THE ROLE OF PHOSPHOLIPIDS IN THE MODULATION OF THE MONOCYTIC OXIDATIVE BURST

BY

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by

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This thesis is dedicated to my father, Ron and my fiancée, Amanda for their continued

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ABSTRACT

Alveolar macrophages play a central role in the pulmonary inflammatory response by generating reactive oxygen intermediates (ROIs). This study investigates how pulmonary surfactant (composed of approximately 90% phospholipids and 10% surfactant specific proteins) modulates the production of ROI's in monocytes and macrophages.

The human monocyte cell line MonoMac-6 (MM6), peripheral blood monocytes, the murine macrophage RAW 264.7 and the rat alveolar macrophage line NR8383 were primed with lipopolysaccharide (LPS) and stimulated to produce ROIs with opsonised zymosan (OpZ). The surfactants Curosurf®, Survanta® and Exosurf Neonatal™ were all shown to significantly inhibit ROI production ($P<0.01$) measured by luminol-enhanced chemiluminescence (LCL). Preincubation of MM6 cells with 1,2-dipalmitoylphosphatidylcholine (DPPC), the major surfactant phospholipid, also significantly inhibited ROI production ($p<0.001$). In contrast, 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC) increased ROI production ($P<0.001$). DPPC modulation was independent of LPS 'priming' and did not affect cell viability. In addition, DPPC (but not PAPC) significantly inhibited the LPS-stimulated release of TNF-α in MM6 cells ($P<0.05$). However, DPPC failed to modulate nitric oxide production, measured by the Griess assay, demonstrating the selective nature of the effect.

Experiments with a cell-free system showed that DPPC had no direct effect on NADPH oxidase assembly and activation. Flow cytometry analysis indicated that the suppressive effects of DPPC on ROI production were not attributed to CD14, complement, or Fcγ receptor down-regulation. Moreover, the binding of radiolabelled LPS was not decreased in MM6 cells preincubated with DPPC.

Transmission electron microscopy demonstrated that DPPC was taken up by MM6 cells and altered the monocyte membrane ultrastructure. Western blotting demonstrated that the mitogen activated protein kinases were not modulated by DPPC. However, the activity of protein kinase C (PKC) measured by ^{32}P radioassay, was shown to be significantly inhibited by DPPC ($P<0.01$). Taken together these findings indicate that surfactant lipids, particularly DPPC, suppress monocyte NADPH oxidase activation by down-regulation of PKC.

ABBREVIATIONS

ANOVA	One way analysis of variance
ARDS	Acute (adult) respiratory distress syndrome
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
CFU-GM	Colony forming unit – granulocyte macrophage
Chol	Cholesterol
Ci	Curie
CPM	Counts per minute
DAG	Diacylglycerol
DEAE	Diethylaminoethyl
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPC	1,2-Dipalmitoylphosphatidylcholine
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylaminediaminetetraacetic acid
EGTA	Ethylene glycol-bis-tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EPR	Electron paramagnetic resonance
ERK	Extracellular regulated kinase

EU	Endotoxin units
FAD	Flavin adenine dinucleotide
FBS	Foetal bovine serum
GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
H₂O₂	Hydrogen peroxide
HMP	Hexose monophosphate shunt
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP₃	Inositol tri-phosphate
IRDS	Infant respiratory distress syndrome
IU	International units
LAL	Limulus amoebocyte lysate
LBP	Lipopolysaccharide binding protein
LCL	Luminol enhanced chemiluminescence
LDS	Lithium dodecyl sulphate
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor
MFI	Mean fluorescence intensity
MM6	MonoMac-6
MOPS	3-(N-morpholino) propane sulphonic acid

MPO	Myeloperoxidase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
NO·	Nitric oxide
NOS	Nitric oxide synthase
NP40	Tergitol-NP40
NR8383	Normal rat alveolar cell
$O_2^{\cdot-}$	Superoxide
OH·	Hydroxyl radical
OpZ	Opsonised zymosan A
PAF	Platelet activating factor
PAGE	Polyacylamide gel electrophoresis
PAP	Pulmonary alveolar proteinosis
PAPC	1-Palmitoyl-2-arachidonoyl phosphatidylcholine
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PD10	Sephadex G-25 medium
PDT	4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-oxyl
PG	L-α-phosphatidyl-DL-glycerol
PGE_2	Prostaglandin E_2
PHOX	Phagocyte oxidase
PKC	Protein kinase C

PLA$_2$, (C), (D)	Phospholipase A$_2$, C or D
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonyl fluoride
RAW 264.7	Mouse monocyte / macrophage cell
RLU	Relative light units
ROIs	Reactive oxygen intermediates
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SH3	Src homology domain 3
SM	Sphingomyelin
SOD	Superoxide dismutase
TEM	Transmission electron micrographs
Thr	Threonine
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
tPC	L-α-phosphatidylcholine (type XVI-E from egg yolk)
tPE	L-α-phosphatidylethanolamine (type III from egg yolk)
Tyr	Tyrosine

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CHAPTER 1: INTRODUCTION

1.0   GENERAL INTRODUCTION

The thin epithelial lining, which comprises the alveolar surface for gaseous exchange, is uniquely vulnerable to damage related to inflammatory changes. These may result from particulate insults, oxidant gases or damage secondary to infection. Respiratory illnesses including asthma, bronchitis, emphysema, pleurisy, tuberculosis, cystic fibrosis and infection are major causes of death and disability in Western civilisations and in many other parts of the world. In the United Kingdom, respiratory illnesses accounted for approximately 180 deaths per 100,000 population in 1995 (Regional Statistics Branch, 1995). Specifically in the Welsh population, nearly one in four adults who took part in the 1998 Welsh Health Survey reported being treated for a respiratory illness (National Assembly for Wales, 1999). Given the levels of morbidity and mortality caused by respiratory illness, consideration should be given to improved prevention and treatment.

A number of innate mechanisms within the airways exist to provide an appropriate and rapid response to particulate or infectious challenge. These include physical barriers such as nasal hair and mucus, which is continuously secreted by the epithelial cells and glands. Particles are trapped in the upward moving mucus (propelled by cilliary movement) and are carried away from the airway to be swallowed or coughed up. Sensory endings within the nasal passages, trachea and bronchi elicit the cough and sneezing reflexes thus reducing the intake of foreign particles. Most important in processing particles reaching the alveoli is the action of alveolar macrophages. These phagocytes co-exist with pulmonary surfactant in the liquid lining layer that covers the

alveolar surface. These cells are thought to play a central role in the inflammatory response by means of phagocytosis and production of a number of specific mediators including reactive oxygen intermediates (ROIs), lipid metabolites and cytokines (Fels and Cohn, 1986). Obviously, a host response is required to protect the lung, however an exaggerated immune response can be damaging to