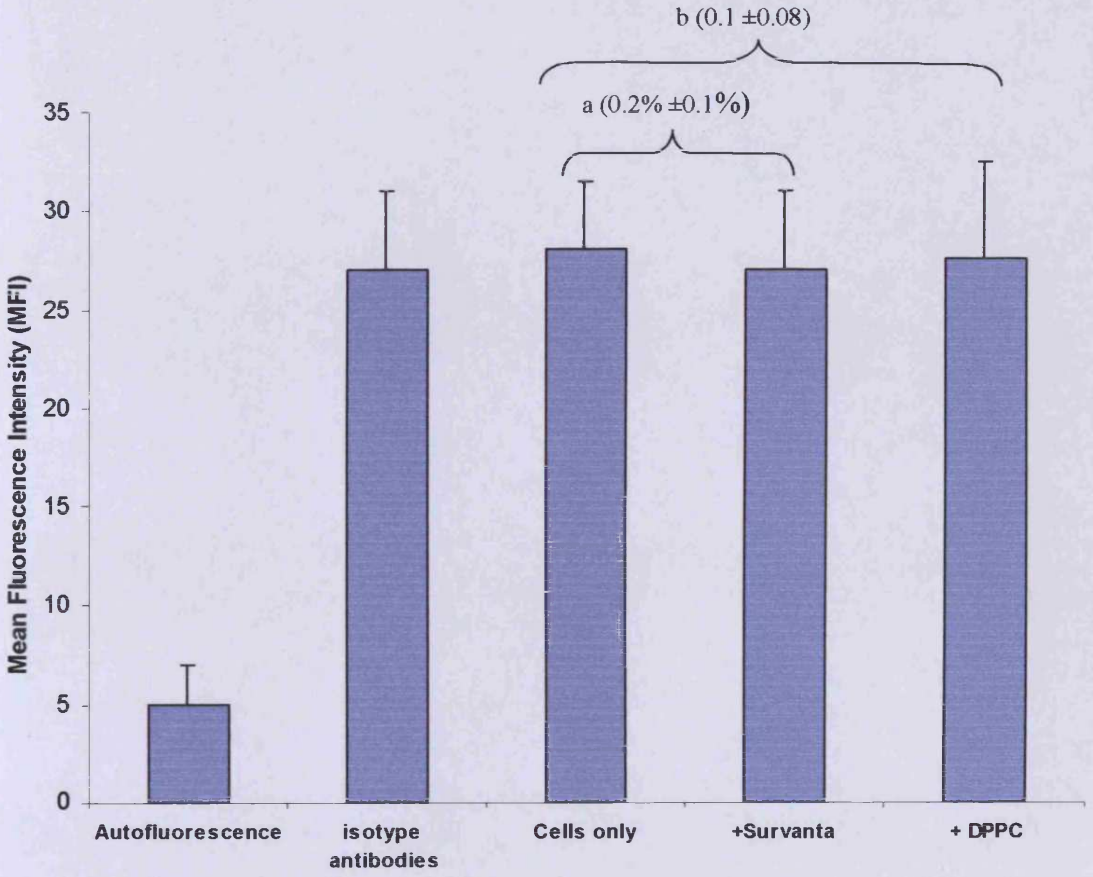


Figure 7.3: Survanta® and DPPC does not significant affect on the expression of TLR4 receptor. A549 cells incubated with Survanta® or DPPC for 2 hours folowed by washing with PBS befor stained with PE anti-human TLR4 as described in methods section. The expression of TLR4 was quantified by flow cytometry. Results are expressed as the mean ± SD of 3 separate experiments. (a) and (b) indicate % difference of MFI between cells only and cells treated with survanta® or DPPC respectively.

7.3 EFFECT OF METHYL β-CYCLODEXTRIN ON IL-8 INDUCED BY LPS AND LIVE BACTERIA

It has been shown that LPS signaling involves lipid raft membrane domains (Triantafillou et al., 2000). The lipid raft disrupting agent methyl β-cyclodextrin, was used to examine the involvement of lipid rafts in IL-8 production induced by live bacteria or LPS by pre-incubating the A549 cells with 5μg/mL of methyl β-cyclodextrin (see 2.2.10) before different bacteria (or LPS) were added. The results show that methyl β-cyclodextrin significantly inhibited the production of IL-8 induced by *P. aeruginosa* (Fig 7.4), *B. cepacia* (Fig 7.5) and LPS components from *P. aeruginosa* clinical, *P. aeruginosa* serotype 10 (Fig 7.6) and from both strains of *B. cepacia* NCTC 10661 and *B. cepacia* ET-12 clinical strain (Fig 7.7). The inhibition was greater than 40% when were compared with stimulated cells/untreated with β-cyclodextrin.

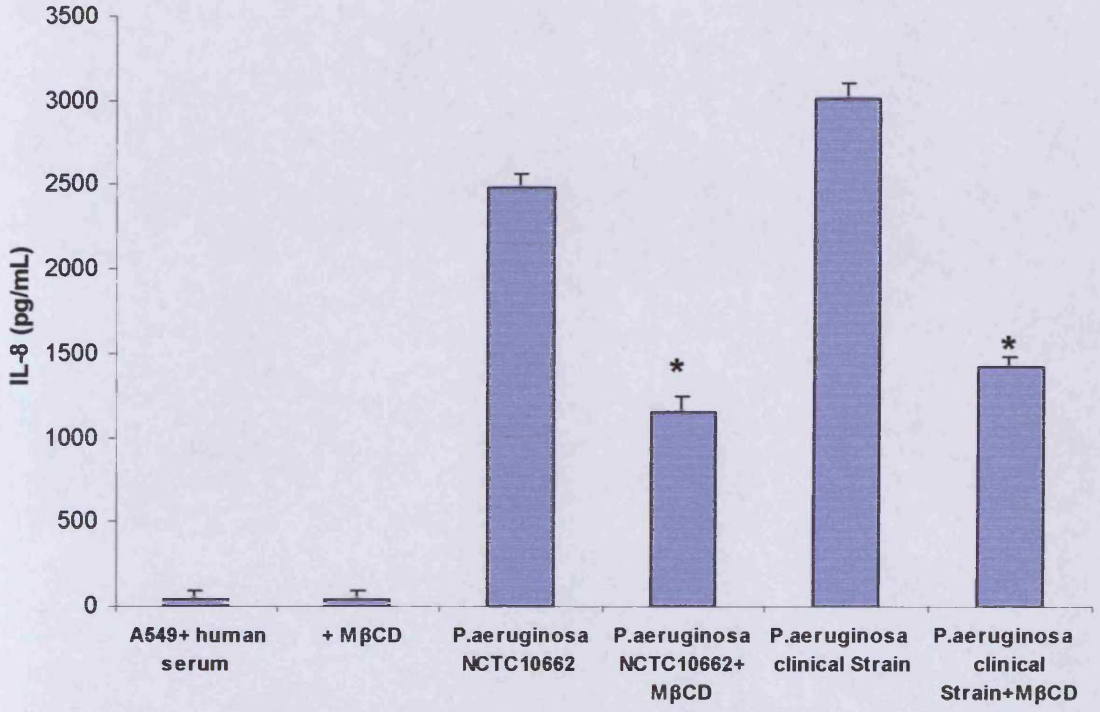


Fig: 7.4 Effect of methyl β-Cyclodextrin on IL-8 induced from A549 cells by *P. aeruginosa* NCTC 10662 *and P. aeruginosa* clinical strain. Confluent A549 cells were pre-incubated with 5μg/mL of MβCD for 2 hours before stimulation with live *P. aeruginosa* in the presence of human serum and incubated for further 18 hours. Supernatants were collected and assayed for IL-8 by ELISA and results were expressed as the mean ± SD and representative of three separate experiments. *P<0.05 vs controls (analysed by Paired T-test).

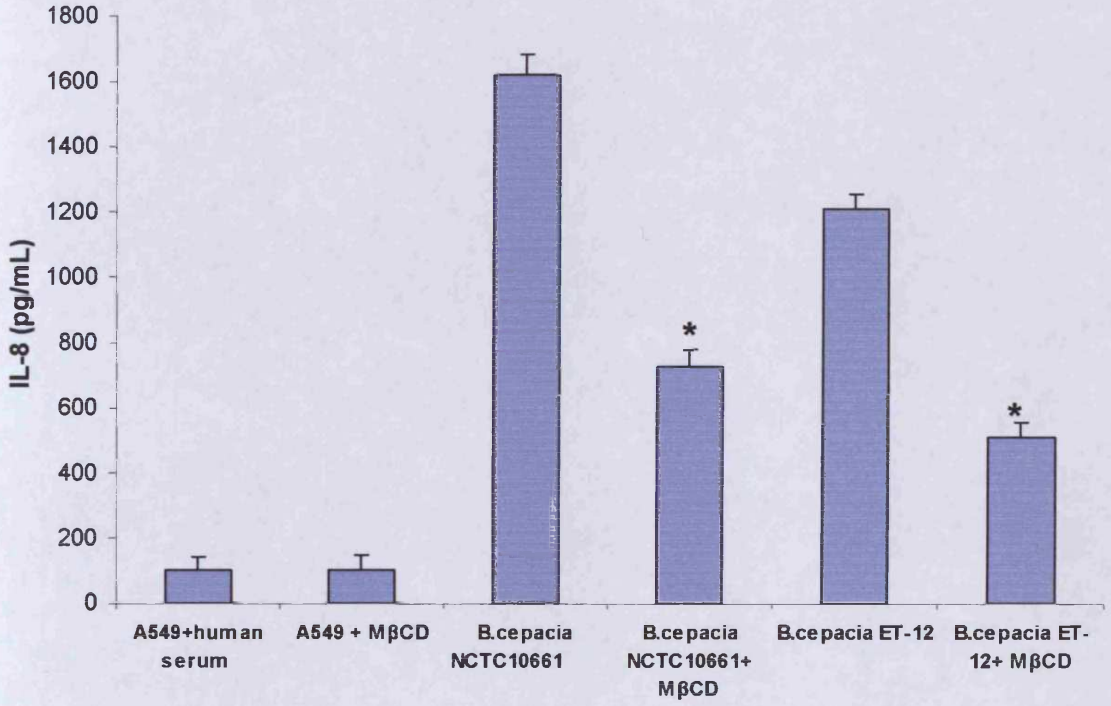


Fig 7.5: Effect of methyl β-Cyclodextrin on IL-8 induced from A549 cells by *B. cepacia* NCTC 10661 and *B. cepacia* ET-12. Confluent A549 cells were pre-incubated with 5μg/mL of MβCD for 2 hours before stimulation with live *B. cepacia* in the presence of human serum. Supernatants were collected after 18 hours and assayed for IL-8 by ELISA. Results were expressed as the mean ± SD and representative of three separate experiments. *P<0.05 vs controls (analysed by Paired T-test).

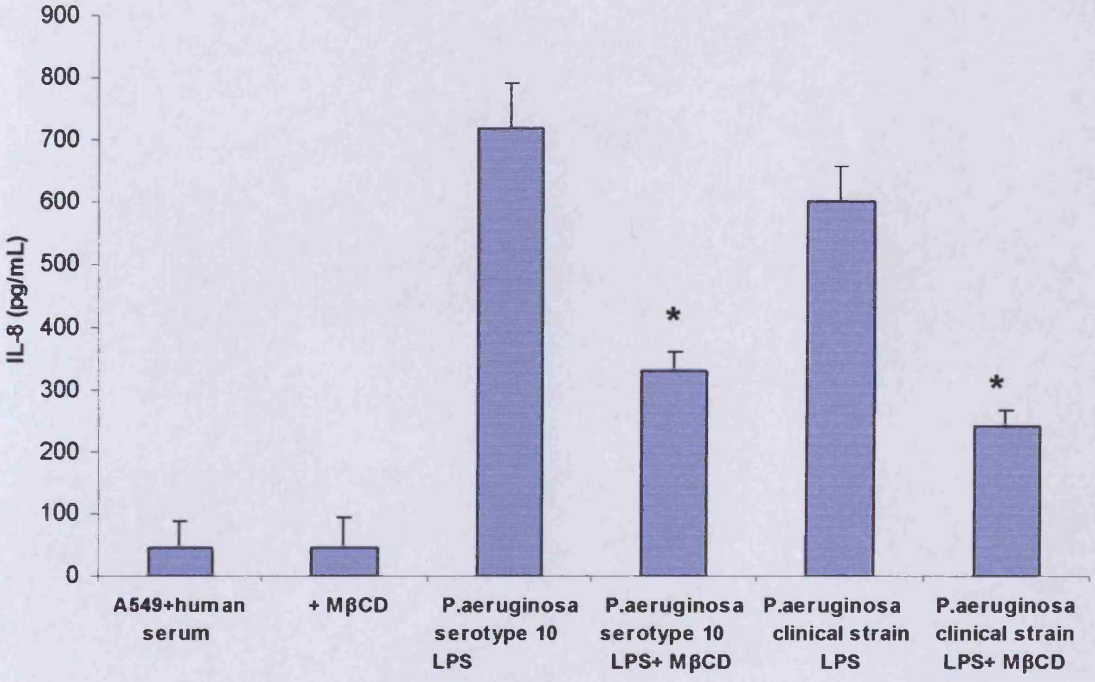


Fig 7.6: Effect of methyl β-Cyclodextrin on IL-8 induced from A549 cells by LPS from *P. aeruginosa* serotype 10 and *P. aeruginosa* clinical strain. Confluent A549 cells were pre-incubated with 5μg/mL of MβCD for 2 hours before stimulation with 100ng/mL of LPS in the presence of human serum. Supernatants were collected after 18 hours and were assayed for IL-8 by ELISA. Results were expressed as the mean ± SD and representative of three separate experiments. *P<0.05 vs control (analysed by Paired T-test).

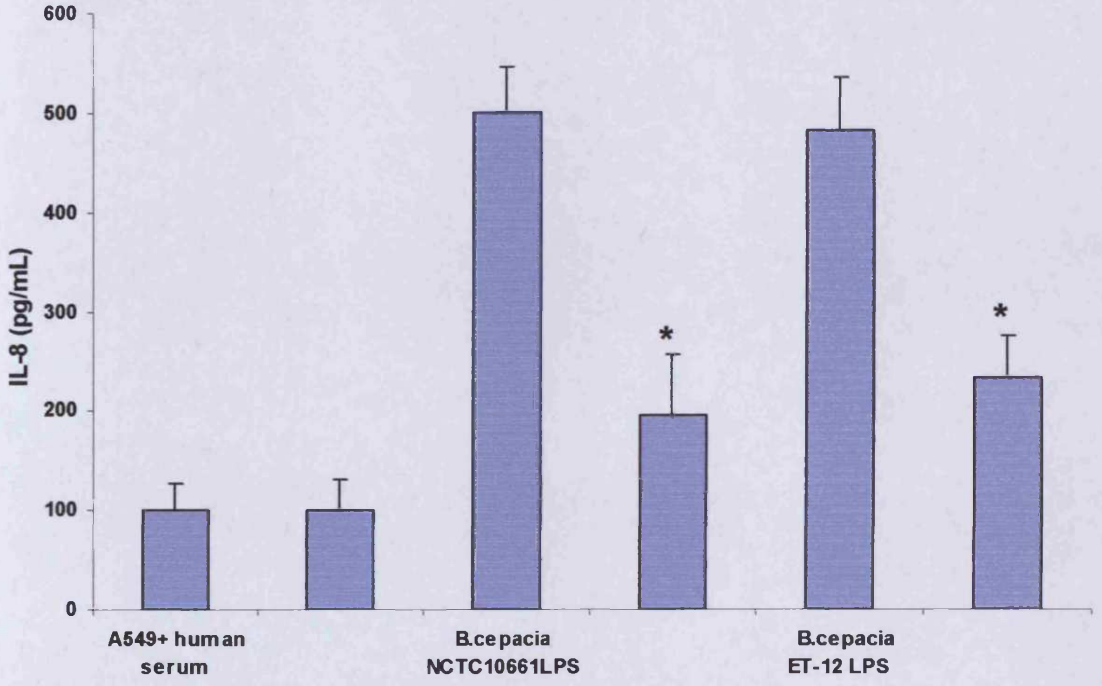


Fig 7.7: Effect of methyl β-Cyclodextrin on IL-8 induced from A549 cells by LPS from *B. cepacia* NCTC 1066 *and B. cepacia* ET-12. Confluent A549 cells were pre-incubated with 5μg/mL of MβCD for 2 hours before stimulation with 100 ng/mL of LPS in the presence of human serum. Supernatants were collected after 18 hours at 37°C with 5% CO_2 before assayed for IL-8 by ELISA. Results were expressed as the mean ± SD and representative of three distinct experiments. *P<0.05 vs controls (analysed by Paired T-test).

7.4 MECHANISM OF ACTION OF SURFACTANT LIPIDS ON MODULATION OF IL-8

The results from chapter 5 and 6 showed that pulmonary surfactants Survanta® and DPPC lipid inhibited the release of IL-8 cytokine from A549 cells when stimulated with LPS or live bacteria. The involvement of lipid rafts was investigated by MβCD and suggests that surfactant lipids might interfere with lipid raft signaling.

It has been recently shown that lipid rafts are used, as special micro-domain platform where proteins involved in the LPS signaling will be assembled. CD14 is constitutively expressed in the cholesterol-rich lipid raft domain whereas TLR4 is recruited to the domain upon LPS stimulation. The mobility of TLR4 into the raft domain has been shown to be crucial for LPS signaling (Triantafillou et al., 2002). Previous work has shown that surfactant lipids can intercalate into the membranes of the cells and alter the phospholipid composition and membrane fluidity (Morris et al., 2000,Tonks et al., 2003). Therefore, it is hypothesised that surfactant lipids may disrupt membrane lipid rafts or prevent the assembly of signaling proteins in them.

To investigate this hypothesis, the lipid raft microdomains were isolated by density gradient ultra-centrifugation and lipid raft containing fractions were identified by dot blot using HRP-conjugated cholera B subunit which binds to the GM1 gangliosides present in lipid rafts. The dot blot results show the fractions containing lipid rafts (Figure 7.7A) before an equivalent portion of each fraction were analysed by SDS-PAGE. The nitrocellulose membranes were probed with anti-TLR4 followed with HRP-conjugated secondary antibodies.

7.4.1 Western blot of TLR4

Fractions of lipid and non-lipid rafts were prepared from different conditions (LPS and Live bacteria in presence and absence of Survanta® or DPPC) before were subjected to SDS-PAGE electrophoresis (2.2.21.1). The membranes were blocked and probed (2.2.21.2-2.2.21.3) with anti-TLR4 antibodies after the gels were electro blotted. The membranes were developed using enhanced chimluminescence (Vangerow et al., 2001) and the protein bands for TLR4 were identified by comparison with pre-stained molecular weight markers.

The results showed that TLR4 normally resides in the non-raft domain in A549 cells during the resting stage (7.8B, 7.9 B) but when A549 cells were stimulated with LPS from *P. aeruginosa* clinical strain, the TLR4 receptor was recruited into the raft domains (7.8 C). Pre-incubation of A549 cells with Survanta® or DPPC significantly interfered with the recruitment of TLR4 into the lipid raft micro domain and localized it towards the non-raft domains (7.8D & E).

Also the results showed that TLR4 recruited into the lipid raft domain upon stimulation with live *P. aeruginosa* clinical strain (7.9 C) and pre-incubation with survanta® or DPPC interfered with the mobilisation of TLR4 and localized it towards the non-raft domains (7.9 D & E).

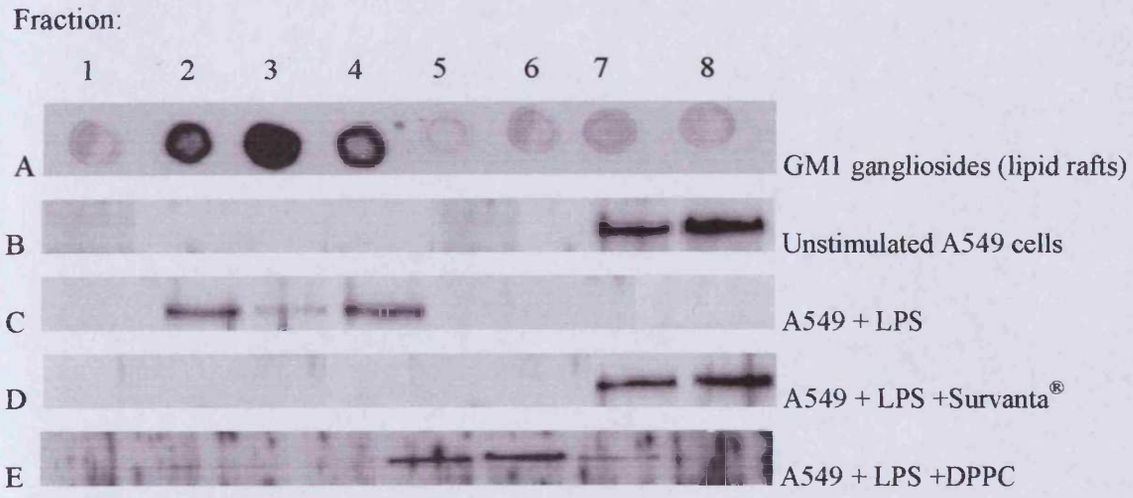


Fig 7.8: Effect of Surfactants on mobilization and translocation of TLR4 receptor to the raft micro-domains of A549 cells stimulated with LPS *P. aeruginosa clinical strain.* A549 epithelial cells with 1% human serum were either not stimulated or pre-treated with survanta® or DPPC for 2 hour prior to stimulation with LPS from *P. aeruginosa* clinical strain. A549 cells were lysed before treatment with 1% Triton X-100 and then subjected to sucrose density gradient centrifugation for overnight. Fractions were collected from the top of the gradient and 2.5μL of each fraction was detected with cholera toxin β sub-unit (CTB) for lipid raft fractions (2-4) and non-lipid raft (1,5-8) by Dot blot (A). Equivalent portion of each fraction were analysed by SDS-PAGE and immunoblotting. The nitrocellulose membranes were probed with anti TLR-4 followed with HRP-conjugated secondary antibodies. B (unstimulated A549 cells), Cells stimulated with 100 ng/mL of LPS alone (C) or pre-treated with Survanta® (D) or DPPC (E) before stimulation.

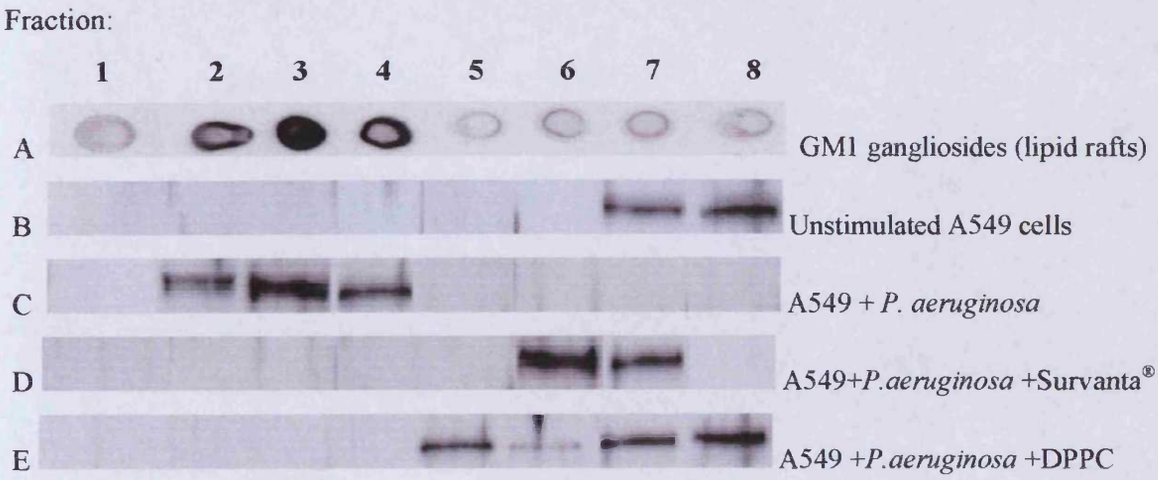


Fig 7.9: Effect of Surfactants on mobilization and translocation of TLR4 receptor to the raft micro-domains of A549 cells stimulated with *P. aeruginosa*. A549 epithelial cells with 1% human serum were either not stimulated or pre-treated with survanta® or DPPC for 2 hour prior to stimulation with *P. aeruginosa* clinical strain. A549 cells were lysed before treatment with 1% Triton X-100 and then subjected to sucrose density gradient centrifugation for overnight. Fractions were collected from the top of the gradient and 2.5μL of each fraction was detected with cholera toxin β sub-unit (CTB) for lipid raft fractions (2-4) and non-lipid raft (1,5-8) by Dot blot (A). Equivalent portion of each fraction were analysed by SDS-PAGE and immunoblotting. The nitrocellulose membranes were probed with anti TLR-4 followed with HRP-conjugated secondary antibodies. B (unstimulated A549 cells), Cells stimulated with 100 ng/mL of LPS alone (C) or pre-treated with Survanta® (D) or DPPC (E) before stimulation.

7.6 SUMMARY OF RESULTS

The results in this chapter demonstrated that:

Survanta® and DPPC act on A549 cells to specifically inhibit bacteria and LPS-induced IL-8 response. Survanta® and® DPPC act at the level of lipid raft microdomains by preventing the translocation of TLR4 into membrane lipid rafts.

CHAPTER 8 DISCUSSION

8.0 GENERAL DISCUSSION

Respiratory tract infections cause considerable morbidity and mortality worldwide and also they are one of the commonest cause of all emergency medical admission and death in the UK (Chung et al., 2002). Lower respiratory tract infection (LRTI) describes a range of symptoms and signs, varying in severity from non-pneumonic LRTI in the young healthy adult through to pneumonia or life-threatening exacerbation in a patient with severe disabling chronic obstructive pulmonary disease (COPD) and cystic fibrosis. Approximately 1.7 million people aged between 16 and 79 years were treated for LRTI in England and Wales in 1991, representing an incidence of 46/1,000 population (Macfarlane et al., 1993). UK studies documenting the frequency of LRTI in the adult population in general practice suggest an overall incidence of 44-84 cases per 1,000 populations per year (Macfarlane et al., 1993, Woodhead et al., 1987).

Bacterial lung infection may be caused by Gram-positive bacteria, such as *S. pneumoniae, S aureus*, and group B streptococci, or Gram-negative bacteria such as *P. aeruginosa,* which are predominant in bacterial infections in the lungs of chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) patients. Ultimately, 80 to 95% of patients with CF succumb to respiratory failure brought on by chronic bacterial infection and concomitant airway inflammation. The ubiquity of *P. aeruginosa* (Green, 1974) in the environment probably underlies the high frequency of recovery of this pathogen from CF patients.

Although *P. aeruginosa* has remained the predominant pulmonary pathogen in CF patients, recent years have seen the emergence of several new pathogens of clinical relevance to CF. An epidemic of infection with *Burkholderia cepacia* was reported in CF patients in the 1980s, and a high mortality was noted and since many reported that a notable number of CF patients were colonized with *B. cepacia* (Gladman et al., 1992,Lewin et al., 1990).

The association of Gram-negative bacteria with the epithelial cells in the lung is important in the establishment of lung infection in CF patients. In addition to antibiotic resistance, growth of bacteria and invasion of the epithelial cells in the lung may be considered key to the predominance and mortality of *P. aeruginosa* and *Burkholderia cepacia* in CF patients. It is established that abnormal composition of airway secretions of the CF lung is the host factor that predisposes CF patients to chronic colonization by *P. aeruginosa* alterations and could derail the functions of several host defense mechanisms. One such mechanism is the phagocytic barrier created by macrophages and neutrophils. It has been reported by Tager et al (Tager et al., 1998) that the neutrophils are defective in phagocytic killing in the lung of CF patients.

The normal host defences of the airway epithelium are proficient in preventing infection of the respiratory tract. Despite repeated exposures to a wide variety of potentially pathogenic organisms, the lower respiratory tract usually remains sterile and the host is unaware of the ongoing clearance mechanisms. However, when bacterial contamination of the airways overwhelms the routine mucociliary defences and polymorphnuclear leukocytes (PMN's) are recruited, the resulting inflammation, whilst serving to clear the infecting organisms, also causes the signs and symptoms of respiratory tract infection.

The alveolar epithelium plays an important role in innate immunity not only by forming an efficient barrier against invading bacteria but also through functions that coordinate other immunological cells such as macrophages and neutrophils. The alveolar epithelium also secretes surfactant, which may have modulatory functions in addition to its major property of lowering the surface tension in the lung. There is increasing recognition that pulmonary surfactant, especially the proteins, may have an important role in innate immune responses in the lung.

Accruing evidence suggest that pulmonary both surfactant protein and surfactant lipids play an important role in immunoregulation of lung defense. The hydrophobic surfactant proteins SP-B and SP-C are important in adsorbing and spreading of the surfactant film at the air

liquid interface. Beside the two hydrophobic proteins two other surfactant proteins was described. The surfactant proteins SP-A and SP-D (collectins) play an important role in lung immune defense by binding to microbial carbohydrate determinants (Holmskove et al., 1994). Both SP-A and SP-D has direct antimicrobial killing in addition to facilitating pathogen uptake and killing by immune cells (Wu et al., 2003). In addition SP-A and SP-D has been shown to inhibit the growth of Gram-negative bacteria directly (Wu et al., 2003).

It has been shown that pre-incubation of SP-A, with smooth LPS in the presence of CD14 has significantly reduced the binding but the association of SP-A with rough LPS is augmented, suggesting different actions of SP-A upon distinct serotypes of LPS (Sano et al., 1999). Thus, a direct interaction of SP-A with CD14 constitutes a likely mechanism by which SP-A modulates LPS-elicited cellular responses. SP-A binds the peptide portion containing the leucine-rich repeats of CD14 whereas SP-D binds the carbohydrate moiety of CD14 (Sano et al., 2000).

Both SP-A and SP-D can directly bind CD14 and deal at several levels with a wide range of potentially pathogenic viruses, bacteria and fungi that generally involves agglutination, enhancement of phagocytosis and killing via oxidative mechanisms, and direct bacteriostatic and fungistatic effects (see Review by Wright, 2005). Similarly, SP-D has been shown to bind a variety of different bacteria via their surface molecules including Gram-negative bacteria (Pikaar et al., 1995; van Iwaarden et al., 1994), LPS expressed by O-serotypes of *K. pneumoniae* (Sahly et al., 2002), purified LPS from *P. aeruginosa* and *E. coli* (Kishore et al., 1996), and cell wall components of Gram-positive bacteria (van de Wetering et al., 2001). SP-D was found to binds to Candida albicans and inhibits the growth in addition to the inhibition of phagocytosis by alveolar macrophage (van Rozendal et al., 2002). Recently it has been shown that SP-D regulated TNF-α production of the alveolar macrophage in response to GBS, *H. influenza* and LPS by reducing the expression of the surface CD14 (Senft et al., 2005). Very recent report showed that SP-D can inhibit non-capsulated *K. pneumoniae* induced IL-6 production from human monocyte-derived macrophage (Kostina et al., 2005).

The effect of SP-D on regulation of anti-inflammatory cytokine has been reported by culturing alveolar macrophages, together with SP-D and allergen, resulted in increased production of IL-10, IL-12, and IFN-α, suggesting a link between macrophages and SP-D mediated suppression of airway inflammation (Takeda et al., 2003). SP-A and SP-D has also been shown to act as an opsonin for respiratory syncytial virus (RSV), binding to the surface of the virion, inhibiting infectivity and enhancing its uptake by peripheral blood mononuclear cells (PBMCs) and alveolar macrophage (Ghildyal et al., 1999, Hickling et al., 1999). In murine model a significant inhibition of nitric oxide and TNF-α induced by *A. fumigatus* has been reported when alveolar macrophages, derived from mice, are preincubated with SP-D (Liu et al., 2005).

The interaction of pulmonary surfactant proteins with bacteria has different effects depending on the bacteria and the surfactant protein involved. It can cause agglutination of bacteria, hindering their entry into host cells and dissemination. It may lead to the killing through permeabilization of their cell walls, increasing the respiratory burst by macrophages and neutrophils, and enhancing their opsonization by phagocytic cells. SP-A binds to *Staphylococcus aureus* (Geertsma et al., 1994), *P. aeruginosa*, *E. coli* J5 (containing O-antigen deficient rough LPS), but not *E. coli* O 111 (with O-antigen containing smooth LPS) (van Iwaarden et al., 1994), *Mycobacterium tuberculosis* (Gaynor et al., 1995; Pasula et al., 1997), *S. pneumonia*, Group A and Group B *Streptococci*, *H. influenzae* via its outer membrane protein (McNeely and Coonrod, 1994), encapsulated *K. pneumoniae* (Kabha et al., 1997), and the bacterial preparation *Bacillus Calmette Guerin* (BCG) (Weikert et al., 1997), cause different levels of aggregation and phagocytosis by macrophages.

Others, however, have shown some degree of SP-A binding to *S. aureus* and *H. influenzae* type b without causing aggregation or phagocytosis (McNeely and Coonrod, 1994). SP-A enhances the attachment of *M. tuberculosis* to alveolar macrophages (Pasula et al., 1997), which may increase its chances of gaining access to alveolar macrophage (Gaynor et al., 1995), whereas SP-D binds to the lipoarabinomannan moiety on the surface of *M. tuberculosis* bacilli and reduces the uptake of bacteria by alveolar macrophages (Ferguson et

al., 1999; Ferguson et al., 2002).

Furthermore, SP-A binds to *Mycoplasma pulmonis* and enhances its phagocytosis and killing by IFN-γ- activated murine alveolar macrophages via generation of peroxynitrite (Hickman-Davis et al., 1999). SP-A alone has been shown to enhance the binding to *E. coli* LPS to the alveolar macrophage by 2-3 fold (Stamme et al., 1999) and to mediate alveolar macrophage generation of peroxynitrite that is necessary for the killing of bacterial pathogen (Hickman-Davis et al., 1999). In addition SP-A also has been shown to regulate the pro-inflammatory cytokine release in pulmonary macrophages (Arias-Diaz et al., 2000). SP-A-mediated uptake of RSV significantly enhances TNF-α production by PBMC and has been shown to reverse the RSV-induced suppression of INF-γ production by U937 macrophages (Barr et al., 2000).

The role of surfactant lipids as integral components of the innate immune system of the lung is relatively little known, but there is growing body evidence that surfactant lipids modulate the innate immunity in the lung. The phospholipid component of surfactant inhibits the production of reactive oxygen species (ROS) in human alveolar macrophage (Hayakawa et al., 1989). The results also show that DPPC phospholipid modulates the production of reactive oxygen intermediate in human monocytes (Tonks et al., 2001), and inhibited neutrophils respiratory burst oxidase response (Suwabe et al., 1998).

It has been shown that phospholipids in surfactant preparation Survanta® inhibit phagocytosis and the association of GBS to rat alveolar macrophage (Golioto and Wright, 2002). It has been reported that DPPC has an inhibitory action on GBS induced injury to A549 cells (Nizet et al., 1996), and to inhibit the ability of GBS to invade A549 cells and to stimulate the production of IL-8 (Doran et al., 2002).

Survanta®, Curosurf® and individual lipid component such as DPPC have also been shown to modulate the production of inflammatory cytokines and mediators (Morris et al., 2000; Thomassen et al., 1992; Thomassen et al, 1994; Walti et al., 1997). Unsaturated phosphatidylcholine have been shown involvement on modulation of the adaptive immune

response (Wilsher et al., 1988). Recently it has been shown that unsaturated phosphatidylcholine PLPC and POPC modulated the induction of IL-8 from A549 epithelial cells (Abate, 2005).

Bacteria that elude these innate defences in the upper respiratory tract may get accesses to the lower respiratory tract and stimulate a brisk inflammatory response. One important factor in the innate immune response in the lung not extensively studied is the role and involvement of pulmonary surfactant, especially the lipid components, on the innate immune response to bacteria in the lung. In addition surfactant lipids may directly influence growth and invasion and may play a role in the elimination of bacteria infection. Such direct effects of pulmonary surfactant need to be determined.

In the alveoli of the lung, pulmonary surfactant forms the initial contact between bacteria or bacterial products and the lung epithelial cells. Under normal conditions, the epithelial cells in the lung must be alert and remain tolerant even to a small amount of bacteria and bacterial products such as LPS. The link between pulmonary surfactant and Gram-negative bacteria and alveolar epithelial cells is considered a key to the investigation of the role the immune response in the lung.

This study, therefore sought to clarify the direct effect of pulmonary surfactants on bacterial survival and growth and on the internalisation of the bacterial by lung epithelial cells. The Gram-negative pathogens *P. aeruginosa* and *B. cepacia* were chosen for their role in infection in chronic airway diseases generally and cystic fibrosis in particular. A further aim of the study was to investigate the potential immunoregulatory role of pulmonary surfactants on the inflammatory responses of lung epithelial cells. The regulation of the important chemokine, IL-8 production from an alveolar type II cells line was studied in this regard.

In the present study the response of alveolar epithelial cells to selected Gram-negative bacteria and their products for IL-8 production, and its possible regulation by pulmonary surfactant was investigated. For this, human A549 epithelial cell line was adopted and used. This cell line, originally isolated from a human in 1976 by Lieber and colleagues, is an adenocarcinoma cell line and has been used by many investigators as a model to study bacterial internalisation and inflammatory responses inducing release of proinflammatory cytokines such IL-6, IL-8, TNF-α and GM-SCF (Chang et al., 2004,Fink et al., 2003, Reddi et al., 2003, Rosseau et al., 2005).

In summary, results from the present study demonstrate that pulmonary surfactants and phospholipids DPPC inhibits the growth of *P. aeruginosa* and *B. cepacia* in the presence of human serum. The inhibitory concentration of surfactants was within the physiological concentration in the lung (Hayakawa et al. 1992;Speer et al. 1991;Thomassen et al. 1992). These bacteria were internalised by the A549 in a cytoskeleton-dependent mechanism.

B. cepacia and *P. aeruginosa* internalisation were increased when bacteria was treated with Curosurf® or DPPC before adding to the A549 cells. Whereas Survanta® increases the internalisation of both strains of *B. cepacia*, it decreased the internalisation *of P. aeruginosa* NCTC and clinical strain. These differences effect of Survanta® on *P. aeruginosa* and *B. cepacia* internalisation to the A549 epithelial cells can not be explained at this level and need to be investigated further.

Clinical and environmental strains of bacteria have often express different virulence factors, and may be expressed *in vivo* in response to hostile environment of the human body (Mims et al 1998). However, it is not known if pulmonary surfactant can regulate innate immune responses differently to clinical or environmental strains, of the same organism. By using bacteria isolated from patients and using factors such as LPS from these strains, the current project has compared the differences between clinical and environmental strains and the effect of pulmonary surfactant on them.

This study shows that Survanta®, Curosurf® and DPPC at concentration of 250μg/mL can modulate both IL-8 protein and IL-8 mRNA induced by both clinical and environmental strains of *P. aeruginosa* and *B. cepacia*. Isolated LPS from these strains also shown to induce IL-8 and these inductions was down modulated with pulmonary surfactant Survanta®, Curosurf® and DPPC phospholipid.

The mechanism of down regulation of IL-8 by pulmonary surfactant and phospholipid DPPC was investigated in contrast to live bacteria and LPS, when A549 epithelial cells were incubated with recombinant IL-1β, it induced IL-8 but this induction was not modulated by surfactants. Since the signaling pathways to these agents only differ at the level of the membrane receptors, the results imply that surfactants can modulate responses specifically to LPS.

Further experiments, revealed that the surfactant used in this study inhibited the translocation of the essential LPS receptor, TLR4 into membrane microdomains (lipid rafts). This could be a mechanism for the immune regulatory properties of pulmonary surfactant lipids for response to Gram-negative pathogens. The results of this study have added additional evidence for the role of surfactant lipids in modulating innate immunity in lung.

8.1 THE ROLE OF PULMONARY SURFACTANT ON THE GROWTH OF GRAM-NEGATIVE BACTERIA

The defence system of the respiratory tract is complex and consists of various well-described cell components that share this defence. The epithelial cells in the lung are covered with pulmonary surfactant and the bacteria that reach the lower respiratory tract first come in contact with surfactant that covers the epithelial cells. Several lines of evidence suggest that surfactant may have a direct effect on inhaled bacteria and can interact with and support the immune defences in the lung. Since the observation that surfactant deficiency in lung is responsible for respiratory distress syndrome (RDS), exogenous surfactant therapy has become an established treatment of RDS. Also surfactant dysfunction has been described in other pulmonary diseases like asthma (Kurashima et al., 1997), COPD (Hohlfeld et al., 1997), pneumonia (Schmidt et al., 2001) and cystic fibrosis (Griese et al., 1997).

Surfactant inactivation probably plays a key role in the pathophysiology of RDS and pneumonia (Somerson et al., 1971). It has been speculated that surfactant given under such circumstances of infection might serve as a nutrient for bacteria and thereby promote the microbial growth (Sherman et al., 1994). Only few reports with divergent results, have been published concerning the direct influence of surfactants on bacterial growth (Laforce and Boose, 1981,Jonsson et al., 1986). Another two studies have analysed bacterial growth in the presence of surfactant preparations currently used for replacement therapy. Sherman et al (Sherman et al 1994) reported that complete natural surfactant derived from human or sheep promoted the growth of GBS, whereas Exosurf®, a synthetic surfactant, was bactericidal. Intermediate effects were observed with surfactants of modified natural surfactants derived from bovine, porcine, or calf origin. It has been reported that modified bovine surfactant, Survanta®, significantly promoted the growth of *E. coli* (Neumeister et al., 1996).

However, these observations were either limited to one bacterial strain and/or one phospholipid concentration. Jonsson et al reported (Jonsson et al., 1986) that supplementation of PBS with purified alveolar lining material exerted slight protection effect to *Streptococcus*

pneumoniae and non-typable *Haemophilus influenza.*

Ovine surfactant was shown to induce killing of *Pasteurella haemolytica*, *E. coli* and *Klebsiella* pneumonia in presence of serum but not *P. aeruginosa* and *Serratia marcescens* (Brogden, 1992). A recent report (Rauprich et al., 2000) showed that Curosurf® inhibited the survival of GBS and killed *S. aureus* in saline, but in contrast *E. coli* was not affected. In nutrient media, this inhibitory effect of Curosurf® was abolished.

The contradictory results of these reports on the influence of surfactant on bacteria could be due to the different strains of bacteria used and the media used in these studies. In addition most of these studies used only one concentration of surfactant and this makes the results hard to compare.

In the current study, these factors were overcome by using saline as a nutrient-free media, TSB as nutrient rich media, and saline supplemented with either different concentrations of human serum or different concentration of TSB. Moreover, we used DPPC, a major surfactant phospholipid, and two different preparations of surfactant, Survanta® and Curosurf® which are composed mainly of phospholipids and DPPC.

The results from the present study demonstrate that growth of the Gram-negative bacteria *P. aeruginosa* and *B. cepacia* is not inhibited in nutrient-rich media when supplemented with Survanta®, Curosurf® or DPPC. From this result, we speculate that the inhibitory effect of surfactants on bacteria when incubated in nutrient-rich media may overcome and abolish any possible inhibitory effect of surfactant lipids especially if these inhibitory effects are small compared with the growth promoting effect of TSB.

Therefore, the effect of surfactants in nutrient-free media was investigated to minimise the bacterial growth promotion of TSB media. In this *in vitro* assay when bacteria were incubated in sterile saline as a nutrient free media, the growth of bacteria was decreased especially after 24 hour incubation but when Survanta® was added the growth of *P.*

aeruginosa and *B. cepacia* were significantly protected from death when was compared to the control without surfactant, whereas Curosurf® and DPPC did not show any effect. This result lead to the speculation that when Survanta® was added to saline, the bacteria might use some of the components in Survanta® as a source of nutrient that protected these bacteria from decline.

Under normal physiological circumstances the alveolar lining fluid may be considered to be relatively poor in nutrient content. However, in the course of infection as in pneumonia and inflammation, serum components, including albumin and glucose, may leak into the bronchoalveolar space and increase the amounts of nutrients available to the bacteria. This amount of nutrient leaked in the lung may be critical for both surfactant inhibition and bacterial growth promoting. Mimicking this situation of serum leakage during inflammation may help to shed light on the role of possible inhibitory effects of surfactant.

In this current study we sought to mimic this situation in the lung where injury and leakage occurs by investigating the effect of surfactant on bacterial growth in presence of human serum. In this study we demonstrate that incubating bacteria with Survanta®, Curosurf® or DPPC inhibited the growth of bacteria. This inhibition appeared after a relatively short time of 6 hours incubation and significantly increased with further incubation. Also this study demonstrated that this inhibition of surfactant was not limited to *P. aeruginosa* but was also seen with *B. cepacia*. When clinical strains from both species were examined, the inhibition of surfactants and DPPC lipid was also observed.

This study demonstrates that this inhibition of bacteria by surfactant was limited in a range of percentage of human serum. The inhibitory effect of surfactant in the presence of human serum started to be seen at 5% and reached a maximum effect at 10% before starting to decline and was abolished at 50%. It can be concluded from this study that surfactant preparations Survanta®, Curosurf® and the associated phospholipid DPPC exert transient inhibitory effect that was not due to the presence of components in human serum since the inhibitory was also shown in 10% of TSB in saline.

In the normal healthy lung therefore, surfactant may play a role by inhibiting bacterial growth but is not bactericidal. This might be beneficial and helpful during surfactant replacement therapy to support the eradication of bacteria and with lung immunity.

8.2 THE ROLE OF PULMONARY SURFACTANT ON BACTERIAL INTERNALISATION BY A549 EPITHELIAL CELLS

Bacteria may gain access to the alveoli and come into contact with the alveolar epithelial cells by attachment and interaction with epithelial cells in the lung. The interaction of the Gram-negative bacteria to the epithelial cells may leads to the internalisation process. Interaction of Gram-negative bacteria with A549 cells has been reported and it was show that the pili or pili components were utilized for the attachment (Chi et al., 1991).

Many studies have investigated the internalisation of bacteria by epithelial cells in the lung. These reports show that different strains of *B. cepacia* could invade and replicate inside A549 epithelial cells (Burns et al., 1996), also different strains of *B. cepacia* have different rates of invasion to the epithelial cells (Burns et al., 1996,Kespichayawattana et al., 2004,Burns et al., 1996,Keig et al., 2002). Internalisation of *P. aeruginosa* to the lung by A549 epithelial cells also was reported but with evidence of poor bacterial survival (Chi et al., 1991).

Only few reports have shown that surfactant protein D (SP-D) enhanced the internalisation of bacteria by rat alveolar macrophages (Ofek et al., 2001) and inhibited the internalisation of *P.aeruginosa* by cornea epithelial cells (Ni et al., 2005). SP-A was shown to inhibit phagocytosis (Ferguson et al., 1999,Pikaar et al., 1995) and recently was reported that SP-A also decrease the binding of *Bordetella* to Cilia (Edwards et al., 2005) and augment the phagocytosis of *S. pneumoniae* by alveolar macrophages (Kuronuma et al., 2004. However, there have been no reports specifically on the role of surfactant lipids on bacterial internalisation by A549 epithelial cells. The role of surfactant and its associated lipids on

internalisation of bacteria to lung immune and epithelial cells is an important aspect not investigated by researchers. This study aimed to investigate the internalisation of bacteria by A549 epithelial cells and the effect of surfactant on this internalisation.

In this study before investigating the effect of surfactant lipids in on bacterial internalisation by A549 epithelial cells, we had to investigate the invasion of *B. cepacia* and *P. aeruginosa* to the A549 epithelial cells of the lung. The internalisation was investigated using the antibiotic protection assay and fluorescent microscopy. The uptake of bacteria by A549 was confirmed by electron microscopy and in this study it was demonstrated that *B. cepacia* could replicate inside the A549 epithelial cells when incubated for 24 hours.

This study demonstrated that both *B. cepacia* and *P. aeruginosa* could be internalised by the A549 epithelial cells. Fluorescent microscopy results showed clearly uptake of bacteria by A549 cells. When bacteria were incubated with the different surfactants, the uptake of bacteria by the A549 epithelial cells was significantly changed. In *P. aeruginosa* the internalisation was decreased by Survanta® and significantly increased by Curosurf® and DPPC, whereas in *B. cepacia* the internalisation was increased by all surfactants. The internalisation of bacteria by the A549 cells was also demonstrated and confirmed using an antibiotic protection assay.

The uptake of bacteria by A549 was confirmed by electron microscopy and this study shows that *B. cepacia* ET-12 strain could replicate inside the A549 epithelial cells when incubated for 24 hours. The different result on the effect of Survanta® on the internalisation of *P. aeruginosa* and *B. cepacia* to A549 epithelial cells may be due to any differences in the binding of these bacteria with Survanta® and needs further investigation.

Many microbial pathogens that invade non-professional phagocytic cells with rigid cytoskeletons could manipulate the cytoskeletal proteins to promote internalisation, intracellular motility and survival (Goldberg and Sansonetti, 1993). In the present study, the functional participation of the cytoskeleton in the internalisation of these bacteria was demonstrated by the use of cytochalasin D. Bacterial entry was prevented by cytochalasin D

indicating that the internalisation process requires actin microfilament function. Cytochalasin D, an inhibitor of actin polymerisation, showed significant reduction in the invasion for both strains of bacteria used and this indicates the functional participation of the microfilament in the internalisation of *B. cepacia* and *P. aeruginosa* by the A549 epithelial cells. These findings are consistent with the eukaryotic cytoskeletal inhibition effects seen with other organisms (Finlay and Falkow, 1988).

This study is the first report on the effect of surfactant on the internalisation of bacteria by A549 epithelial cells. Indeed, the increase of the uptake of bacteria by the A549 cells by surfactant may serve to help in the elimination of bacteria by entrapping bacteria and providing a mechanism for removal from the lung.

8.3 BACTERIA AND BACTERIAL COMPONENTS INDUCED IL-8 RESPONSES FROM LUNG EPITHELIAL CELLS

It is known that alveolar epithelial cells in the lung are capable of secreting chemoattractants and proinflammatory cytokines, which are important mediators in both lung defence and inflammation (Levine, 1995). In the presence of bacterial infection, the epithelial cells may act as an early warning system for local immune and inflammatory cells. Previous studies have demonstrated constant responses of A549 cells to bacteria as measured by the ability of these cells to release cytokines as a response to bacteria especially Gram-negative bacteria (Palfreyman et al., 1997, Fink et al., 2003, Utaisincharoen et al., 2004). One of these cytokines concerned in the present study is IL-8, which play role in recruiting neutrophils to site of infection in the lung.

Many investigators have reported that A549 epithelial cells are a source of IL-8 induced by bacterial lung pathogens such as S. *pneumoniae* (Madsen et al., 1999), *S. agalactiae* (Doran et al., 2002) and *Leigionella pneumophila* (Chang et al., 2004). Many studies have been published about the release of IL-8 from A549 epithelial cells induced by Gram-negative bacteria such as *B. cepacia* (Palfreyman et al., 1997,Fink et al., 2003) and other Burkholderia such as *Burkholderia pseudomallei* (Utaisincharoen et al., 2004).

There are no published reports about the release of IL-8 from A549 cells by *P. aeruginosa* but there are few reports on other human bronchial epithelial cells such as (HBE4-E6) (Massengale et al., 1999) and from 16HBE cells, human bronchial epithelial cells (Reiniger et al., 2005), human tracheal epithelial, and SV40-transformed human airway cell line (Kube et al., 2001).

Internalisation of bacteria by the A549 cells was discussed earlier and many investigators have reported that cytochalasin D inhibited the internalisation of bacteria in other cells, so the current results are in agreement with these reports by (Finlay and Falkow, 1988). The relation

between bacterial internalisation by A549 cells and IL-8 cytokine release have been reported by many investigators by using inhibitor such as cytochalasin D, an inhibitor of actin polymerization needed for internalisation of bacteria. Utaisincharoen et al (2004) reported that cytochalasin D did not interfere with IL-8 production from A549 epithelial cells induced by *Burkholderia pseudomallei* indicating that bacterial uptake is not required for the production of this chemokine. It is also reported that cytochalasin D, an inhibitor is needed for internalisation of bacteria, and it did not have any effect on the IL-8 released by A549 cells stimulated by *Legionella pneumophila* whereas IL-6 and TNF-α were affected (Chang et al., 2004).

In this study, it was demonstrated that live bacteria (*P. aeruginosa* and *B. cepacia*) both NCTC and clinical strains, could invade the epithelial A549 epithelial cells and induce IL-8 production. This invasion was inhibited significantly by cytochalasin D, but with no affect on the amount of IL-8 production. These results also revealed that the internalisation of both NCTC and clinical strains of *P. aeruginosa* and *B. cepacia* were independent of the IL-8 release. This is in agreement with results by Chang et al., (2004) and Utaisincharoen et al., (2004).

Other cytokines released from A549 epithelial cells upon stimulation with bacteria have been also reported. Chang et al (2004) have reported that *Legionella pneumophila* could stimulate A549 epithelial cells to release IL-6, IL-8 and TNF-α. Recently It has been reported that *Moraxella catarrhalis* provoke monocyte-specific chemotactic (MCP-1) activity and noted different induction of inflammatory cytokines of IL-8, IL-6, and GM-CSF from A549 cells (Rosseau et al., 2005).

Also very recently it has been reported that A549 cells grown in 3 dimensional (3-D) aggregates responded to *P. aeruginosa* challenge by increasing the production of the cytokines IL-12, TNF-α, IL-10, and IL-6 (Carterson et al., 2005).

Previously we discussed the release of different cytokines upon stimulation with live bacteria. Incubation of A549 epithelial cells with bacteria induced IL-8 so this IL-8 induction was due to some of bacterial components. Bacterial cell components such LPS, PGN, flagella, pilli participate in this induction. In addition to that secreted components might be released by bacteria and may share the induction of IL-8 from A549 cells, an example of these components is the exotoxin from *P. aeruginosa* and type III secretion from *B. cepacia.*

On of these components, LPS, which is part of the cell wall of the bacteria, is one of the most potent virulent factors in Gram-negative bacteria. Several investigators (Koyama et al., 2000,Reddi et al., 2003) have reported that LPS induced IL-8 from A549 epithelial cells, whereas (Standiford et al 1990) have reported that LPS did not stimulate the release of IL-8 from A549 cells. These conflicting results may be due the passage of A549 cells or may be due to other condition such as the situation how these cells were stimulated.

Peptidoglycan (PGN) is one of the components of the cell wall. Virtually all bacteria contain a layer of PGN, but the amount, location, and specific composition vary. For example, PGN is found as a thick exposed layer comprising the bacterial cell wall of Gram-positive bacteria in association with LTA, whereas in Gram-negative bacteria it is present as thin layer overlaid by a thick layer of LPS. In Gram-positive and Gram-negative bacteria, PGN as well as LTA and LPS are released spontaneously into the culture medium during the growth of bacteria (Pollack et al., 1992,Soto et al., 1996).

Previous studies demonstrated that Peptidoglycan stimulated the release of IL-8 from vaginal cell line epithelial cells (Pivarcsi et al., 2005,Kumar et al., 2004). Also it has demonstrated by (Shimizu et al., 2004) that human uroepithelial cell lines release IL-8 cytokine and the requirement of sCD14 upon stimulation with PGN. It has been reported that human corneal epithelial cells fail to secrete IL-6 and IL-8 stimulated with PGN (Ueta et al., 2004). Recently it has demonstrated that A549 lung and bronchial epithelial cell line showed BEAS-2B showed response by expressing IL-6, IL-8 mRNA and TLR2 upon the stimulation with PGN (Homma et al., 2004)

Flagella a major virulent component in Gram-negative bacteria and during normal growth and infection, many bacteria secrete flagellin, the structural component of the bacterial flagellum (Macnab, 1999). In epithelial cells, flagellin from different bacterial species elicits a strong inflammatory induction including IL-6, IL-8 secretion and inducible NO synthase activity (DiMango et al., 1995,Gewirtz et al., 2001).

Recent investigations have indicated that flagellin, the principal component of bacterial flagella (Samatey et al., 2001), can induce the expression of proinflammatory cytokine by intestinal epithelial cells (Eaves-Pyles et al., 2001,Steiner et al., 2000) *in vitro*. It has been reported that flagellin is a unique ligand of TLR5 (Hayashi et al., 2001). Recent report by Liaudet and his colleagues showed that very small concentrations of flagellin are potent and induced IL-8 cytokine (Liaudet et al., 2003) from A549 epithelial cells and increase the expression of ICAM-1. Recently it has been demonstrated that Flagellin from *P. aeruginosa* induced IL-8 cytokine from lung epithelial cells CALU-3 and A549 cells (Cobb et al., 2004).

Other example of bacterial components may contribute in the inflammatory induction in the lung. These specific components are released in the culture media during the growth of bacteria. Supernatant from Gram-negative bacteria have different components that stimulate the epithelial cells to secrete different proinflammatory cytokines. Like many other Gram-negative bacteria, *Burkholderia cepacia* naturally releases these components during normal growth.

Studies have demonstrated the ability of *B. cepacia* exoproducts, including LPS, to elicit cytokines released from lung epithelial cells (Palfreyman et al., 1997). The release of phospholipase C was reported by (Weingart and Hooke, 1999). It was demonstrated that supernatant from *B. cepacia* induced IL-8 from A549 epithelial cells (Palfreyman et al., 1997). Also it has reported that *B. cepacia* cell-free culture supernatants induce the release of IL-6 and IL-8, but not IL-1β or GM-CSF, from A549 epithelial cells (Fink, Steer et al., 2003). *P. aeruginosa* also secretes different components during their normal growth.

Previous studies investigated and identify of the *P. aeruginosa* secretory factor with the properties of Pyocyanin has increased IL-8 release by A549 epithelial cells (Denning et al., 1998). Small molecular weight secretory factors was reported to induce IL-8 from A549 epithelial cells (Leidal et al., 2001). In *P. aeruginosa* infections, exoenzyme S (ExoS) and type III secreted toxins (ExoT, ExoU, and ExoY) are responsible for causing acute lung injury and sepsis (Finck-Barbancon et al., 1997,Roy-Burman et al., 2001). These secretory products such as type III proteins ExoS, ExoU and ExoY from *P. aeruginosa* were not investigated for cytokine stimulation.

In this study we demonstrated that both intact components from heat killed bacteria and secreted from conditioned media from *P. aeruginosa* and *B. cepacia* participated in IL-8 induction from A549 epithelial cells. We know that heat killed bacteria have the intact components such as LPS and PGN in addition to flagella and any cell contents like DNA, whereas IL-8 induction by conditioned media from bacteria is supposed to be due to any components might secreted by the bacteria such as Pyocyanin and secreted proteins in addition to LPS, PGN and other secretory products discussed earlier.

8.4 LPS INDUCED IL-8 RESPONSE FROM LUNG EPITHELIAL CELLS

In our previous discussion we demonstrated that live bacteria, heat killed and conditioned media from *P. aeruginosa* and *B. cepacia* induced IL-8 from A549 lung epithelial cells. This study also charaterised that the IL-8 released was partially due to the LPS involvement in the induction by live bacteria and heated killed bacteria (HK). This conclusion is based on the presence of LPS determined by LAL assay and on the inhibition by Polymyxin B for IL-8 release in presence of human serum. Also neutralising TLR4 and CD14 receptors showed significant reduction of IL-8 induced by live bacteria and this suggest the involvement of LPS in induction of IL-8 by bacteria. To validate and characterise this concluded results, LPS isolation was made from different strains of *P. aeruginosa* and *B. cepacia* and the responses of the A549 epithelial cells to these LPS were investigated.

Previous studies on A549 response to LPS induction of IL-8 cytokine have demonstrated variable results. Some of these investigations were comparable and others were differential with contradictory results. Some studies have been shown that lung epithelial A549 cells do not respond to LPS (Standiford et al., 1990, Palfreyman, Watson et al, 1997, Fink, Steer et al., 2003), while others have described IL-8 release from A549 cells following stimulation with *P. aeruginosa* LPS (Koyama et al., 2000), and *B. cepacia* (Reddi et al., 2003). Recent report demonstrated that A549 release IL-8 protein when stimulation with LPS (Sachse et al., 2005).

Two important points must be discussed here; one is the concentration of LPS that has been used to stimulate the lung epithelial cells and the second point is the condition used in stimulation. The concentration of LPS is critical and important and either should reflect the number of bacteria during lung infection or reflect the amount of LPS in the lung of patients with lower respiratory diseases. The concentration of LPS in the lung of patients with bacterial lung infection is not reported but the LPS concentration in bronchoalveolar lavage (BAL) samples from patients with acute respiratory distress syndrom (ARDS) have been reported to have 0.001-1.6 ng/mL by (Martin et al., 1994).

The stimulation is also critical, some investigators stimulated the A549 cells with LPS in absence of human serum or in incomplete media and demonstrated no IL-8 release from A549 epithelial cells (Standiford et al., 1990). Other investigator used human serum in their investigation but with different responses (Reddi et al., 2003) and reported responses of release of IL-8 from A549 cells in presence of human serum whereas Standiford et al., 1990 and others reported no IL-8 release even in the presence of human serum.

The concentration of LPS based on the number of bacteria also should be taken into consideration in these studies. In studies where IL-8 release was reported, high concentration of LPS was used (100 μg/mL) (Koyama et al., 2000). High concentration of LPS used in the stimulation A549 cells is not relevant to the concentration of LPS in the lung and might put the viability of theses cells not only under doubt but also under extra potential stress. In addition that commercially available LPS preparations are frequently contaminated with lipoproteins and lipopeptide (Hirschfeld et al., 2000) and this might induce inflammatory responses through a pathway other than the TLR4, like TLR2 (Abreu et al., 2001,Hirschfeld et al., 2000).

Using Polymyxin B as an inhibitor of LPS stimulatory response is beneficial and many studies demonstrated that Polymyxin B inhibits LPS induced cytokine. (Uehara et al., 2002) has demonstrated that Polymyxin B did not affect the activity of LTA, PGN on cytokine induction from human epithelial cells, whereas the activity of LPS was completely inhibited. Another study demonstrated that IL-8 induced from A549 epithelial cells by LPS from different strains of *B. cepacia* was completely abolished by coincubation with Polymyxin B (Palfreyman et al., 1997). The activity of *P. aeruginosa* LPS on cytokines induction from A549 cells also was inhibited by PMB (Koyama et al., 1999). LAL assay is also used to determine the LPS bioactivity but does not show the contamination content of LPS.

In this study we investigated the responses of the A549 cells to different concentrations of LPS in different conditions in presence and absence of human serum. The concentration used

in our study for the time course is quite moderate and did not exceed 1μg/mL trying to lower any possible responses by contaminants and to keep the epithelial cells far from any possible extra potential and sudden chock might happened when very high concentration of LPS are used. Another reason high dose of LPS or infection in the lung does not happened suddenly but with gradual exposure to LPS or bacteria. Using different condition of stimulation is to mimic the reality in the lung in the early or the beginning of inflammation before the leakage of serum and also at the stage after leakage of serum in the lung.

The present study demonstrated that A549 epithelial cells responded and released IL-8 cytokine only to high concentration of LPS in the absence of human serum. So in the absence of human serum or in the early stage of infection, the A549 lung epithelial cells respond poorly to LPS. At the same time, it has been put in account that during bacterial lung infection not only LPS but other components PGN and any another components such flagellin and excreted components participate in the inflammation. The A549 epithelial cells response to bacterial infection should be judged to all components in order to have a full and complete view about A549 epithelial cells response especially in early infection where human serum is not present.

When human serum was added, the A549 cells became more responsive to LPS and released IL-8 cytokine to lesser concentration of LPS. The A549 epithelial cells responded to all isolated LPS from NCTC and clinical strain of *P. aeruginosa* and *B. cepacia* and commercial LPS from *P. aeruginosa* serotype 10 but with differences in the amount of IL-8 released. LPS isolated from *P. aeruginosa* clinical strain was the more potent than the commercially serotype 10 from *P. aeruginosa*, whereas LPS from *B. cepacia* ET-12 clinical strain is less potent than LPS from *B. cepacia* NCTC 10661 strain. To examine whether the endotoxin activity of commercial LPS preparation from *P. aeruginosa* is similar to isolated LPS in our lab, the activities were assessed using LAL assay. Polymyxin B showed inhibited the bioactivity of LPS from clinical and NCTC strains, so combining LAL assay and PMB neutralising of LPS activity reveal that these LPS has endotoxin bioactivity.

Understanding the hyporesponsiveness of the A549 epithelial cells to LPS in the absence of human serum was a good question to be answered and a task to be solved in this issue. The A549 cells hyporesponsive to LPS might be because of the functional deficiency of one or more necessary elements in the signaling pathway including the TLR activation. Adding human serum abolished the hyporesponsivity of A549 cells and the cells become responsive to the LPS. This hyporesponsive give special characteristic to A549 cells to be selective in responses to LPS.

To investigate the pathway, the A549 cells were stimulated with IL-1β which is known to share the common pathway with LPS downstream in the LPS receptor complex (Martin and Wesche, 2002,Zhang and Ghosh, 2001). In this study when A549 cells were stimulated with rIL-1β, the cells released high level of IL-8 cytokine.

These results concluded that A549 cells responsive to rIL-1β in the absence of human serum are present in the intact A549 cell, whereas in LPS signaling pathway seems to be not sharing this specific part in the signaling pathway. The hyporesponsiveness of A549 cells to LPS probably occurs of the deficiency of necessary component/s of the LPS receptor complex in the upstream of MyD88 adaptor protein.

Several studies have demonstrated that a lack of or the low expression of LPS receptor complex components (MD-2, TLR4 or CD14) makes intestinal epithelial cells hyporesponsive to LPS (Cario et al., 2000), Vora et al., 2001), and in the bladder mucosa (Backhed and Hornef, 2003). Also several investigators have suggested a similar lacking of the expression components in responding of LPS receptors complex in the lung epithelial cells, and relating the reason of the hyporesponsive to LPS (Jia et al., 2004). Few studies have reported that lung epithelial cells lack TLR4 and this has been suggested why these cells are hyporesponsive to LPS (Hamann et al., 2002,Tsutsumi-Ishii and Nagaoka, 2003). One recent report also suggested the lack of MD-2 expression was the reason behind the hyporesponsive of the epithelial cells to LPS (Jia et al., 2004).

This study demonstrated the hyporesponsivness of the A549 epithelial cells were not due to the lack of TLR4 (see result of Westen blot), and this likely to be due to the lack optimal amount of surface CD14 since this hyporesponsivity disappeared with adding of human serum which is a rich source of soluble CD14. In addition to this if the A549 epithelial cells is lacking the TLR4 as suggested by (Tsutsumi-Ishii & Nagaoka, 2003) which is the core in receptors in LPS signalling, the cells will not respond to LPS even in the presence of CD14 in the human serum. Moreover the A549 epithelial cells respond to small concentration of LPS when human serum was added and this indicate the need of CD14 and also blocking CD14 in the human serum indicated the involvement of CD14 in human serum for this induction. These results are in agreement with a study by (Schulz et al, 2002) which demonstrated the necessary of soluble CD14 in the human serum in LPS induced IL-8 by A549 cells.

These results explain the hyporesponsiveness of the A549 epithelial cells in the lung in the early stage of bacterial inflammations where the LPS concentration will be limited and where there is no human serum. In this situation the A549 epithelial cells act as hyporesponsive to even high LPS concentration. Whereas with progression of the inflammation, injuries occur and components which include serum will leak into the environment of inflammation, where the epithelial cells become more responsive and the LPS acts to stimulate the cells. The A549 epithelial cells in the lung seem to play very distinct and well balanced role in LPS responsiveness and controlling the activation in the lung.

This study demonstrated that A549 cells express a low molecular weight (100 kDa) isoform of TLR4 protein when examined by Western blot. The expression of TLR4 also was detected by flow cytometry. The expression of the other receptors mCD14, LBP and MD-2 was investigated in order to have more overview about the role of these components on LPS induced IL-8 from A549 epithelial cells (Abate, 2005) and has shed some light on the responsiveness of the epithelial cells in the lung.

8.5 EFFECT OF PULMONARY SURFACTANT & SURFACTANT LIPIDS ON IL-8 INDUCED FROM A549 EPITHELIAL CELLS

Lung epithelial cells play an important role in inflammation, releasing proinflammatory cytokines such as IL-8. IL-8 is a principal chemokine released from lung epithelial cells and is considered to play a pivotal role in recruitment of inflammatory cells to the area of infection in the lung and promote their transendothelial migration. Human IL-8 can also bind to endothelial cell surfaces (Rot et al., 1996) and even be transported across endothelial cells to be presented at the luminal surface (Quinton et al., 2004). Quinton et al. described neutrophil recruitment to the airways is profound and their work suggests that preactivated neutrophils have an inherent tendency to end up in the airways. Of course, preferential homing of neutrophils to the lungs has been known for at least more than 50 years (Bierman et al., 1952), and IL-8 induced neutrophil accumulation in the lung was described more than a decade ago (Van Zee et al., 1992). Neutrophil trafficking to the lung and airways is not well understood.

In cystic fibrosis (CF), an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, altered mucus viscosity is associated with recurrent bronchial bacterial infections, and chronic lung inflammation. Most morbidity and mortality associated with chronic lung infections are due to chronic, progressive airway destruction.

A hallmark of lung inflammation in CF is the accumulation of neutrophils into the airway lumen. Recruitment of neutrophils to the airway surface is a two-edged sword, necessary for surveillance and removal of local infection, but it exposes vulnerable epithelium to potent, damaging mediators including neutrophil elastase and reactive oxygen species. Dysregulation of neutrophil influx results in excessive protease release and tissue damage (Wilmott et al., 1990,Birrer et al., 1994). Although protease damage is a key component of airway

devastation, it alone does not account for all of the inflammation present in lungs.

Protease is also responsible for sepsis-induced microvascular permeability and alveolar epithelial cell damage. Indeed, marked increases in proinflammatory cytokines and chemokines in particular, the chemokine interleukin 8 (IL-8) in patients inflamed lungs have been reported (Dean et al., 1993). An overproduction of IL-8 leads to an influx of neutrophils, ultimately contributing to the chronic, debilitating lung damage observed in CF patients (Dinwiddie, 2000).

The predominant pathogens that colonize CF patients are *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia*. This study has focused on lower respiratory infections by Gram-negative bacteria since *P. aeruginosa* is the predominant CF pathogen and is commonly isolated from 80% of CF patients and *B. cepacia* is a highly invasive organism that can migrate across the epithelial barrier of the lung and invade the parenchyma and capillaries. *B. cepacia* is increasingly becoming a deadly human pathogen in the CF community. Also there is accumulating evidence that gram negative bacterial infections play a role in the pathology and in the inflammatory responses in chronic lung diseases (Sethi, 2000a,Sethi, 2000b).

In the previous section in this chapter, the induction of IL-8 cytokine by both NCTC and clinical strains of *P. aeruginosa* and *B. cepacia* was discussed. Also this study has demonstrated the induction of IL-8 by LPS, heat-killed and conditioned media from bacteria from the same NCTC and clinical strains. The induction of IL-8 cytokines by bacteria and bacterial products and release from the epithelial cells in the lung needs to be highly regulated to control the influx of neutrophils and prevent damage to the inflamed lung. Neutralizing antibodies against IL-8 prevented lung injury in animal models of lung disease, indicating that IL-8 is an important mediator of lung injury (Broaddus et al., 1994).

Many studies has been reported the involvement of pulmonary surfactant protein in the immune regulation defence in the lung (Whitsett JA, 2005). These reports showed the pulmonary surfactant proteins SP-A and SP-D could bind with bacteria and induce direct killing or facilitating the uptake and killing by immune cells (Wu et al., 2003). SP-A and SP-D bind to LPS (Sano et al., 1999) and to CD14 (Sano et al., 2000) constitute a likely mechanism by which SP-A modulates LPS-elicited cellular responses. Also it has been reported that SP-A binds to bacteria such as *S. aureus* (Geertsma et al., 1994), *P. aeruginosa*, *E. coli* (van Iwaarden et al., 1994), *Mycobacterium tuberculosis* (Pasula et al., 1997), *S. pneumonia*, Group A, GBS, *H. influenzae* and encapsulated *K. pneumoniae* via the outer membrane protein (McNeely and Coonrod, 1994, Kabha et al., 1997). Similarly, SP-D has been shown to bind a variety of different bacteria including Gram-negative bacteria (Pikaar et al., 1995, van Iwaarden et al., 1994), and to LPS from *K. pneumoniae* (Sahly et al., 2002), LPS from *P. aeruginosa* and *E. coli* (Kishore et al., 1996), and to cell wall components of Gram-positive bacteria such as LTA (van de Wetering et al., 2001).

Recently it has been shown that SP-D and regulates metaloproteinase-12 (MMP-12) production that in turn alters the surface expression of phagocytic receptors (sCD14) and LPS induced TNF-α induced by GBS, *H. influenzam* and LPS (Senft et al., 2005). Also SP-D was shown to inhibit IL-6 induced by non-capsulated *K. pneumoniae* (Kostina et al., 2005). There is also evidence that surfactant lipids could modulate the innate immunity in the lung. The phospholipid component DPPC inhibits the production of reactive oxygen species (ROS) in human alveolar macrophage (Hayakawa et al., 1989).

The results also show that DPPC phospholipid modulates the production of reactive oxygen species intermediate and TNF-α in human monocytes (Tonks et al., 1999, Tonks et al., 2001). DPPC alone also shows to suppress neutrophils respiratory burst oxidase response (Suwabe et al., 1998). It has been shown that DPPC regulated PAF in human monocytes (Tonks et al, 2003). Recently it has been shown that other phospholipids such as PLPC and POPC can modulate the induction of IL-8 from A549 epithelial cells (Abate, 2005).

Surfactant preparation Survanta® inhibits phagocytosis and the association of GBS to rat alveolar macrophage (Golioto and Wright, 2002). DPPC inhibited the ability of GBS to invade A549 cells and to stimulate the production of IL-8 (Doran et al., 2002) and inhibited GBS induced injury to A549 cells (Nizet et al., 1996). It has been reported that Survanta®, Curosurf® and individual lipid component such as DPPC have also been shown to modulate the production PGE_2 (Morris et al., 2000), and inflammatory cytokines (Thomassen et al., 1992; Thomassen et al, 1994; Walti et al., 1997). Recent report by has shown that surfactant preparation of (Alveofact) blocked the TNF-α released from human monocytic cells in response to *P.aeruginosa* and *P.aeruginosa* LPS (Bufler et al., 2004).

The down regulation of IL-8 cytokine induced by bacteria and bacterial products such as LPS in the airways and alveolus may be a helpful tool to improve the clinical situation of patients suffering from pulmonary chronic infections. The down regulation of IL-8 cytokine released in the lung would prevent recruitment of neutrophils and control the release of the overproduction of proteases that are responsible for debilitating lung damage in the lung. In other word the orchestra of IL-8 induction and down regulation of the protease produced by the neutrophils recruited in the lung must be controlled to a stage that it will prevent the responses and may to keep it in a balanced state in a way that damage to the lung will be prevented and not occur.

In the lung epithelium, IL-8 expression is induced by a wide variety of agents, including IL-1, bacterial cell wall products (Massion et al., 1994), and TNF-α (Standiford e al., 1990). The airway and respiratory epithelia play important roles in the initiation and modulation of inflammatory responses via production of cytokines and surfactant.

In this current investigation as hypothesised that pulmonary surfactant and particularly surfactant phospholipids components could play an important role in down-modulating the production of IL-8 cytokine released by the lung epithelial cells and thereby consequently suppressing the inflammatory responses in the lung.

This hypothesis was based on studies reported that pulmonary surfactants down modulate different immune responses in human monocytes (Morris et al., 2000, Tonks et al., 1999, Tonks et al 2005). Also the hypothesis is based on the growing evidence that pulmonary surfactant not only confers the mechanical stability in the alveoli and in the small airways (Bastacky et al., 1995, Bernhard et al., 1997, Hills, 1999,Thomassen et al., 1994) but also has a potential role in modulating inflammation in normal and injured lung (Coonrod, 1987,Crouch and Wright, 2001,Wu et al., 2003).

In this study, the results support the hypothesis. It has been shown that Survanta® and Curosurf® which are pulmonary surfactant preparations used for replacement therapy, as well as the phospholipid DPPC, modulated inflammation by suppression of IL-8 induced from A549 epithelial cells. This down modulation of IL-8 cytokine was seen for environmental and clinical strains of *P. aeruginosa* and *B. cepacia.* It was also demonstrated in this study that the down modulation of IL-8 cytokine induced by live bacteria from A549 epithelial cells by Survanta® and DPPC was at the gene expression level as documented by the suppression of mRNA of IL-8.

To identify the bacterial components induced IL-8 and modulated by surfactant in presence and absence of human serum. One of the serum dependent components is LPS, which secreted and harbored by *P. aeruginosa* and *B. cepacia* was hypothesised and investigated later in this study. Other components such as the pilli and flagella are serum independent and contributed on IL-8 induction and might be modulated by surfactant lipids. This study focused on serum dependent component that was down regulated by surfactants. Isolation and identification of this component are essential need for future investigation of possible modulation of IL-8 by surfactant lipids.

To identify the serum dependent components that is modulated by Survanta®, Curosurf® or DPPC, heated-killed bacteria were investigated and showed that IL-8 induced by HK from both bacteria in presence of human serum was down modulated by surfactant. LAL assay and Polymyxin B results revealed that one of the components participated in IL-8 induction was

LPS. Further more, the IL-8 activity was inhibited with anti-TLR4 but not anti-TLR2 antibody providing further evidence that the active component is LPS. In addition, peptidoglycan-mediated IL-8 production was not modulated by the surfactants again supporting LPS as the major component inhibited by the surfactant lipids.

To characterise some of these components that their IL-8 induction was modulated by surfactant, LPS from environmental and clinical isolates from the same bacteria were isolated and the effect of surfactant preparations Survanta®, Curosurf® (with their constituent of SP-B, SP-C) and phospholipids DPPC on IL-8 induced was investigated. The results in this study showed that Survanta® and Curosurf® down modulated IL-8 induced by all isolated LPS from clinical strains of *P. aeruginosa* and *B. cepacia* ET-12 as well as the LPS from *P. aeruginosa* serotype 10. In addition to that Polymyxin B inhibited bacterial and LPS induced IL-8. This evidence leads the investigation of our study toward focusing on the effect of pulmonary surfactants on LPS induction from A549 epithelial cells.

Pulmonary surfactant is composed of proteins and phospholipids of which dipalmitoylphosphatidylcholine (DPPC) is the major constituent of pulmonary surfactant, accounting for 60% by weight of total lipids. This current study also investigated the phospholipid DPPC effect alone on the release of IL-8 from A549 epithelial cells upon the induction with both live bacteria and cell wall components like LPS and PGN. The results from this study demonstrated that DPPC down regulated the production of IL-8 induced by live bacteria of both environmental and clinical strains of *P. aeruginosa* and *B. cepacia.* When live bacteria of both environmental and clinical strains of *P. aeruginosa* and *B. cepacia* was investigated, the DPPC also down regulated the production of IL-8 proteins and IL-8 mRNA.

Also DPPC down regulated IL-8 induced by LPS from *P. aeruginosa* clinical strain, *B. cepacia* NCTC10661, *B. cepacia* ET-12 and the commercially LPS from *P. aeruginosa* serotype 10. When LPS from clinical strains of *P. aeruginosa* and *B. cepacia* and LPS from *P. aeruginosa* clinical serotype 10 was investigated, the DPPC also down regulated the

production of IL-8 proteins and IL-8 mRNA.

This current study has demonstrated, for the first time the modulatory, action of Survanta®, Curosurf® and DPPC on Gram-negative bacteria-induced IL-8 from A549 lung epithelial cells, and also for the first time it was demonstrated that this down regulation was similar for both environmental and clinical strains of *P. aeruginosa* and *B. cepacia*. This down regulation of IL-8 by surfactant Survanta® and Curosurf® may be shared by action of the proteins constituent (SP-B and SP-C) in addition to the phospholipids such as DPPC or any other lipids contained in these surfactant preparations. SP-A has been shown to modulate inflammatory responses through the binding to microbial carbohydrate determinants (Holmskove et al., 1994), also SP-A and SP-D bind to some well known inflammation causing ligands from bacterial cell walls such as LPS, PGN, LTA (Stamme et al., 1999, Crouch and Wright, 2001). In addition it was reported that SP-A inhibits the binding of LBP with LPS induced TNF-α from human embryonic cells (HEK293) (Stamme et al., 2002), and down regulate LPS induced IL-10 from mice alveolar macrophage (Chabot et al., 2003.

It is well known that SP-B and SP-C stabilizing the phospholipid monolayer but not known if these proteins have any modulatory effect on cytokines production. In addition the presence of SP-B and SP-C in surfactant preparations Survanta® and Curosurf® is very low, and this put the possibility of these proteins on the contribution of IL-8 inhibition in less important but with possible consideration. In addition to that phospholipids other than DPPC and other lipids content in both surfactant preparations may have possible contribution to the down regulation of IL-8 production since it has been shown recently that palmitoyllinoleoyl phosphatidylcholine (PLPC) and palmitoyloleoylphosphatidylcholine (POPC) down regulate LPS induced IL-8 from A549 cells (Abate, 2005). In this study Wheras DPPC has been charaterised as individual component on IL-8 down regulation, the elucidating of the real participation of each individual components of surfactant proteins and lipids other than DPPC in Survanta® and Curosurf® preparations need characterisation and further studies.

Identifying the components involved in this IL-8 induction and modulation was also investigated and this study demonstrated that there was at least two major components from gram negative bacteria were involved on this orchestra; Serum dependent components in which LPS was identified later as one of these components and serum independent components in which flagellin and other components that need to be fully identified.

In support of the modulatory action of DPPC and surfactant preparations Survanta®, Curosurf® in this study there have been several studies demonstrating the modulatory action of DPPC on TNF-α release by monocytes (Morris et al., 2000,Suwabe et al., 1998,Tonks et al., 1999,Walti et al., 1997). Recent reports demonstrated that Survanta® inhibits proinflammatory cytokines secreted from LPS-stimulated human alveolar macrophages (Raychaudhuri et al., 2004) and a very recent report that surfactant preparations and their major constituent DPPC can downregulate oxidative functions in human monocytes by a mechanism that may involve PKC regulation (Tonks et al., 2005). These studies support a general immunomodulatory role for pulmonary surfactants that agree with the role of surfactant on IL-8 induction in lung epithelial cells found in this work.

In summary, the current study has shown that surfactant can down regulate IL-8 cytokine released by A549 epithelial cells induced by Gram-negative bacteria and LPS. This immunoregulatory role of surfactant preparations and their constituent DPPC on proinflammatory responses of the lung epithelium will act to control the release of neutrophils and further control the release of proteases, which are responsible for the destruction of the lung parenchyma during lower respiratory tract bacterial infections.

Indeed, down regulation of the IL-8 released from the lung epithelium by surfactant lipids has clarified one of the discovered functional properties of surfactant lipids and added to its major functions in the lung. The new insight of down regulation by surfactant lipids may be a potential therapeutic use in lung injury by acting terminating the acute inflammatory responses. Understanding this down regulatory effect of surfactant lipids may open new therapeutic area in lower respiratory inflammations.

8.6 THE MECHANISMS OF ACTION OF SURFACTANT LIPIDS ON IL-8 INDUCED FROM A549 EPITHELIAL CELLS

An important question raised by the current study is how surfactant lipids down regulate the induction of IL-8 from A549 epithelial cells in the lung. Generally pulmonary surfactant plays an important role in immunoregulation of many cytokines in the lung. Surfactant proteins A and D have distinct functions in the innate immune response to microbial pathogens. Both SP-A and SP-D has direct antimicrobial killing in addition to facilitating pathogen uptake and killing by immune cells (Wu et al., 2003). Studies with SP-A knockout mice revealed that these animals are more sensitive to Bacterial infection (Korthagen et al., 1998). SP-A shown to modulate the cellular response to LPS (Sano et al., 1999) and also regulates the TNF-α and other pro-inflammatory cytokine induced by LPS in alveolar macrophage (Arias-Diaz et al., 2000, Alcorn F and Wright, 2004). In addition, the surfactant lipids suppress variety immune responses of lymphocytes and alveolar macrophages (Speer et al., 1991, Tonks et al., 2003, Raychaudhuri et al., 2004, Tonks et al 2005)

The majority of previous studies of the immunomodulatory properties of whole surfactant preparations, or the constituent proteins or phospholipids have investigated the mechanism of action (Tonks et al., 1999, Tonks et al., 2003, Raychaudhuri et al. 2004, Tonks et al., 2005). Some of these studies demonstrated that DPPC modulated PAF by acting and decreasing the membrane fluidity of MM6 (Tonks et al., 2003), Wheras other reports demonstrated that DPPD down regulated the inflammatory (ROI) of monocytes is independent of MAPK (ERK 1, ERK 2 and p38) (Tonks et al., 2001). Two recent studies have demonstrated that Survanta® blocks lipopolysaccharide signaling by inhibiting both mitogen-activated protein and IκB in human alveolar macrophages (Raychaudhuri et al. 2004), and DPPC modulated the ROI through PKC mechanism (Tonks et al., 2005).

To elucidate the mechanism by which the surfactant and phospholipid DPPC down-regulated IL-8 induced by bacteria and LPS in the lung epithelial cells, A549 cells was stimulated with exogenous IL-1β in presence or absence of Survanta®, Curosurf® or DPPC. When the cells

were pre-incubated with surfactant or DPPC prior stimulation with IL-1β, IL-8 induction was not inhibited. Based on this and knowing that IL-1β and LPS share similarity in their signaling pathway (Muzio et al., 2000), it suggests that the surfactants action is not involved in the down stream of pathways from MyD88 to NF-kB activation.

These observations, in addition to previous investigation done on the effect of DPPC on the plasma membrane fluidity on other cell line by (Tonks et al., 2001,Tonks et al., 2003) have driven our attention to the area of the lipid raft membrane. The possible interference of surfactant lipids with the plasma membrane of A549 epithelial cells, which may affect the membrane and may lead to changes in membrane fluidity and on the trans membrane receptors, was predicted and hypothesised. This hypothesis is supported by a report that showed DPPC significantly decreases membrane fluidity in MM6 cells (Tonks et al., 2003). In addition, several studies have shown the involvement of lipid rafts in the innate immune response (Pfeiffer et al., 2001,Triantafilou et al., 2002). The lipid raft domains are composed of cholesterol and sphingolipids that float as platforms in the glycerophospholipid plasma membrane (Munro, 2003).

It has been proposed that lipid rafts are used as a platform where the LPS and signaling proteins are assembled and initiate cell activation. Upon stimulation with LPS, TLR4 translocates to the raft domains and co-clusters with other glycoproteins, such as CD14 which is normally located in the raft micro-domain of the plasma membrane (Triantafilou et al., 2002).

In the current study, the involvement of lipid rafts in IL-8 induction from A549 cells upon stimulation with LPS and bacteria was investigated by using raft disruption agents such as MβCD and the results demonstrated that MβCD significantly suppressed the release of IL-8 from A549 epithelial cells induced by LPS and live bacteria.

Based on these results the mobilisation of TLR4 was investigated and it was demonstrated that upon stimulation with LPS and live bacteria, TLR4 translocated from non-raft to raft micro-domains in lung epithelial cells. These results in agreement with a study on monocytes by (Triantafilou et al., 2002).

Taken together, these findings, suggest that surfactant lipids may act, at least in part, on down regulation of IL-8 induced by LPS and live bacteria by interfering with lipid rafts and mobilisation of TLR4. These finding on the mechanism of action of surfactant on IL-8 induction were further enhanced by considering the possible of other factors which may interfere with this down regulation of IL-8 by surfactant lipids. The effect of surfactant lipids Survanta®, Curosurf® and DPPC on the viability of A549 epithelial cells was investigated and the results excluded any significant reduction on viability or possible toxicity to the cells when compared with untreated cells. Also the viability of the cells with incubation with LPS or live bacteria either alone or together with surfactant preparation and lipids was investigated and the possible effect on the viability was excluded.

The possibility that the surfactant preparations could directly down regulate the expression of TLR4 was investigated by flow cytometry. It was shown that Survanta® or DPPC did not alter the expression of TLR4 directly in A549 cells suggesting that surfactants do not alter signaling via direct modulation of TLR4 expression. The results from this study demonstrate, for the first time, that DPPC and surfactant preparation Survanta® or its constituent lipids, inhibit the action of LPS and live bacteria on IL-8 release from A549 lung epithelial cells by interfering with the recruitment of components necessary for LPS signaling.

In conclusion this study demonstrated that Survanta® and DPPC can modulate bacterial and LPS induced IL-8 from A549 epithelial cells by interfering with the recruitment of TLR4 to the lipid raft microdomains of the epithelial cells. These actions of surfactant lipids consequently affect IL-8 mRNA transcription and further down-regulate the neutrophil recruitment in the lung. This study demonstrated for the first time the mechanism of action of surfactant lipids on IL-8 induced by live Gram-negative bacteria, however the result of

surfactant mechanism action on LPS induced IL-8 agrees with recent investigation (Abate, 2005). Other possible mechanism of actions of DPPC and surfactant preparations may be involved and their involvements are considered but need further investigation especially in A549 lung epithelial cells.

8.7 GENERAL CONCLUSIONS

The study of this project has provided evidence that pulmonary surfactant Survanta®, Curosurf® and phospholipid DPPC may play a pivotal role in bacterial lower respiratory tract infections. Pulmonary surfactant impairment and deficiency of phospholipids composition in cystic fibrosis has been reported (Mander et al., 2002, Meyer et al., 2000, Griese et al 2004), also abnormalities of surfactant have been described in association with variety of infections and pathogens, including bacteria (Baughman et al., 1993, Gunther et al., 1996).

The type II epithelial cells in the lung have a role as a mechanical barrier and play additional important roles by producing surfactant, which play an immunomodulatory role in bacterial infections of the lung. Bacteria reaching the alveoli in the lung come in contact with surfactant and in certain circumstances such as surfactant dysfunction or reduction, will contact the alveolar epithelial cells directly. This would be expected to increase the inflammatory response of these cells and contribute to lung inflammation damage. This bacterial contact with surfactant and lung epithelial cells is one of the important issues investigated in this study.

The present work demonstrated that pulmonary surfactant lipids have an inhibitory effect on Gram-negative bacteria *P. aeruginosa* and *B. cepacia* and this inhibitory action seem to be during the leakage of human serum. This inhibitory action of surfactant lipids on bacterial growth showed to be within the physiological concentration in the lung. This study concluded that the inhibitory action of DPPC and surfactants Survanta®, Curosurf® with their lipids constituent on bacteria has to be in a situation where bacteria should be in a state where

the nutrition is limited, which may be as same in the lung during the early bacterial infection. These roles of surfactants lipids added new insight to the modulatory properties of surfactant concept in general.

This bacterial contact is one of the important issues investigated in this study and show that *P. aeruginosa* and *B. cepacia* are internalised by A549 epithelial cells. This surfactant-mediated bacterial uptake by the epithelial cells might benefit the host aiding the eradication of bacteria from the lung.

The present work also provides *in vitro* data for the first time that surfactant lipids down-regulate bacteria-induced IL-8 release from lung epithelial cells. Also the down regulation was demonstrated in LPS-induced IL-8 from the same epithelial cells. It was shown that the action of surfactant lipids interferes with the translocation of TLR4 receptors from non-raft to raft microdomain on the plasma membrane following bacteria and LPS stimulation. This suggests, one probable mechanism of surfactant on the down regulation of IL-8 from lung epithelial cells and may be from other cells.

Surfactant Survanta®, Curosurf® and phospholipid DPPC down regulate of bacteria and LPS induced IL-8 from A549 epithelial cells. This regulation may lead to the down regulation of neutrophils recruitment and this suggests further affect on the release of destructive neutrophil proteases production that are responsible of tissue damage in the lung. In this situation surfactants may play an indirect role in correcting the overproduction of proteases during lung infection and inflammation.

This study has demonstrated that surfactant attenuates IL-8 secretion from alveolar epithelial cells, suggesting that surfactant may serve an endogenous immunoregulatory role in the lung. This may be of significance, because surfactant function is disrupted in many lung diseases, including acute respiratory disease such as cystic fibrosis, pneumonia and asthma (Griese, 1999). Excess cytokine and chemokine production have been implicated as contributors to the pathology of all of these diseases (Gauldie et al., 1993, Lukacs, 2001).

These data contributes information on the role of surfactant especially the phospholipids in lung, which may resemble the function of surfactant lipids in normal and may shed some light on the need of surfactant lipids in which surfactant may be modified, altered during infection and inflammation of the lung. Also this data may contribute to the possible use of surfactant as replacement therapy in lower respiratory infections caused by Gram-negative bacteria or as alternative strategy to correct the excessive inflammation and tissue damage produced as a result of high production of IL-8 by the epithelial cells in the lung.

8.8 RECOMMENDATION FOR FUTURE STUDIES

Although the current study has provided useful information and insights in the potential immunomodulatory roles of pulmonary surfactant lipids in health and disease, this work could be usefully extended if further time was available. For example, the following investigation could be undertaken:

- The role of surfactant lipids on IL-8 released from lung epithelial cells and induced by other bacterial components other than LPS.
- The effect of surfactant proteins in the uptake of the bacteria by lung epithelial cells.
- The role of pulmonary surfactant lipids on IL-8 induced by Gram-positive bacteria

REFERENCES

Reference List

Abate W. Liopolysaccharide-induced interleukine-8 releas in lung epithelial cells and its regulation by pulmonary surfactant lipid (Ph.D thesis 2005), Universiry of Wales, Cardiff, UK.

Abreu MT, Vora P, Faure E, Thomas LS, Arnold ET, Arditi M. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. J Immunol 2001; 167(3):1609-1616.

Adamo R, Sokol S, Soong G, Gomez MI, Prince A. Pseudomonas aeruginosa flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor. Am J Respir Cell Mol Biol 2004; 30(5):627-634.

Ader F, Le Berre R, Faure K, Gosset P, Epaulard O, Toussaint B, Polack B, Nowak E, Viget NB, Kipnis E, Guery BP. Alveolar response to Pseudomonas aeruginosa: role of the type III secretion system. Infect Immun 2005; 73(7):4263-4271.

Ahuja A, Oh N, Chao W, Spragg RG, Smith RM. Inhibition of the human neutrophil respiratory burst by native and synthetic surfactant. Am J Respir Cell Mol Biol 1996; 14(5):496-503.

Akashi S, Shimazu R, Ogata H, Nagai Y, Takeda K, Kimoto M, Miyake K. Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. J Immunol 2000; 164(7):3471-3475.

Akashi S, Nagai Y, Ogata H, Oikawa M, Fukase K, Kusumoto S, Kawasaki K, Nishijima M, Hayashi S, Kimoto M, Miyake K. Human MD-2 confers on mouse Toll-like receptor 4 species-specific lipopolysaccharide recognition. Int Immunol 2001; 13(12):1595-1599.

Akira S. Mammalian Toll-like receptors. Curr Opin Immunol 2003; (15): 5-11.

Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001; 2(8):675-680.

Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004; 4(7):499-511.

Albert AD, Young JE, Paw Z. Phospholipid fatty acyl spatial distribution in bovine rod outer segment disk membranes. Biochim Biophys Acta 1998; 1368(1):52-60.

Allison DG, Goldsbrough MJ. Polysaccharide production in Pseudomonas cepacia 438. J Basic Microbiol 1994; 34(1):3-10.

Amin RS, Wert SE, Baughman RP, Tomashefski JF, Jr., Nogee LM, Brody AS, Hull WM, Whitsett JA. Surfactant protein deficiency in familial interstitial lung disease. J Pediatr 2001; 139(1):85-92.

Anderson K. V., Bkla L., Nusselein-Volhaed C. Establishment of dorsal-ventral polarity in drosophila embryo: the induction of polarity by the Toll gene product. Cell (1985); (42): 791-798.

Anderson RG. The caveolae membrane system. Annu Rev Biochem 1998; 67:199-225.

Anwar H, Brown MR, Cozens RM, Lambert PA. Isolation and characterization of the outer and cytoplasmic membranes of Pseudomonas cepacia. J Gen Microbiol 1983; 129(2):499-507.

Arbibe L, Mira JP, Teusch N, Kline L, Guha M, Mackman N, Godowski PJ, Ulevitch RJ, Knaus UG. Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway 397. Nat Immunol 2000; 1(6):533-540.

Arenberg DA, Keane MP, DiGiovine B, Kunkel SL, Morris SB, Xue YY, Burdick MD, Glass MC, Iannettoni MD, Strieter RM. Epithelial-neutrophil activating peptide (ENA-78) is an important angiogenic factor in non-small cell lung cancer. J Clin Invest 1998; 102(3):465-472.

Arita Y, Ansamma J, Hshi-Chi K, Yuchi L, Thomas A. Palaia, JM, Jeffrey A. Kazzaz Superoxide dismutase moderates basal and induced bacterial adherence and interleukin-8 expression in airway epithelial cells. *AJP- Lung* 2004 (287):1199-1206.

Atochina EN, Beck JM, Scanlon ST, Preston AM, Beers MF. Pneumocystis carinii pneumonia alters expression and distribution of lung collectins SP-A and SP-D. J Lab Clin Med. 2001 (137): 429-39.

Asano K, Chee CB, Gaston B, Lilly CM, Gerard C, Drazen JM, Stamler JS. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. Proc Natl Acad Sci U S A 1994; 91(21):10089-10093.

Augusto LA, Synguelakis M, Johansson J, Pedron T, Girard R, Chaby R. Interaction of pulmonary surfactant protein C with CD14 and lipopolysaccharide. Infect Immun 2003; 71(1):61-67.

Azghani AO, Idell S, Bains M, Hancock RE. Pseudomonas aeruginosa outer membrane protein F is an adhesin in bacterial binding to lung epithelial cells in culture. Microb Pathog 2002; 33(3):109-114.

Backhed F, Hornef M. Toll-like receptor 4-mediated signaling by epithelial surfaces: necessity or threat? Microbes Infect 2003; (5): 951-959.

Baddiley J. Bacterial cell walls and membranes. Discovery of the teichoic acids. Bioessays 1989; 10(6):207-210.

Bals R, Weiner DJ, Wilson JM. The innate immune system in cystic fibrosis lung disease 134. J Clin Invest 1999; 103(3):303-307.

Bals R, Hiemstra PS. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. Eur Respir J 2004; 23(2):327-333.

Bastacky J, Lee CY, Goerke J, Koushafar H, Yager D, Kenaga L, Speed TP, Chen Y, Clements JA. Alveolar lining layer is thin and continuous: low-temperature scanning electron microscopy of rat lung. J Appl Physiol 1995; 79(5):1615-1628.

Beardsall K, Thompson MH, Mulla RJ. Neonatal group B streptococcal infection in South Bedfordshire, 1993-1998. Arch Dis Child Fetal Neonatal Ed 2000; 82(3):F205-F207.

Beers MF, Hamvas A, Moxley MA, Gonzales LW, Guttentag SH, Solarin KO, Longmore WJ, Nogee LM, Ballard PL. Pulmonary surfactant metabolism in infants lacking surfactant protein B . Am J Respir Cell Mol Biol 2000; 22(3):380-391.

Bernhard W, Haagsman HP, Tschernig T, Poets CF, Postle AD, van Eijk ME, von der HH. Conductive airway surfactant: surface-tension function, biochemical composition, and possible alveolar origin. Am J Respir Cell Mol Biol 1997; 17(1):41-50.

Bernhard W, Hoffmann S, Dombrowsky H, Rau GA, Kamlage A, Kappler M, Haitsma JJ, Freihorst J, von der HH, Poets CF. Phosphatidylcholine molecular species in lung surfactant: composition in relation to respiratory rate and lung development. Am J Respir Cell Mol Biol 2001; 25(6):725-731.

Bernhard W, Haslam PL, Floros J. From birds to humans: new concepts on airways relative to alveolar surfactant. Am J Respir Cell Mol Biol 2004; 30(1):6-11.

Beutler B, Hoebe K, Du X, Ulevitch RJ. How we detect microbes and respond to them: the Toll-like receptors and their transducers. J Leukoc Biol 2003; 74(4):479-485.

Bhakdi S, Klonisch T, Nuber P, Fischer W. Stimulation of monokine production by lipoteichoic acids. Infect Immun 1991; 59(12):4614-4620.

Bianchi M, Fantuzzi G, Bertini R, Perin L, Salmona M, Ghezzi P. The pneumotoxicant paraquat induces IL-8 mRNA in human mononuclear cells and pulmonary epithelial cells. Cytokine 1993; (5): 525-530.

Bierman HR, Kelly KH, Cordes FL, Byron RL, Jr., Polhenus JA, Rppoport S. The release of leukocytes and platelets from the pulmonary circulation by epinephrine. Blood 1952; 7(7):683-692.

Birrer P, McElvaney NG, Rudeberg A, Sommer CW, Liechti-Gallati S, Kraemer R, Hubbard R, Crystal RG. Protease-antiprotease imbalance in the lungs of children with cystic fibrosis. Am J Respir Crit Care Med 1994; 150(1):207-213.

Black RE, Morris SS, Bryce J. Where and why are 10 million children dying every year?. Lancet 2003; 361(9376):2226-2234.

Broaddus V C, Boylan A M, Hoeffel J M, Kim K J, Sadick M, Chuntharapai A, Hebert C A. Neutralization of IL-8 inhibits neutrophil influx in a rabbit model of endotoxin-induced pleurisy. J Immunol 1994; (152): 2960-2967.

Boom R. SOL CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, and Van Der Noordaa J. Rapid and simple method for purification of Nucleic Acid. J. clin Microbiology 1990; (28): 495-503.

Borchers MT, Carty MP, Leikauf GD. Regulation of human airway mucins by acrolein and inflammatory mediators. Am J Physiol 1999; 276(4 Pt 1):L549-L555.

Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 1997; 89(10):3503-3521.

Borron P, McIntosh JC, Korfhagen TR, Whitsett JA, Taylor J, Wright JR. Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production in vivo. Am J Physiol Lung Cell Mol Physiol 2000; 278(4):L840-L847.

Bouhafs RK, Jarstrand C. Lipid peroxidation of lung surfactant by bacteria. Lung 1999; 177(2):101-110.

Broaddus VC, Boylan AM, Hoeffel JM, Kim KJ, Sadick M, Chuntharapai A, Hebert CA. Neutralization of IL-8 inhibits neutrophil influx in a rabbit model of endotoxin-induced pleurisy. J Immunol 1994; 152(6):2960-2967.

Brogden KA. Ovine pulmonary surfactant induces killing of Pasteurella haemolytica, Escherichia coli, and Klebsiella pneumoniae by normal serum. Infect Immun 1992; 60(12):5182-5189.

Bufler P, Schikor D, Schmidt B, Griese M. Cytokine stimulation by Pseudomonas aeruginosa strain variation and modulation by pulmonary surfactant. Exp Lung Res 2004; 30(3):163-179.

Burns JL, Jonas M, Chi EY, Clark DK, Berger A, Griffith A. Invasion of respiratory epithelial cells by Burkholderia (Pseudomonas) cepacia. Infect Immun 1996; 64(10):4054-4059.

Burns J L, Gibson R L, McNamara S, Yim D, Emerson J, Rosenfeld M, Hiatt P, McCoy K, Castile R, Smith A L, Ramsey B W. Longitudinal assessment of Pseudomonas aeruginosa in young children with cystic fibrosis. J Infect Dis 2001; (183): 444-452

Chandra R K, Heresi G, Schlesinger L. In defence of the lung. Indian J Pediatr 1981; (48): 9-12.

Cao Z, Henzel WJ, Gao X. IRAK: a kinase associated with the interleukin-1 receptor. Science 1996; 271(5252):1128-1131.

Cario E, Rosenberg I M, Brandwein S L, Beck P L, Reinecker H C, Podolsky D K. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. J Immunol 2000; (164): 966-972.

Caroff M, Aussel L, Zarrouk H, Martin A, Richards JC, Therisod H, Perry MB, Karibian D. Structural variability and originality of the Bordetella endotoxins. J Endotoxin Res 2001; 7(1):63-68.

Caroff M, Karibian D, Cavaillon JM, Haeffner-Cavaillon N. Structural and functional analyses of bacterial lipopolysaccharides. Microbes Infect 2002; 4(9):915-926.

Carterson AJ, Honer zu BK, Ott CM, Clarke MS, Pierson DL, Vanderburg CR, Buchanan KL, Nickerson CA, Schurr MJ. A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue culture model for Pseudomonas aeruginosa pathogenesis. Infect Immun 2005; 73(2):1129-1140.

Chabot S, Salez L, McCormack FX, Touqui L, Chignard M. Surfactant protein A inhibits lipopolysaccharide-induced in vivo production of interleukin-10 by mononuclear phagocytes during lung inflammation. Am J Respir Cell Mol Biol 2003; 28(3):347-353.

Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, Ogura Y, Kawasaki A, Fukase K, Kusumoto S, Valvano MA, Foster SJ, Mak TW, Nunez G, Inohara N. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat Immunol 2003; 4(7):702-707.

Chang B, Amemura-Maekawa J, Kura F, Kawamura I, Watanabe H. Expression of IL-6 and TNF-alpha in human alveolar epithelial cells is induced by invading, but not by adhering, Legionella pneumophila. Microb Pathog 2004; 37(6):295-302.

Chi E, Mehl T, Nunn D, Lory S. Interaction of Pseudomonas aeruginosa with A549 pneumocyte cells. Infect Immun 1991; 59(3):822-828.

Chimote G, Banerjee R. Lung surfactant dysfunction in tuberculosis: effect of mycobacterial tubercular lipids on dipalmitoylphosphatidylcholine surface activity. Colloids Surf B Biointerfaces 2005; 45(3-4):215-223.

Chung F, Barnes N, Allen M, Angus R, Corris P, Knox A, Miles J, Morice A, O'Reilly J, Richardson M. Assessing the burden of respiratory disease in the UK. Respir Med 2002; 96(12):963-975.

Cieri MV, Mayer-Hamblett N, Griffith A, Burns JL. Correlation between an in vitro invasion assay and a murine model of Burkholderia cepacia lung infection. Infect Immun 2002; 70(3):1081-1086.

Clements JA. Pulmonary edema and permeability of alveolar membranes 125. Arch Environ Health 1961; 2:280-283.

Clerc P, Sansonetti P J. Entry of Shigella flexneri into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. Infect Immun 1987; (55): 2681-2688.

Cleveland MG, Gorham JD, Murphy TL, Tuomanen E, Murphy KM. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect Immun 1996; 64(6):1906-1912.

Clode FE, Kaufmann ME, Malnick H, Pitt TL. 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 2000; 38(5):1763-1766.

Cobb LM, Mychaleckyj JC, Wozniak DJ, Lopez-Boado YS. Pseudomonas aeruginosa flagellin and alginate elicit very distinct gene expression patterns in airway epithelial cells: implications for cystic fibrosis disease. J Immunol 2004; 173(9):5659-5670.

Collins LV, Kristian SA, Weidenmaier C, Faigle M, Van Kessel KP, van Strijp JA, Gotz F, Neumeister B, Peschel A. Staphylococcus aureus strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. J Infect Dis 2002; 186(2):214-219.

Coonrod JD. Role of surfactant free fatty acids in antimicrobial defenses. Eur J Respir Dis Suppl 1987; 153:209-214.

Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999; 284(5418):1318-1322.

Cox G, Gauldie J, Jordana M. Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. Am J Respir Cell Mol Biol 1992; 7(5):507-513.

Crapo JD, Barry BE, Gehr P, Bachofen M, Weibel ER. Cell number and cell characteristics of the normal human lung. Am Rev Respir Dis 1982; 126(2):332-337.

Creuwels LA, van Golde LM, Haagsman HP. The pulmonary surfactant system: biochemical and clinical aspects. Lung 1997; 175(1):1-39.

Cristofaro P, Opal SM. The Toll-like receptors and their role in septic shock. Expert Opin Ther Targets 2003; 7(5):603-612.

Crouch E, Wright JR. Surfactant proteins a and d and pulmonary host defense. Annu Rev Physiol 2001; 63:521-554.

da Silva CJ, Soldau K, Christen U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. J Biol Chem 2001; 276(24):21129-21135.

de Bentzmann S, Plotkowski C, Puchelle E. Receptors in the Pseudomonas aeruginosa adherence to injured and repairing airway epithelium. Am J Respir Crit Care Med 1996; 154(4 Pt 2):S155-S162.

Dean TP, Dai Y, Shute JK, Church MK, Warner JO. Interleukin-8 concentrations are elevated in bronchoalveolar lavage, sputum, and sera of children with cystic fibrosis. Pediatr Res 1993; 34(2):159-161.

DeForge LE, Preston AM, Takeuchi E, Kenney J, Boxer LA, Remick DG. Regulation of interleukin 8 gene expression by oxidant stress. J Biol Chem 1993; 268(34):25568-25576.

Denning GM, Railsback MA, Rasmussen GT, Cox CD, Britigan Be. Pseudomonas pyocyanine alters calcium signaling in human airway epithelial cells. Am J Physiol 1998; 274(6 Pt 1):893-900

Diamond G, Legarda D, Ryan LK. The innate immune response of the respiratory epithelium. Immunol Rev 2000; 173:27-38.

DiMango E, Zar HJ, Bryan R, Prince A. Diverse Pseudomonas aeruginosa gene products stimulate respiratory epithelial cells to produce interleukin-8. J Clin Invest 1995; 96(5):2204-2210.

Dinwiddie R. Pathogenesis of lung disease in cystic fibrosis. Respiration 2000; 67(1):3-8.

Doran KS, Chang JC, Benoit VM, Eckmann L, Nizet V. Group B streptococcal beta-hemolysin/cytolysin promotes invasion of human lung epithelial cells and the release of interleukin-8. J Infect Dis 2002; 185(2):196-203.

Dorger M, Krombach F. Interaction of alveolar macrophages with inhaled mineral particulates. J Aerosol Med 2000; 13(4):369-380.

Dreyfuss D, Ricard JD. Acute lung injury and bacterial infection. Clin Chest Med 2005; 26(1):105-112.

Droemann D, Goldmann T, Branscheid D, Clark R, Dalhoff K, Zabel P, Vollmer E. Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung. Histochem Cell Biol 2003; 119(2):103-108.

Drost EM, MacNee W. Potential role of IL-8, platelet-activating factor and TNF-alpha in the sequestration of neutrophils in the lung: effects on neutrophil deformability, adhesion receptor expression, and chemotaxis. Eur J Immunol 2002; 32(2):393-403.

Dunne A, Ejdeback M, Ludidi PL, O'Neill LA, Gay NJ. Structural complementarity of Toll/interleukin-1 receptor domains in Toll-like receptors and the adaptors Mal and MyD88. J Biol Chem 2003; 278(42):41443-41451.

Dziarski R, Tapping RI, Tobias PS. Binding of bacterial peptidoglycan to CD14. J Biol Chem 1998; 273(15):8680-8690.

Dziarski R, Viriyakosol S, Kirkland TN, Gupta D. Soluble CD14 enhances membrane CD14-mediated responses to peptidoglycan: structural requirements differ from those for responses to lipopolysaccharide. Infect Immun 2000; 68(9):5254-5260.

Dziarski R, Wang Q, Miyake K, Kirschning CJ, Gupta D. MD-2 enables Toll-like receptor 2 (TLR2)-mediated responses to lipopolysaccharide and enhances TLR2-mediated responses to Gram-positive and Gram-negative bacteria and their cell wall components. J Immunol 2001; 166(3):1938-1944.

Eaves-Pyles TD, Wong HR, Odoms K, Pyles RB. Salmonella flagellin-dependent proinflammatory responses are localized to the conserved amino and carboxyl regions of the protein. J Immunol 2001; 167(12):7009-7016.

Eckmann L, Kagnoff MF, Fierer J. Intestinal epithelial cells as watchdogs for the natural immune system. Trends Microbiol 1995; 3(3):118-120.

Edwards JA, Groathouse NA, Boitano S. Bordetella bronchiseptica adherence to cilia is mediated by multiple adhesin factors and blocked by surfactant protein A. Infect Immun 2005; 73(6):3618-3626.

Elborn JS, Nixon L, Bell SC, Shale DJ. On tumor necrosis factor alpha in CF. Pediatr Pulmonol 1994; 17(6):411-412.

Ernst RK, Yi EC, Guo L, Lim KB, Burns JL, Hackett M, Miller SI. Specific lipopolysaccharide found in cystic fibrosis airway Pseudomonas aeruginosa. Science 1999; 286(5444):1561-1565.

Esen N, Tanga FY, DeLeo JA, Kielian T. Toll-like receptor 2 (TLR2) mediates astrocyte activation in response to the Gram-positive bacterium Staphylococcus aureus. J Neurochem 2004; 88(3):746-758.

Fehrenbach H. Alveolar epithelial type II cell: defender of the alveolus revisited. Respir Res 2001; 2(1):33-46.

Feldman M, Bryan R, Rajan S, Scheffler L, Brunnert S, Tang H, Prince A. Role of flagella in pathogenesis of Pseudomonas aeruginosa pulmonary infection. Infect Immun 1998; 66(1):43-51.

Ferguson JS, Voelker DR, Ufnar JA, Dawson AJ, Schlesinger LS. Surfactant protein D inhibition of human macrophage uptake of Mycobacterium tuberculosis is independent of bacterial agglutination. J Immunol 2002; 168(3):1309-1314.

Ferguson J S, Voelker D R, McCormack F X, Schlesinger L S. Surfactant protein D binds to Mycobacterium tuberculosis bacilli and lipoarabinomannan via carbohydrate-lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages. J Immunol 1999; (163): 312-321.

Ferrara A, Dos SC, Lupi A. Effect of some fractions of alveolar surfactant (phospholipids and SP-A) on the bactericidal activity of different antimicrobials against some respiratory pathogens. Clin Microbiol Infect 2001; 7(3):114-119.

Finck-Barbancon V, Goranson J, Zhu L, Sawa T, Wiener-Kronish JP, Fleiszig SM, Wu C, Mende-Mueller L, Frank DW. ExoU expression by Pseudomonas aeruginosa correlates with acute cytotoxicity and epithelial injury. Mol Microbiol 1997; 25(3):547-557.

Finck-Barbancon V, Frank DW. Multiple domains are required for the toxic activity of Pseudomonas aeruginosa ExoU. J Bacteriol 2001; 183(14):4330-4344.

Fink J, Steer JH, Joyce DA, McWilliam AS, Stewart GA. Pro-inflammatory effects of Burkholderia cepacia on cystic fibrosis respiratory epithelium. FEMS Immunol Med Microbiol 2003; 38(3):273-282.

Finlay B B, Cossart P. Exploitation of mammalian host cell functions by bacterial pathogens. Science 1997; (276): 718-725.

Finlay BB, Falkow S. Comparison of the invasion strategies used by Salmonella cholerae-suis, Shigella flexneri and Yersinia enterocolitica to enter cultured animal cells: endosome acidification is not required for bacterial invasion or intracellular replication. Biochimie 1988; 70(8):1089-1099.

Finlay B B, Ruschkowski S, Dedhar S. Cytoskeletal rearrangements accompanying salmonella entry into epithelial cells. J Cell Sci 1991; (99 (Pt 2)): 283-296.

Fischer W. Physiology of lipoteichoic acids in bacteria. Adv Microb Physiol 1988; 29:233-302.

Fischer W. Lipoteichoic acid and lipids in the membrane of Staphylococcus aureus. Med Microbiol Immunol (Berl) 1994; 183(2):61-76.

Froh D, Ballard Pl, Williams MC, Gonzales J, Goerke J, Odom MW, Gonzales LW. Lamellar bodies of cultured human fetal lung: content of surfactant protein A (SP-A), surface film formation and structural transformation in vitro. Biochem Biophys Acta. 1990 (1): 78-89.

Fitzgerald K.A, Palsson-McDwemott E. M, Bowie A.G, Jefferies C.A, Mansell A., Brady G, Brint E, Dunne A, O'Neill L.A. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature 2001; 413:78-83.

Fitz Simmons SC. The changing epidemiology of cystic fibrosis. J Pediatr 1993; 122(1):1-9.

Fuchs S. Gumbleton, U, Schafer F, Lehr CM: Models of alveolar epithelium, In cell culture models of biological barrier- In vitro test system for drug absorption and delivery. 2002, New York.

Gangloff S.C, Zähringer U, Blondin C, Guenounou M, Silver J and Goyert S.G: Influence of CD14 on Ligand Interactions between Lipopolysaccharide and Its Receptor Complex. The Journal of Immunology 2005: 175: 3940-3945.

Ganz T. Epithelia: not just physical barriers. Proc Natl Acad Sci U S A 2002; 99(6):3357-3358.

Garenne M, Ronsmans C, Campbell H. The magnitude of mortality from acute respiratory infections in children under 5 years in developing countries. World Health Stat Q 1992; 45(2-3):180-191.

Gaynor CD, McCormack FX, Voelker DR, McGowan SE, Schlesinger LS. Pulmonary surfactant protein A mediates enhanced phagocytosis of Mycobacterium tuberculosis by a direct interaction with human macrophages. J Immunol 1995; 155(11):5343-5351.

Geertsma MF, Broos HR, van den Barselaar MT, Nibbering PH, van Furth R. Lung surfactant suppresses oxygen-dependent bactericidal functions of human blood monocytes by inhibiting the assembly of the NADPH oxidase. J Immunol 1993; 150(6):2391-2400.

Geertsma MF, Nibbering PH, Haagsman HP, Daha MR, van Furth R. Binding of surfactant protein A to C1q receptors mediates phagocytosis of Staphylococcus aureus by monocytes. Am J Physiol 1994; 267(5 Pt 1):L578-L584.

Gehr P, Bachofen M, Weibel ER. The normal human lung: ultrastructure and morphometric estimation of diffusion capacity. Respir Physiol 1978; 32(2):121-140.

Gehr P, Geiser M, Im H, V, Schurch S, Waber U, Baumann M. Surfactant and inhaled particles in the conducting airways: structural, stereological, and biophysical aspects. Microsc Res Tech 1993; (26): 423-436.

Gelb AF, Schein M, Kuei J, Tashkin DP, Muller NL, Hogg JC, Epstein JD, Zamel N. Limited contribution of emphysema in advanced chronic obstructive pulmonary disease. Am Rev Respir Dis 1993; 147(5):1157-1161.

Gessner AR, Mortensen JE. Pathogenic factors of Pseudomonas cepacia isolates from patients with cystic fibrosis. J Med Microbiol 1990; 33(2):115-120.

Gewirtz A T, Simon P O, Jr., Schmitt C K, Taylor L J, Hagedorn C H, O'Brien A D, Neish A S, Madara J L. Salmonella typhimurium translocates flagellin across intestinal epithelia, inducing a proinflammatory response. J Clin Invest 2001; (107): 99-109.

Giard D J, Aaronson S A, Todaro G J, Arnstein P, Kersey J H, Dosik H, Parks W P. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 1973; (51): 1417-1423.

Gilmour PS, Rahman I, Hayashi S, Hogg JC, Donaldson K, MacNee W. Adenoviral E1A primes alveolar epithelial cells to PM(10)-induced transcription of interleukin-8. Am J Physiol Lung Cell Mol Physiol 2001; 281(3):L598-L606.

Ginsburg I. Role of lipoteichoic acid in infection and inflammation. Lancet Infect Dis 2002; 2(3):171-179.

Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J, Tedin K, Taha MK, Labigne A, Zahringer U, Coyle AJ, DiStefano PS, Bertin J, Sansonetti PJ, Philpott DJ. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science 2003a; 300(5625):1584-1587.

Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, Sansonetti PJ. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003b; 278(11):8869-8872.

Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ, Sansonetti PJ, Mengin-Lecreulx D. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. J Biol Chem 2003c; 278(43):41702-41708.

Glauert, A. M. 1975. . Practical methods in electron microscopy, vol. 3/I. Fixation, dehydration and embedding of biological specimens. North-Holland Publishing Company, Amsterdam, The Netherlands.

Gobran LI, Rooney SA. Regulation of SP-B and SP-C secretion in rat type II cells in primary culture. Am J Physiol Lung Cell Mol Physiol 2001; 281(6):L1413-L1419.

Goding, J. W. Monoclonal Antibodies: principles and practice, 1996. 3rd edition, San Diego, CA, Academic Press.

Goerke J. Pulmonary surfactant: functions and molecular composition. Biochim Biophys Acta 1998; 1408(2-3):79-89.

Goldberg M B, Sansonetti P J. Shigella subversion of the cellular cytoskeleton: a strategy for epithelial colonization. Infect Immun 1993; (61): 4941-4946.

Goldstein R, Sun L, Jiang RZ, Sajjan U, Forstner JF, Campanelli C. Structurally variant classes of pilus appendage fibers coexpressed from Burkholderia (Pseudomonas) cepacia. J Bacteriol 1995; 177(4):1039-1052.

Golioto A, Wright J R. Effects of surfactant lipids and surfactant protein a on host defense functions of rat alveolar macrophages. Pediatr Res 2002; (51): 220-227.

Gon Y, Asai Y, Hashimoto S, Mizumura K, Jibiki I, Machino T, Ra C, Horie T. A20 inhibits toll-like receptor 2- and 4-mediated interleukin-8 synthesis in airway epithelial cells. Am J Respir Cell Mol Biol 2004; 31(3):330-336.

Govan JR, Nelson JW. Microbiology of lung infection in cystic fibrosis. Br Med Bull 1992; 48(4):912-930.

Govan JR, Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. Microbiol Rev 1996; 60(3):539-574.

Govan JR, Hughes JE, Vandamme P. Burkholderia cepacia: medical, taxonomic and ecological issues. J Med Microbiol 1996; 45(6):395-407.

Green SK, Schroth MN, Cho JJ, Kominos SK, Vitanza-jack VB. Agricultural plants and soil as a reservoir for Pseudomonas aeruginosa. Appl Microbiol 1974; 28(6):987-991.

Gregory TJ, Longmore WJ, Moxley MA, Whitsett JA, Reed CR, Fowler AA, III, Hudson LD, Maunder RJ, Crim C, Hyers TM. Surfactant chemical composition and biophysical activity in acute respiratory distress syndrome. J Clin Invest 1991; 88(6):1976-1981.

Griese M, Birrer P, Demirsoy A. Pulmonary surfactant in cystic fibrosis. Eur Respir J 1997; 10(9):1983-1988.

Griese M. Pulmonary surfactant in health and human lung diseases: state of the art. Eur Respir J 1999; 13(6):1455-1476.

Griese M, Essl R, Schmidt R, Rietschel E, Ratjen F, Ballmann M, Paul K. Pulmonary surfactant, lung function, and endobronchial inflammation in cystic fibrosis. Am J Respir Crit Care Med 2004; 170(9):1000-1005.

Guest JF, Morris A. Community-acquired pneumonia: the annual cost to the National Health Service in the UK. Eur Respir J 1997; 10(7):1530-1534.

Gunther A, Siebert C, Schmidt R, Ziegler S, Grimminger F, Yabut M, Temmesfeld B, Walmrath D, Morr H, Seeger W. Surfactant alterations in severe pneumonia, acute respiratory distress syndrome, and cardiogenic lung edema. Am J Respir Crit Care Med 1996; 153(1):176-184.

Gupta D, Kirkland TN, Viriyakosol S, Dziarski R. CD14 is a cell-activating receptor for bacterial peptidoglycan. J Biol Chem 1996; 271(38):23310-23316.

Gustafsson M, Curstedt T, Jornvall H, Johansson J. Reverse-phase HPLC of the hydrophobic pulmonary surfactant proteins: detection of a surfactant protein C isoform containing Nepsilon-palmitoyl-lysine. Biochem J 1997; 326 (Pt 3):799-806.

Harder T, Scheiffele P, Verkade P, Simons K. Lipid domain structure of the plasma membrane revealed by patching of membrane components. J Cell Biol 1998; 141(4):929-942.

Hart SP, Dougherty GJ, Haslett C, Dransfield I. CD44 regulates phagocytosis of apoptotic neutrophil granulocytes, but not apoptotic lymphocytes, by human macrophages. J Immunol 1997; 159(2):919-925.

Hartshorn KL, Crouch E, White MR, Colamussi ML, Kakkanatt A, Tauber B, Shepherd V, Sastry KN. Pulmonary surfactant proteins A and D enhance neutrophil uptake of bacteria. Am J Physiol 1998; 274(6 Pt 1):L958-L969.

Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. Am J Respir Crit Care Med 1999; 160(5 Pt 2):S5-11.

Hao M, Lin S X, Karylowski O J, Wustner D, McGraw T E, Maxfield F R. Vesicular and non-vesicular sterol transport in living cells. The endocytic recycling compartment is a major sterol storage organelle. J Biol Chem 2002; (277): 609-617.

Hatchette TF, Gupta R, Marrie TJ. Pseudomonas aeruginosa community-acquired pneumonia in previously healthy adults: case report and review of the literature. Clin Infect Dis 2000; 31(6):1349-1356.

Hauser AR, Fleiszig S, Kang PJ, Mostov K, Engel JN. Defects in type III secretion correlate with internalization of Pseudomonas aeruginosa by epithelial cells. Infect Immun 1998; 66(4):1413-1420.

Hayakawa H, Myrvik QN, St Clair RW. Pulmonary surfactant inhibits priming of rabbit alveolar macrophage. Evidence that surfactant suppresses the oxidative burst of alveolar macrophage in infant rabbits. Am Rev Respir Dis 1989; 140(5):1390-1397.

Hayakawa H, Giridhar G, Myrvik QN, Kucera L. Pulmonary surfactant phospholipids modulate priming of rabbit alveolar macrophages for oxidative responses. J Leukoc Biol 1992; 51(4):379-385.

Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001; 410(6832):1099-1103.

Hazenbos WL, van den Berg BM, van't Wout JW, Mooi FR, van Furth R. Virulence factors determine attachment and ingestion of nonopsonized and opsonized Bordetella pertussis by human monocytes. Infect Immun 1994; 62(11):4818-4824.

Heeckeren A, Walenga R, Konstan MW, Bonfield T, Davis PB, Ferkol T. Excessive inflammatory response of cystic fibrosis mice to bronchopulmonary infection with Pseudomonas aeruginosa 245. J Clin Invest 1997; 100(11):2810-2815.

Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408(6813):740-745.

Hickman-Davis J, Gibbs-Erwin J,Lindsey J, Matalon S. Surfactant protein A mediates mycoplasmacidal activity of alveolar macrophages by production of peroxinitrite. Proc.Natl.Acad.sci.USA 1999; (96): 4953-4958.

Henneke P, Takeuchi O, van Strijp JA, Guttormsen HK, Smith JA, Schromm AB, Espevik TA, Akira S, Nizet V, Kasper DL, Golenbock DT. Novel engagement of CD14 and multiple toll-like receptors by group B streptococci. J Immunol 2001; 167(12):7069-7076.

Hermann C, Spreitzer I, Schroder NW, Morath S, Lehner MD, Fischer W, Schutt C, Schumann RR, Hartung T. Cytokine induction by purified lipoteichoic acids from various bacterial species--role of LBP, sCD14, CD14 and failure to induce IL-12 and subsequent IFN-gamma release. Eur J Immunol 2002; 32(2):541-551.

Herting E, Jarstrand C, Rasool O, Curstedt T, Sun B, Robertson B. Experimental neonatal group B streptococcal pneumonia: effect of a modified porcine surfactant on bacterial proliferation in ventilated near-term rabbits. Pediatr Res 1994; 36(6):784-791.

Heumann D, Roger T. Initial responses to endotoxins and Gram-negative bacteria. Clin Chim Acta 2002; 323(1-2):59-72.

Hills BA. An alternative view of the role(s) of surfactant and the alveolar model. J Appl Physiol 1999; 87(5):1567-1583.

Hills BA, Chen Y. Suppression of neural activity of bronchial irritant receptors by surface-active phospholipid in comparison with topical drugs commonly prescribed for asthma. Clin Exp Allergy 2000; 30(9):1266-1274.

Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J Immunol 2000; 165(2):618-622.

Hitoshi S, Kusunoki S, Kanazawa I, Tsuji S. Molecular cloning and expression of two types of rabbit beta-galactoside alpha 1,2-fucosyltransferase. J Biol Chem 1995; 270(15):8844-8850.

Hoffman A, Lawrence M, Orgnibene F, suffredini A, Lipschik G, Kovacs J, Masur H, Sherman J. Reduction of pulmonary surfactant in patient with human immynodeficiency virus infection and Pneumocystis carini pneumonia. Chest 1992; (102): 1730-1736.

Hoebe K, Janssen EM, Kim SO, Alexopoulou L, Flavell RA, Han J, Beutler B. Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. Nat Immunol 2003a; 4(12):1223-1229.

Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, Goode J, Lin P, Mann N, Mudd S, Crozat K, Sovath S, Han J, Beutler B. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature 2003b; 424(6950):743-748.

Hohlfeld J, Fabel H, Hamm H. The role of pulmonary surfactant in obstructive airways disease. Eur Respir J 1997; 10(2):482-491.

Holmskov U, Malhotra R, Sim RB, Jensenius JC. Collectins: collagenous C-type lectins of the innate immune defense system. Immunol Today 1994; 15(2):67-74.

Homey B, Zlotnik A. Chemokines in allergy. Curr Opin Immunol 1999; 11(6):626-634.

Homma T, Kato A, Hashimoto N, Batchelor J, Yoshikawa M, Imai S, Wakiguchi H, Saito H, Matsumoto K. Corticosteroid and cytokines synergistically enhance toll-like receptor 2 expression in respiratory epithelial cells. Am J Respir Cell Mol Biol 2004; 31(4):463-469.

Honda Y, Takahashi H, Kuroki Y, Akino T, Abe S. Decreased contents of surfactant proteins A and D in BAL fluids of healthy smokers. Chest 1996; 109(4):1006-1009.

Hoppe HJ, Reid KB. Collectins--soluble proteins containing collagenous regions and lectin domains--and their roles in innate immunity. Protein Sci 1994; 3(8):1143-1158.

Houston MS, Silverstein MD, Suman VJ. Community-acquired lower respiratory tract infection in the elderly: a community-based study of incidence and outcome. J Am Board Fam Pract 1995; 8(5):347-356.

Hughes D, Haslam P. Changes in phosphatidylglycerol in bronchalveolar lavage fluids from patients with cryptogenic fibrosing alveolitis. Chest 1999; (95): 82-89.

Hutchison M L, Poxton I R, Govan J R. Burkholderia cepacia produces a hemolysin that is capable of inducing apoptosis and degranulation of mammalian phagocytes. Infect Immun 1998; (66): 2033-2039.

Hyde DM, Miller LA, McDonald RJ, Stovall MY, Wong V, Pinkerton KE, Wegner CD, Rothlein R, Plopper CG. Neutrophils enhance clearance of necrotic epithelial cells in ozone-induced lung injury in rhesus monkeys. Am J Physiol 1999; 277(6 Pt 1):L1190-L1198.

Imundo L, Barasch J, Prince A, Al Awqati Q. Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface. Proc Natl Acad Sci U S A 1995; (92): 3019-3023.

Inohara N, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, Fukase K, Inamura S, Kusumoto S, Hashimoto M, Foster SJ, Moran AP, Fernandez-Luna JL, Nunez G. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J Biol Chem 2003; 278(8):5509-5512.

Isles A, Maclusky I, Corey M, Gold R, Prober C, Fleming P, Levison H. Pseudomonas cepacia infection in cystic fibrosis: an emerging problem. J Pediatr 1984; 104(2):206-210.

Janes PW, Ley SC, Magee AI. Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. J Cell Biol 1999; 147(2):447-461.

Janssens S, Beyaert R. Auniversal role of MYD88 in TLR/IL-1R-mediated signalling. Trends Biochem.Sci 2002; (27): 174-482.

Janeway C, Jr., Medzhitov R. Viral interference with IL-1 and toll signaling. Proc Natl Acad Sci U S A 2000; 97(20):10682-10683.

Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 1989; 54 Pt 1:1-13.

Jia HP, Kline JN, Penisten A, Apicella MA, Gioannini TL, Weiss J, McCray PB, Jr. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. Am J Physiol Lung Cell Mol Physiol 2004; 287(2):L428-L437.

Johansson J, Curstedt T. Molecular structures and interactions of pulmonary surfactant components. Eur J Biochem 1997; 244(3):675-693.

Jonsson S, Musher DM, Goree A, Lawrence EC. Human alveolar lining material and antibacterial defenses. Am Rev Respir Dis 1986; 133(1):136-140.

Kabha K, Schmegner J, Keisari Y, Parolis H, Schlepper-Schaeffer J, Ofek I. SP-A enhances phagocytosis of Klebsiella by interaction with capsular polysaccharides and alveolar macrophages. Am J Physiol 1997; 272(2 Pt 1):L344-L352.

Kagnoff MF, Eckmann L. Epithelial cells as sensors for microbial infection. J Clin Invest 1997; 100(1):6-10.

Kalina M, Blau H, Riklis S, Kravtsov V. Interaction of surfactant protein A with bacterial lipopolysaccharide may affect some biological functions. Am J Physiol 1995; 268(1 Pt 1):L144-L151.

Kalliola S, Vuopio-Varkila J, Takala AK, Eskola J. Neonatal group B streptococcal disease in Finland: a ten-year nationwide study. Pediatr Infect Dis J 1999; 18(9):806-810.

Kang BH, Crapo JD, Wegner CD, Letts LG, Chang LY. Intercellular adhesion molecule-1 expression on the alveolar epithelium and its modification by hyperoxia. Am J Respir Cell Mol Biol 1993; 9(4):350-355.

Karin M, Delhase M. The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin Immunol 2000; 12(1):85-98.

Keig PM, Ingham E, Kerr KG. Invasion of human type II pneumocytes by Burkholderia cepacia 413. Microb Pathog 2001; 30(3):167-170.

Keig PM, Ingham E, Vandamme PA, Kerr KG. Differential invasion of respiratory epithelial cells by members of the Burkholderia cepacia complex. Clin Microbiol Infect 2002; 8(1):47-49.

Keller R, Fischer W, Keist R, Bassetti S. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. Infect Immun 1992; 60(9):3664-3672.

Kenworthy A. Peering inside lipid rafts and caveolae. Trends Biochem Sci 2002; 27(9):435-437.

Khan TZ, Wagener JS, Bost T, Martinez J, Accurso FJ, Riches DW. Early pulmonary inflammation in infants with cystic fibrosis. Am J Respir Crit Care Med 1995; 151(4):1075-1082.

King RJ, Clements JA. Surface active materials from dog lung. II. Composition and physiological correlations. Am J Physiol 1972; 223(3):715-726.

Kishore U, Reid KB. C1q: structure, function, and receptors. Immunopharmacology 2000; 49(1-2):159-170.

Kishore U, Strong P, Perdikoulis MV, Reid KB. A recombinant homotrimer, composed of the alpha helical neck region of human surfactant protein D and C1q B chain globular domain, is an inhibitor of the classical complement pathway. J Immunol 2001; 166(1):559-565.

Knowles MR, Boucher RC. Mucus clearance as a primary innate defense mechanism for mammalian airways. J Clin Invest 2002; 109(5):571-577.

Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R, Flavell RA. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature 2002; 416(6877):194-199.

Korfhagen TR, Sheftelyevich V, Burhans MS, Bruno MD, Ross GF, Wert SE, Stahlman MT, Jobe AH, Ikegami M, Whitsett JA, Fisher JH. Surfactant protein-D regulates surfactant phospholipid homeostasis in vivo. J Biol Chem. 1998 (43):28438-43.

Kostina E, Ofek I, Crouch E, Friedman R, Sirota L, Klinger G, Sahly H, Keisari Y. Noncapsulated Klebsiella pneumoniae bearing mannose-containing O antigens is rapidly eradicated from mouse lung and triggers cytokine production by macrophages following opsonization with surfactant protein D. Infect Immun 2005; 73(12):8282-8290.

Kowalski MP, Pier GB. Localization of cystic fibrosis transmembrane conductance regulator to lipid rafts of epithelial cells is required for Pseudomonas aeruginosa-induced cellular activation. J Immunol 2004; 172(1):418-425.

Koyama S, Sato E, Nomura H, Kubo K, Miura M, Yamashita T, Nagai S, Izumi T. The potential of various lipopolysaccharides to release monocyte chemotactic activity from lung epithelial cells and fibroblasts. Eur Respir J 1999; 14(3):545-552.

Koyama S, Sato E, Nomura H, Kubo K, Miura M, Yamashita T, Nagai S, Izumi T. The potential of various lipopolysaccharides to release IL-8 and G-CSF. Am J Physiol Lung Cell Mol Physiol 2000; 278(4):L658-L666.

Krakauer T. Pentoxifylline inhibits ICAM-1 expression and chemokine production induced by proinflammatory cytokines in human pulmonary epithelial cells. Immunopharmacology 2000; 46(3):253-261.

Kremlev S G, Phelps D S. Surfactant protein A stimulation of inflammatory cytokine and immunoglobulin production. Am J Physiol 1994; (267): L712-L719.

Kremlev SG, Umstead TM, Phelps DS. Surfactant protein A regulates cytokine production in the monocytic cell line THP-1. Am J Physiol 1997; 272(5 Pt 1):L996-1004.

Krivan HC, Roberts DD, Ginsburg V. Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids. Proc Natl Acad Sci U S A 1988; 85(16):6157-6161.

Kronborg G, Hansen MB, Svenson M, Fomsgaard A, Hoiby N, Bendtzen K. Cytokines in sputum and serum from patients with cystic fibrosis and chronic Pseudomonas aeruginosa infection as markers of destructive inflammation in the lungs. Pediatr Pulmonol 1993; 15(5):292-297.

Kube D, Sontich U, Fletcher D, Davis P B. Proinflammatory cytokine responses to P. aeruginosa infection in human airway epithelial cell lines. Am J Physiol Lung Cell Mol Physiol 2001; (280): L493-L502.

Kumar A, Zhang J, Yu F S. Innate immune response of corneal epithelial cells to Staphylococcus aureus infection: role of peptidoglycan in stimulating proinflammatory cytokine secretion. Invest Ophthalmol Vis Sci 2004; (45): 3513-3522.

Kunkel SL, Standiford T, Kasahara K, Strieter RM. Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. Exp Lung Res 1991; 17(1):17-23.

Kurashima K, Fujimura M, Matsuda T, Kobayashi T. Surface activity of sputum from acute asthmatic patients. Am J Respir Crit Care Med 1997; 155(4):1254-1259.

Kuronuma K, Sano H, Kato K, Kudo K, Hyakushima N, Yokota S, Takahashi H, Fujii N, Suzuki H, Kodama T, Abe S, Kuroki Y. Pulmonary surfactant protein A augments the phagocytosis of Streptococcus pneumoniae by alveolar macrophages through a casein kinase 2-dependent increase of cell surface localization of scavenger receptor A. J Biol Chem 2004; 279(20):21421-21430.

Kusunoki T, Hailman E, Juan TS, Lichenstein HS, Wright SD. Molecules from Staphylococcus aureus that bind CD14 and stimulate innate immune responses. J Exp Med 1995; 182(6):1673-1682.

Kwon S, Newcomb RL, George SC. Mechanisms of synergistic cytokine-induced nitric oxide production in human alveolar epithelial cells. Nitric Oxide 2001; 5(6):534-546.

Laforce FM, Boose DS. Sublethal damage of Escherichia coli by lung lavage. Am Rev Respir Dis 1981; 124(6):733-737.

Lamblin C, Gosset P, Tillie-Leblond I, Saulnier F, Marquette CH, Wallaert B, Tonnel AB. Bronchial neutrophilia in patients with noninfectious status asthmaticus. Am J Respir Crit Care Med 1998; 157(2):394-402.

Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML. Siderophore-mediated signaling regulates virulence factor production in Pseudomonasaeruginosa. Proc Natl Acad Sci U S A 2002; 99(10):7072-7077.

Lee SC, Hsu JY, Fu LS, Chu JJ, Fan SJ, Chi CS. Comparison of the activities of granulocyte-macrophage colony-stimulating factor and interleukin-8 secretion between two lung epithelial cell lines. J Microbiol Immunol Infect 2005; 38(5):327-331.

Leidal KG, Munson KL, Denning GM. Small molecular weight secretory factors from Pseudomonas aeruginosa have opposite effects on IL-8 and RANTES expression by human airway epithelial cells. Am J Respir Cell Mol Biol 2001; 25(2):186-195.

Lemitre B, Nicolas E, Michhart J.M, Hoffman J.A. The dorsoventral regulatory gene cassette spatzle/Toll/cactuscontrols the potent antifungal response in Drosophila adults. Cell 1996; (86) 973-983

Leowski J. Mortality from acute respiratory infections in children under 5 years of age: global estimates. World Health Stat Q 1986; 39(2):138-144.

Lewin L O, Byard P J, Davis P B. Effect of Pseudomonas cepacia colonization on survival and pulmonary function of cystic fibrosis patients. J Clin Epidemiol 1990; (43): 125-131.

LeVine AM, Whitsett JA, Hartshorn KL, Crouch EC, Korfhagen TR. Surfactant protein D enhances clearance of influenza A virus from the lung in vivo. J Immunol 2001; 167(10):5868-5873.

Liaudet L, Szabo C, Evgenov OV, Murthy KG, Pacher P, Virag L, Mabley JG, Marton A, Soriano FG, Kirov MY, Bjertnaes LJ, Salzman AL. Flagellin from gram-negative bacteria is a potent mediator of acute pulmonary inflammation in sepsis. Shock 2003; 19(2):131-137.

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

Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW, Carroll JD, Espevik T, Ingalls RR, Radolf JD, Golenbock DT. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem 1999; 274(47):33419-33425.

Lien E, Means TK, Heine H, Yoshimura A, Kusumoto S, Fukase K, Fenton MJ, Oikawa M, Qureshi N, Monks B, Finberg RW, Ingalls RR, Golenbock DT. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. J Clin Invest 2000; 105(4):497-504.

Lin CH, Kuan IH, Lee HM, Lee WS, Sheu JR, Ho YS, Wang CH, Kuo HP. Induction of cyclooxygenase-2 protein by lipoteichoic acid from Staphylococcus aureus in human pulmonary epithelial cells: involvement of a nuclear factor-kappa B-dependent pathway. Br J Pharmacol 2001; 134(3):543-552.

Lin CH, Kuan IH, Wang CH, Lee HM, Lee WS, Sheu JR, Hsiao G, Wu CH, Kuo HP. Lipoteichoic acid-induced cyclooxygenase-2 expression requires activations of p44/42 and p38 mitogen-activated protein kinase signal pathways. Eur J Pharmacol 2002; 450(1):1-9.

London E, Brown D A. Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). Biochim Biophys Acta 2000; (1508): 182-195.

LiPuma JJ, Spilker T, Gill LH, Campbell PW, III, Liu L, Mahenthiralingam E. Disproportionate distribution of Burkholderia cepacia complex species and transmissibility markers in cystic fibrosis. Am J Respir Crit Care Med 2001; 164(1):92-96.

Liu CF, Chen YL, Shieh CC, Yu CK, Reid KB, Wang JY Therapeutic effect of surfactant protein D in allergic inflammation of mite-sensitized mice. Clin Exp Allergy. 2005(4):515-21

Liu L, Wang M, Fisher AB, Zimmerman UJ. Involvement of annexin II in exocytosis of lamellar bodies from alveolar epithelial type II cells. Am J Physiol 1996; 270(4 Pt 1):L668-L676.

Lloyd C. Chemokines in allergic lung inflammation. Immunology 2002; 105(2):144-154.

Lukasiewicz J, Lugowski C. [Biologic activity of lipopolysaccharides]. Postepy Hig Med Dosw 2003; 57(1):33-53.

Luster AD. Chemokines--chemotactic cytokines that mediate inflammation. N Engl J Med 1998; 338(7):436-445.

Macfarlane JT, Colville A, Guion A, Macfarlane RM, Rose DH. Prospective study of aetiology and outcome of adult lower-respiratory-tract infections in the community. Lancet 1993; 341(8844):511-514.

Maclin CC. The pulmonary alveolar mucoid film and the pneumonocytes. Lancet 1954; 266(6822):1099-1104.

Macnab RM. The bacterial flagellum: reversible rotary propellor and type III export apparatus. J Bacteriol 1999; 181(23):7149-7153.

Madsen M, Lebenthal Y, Cheng Q, Smith BL, Hostetter MK. A pneumococcal protein that elicits interleukin-8 from pulmonary epithelial cells. J Infect Dis 2000; 181(4):1330-1336.

Madsen MT, Uppaluri R, Hoffman EA, McLennan G. Pulmonary CT image classification with evolutionary programming. Acad Radiol 1999; 6(12):736-741.

Majcherczyk PA, Rubli E, Heumann D, Glauser MP, Moreillon P. Teichoic acids are not required for Streptococcus pneumoniae and Staphylococcus aureus cell walls to trigger the release of tumor necrosis factor by peripheral blood monocytes. Infect Immun 2003; 71(7):3707-3713.

Malhotra R, Lu J, Holmskov U, Sim RB. Collectins, collectin receptors and the lectin pathway of complement activation. Clin Exp Immunol 1994; 97 Suppl 2:4-9.

Malloy JL, Veldhuizen RA, Thibodeaux BA, O'Callaghan RJ, Wright JR. Pseudomonas aeruginosa protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions. Am J Physiol Lung Cell Mol Physiol 2005; 288(2):L409-L418.

Mandell GL, Douglas RG, Bennett JE, Dolin R. Mandell, Douglas, and Bennett's principles and practice of infectious diseases. 5th ed ed. Philadelphia: Churchill Livingstone, 2000.

Mander A, Langton-Hewer S, Bernhard W, Warner JO, Postle AD. Altered phospholipid composition and aggregate structure of lung surfactant is associated with impaired lung function in young children with respiratory infections. Am J Respir Cell Mol Biol 2002; 27(6):714-721.

Manniello JM, Heymann H, Adair FW. Isolation of atypical lipopolysaccharides from purified cell walls of Pseudomonas cepacia. J Gen Microbiol 1979; 112(2):397-400.

Manno G, Dalmastri C, Tabacchioni S, Vandamme P, Lorini R, Minicucci L, Romano L, Giannattasio A, Chiarini L, Bevivino A. Epidemiology and clinical course of Burkholderia cepacia complex infections, particularly those caused by different Burkholderia cenocepacia strains, among patients attending an Italian Cystic Fibrosis Center. J Clin Microbiol 2004; 42(4):1491-1497.

Manthey C L, Perera P Y, Henricson B E, Hamilton T A, Qureshi N, Vogel S N. Endotoxin-induced early gene expression in C3H/HeJ (Lpsd) macrophages. J Immunol 1994; (153): 2653-2663.

Marchlewicz BA, Duncan JL. Properties of a hemolysin produced by group B streptococci. Infect Immun 1980; 30(3):805-813.

Mariencheck WI, Alcorn JF, Palmer SM, Wright JR. Pseudomonas aeruginosa elastase degrades surfactant proteins A and D. Am J Respir Cell Mol Biol 2003; 28(4):528-537.

Marko M, Chipperfield R, and Birnboim HC. A procedure for the large scale isolation of high purified plasmid DNA using alkaline extraction and binding to glass powder. Analytical Biochem 1982; (121): 382-387.

Martin MU, Wesche H. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. Biochim Biophys Acta 2002; 1592(3):265-280.

Martin TR, Rubenfeld G, Steinberg KP, Hudson LD, Raghu G, Moriarty AM, Leturcq DJ, Tobias PS, Ulevitch RJ. Endotoxin, endotoxin-binding protein, and soluble CD14 are present in bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome. Chest 1994; 105(3 Suppl):55S-56S.

Martin TR. Recognition of bacterial endotoxin in the lungs. Am J Respir Cell Mol Biol 2000; 23(2):128-132.

Maschera B, Ray K, Burns K, Volpe F. Overexpression of an enzymically inactive interleukin-1-receptor-associated kinase activates nuclear factor-kappaB. Biochem J 1999; 339 (Pt 2):227-231.

Mason RJ, Voelker DR. Regulatory mechanisms of surfactant secretion. Biochim Biophys Acta 1998; 1408(2-3):226-240.

Massengale AR, Quinn F, Jr., Yankaskas J, Weissman D, McClellan WT, Cuff C, Aronoff SC. Reduced interleukin-8 production by cystic fibrosis airway epithelial cells. Am J Respir Cell Mol Biol 1999; 20(5):1073-1080.

Massion PP, Inoue H, Richman-Eisenstat J, Grunberger D, Jorens PG, Housset B, Pittet JF, Wiener-Kronish JP, Nadel JA. Novel Pseudomonas product stimulates interleukin-8 production in airway epithelial cells in vitro. J Clin Invest 1994; 93(1):26-32.

Mathers CD, Vos ET, Stevenson CE, Begg SJ. The burden of disease and injury in Australia 547. Bull World Health Organ 2001; 79(11):1076-1084.

McCormack F X, Whitsett J A. The pulmonary collectins, SP-A and SP-D, orchestrate innate immunity in the lung. J Clin Invest 2002; (109): 707-712.

McCurdy JD, Olynych TJ, Maher LH, Marshall JS. Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. J Immunol 2003; 170(4):1625-1629.

McDowell A, Mahenthiralingam E, Dunbar KE, Moore JE, Crowe M, Elborn JS. Epidemiology of Burkholderia cepacia complex species recovered from cystic fibrosis patients: issues related to patient segregation. J Med Microbiol 2004; 53(Pt 7):663-668.

McKevitt AI, Woods DE. Characterization of Pseudomonas cepacia isolates from patients with cystic fibrosis. J Clin Microbiol 1984; 19(2):291-293.

McNeely TB, Coonrod JD. Comparison of the opsonic activity of human surfactant protein A for Staphylococcus aureus and Streptococcus pneumoniae with rabbit and human macrophages. J Infect Dis 1993; 167(1):91-97.

Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997; 388(6640):394-397.

Medzhitov R, Janeway C, Jr. Innate immunity. N Engl J Med 2000; 343(5):338-344.

Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol 2001; 1(2):135-145.

Melton KR, Nesslein LL, Ikegami M, Tichelaar JW, Clark JC, Whitsett JA, Weaver TE. SP-B deficiency causes respiratory failure in adult mice. Am J Physiol Lung Cell Mol Physiol 2003; 285(3):L543-L549.

Meyer k, Sharma A, Brown R, Weatherly M, Moya F, Lewandoski, Zimmerman J. Function and Composition of Pulmonary Surfactant and Surfactant-Derived Fatty Acid Profiles Are Altered in Young Adults With Cystic Fibrosis. Chest 2000;118:164-174.Mol Physiol 2003; (285): L543-L549.

Meyer JM, Neely A, Stintzi A, Georges C, Holder IA. Pyoverdin is essential for virulence of Pseudomonas aeruginosa. Infect Immun 1996; 64(2):518-523.

Mezzetti A, Schrag JD, Cheong CS, Kazlauskas RJ. Mirror-image packing in enantiomer discrimination molecular basis for the enantioselectivity of B.cepacia lipase toward 2-methyl-3-phenyl-1-propanol. Chem Biol 2005; 12(4):427-437.

Mikamo H, Johri AK, Paoletti LC, Madoff LC, Onderdonk AB. Adherence to, invasion by, and cytokine production in response to serotype VIII group B Streptococci. Infect Immun 2004; 72(8):4716-4722.

Miles PR, Bowman L, Rao KM, Baatz JE, Huffman L. Pulmonary surfactant inhibits LPS-induced nitric oxide production by alveolar macrophages. Am J Physiol 1999; 276(1 Pt 1):L186-L196.

Miller MD, Krangel MS. Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. Crit Rev Immunol 1992; 12(1-2):17-46.

Mims C, Playfair J RWWR. Medical microbiology. Mosby International limited, London, 1998.

Monton C, Torres A. Lung inflammatory response in pneumonia. Monaldi Arch Chest Dis 1998; 53(1):56-63.

Moore KJ, Andersson LP, Ingalls RR, Monks BG, Li R, Arnaout MA, Golenbock DT, Freeman MW. Divergent response to LPS and bacteria in CD14-deficient murine macrophages. J Immunol 2000; 165(8):4272-4280.

Moreillon P, Majcherczyk PA. Proinflammatory activity of cell-wall constituents from gram-positive bacteria. Scand J Infect Dis 2003; 35(9):632-641.

Morgenroth K, Bolz J. Morphological features of the interaction between mucus and surfactant on the bronchial mucosa. Respiration 1985; 47(3):225-231.

Mori N, Oishi K, Sar B, Mukaida N, Nagatake T, Matsushima K, Yamamoto N. Essential role of transcription factor nuclear factor-kappaB in regulation of interleukin-8 gene expression by nitrite reductase from Pseudomonas aeruginosa in respiratory epithelial cells. Infect Immun 1999; 67(8):3872-3878.

Morris RH, Price AJ, Tonks A, Jackson SK, Jones KP. Prostaglandin E(2) and tumour necrosis factor-alpha release by monocytes are modulated by phospholipids. Cytokine 2000; 12(11):1717-1719.

Muhlebach MS, Noah TL. Endotoxin activity and inflammatory markers in the airways of young patients with cystic fibrosis. Am J Respir Crit Care Med 2002; 165(7):911-915.

Muller-Loennies S, Zahringer U, Seydel U, Kusumoto S, Ulmer A J, Rietschel E T. What we know and don't know about the chemical and physical structure of lipopolysaccharide in relation to biological activity. Prog Clin Biol Res 1998; (397): 51-72.

Munro S. Lipid rafts: elusive or illusive? Cell 2003; (115): 377-388.

Muzio M, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science 1997; 278(5343):1612-1615.

Muzio M, Polentarutti N, Bosisio D, Manoj Kumar P P, Mantovani A. Toll-like receptor family and signalling pathway. Biochem Soc Trans 2000; (28): 563-566.

Myhre AE, Stuestol JF, Dahle MK, Overland G, Thiemermann C, Foster SJ, Lilleaasen P, Aasen AO, Wang JE. Organ injury and cytokine release caused by peptidoglycan are dependent on the structural integrity of the glycan chain. Infect Immun 2004; 72(3):1311-1317.

Nakamura H, Yoshimura K, McElvaney NG, Crystal RG. Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. J Clin Invest 1992; 89(5):1478-1484.

Nelson S, Summer WR. Innate immunity, cytokines, and pulmonary host defense. Infect Dis Clin North Am 1998; 12(3):555-67, vii.

Neumeister B, Woerndle S, Bartmann P. Effects of different surfactant preparations on bacterial growth in vitro. Biol Neonate 1996; 70(2):128-134.

Ni M, Evans DJ, Hawgood S, Anders EM, Sack RA, Fleiszig SM. Surfactant protein D is present in human tear fluid and the cornea and inhibits epithelial cell invasion by Pseudomonas aeruginosa. Infect Immun 2005; 73(4):2147-2156.

Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z, Matsumoto K. The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature 1999; (398): 252-256.

Nikaido H, Nakae T. The outer membrane of Gram-negative bacteria. Adv Microb Physiol 1979; 20:163-250.

Nogee LM. Alterations in SP-B and SP-C expression in neonatal lung disease. Annu Rev Physiol 2004; 66:601-623.

O'Connor W, Jr., Harton JA, Zhu X, Linhoff MW, Ting JP. Cutting edge: CIAS1/cryopyrin/PYPAF1/NALP3/CATERPILLER 1.1 is an inducible inflammatory mediator with NF-kappa B suppressive properties. J Immunol 2003; 171(12):6329-6333.

O'Neill LA. The role of MyD88-like adapters in Toll-like receptor signal transduction. Biochem Soc Trans 2003; 31(Pt 3):643-647.

Ofek I, Mesika A, Kalina M, Keisari Y, Podschun R, Sahly H, Chang D, McGregor D, Crouch E. Surfactant protein D enhances phagocytosis and killing of unencapsulated phase variants of Klebsiella pneumoniae. Infect Immun 2001; 69(1):24-33.

Ohmer-Schrock D, Schlatterer C, Plattner H, Schlepper-Schafer J. Lung surfactant protein A (SP-A) activates a phosphoinositide/calcium signaling pathway in alveolar macrophages 298. J Cell Sci 1995; 108 (Pt 12):3695-3702.

Ormerod MG. Introduction& Instrumentation. In: Ormerod MG, editor. Flow Cytometry. Oxford: Bios Scientific, 1999: 1-21.

Palfreyman RW, Watson ML, Eden C, Smith AW. Induction of biologically active interleukin-8 from lung epithelial cells by Burkholderia (Pseudomonas) cepacia products. Infect Immun 1997; 65(2):617-622.

Pasula R, Downing JF, Wright JR, Kachel DL, Davis TE, Jr., Martin WJ. Surfactant protein A (SP-A) mediates attachment of Mycobacterium tuberculosis to murine alveolar macrophages. Am J Respir Cell Mol Biol 1997; 17(2):209-217.

Pechkovsky DV, Zissel G, Ziegenhagen MW, Einhaus M, Taube C, Rabe KF, Magnussen H, Papadopoulos T, Schlaak M, Muller-Quernheim J. Effect of proinflammatory cytokines on interleukin-8 mRNA expression and protein production by isolated human alveolar epithelial cells type II in primary culture. Eur Cytokine Netw 2000; 11(4):618-625.

Perlmann, H. and Perlmann, P. Enzyme-Linked Immunosorbent Assay. In: Cell Biology: A Laboratory Handbook (1994). San Diego, CA, Academic Press, Inc., 322-328.

Persson A, Rust K, Chang D, Moxley M, Longmore W, Crouch E. CP4: a pneumocyte-derived collagenous surfactant-associated protein. Evidence for heterogeneity of collagenous surfactant proteins. Biochemistry 1988; 27(23):8576-8584.

Pfeiffer A, Bottcher A, Orso E, Kapinsky M, Nagy P, Bodnar A, Spreitzer I, Liebisch G, Drobnik W, Gempel K, Horn M, Holmer S, Hartung T, Multhoff G, Schutz G, Schindler H, Ulmer AJ, Heine H, Stelter F, Schutt C, Rothe G, Szollosi J, Damjanovich S, Schmitz G. Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptor clustering in rafts. Eur J Immunol 2001; 31(11):3153-3164.

Phelps DS, Rose RM. Increased recovery of surfactant protein A in AIDS-related pneumonia. Am Rev Respir Dis 1991; 143(5 Pt 1):1072-1075.

Pier GB. Pulmonary disease associated with Pseudomonas aeruginosa in cystic fibrosis: current status of the host-bacterium interaction. J Infect Dis 1985; 151(4):575-580.

Pier GB, Grout M, Zaidi TS. Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of Pseudomonas aeruginosa from the lung. Proc Natl Acad Sci U S A 1997; 94(22):12088-12093.

Pikaar J C, Voorhout W F, van Golde L M, Verhoef J, Van Strijp J A, van Iwaarden J F. Opsonic activities of surfactant proteins A and D in phagocytosis of gram-negative bacteria by alveolar macrophages. J Infect Dis 1995; (172): 481-489.

Pison U, Obertacke U, Brand M, Seeger W, Joka T, Bruch J, Schmit-Neuerburg KP. Altered pulmonary surfactant in uncomplicated and septicemia-complicated courses of acute respiratory failure. J Trauma 1990; 30(1):19-26.

Pivarcsi A, Nagy I, Koreck A, Kis K, Kenderessy-Szabo A, Szell M, Dobozy A, Kemeny L. Microbial compounds induce the expression of pro-inflammatory cytokines, chemokines and human beta-defensin-2 in vaginal epithelial cells. Microbes Infect 2005.

Platz J, Beisswenger C, Dalpke A, Koczulla R, Pinkenburg O, Vogelmeier C, Bals R. Microbial DNA induces a host defense reaction of human respiratory epithelial cells. J Immunol 2004; (173): 1219-1223.

Plotkowski M C, Costa A O, Morandi V, Barbosa H S, Nader H B, de Bentzmann S, Puchelle E. Role of heparan sulphate proteoglycans as potential receptors for non-piliated Pseudomonas aeruginosa adherence to non-polarised airway epithelial cells. J Med Microbiol 2001; (50): 183-190.

Poltorak A, He X, Smirnova I, Liu M Y, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998; (282): 2085-2088.

Poltorak A, Ricciardi-Castagnoli P, Citterio S, Beutler B. Physical contact between lipopolysaccharide and toll-like receptor 4 revealed by genetic complementation. Proc Natl Acad Sci U S A 2000; (97): 2163-2167.

Plotkowski MC, Tournier JM, Puchelle E. Pseudomonas aeruginosa strains possess specific adhesins for laminin. Infect Immun 1996; 64(2):600-605.

Pollack JH, Ntamere AS, Neuhaus FC. D-alanyl-lipoteichoic acid in Lactobacillus casei: secretion of vesicles in response to benzylpenicillin. J Gen Microbiol 1992; 138(5):849-859.

Polotsky VY, Fischer W, Ezekowitz RA, Joiner KA. Interactions of human mannose-binding protein with lipoteichoic acids. Infect Immun 1996; 64(1):380-383.

Possmayer F. A proposed nomenclature for pulmonary surfactant-associated proteins. Am Rev Respir Dis 1988; 138(4):990-998.

Poyart C, Pellegrini E, Marceau M, Baptista M, Jaubert F, Lamy MC, Trieu-Cuot P. Attenuated virulence of Streptococcus agalactiae deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells. Mol Microbiol 2003; 49(6):1615-1625.

Projan SJaRPN. The molecular basis of pathogenicity. he staphylococci in human disease. Churchill Livingstone, New-York, N.Y. 1997.

Quinton LJ, Nelson S, Zhang P, Boe DM, Happel KI, Pan W, Bagby GJ. Selective transport of cytokine-induced neutrophil chemoattractant from the lung to the blood facilitates pulmonary neutrophil recruitment. Am J Physiol Lung Cell Mol Physiol 2004; 286(3):L465-L472.

Qureshi N, Takayama K, Ribi E. Purification and structural determination of nontoxic lipid A obtained from the lipopolysaccharide of Salmonella typhimurium. J Biol Chem 1982; (19): 11808-15.

Rauprich P, Moller O, Walter G, Herting E, Robertson B. Influence of modified natural or synthetic surfactant preparations on growth of bacteria causing infections in the neonatal period. Clin Diagn Lab Immunol 2000; 7(5):817-822.

Ray P, Yang L, Zhang DH, Ghosh SK, Ray A. Selective up-regulation of cytokine-induced RANTES gene expression in lung epithelial cells by overexpression of IkappaBR. J Biol Chem 1997; 272(32):20191-20197.

Raychaudhuri B, Abraham S, Bonfield TL, Malur A, Deb A, DiDonato JA, Kavuru MS, Thomassen MJ. Surfactant blocks lipopolysaccharide signaling by inhibiting both mitogen-activated protein and IkappaB kinases in human alveolar macrophages. Am J Respir Cell Mol Biol 2004; 30(2):228-232.

Re F, Strominger JL. Separate functional domains of human MD-2 mediate Toll-like receptor 4-binding and lipopolysaccharide responsiveness. J Immunol 2003; 171(10):5272-5276.

Read RC, Wilson R, Rutman A, Lund V, Todd HC, Brain AP, Jeffery PK, Cole PJ. Interaction of nontypable Haemophilus influenzae with human respiratory mucosa in vitro. J Infect Dis 1991; 163(3):549-558.

Reddi K, Phagoo SB, Anderson KD, Warburton D. Burkholderia cepacia-induced IL-8 gene expression in an alveolar epithelial cell line: signaling through CD14 and mitogen-activated protein kinase. Pediatr Res 2003; 54(3):297-305.

Reiniger N, Ichikawa JK, Pier GB. Influence of cystic fibrosis transmembrane conductance regulator on gene expression in response to Pseudomonas aeruginosa infection of human bronchial epithelial cells. Infect Immun 2005; 73(10):6822-6830.

Resh MD. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim Biophys Acta 1999; 1451(1):1-16.

Rietschel ET, Brade H. Bacterial endotoxins. Sci Am 1992; 267(2):54-61.

Rollins BJ. Chemokines. Blood 1997; 90(3):909-928.

Rooney SA, Young SL, Mendelson CR. Molecular and cellular processing of lung surfactant. FASEB J 1994; 8(12):957-967.

Rosenberger CM, Brumell JH, Finlay BB. Microbial pathogenesis: lipid rafts as pathogen portals. Curr Biol 2000; 10(22):R823-R825.

Rosenstein BJ, Hall DE. Pneumonia and septicemia due to Pseudomonas cepacia in a patient with cystic fibrosis. Johns Hopkins Med J 1980; 147(5):188-189.

Rosseau S, Hammerl P, Maus U, Gunther A, Seeger W, Grimminger F, Lohmeyer J. Surfactant protein A down-regulates proinflammatory cytokine production evoked by Candida albicans in human alveolar macrophages and monocytes. J Immunol 1999; 163(8):4495-4502.

Rosseau S, Wiechmann K, Moderer S, Selhorst J, Mayer K, Krull M, Hocke A, Slevogt H, Seeger W, Suttorp N, Seybold J, Lohmeyer J. Moraxella catarrhalis--infected alveolar epithelium induced monocyte recruitment and oxidative burst. Am J Respir Cell Mol Biol 2005; 32(2):157-166.

Rossi D, Zlotnik A. The biology of chemokines and their receptors. Annu Rev Immunol 2000; 18:217-242.

Rot A, Hub E, Middleton J, Pons F, Rabeck C, Thierer K, Wintle J, Wolff B, Zsak M, Dukor P. Some aspects of IL-8 pathophysiology. III: Chemokine interaction with endothelial cells. J Leukoc Biol 1996; 59(1):39-44.

Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J, Sawa T, Frank DW, Wiener-Kronish JP. Type III protein secretion is associated with death in lower respiratory and systemic Pseudomonas aeruginosa infections. J Infect Dis 2001; 183(12):1767-1774.

Rubens CE, Smith S, Hulse M, Chi EY, van Belle G. Respiratory epithelial cell invasion by group B streptococci. Infect Immun 1992; 60(12):5157-5163.

Sabroe I, Prince LR, Jones EC, Horsburgh MJ, Foster SJ, Vogel SN, Dower SK, Whyte MK. Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. J Immunol 2003; 170(10):5268-5275.

Sachse F, Ahlers F, Stoll W, Rudack C. Neutrophil chemokines in epithelial inflammatory processes of human tonsils. Clin Exp Immunol 2005; 140(2):293-300.

Sahly H, Ofek I, Podschun R, Brade H, He Y, Ullmann U, Crouch E. Surfactant protein D binds selectively to Klebsiella pneumoniae lipopolysaccharides containing mannose-rich O-antigens. J Immunol 2002; 169(6):3267-3274.

Saiman L, Prince A. Pseudomonas aeruginosa pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. J Clin Invest 1993; 92(4):1875-1880.

Sajjan U, Corey M, Humar A, Tullis E, Cutz E, Ackerley C, Forstner J. Immunolocalisation of Burkholderia cepacia in the lungs of cystic fibrosis patients. J Med Microbiol 2001; 50(6):535-546.

Sajjan US, Sun L, Goldstein R, Forstner JF. 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 1995; 177(4):1030-1038.

Sajjan US, Sylvester FA, Forstner JF. Cable-piliated Burkholderia cepacia binds to cytokeratin 13 of epithelial cells. Infect Immun 2000a; 68(4):1787-1795.

Sajjan U, Wu Y, Kent G, Forstner J. Preferential adherence of cable-piliated burkholderia cepacia to respiratory epithelia of CF knockout mice and human cystic fibrosis lung explants. J Med Microbiol 2000b; (49): 875-885.

Samatey FA, Imada K, Nagashima S, Vonderviszt F, Kumasaka T, Yamamoto M, Namba K. Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling. Nature 2001; 410(6826):331-337.

Scanlin T F, Glick M C. Terminal glycosylation in cystic fibrosis. Biochim Biophys Acta 1999; (1455): 241-253.

Sano H, Sohma H, Muta T, Nomura S, Voelker DR, Kuroki Y. Pulmonary surfactant protein A modulates the cellular response to smooth and rough lipopolysaccharides by interaction with CD14. J Immunol 1999; 163(1):387-395.

Sano H, Chiba H, Iwaki D, Sohma H, Voelker DR, Kuroki Y. Surfactant proteins A and D bind CD14 by different mechanisms. J Biol Chem 2000; 275(29):22442-22451.

Sano H, Kuronuma K, Kudo K, Mitsuzawa H, Sato M, Murakami S, Kuroki Y. Regulation of inflammation and bacterial clearance by lung collectins. Respirology 2006; 11 Suppl 1:S46-S50.

Saraf A, Larsson L, Larsson BM, Larsson K, Palmberg L. House dust induces IL-6 and IL-8 response in A549 epithelial cells. Indoor Air 1999; 9(4):219-225.

Sato H, Frank DW. ExoU is a potent intracellular phospholipase. Mol Microbiol 2004; 53(5):1279-1290.

Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. J Clin Invest 1989; 83(3):865-875.

Sawa T, Yahr TL, Ohara M, Kurahashi K, Gropper MA, Wiener-Kronish JP, Frank DW. Active and passive immunization with the Pseudomonas V antigen protects against type III intoxication and lung injury. Nat Med 1999; 5(4):392-398.

Scheiffele P, Roth MG, Simons K. Interaction of influenza virus haemagglutinin with sphingolipid-cholesterol membrane domains via its transmembrane domain. EMBO J 1997; 16(18):5501-5508.

Schmidt R, Meier U, Yabut-Perez M, Walmrath D, Grimminger F, Seeger W, Gunther A. Alteration of fatty acid profiles in different pulmonary surfactant phospholipids in acute respiratory distress syndrome and severe pneumonia. Am J Respir Crit Care Med 2001; (163): 95-100.

Schroder NW, Pfeil D, Opitz B, Michelsen KS, Amberger J, Zahringer U, Gobel UB, Schumann RR. Activation of mitogen-activated protein kinases p42/44, p38, and stress-activated protein kinases in myelo-monocytic cells by Treponema lipoteichoic acid. J Biol Chem 2001; 276(13):9713-9719.

Schroder NW, Morath S, Alexander C, Hamann L, Hartung T, Zahringer U, Gobel UB, Weber JR, Schumann RR. Lipoteichoic acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. J Biol Chem 2003; 278(18):15587-15594.

Schroeder TH, Lee MM, Yacono PW, Cannon CL, Gerceker AA, Golan DE, Pier GB. 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 U S A 2002; 99(10):6907-6912.

Schuchat A, Deaver-Robinson K, Plikaytis BD, Zangwill KM, Mohle-Boetani J, Wenger JD. Multistate case-control study of maternal risk factors for neonatal group B streptococcal disease. The Active Surveillance Study Group. Pediatr Infect Dis J 1994; 13(7):623-629.

Schulz C, Farkas L, Wolf K, Kratzel K, Eissner G, Pfeifer M. Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type II-like pneumocytes (A-549). Scand J Immunol 2002; 56(3):294-302.

Schumann R R, Zweigner J. A novel acute-phase marker: lipopolysaccharide binding protein (LBP). Clin Chem Lab Med 1999; (37): 271-274.

Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ. Structure and function of lipopolysaccharide binding protein. Science 1990; 249(4975):1429-1431.

Schumann RR, Kirschning CJ, Unbehaun A, Aberle HP, Knope HP, Lamping N, Ulevitch RJ, Herrmann F. The lipopolysaccharide-binding protein is a secretory class 1 acute-phase protein whose gene is transcriptionally activated by APRF/STAT/3 and other cytokine-inducible nuclear proteins. Mol Cell Biol 1996; 16(7):3490-3503.

Schwab U, Leigh M, Ribeiro C, Yankaskas J, Burns K, Gilligan P, Sokol P, Boucher R. Patterns of epithelial cell invasion by different species of the Burkholderia cepacia complex in well-differentiated human airway epithelia. Infect Immun 2002; 70(8):4547-4555.

Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem 1999; 274(25):17406-17409.

Senft AP, Korfhagen TR, Whitsett JA, Shapiro SD, LeVine AM. Surfactant protein-D regulates soluble CD14 through matrix metalloproteinase-12. J Immunol 2005; 174(8):4953-4959.

Sethi S, Murphy TF. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. Clin Microbiol Rev 2001; 14(2):336-363.

Seydel U, Oikawa M, Fukase K, Kusumoto S, Brandenburg K. Intrinsic conformation of lipid A is responsible for agonistic and antagonistic activity. Eur J Biochem 2000; (267): 3032-3039.

Shapiro HM. Overture. In: Shapiro HM, editor. Practical flow cytometry. New York: Wiley-Liss, 1995: 1-31.

Sherman MP, Campbell LA, Merritt TA, Long WA, Gunkel JH, Curstedt T, Robertson B. Effect of different surfactants on pulmonary group B streptococcal infection in premature rabbits. J Pediatr 1994; 125(6 Pt 1):939-947.

Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 1999; 189(11):1777-1782.

Shimizu T, Yokota S, Takahashi S, Kunishima Y, Takeyama K, Masumori N, Takahashi A, Matsukawa M, Itoh N, Tsukamoto T, Fujii N. Membrane-anchored CD14 is important for induction of interleukin-8 by lipopolysaccharide and peptidoglycan in uroepithelial cells. Clin Diagn Lab Immunol 2004; 11(5):969-976.

Shirtliff ME, Mader JT, Camper AK. Molecular interactions in biofilms. Chem Biol 2002; 9(8):859-871.

Simons K, Ikonen E. Functional rafts in cell membranes. Nature 1997; 387(6633):569-572.

Simons K, Toomre D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 2000; 1(1):31-39.

Singh PK, Jia HP, Wiles K, Hesselberth J, Liu L, Conway BA, Greenberg EP, Valore EV, Welsh MJ, Ganz T, Tack BF, McCray PB, Jr. Production of beta-defensins by human airway epithelia. Proc Natl Acad Sci U S A 1998; 95(25):14961-14966.

Singer, S.J. and G.L.Nicolson. The fluid mosaic model of the structure of cell membranes. Science (1972). 175 (23): 720-731

Sokol PA. Production and utilization of pyochelin by clinical isolates of Pseudomonas cepacia. J Clin Microbiol 1986; 23(3):560-562.

Somerson NL, Kontras SB, Pollack JD, Weiss HS. Pulmonary compliance: alteration during infection. Science 1971; 171(966):66-68.

Song Z, Wu H, Ciofu O, Kong KF, Hoiby N, Rygaard J, Kharazmi A, Mathee K. Pseudomonas aeruginosa alginate is refractory to Th1 immune response and impedes host immune clearance in a mouse model of acute lung infection. J Med Microbiol 2003; 52(Pt 9):731-740.

Soong G, Reddy B, Sokol S, Adamo R, Prince A. TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. J Clin Invest 2004; 113(10):1482-1489.

Speer C P, Gotze B, Curstedt T, Robertson B. Phagocytic functions and tumor necrosis factor secretion of human monocytes exposed to natural porcine surfactant (Curosurf). Pediatr Res 1991; (30): 69-74.

Soto A, Evans TJ, Cohen J. Proinflammatory cytokine production by human peripheral blood mononuclear cells stimulated with cell-free supernatants of Viridans streptococci. Cytokine 1996; 8(4):300-304.

Stamme C, Wright JR. Surfactant protein A enhances the binding and deacylation of E. coli LPS by alveolar macrophages. Am J Physiol 1999; 276(3 Pt 1):L540-L547.

Stamme C, Muller M, Hamann L, Gutsmann T, Seydel U. Surfactant protein a inhibits lipopolysaccharide-induced immune cell activation by preventing the interaction of lipopolysaccharide with lipopolysaccharide-binding protein. Am J Respir Cell Mol Biol 2002; 27(3):353-360.

Standiford TJ, Arenberg DA, Danforth JM, Kunkel SL, VanOtteren GM, Strieter RM. Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: a cellular and molecular analysis. Infect Immun 1994; 62(1):119-125.

Stefanova I, Horejsi V, Ansotegui IJ, Knapp W, Stockinger H. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science 1991; 254(5034):1016-1019.

Steiner TS, Nataro JP, Poteet-Smith CE, Smith JA, Guerrant RL. Enteroaggregative Escherichia coli expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. J Clin Invest 2000; 105(12):1769-1777.

Stewart-Tull DE. The immunological activities of bacterial peptidoglycans. Annu Rev Microbiol 1980; 34:311-340.

Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV. Complete genome sequence of Pseudomonas aeruginosa PA01, an opportunistic pathogen. Nature 2000; 406(6799):959-964.

Straus DC, Lonon MK, Woods DE, Garner CW. Production of an extracellular toxic complex by various strains of Pseudomonas cepacia. J Med Microbiol 1989; 30(1):17-22.

Straus DC, Lonon MK, Woods DE, Garner CW. 3-deoxy-D-manno-2-octulosonic acid in the lipopolysaccharide of various strains of Pseudomonas cepacia. J Med Microbiol 1990; 33(4):265-269.

Stringer B, Imrich A, Kobzik L. Lung epithelial cell (A549) interaction with unopsonized environmental particulates: quantitation of particle-specific binding and IL-8 production. Exp Lung Res 1996; (22): 495-508.

Strunk RC, Eidlen DM, Mason RJ. Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. J Clin Invest 1988; 81(5):1419-1426.

Stryer, L. Intoduction to Biological membrane (ed). Biochemistry. New york; W.H. Freeman and company 1988: 283-312.

Sutcliffe I C, Shaw N. Atypical lipoteichoic acids of gram-positive bacteria. J Bacteriol 1991; (173): 7065-7069.

Suwabe A, Otake K, Yakuwa N, Suzuki H, Ito M, Tomoike H, Saito Y, Takahashi K. Artificial surfactant (Surfactant TA) modulates adherence and superoxide production of neutrophils 317. Am J Respir Crit Care Med 1998; 158(6):1890-1899.

Sweet M J, Hume D A. Endotoxin signal transduction in macrophages. J Leukoc Biol 1996; (60): 8-26.

Sylvester FA, Sajjan US, Forstner JF. Burkholderia (basonym Pseudomonas) cepacia binding to lipid receptors. Infect Immun 1996; 64(4):1420-1425.

Tager A M, Wu J, Vermeulen M W. The effect of chloride concentration on human neutrophil functions: potential relevance to cystic fibrosis. Am J Respir Cell Mol Biol 1998; (19): 643-652.

Takase H, Nitanai H, Hoshino K, Otani T. Impact of siderophore production on Pseudomonas aeruginosa infections in immunosuppressed mice. Infect Immun 2000; 68(4):1834-1839.

Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003; 21:335-376.

Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005; 17(1):1-14.

Teder P, Vandivier RW, Jiang D, Liang J, Cohn L, Pure E, Henson PM, Noble PW. Resolution of lung inflammation by CD44. Science 2002; 296(5565):155-158.

Tokieda K, Whitsett JA, Clark JC, Weaver TE, Ikeda K, McConnell KB, Jobe AH, Ikegami M, Iwamoto HS. Pulmonary dysfunction in neonatal SP-B-deficient mice. Am J Physiol 1997; 273(4 Pt 1):L875-L882.

Thomassen M J, Meeker D P, Antal J M, Connors M J, Wiedemann H P. Synthetic surfactant (Exosurf) inhibits endotoxin-stimulated cytokine secretion by human alveolar macrophages. Am J Respir Cell Mol Biol 1992; (7): 257-260.

Tomich, CA. Herfst, JW. Golden, Christian D.M. Role of Flagella in Host Cell Invasion by *Burkholderia cepacia* Mladen. Infection and Immunity 2002 (70): 1799-1806.

Tonks A, Jones KP, Jackson SK. Phospholipid modulation of monocyte oxidative activity measured by luminol-enhanced chemiluminescence. Lipids 1999; 34 Suppl:S147.

Tonks A, Morris RH, Price AJ, Thomas AW, Jones KP, Jackson SK. Dipalmitoylphosphatidylcholine modulates inflammatory functions of monocytic cells independently of mitogen activated protein kinases. Clin Exp Immunol 2001; 124(1):86-94.

Tonks A, Parton J, Tonks AJ, Morris RH, Finall A, Jones KP, Jackson SK. Surfactant phospholipid DPPC downregulates monocyte respiratory burst via modulation of PKC. Am J Physiol Lung Cell Mol Physiol 2005; 288(6):L1070-L1080.

Tonks AJ, Tonks A, Morris RH, Jones KP, Jackson SK. Regulation of platelet-activating factor synthesis in human monocytes by dipalmitoyl phosphatidylcholine. J Leukoc Biol 2003; 74(1):95-101.

Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori MA, Werts C, Boneca IG. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 2004; 5(10):1000-1006.

Triantafilou M, Miyake K, Golenbock DT, Triantafilou K. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J Cell Sci 2002; 115(Pt 12):2603-2611.

Triantafilou M, Manukyan M, Mackie A, Morath S, Hartung T, Heine H, Triantafilou K. Lipoteichoic acid and toll-like receptor 2 internalization and targeting to the Golgi are lipid raft-dependent. J Biol Chem 2004; 279(39):40882-40889.

Tsilibary EC, Williams MC. Actin and secretion of surfactant. J Histochem Cytochem 1983; 31(11):1298-1304.

Tsutsumi-Ishii Y, Nagaoka I. Modulation of human beta-defensin-2 transcription in pulmonary epithelial cells by lipopolysaccharide-stimulated mononuclear phagocytes via proinflammatory cytokine production. J Immunol 2003; (170): 4226-4236.

Tuomanen EI, Austrian R, Masure HR. Pathogenesis of pneumococcal infection. N Engl J Med 1995; 332(19):1280-1284.

Twigg H L, III. Pulmonary host defenses. J Thorac Imaging 1998; (13): 221-233.

Uehara A, Sugawara S, Takada H. Priming of human oral epithelial cells by interferon-gamma to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids and peptidoglycans. J Med Microbiol 2002; 51(8):626-634.

Ueta M, Nochi T, Jang M H, Park E J, Igarashi O, Hino A, Kawasaki S, Shikina T, Hiroi T, Kinoshita S, Kiyono H. Intracellularly expressed TLR2s and TLR4s contribution to an immunosilent environment at the ocular mucosal epithelium. J Immunol 2004; (173): 3337-3347.

Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, Aderem A. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 1999; 401(6755):811-815.

Utaisincharoen P, Anuntagool N, Arjcharoen S, Lengwehasatit I, Limposuwan K, Chaisuriya P, Sirisinha S. Burkholderia pseudomallei stimulates low interleukin-8 production in the human lung epithelial cell line A549. Clin Exp Immunol 2004; 138(1):61-65.

van de Wetering JK, van Eijk M, van Golde LM, Hartung T, van Strijp JA, Batenburg JJ. Characteristics of surfactant protein A and D binding to lipoteichoic acid and peptidoglycan, 2 major cell wall components of gram-positive bacteria. J Infect Dis 2001; 184(9):1143-1151.

van Iwaarden F, Welmers B, Verhoef J, Haagsman HP, van Golde LM. Pulmonary surfactant protein A enhances the host-defense mechanism of rat alveolar macrophages. Am J Respir Cell Mol Biol 1990; 2(1):91-98.

Van Iwaarden JF, Pikaar JC, Storm J, Brouwer E, Verhoef J, Oosting RS, van Golde LM, van Strijp JA. Binding of surfactant protein A to the lipid A moiety of bacterial lipopolysaccharides. Biochem J 1994; 303 (Pt 2):407-411.

Vandamme P, Holmes B, Coenye T, Goris J, Mahenthiralingam E, LiPuma JJ, Govan JR. Burkholderia cenocepacia sp. nov.--a new twist to an old story. Res Microbiol 2003; 154(2):91-96.

Vanderzwan J, McCaig L, Mehta S, Joseph M, Whitsett J, McCormack DG, Lewis JF. Characterizing alterations in the pulmonary surfactant system in a rat model of Pseudomonas aeruginosa pneumonia. Eur Respir J 1998; 12(6):1388-1396.

Vangerow B, Hafner D, Rueckoldt H, Marx G, Ott N, Leuwer M, Frerking I, Germann PG. Effects of C1 inhibitor and r-SP-C surfactant on oxygenation and histology in rats with lavage-induced acute lung injury. Intensive Care Med 2001; 27(9):1526-1531.

van Rozendaal B A, van Spriel A B, van De Winkel J G, Haagsman H P. Role of pulmonary surfactant protein D in innate defense against Candida albicans. J Infect Dis 2000; (182): 917-922.

Vasil ML, Chamberlain C, Grant CC. Molecular studies of Pseudomonas exotoxin A gene. Infect Immun 1986; 52(2):538-548.

Vasil ML, Ochsner UA. The response of Pseudomonas aeruginosa to iron: genetics, biochemistry and virulence. Mol Microbiol 1999; 34(3):399-413.

Vasselon T, Hanlon WA, Wright SD, Detmers PA. Toll-like receptor 2 (TLR2) mediates activation of stress-activated MAP kinase p38. J Leukoc Biol 2002; 71(3):503-510.

Veldhuizen R, Nag K, Orgeig S, Possmayer F. The role of lipids in pulmonary surfactant. Biochim Biophys Acta 1998; (1408): 90-108.

Viriyakosol S, Tobias P S, Kitchens R L, Kirkland T N. MD-2 binds to bacterial lipopolysaccharide. J Biol Chem 2001; (276): 38044-38051.

Visintin A, Mazzoni A, Spitzer JA, Segal DM. Secreted MD-2 is a large polymeric protein that efficiently confers lipopolysaccharide sensitivity to Toll-like receptor 4. Proc Natl Acad Sci U S A 2001; 98(21):12156-12161.

Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of Toll-like receptors in human monocytes and dendritic cells. J Immunol 2001; 166(1):249-255.

Visintin A, Latz E, Monks BG, Espevik T, Golenbock DT. Lysines 128 and 132 enable lipopolysaccharide binding to MD-2, leading to Toll-like receptor-4 aggregation and signal transduction. J Biol Chem 2003; 278(48):48313-48320.

Viviano CJ, Bakewell WE, Dixon D, Dethloff LA, Hook GE. Altered regulation of surfactant phospholipid and protein A during acute pulmonary inflammation. Biochim Biophys Acta 1995; 1259(3):235-244.

von Aulock S, Morath S, Hareng L, Knapp S, Van Kessel KP, van Strijp JA, Hartung T. Lipoteichoic acid from Staphylococcus aureus is a potent stimulus for neutrophil recruitment. Immunobiology 2003; 208(4):413-422.

von Aulock S, Schroder NW, Traub S, Gueinzius K, Lorenz E, Hartung T, Schumann RR, Hermann C. Heterozygous toll-like receptor 2 polymorphism does not affect lipoteichoic acid-induced chemokine and inflammatory responses. Infect Immun 2004; 72(3):1828-1831.

Walti H, Polla BS, Bachelet M. Modified natural porcine surfactant inhibits superoxide anions and proinflammatory mediators released by resting and stimulated human monocytes. Pediatr Res 1997; 41(1):114-119.

Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 2001; 412(6844):346-351.

Wang JY, Kishore U, Lim BL, Strong P, Reid KB. Interaction of human lung surfactant proteins A and D with mite (Dermatophagoides pteronyssinus) allergens. Clin Exp Immunol 1996; 106(2):367-373.

Wang Q, Dziarski R, Kirschning CJ, Muzio M, Gupta D. Micrococci and peptidoglycan activate TLR2-->MyD88-->IRAK-->TRAF-->NIK-->IKK-->NF-kappaB signal transduction pathway that induces transcription of interleukin-8. Infect Immun 2001; 69(4):2270-2276.

Wang ZM, Liu C, Dziarski R. Chemokines are the main proinflammatory mediators in human monocytes activated by Staphylococcus aureus, peptidoglycan, and endotoxin. J Biol Chem 2000; 275(27):20260-20267.

Watarai M, Makino S, Fujii Y, Okamoto K, Shirahata T. Modulation of Brucella-induced macropinocytosis by lipid rafts mediates intracellular replication. Cell Microbiol 2002; 4(6):341-355.

Watford WT, Ghio AJ, Wright JR. Complement-mediated host defense in the lung. Am J Physiol Lung Cell Mol Physiol 2000; 279(5):L790-L798.

Weaver TE, Conkright JJ. Function of surfactant proteins B and C. Annu Rev Physiol 2001; 63:555-578.

Webb DS, Jeska EL. Enhanced luminol-dependent chemiluminescence of stimulated rat alveolar macrophages by pretreatment with alveolar lining material. J Leukoc Biol 1986; 40(1):55-64.

Weidel W, Pelzer H. Bagsaped macromolecule-A new otlookon bacterial cell walls. Adv Enzymol Relat Areas Mol Biol 1964; 26:193-232.

Weidemann B, Brade H, Rietschel ET, Dziarski R, Bazil V, Kusumoto S, Flad HD, Ulmer AJ. Soluble peptidoglycan-induced monokine production can be blocked by anti-CD14 monoclonal antibodies and by lipid A partial structures. Infect Immun 1994; 62(11):4709-4715.

Weikert LF, Edwards K, Chroneos ZC, Hager C, Hoffman L, Shepherd VL. SP-A enhances uptake of bacillus Calmette-Guerin by macrophages through a specific SP-A receptor. Am J Physiol 1997; 272(5 Pt 1):L989-L995.

Weingart C L, Hooke A M. A nonhemolytic phospholipase C from Burkholderia cepacia. Curr Microbiol 1999; (38): 233-238.

Westphal O, Luderitz O, Keiderling W. Effect of bacterial toxins; biochemical analysis of inflammation. Infektionskr Hyg 1952 (158): 152-60.

Whitsett JA. Intrinsic and innate defenses in the lung: intersection of pathways regulating lung morphogenesis, host defense, and repair. J Clin Invest 2002; 109(5):565-569.

Whitsett JA. Surfactant proteins in innate host defense of the lung. Biol Neonate 2005; 88(3):175-180.

Wicken AJ, Knox KW. Lipoteichoic acids: a new class of bacterial antigen. Science 1975; 187(4182):1161-1167.

Wilmott RW, Kassab JT, Kilian PL, Benjamin WR, Douglas SD, Wood RE. Increased levels of interleukin-1 in bronchoalveolar washings from children with bacterial pulmonary infections. Am Rev Respir Dis 1990; 142(2):365-368.

Wilsher ML, Hughes DA, Haslam PL. Immunoregulatory properties of pulmonary surfactant: influence of variations in the phospholipid profile. Clin Exp Immunol 1988; 73(1):117-122.

Wilson R, Pitt T, Taylor G, Watson D, MacDermot J, Sykes D, Roberts D, Cole P. Pyocyanin and 1-hydroxyphenazine produced by Pseudomonas aeruginosa inhibit the beating of human respiratory cilia in vitro. J Clin Invest 1987; 79(1):221-229.

Wine JJ. The genesis of cystic fibrosis lung disease. J Clin Invest 1999; 103(3):309-312.

Wong MM, Fish EN. Chemokines: attractive mediators of the immune response. Semin Immunol 2003; 15(1):5-14.

Woodhead MA, Macfarlane JT, McCracken JS, Rose DH, Finch RG. Prospective study of the aetiology and outcome of pneumonia in the community. Lancet 1987; 1(8534):671-674.

Wright JR. Clearance and recycling of pulmonary surfactant. Am J Physiol 1990; 259(2 Pt 1):L1-12.

Wright JR. Immunomodulatory functions of surfactant. Physiol Rev 1997; 77(4):931-962.

Wright JR. Pulmonary surfactant: a front line of lung host defense. J Clin Invest 2003; 111(10):1453-1455.

Wright JR. Immunoregulatory functions of surfactant proteins. Nat Rev Immunol 2005; 5(1):58-68.

Wu H, Kuzmenko A, Wan S, Schaffer L, Weiss A, Fisher JH, Kim KS, McCormack FX. Surfactant proteins A and D inhibit the growth of Gram-negative bacteria by increasing membrane permeability. J Clin Invest 2003; 111(10):1589-1602.

Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, Takeuchi O, Takeda K, Akira S. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol 2003a; 4(11):1144-1150.

Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003b; 301(5633):640-643.

Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 1999; 163(1):1-5.

Yoshimura A, Takada H, Kaneko T, Kato I, Golenbock D, Hara Y. Structural requirements of muramylpeptides for induction of Toll-like receptor 2-mediated NF-kappaB activation in CHO cells. J Endotoxin Res 2000; 6(5):407-410.

Yu B, Wright SD. Catalytic properties of lipopolysaccharide (LPS) binding protein. Transfer of LPS to soluble CD14. J Biol Chem 1996; 271(8):4100-4105.

Zahringer U, Lindner B, Rietschel ET. Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. Adv Carbohydr Chem Biochem 1994; 50:211-276.

Zeligs BJ, Nerurkar LS, Bellanti JA. Chemotactic and candidacidal responses of rabbit alveolar macrophages during postnatal development and the modulating roles of surfactant in these responses. Infect Immun 1984; 44(2):379-385.

Zhang G, Ghosh S. Toll-like receptor-mediated NF-kappaB activation: a phylogenetically conserved paradigm in innate immunity. J Clin Invest 2001; 107(1):13-19.

Zhang P, Summer WR, Bagby GJ, Nelson S. Innate immunity and pulmonary host defense 433. Immunol Rev 2000; 173:39-51.

Zhao N, Qu C, Wang E, Chen W. Phylogenetic evidence for the transfer of Pseudomonas cocovenenans (van Damme et al. 1960) to the genus Burkholderia as Burkholderia cocovenenans (van Damme et al. 1960) comb. nov. Int J Syst Bacteriol 1995; 45(3):600-603.

Zhao YX, Andoh A, Shimada M, Takaya H, Hata K, Fujiyama Y, Bamda T. Secretion of complement components of the alternative pathway (C3 and factor B) by the human alveolar type II epithelial cell line A549. Int J Mol Med 2000; 5(4):415-419.

Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity 156. Immunity 2000; 12(2):121-127.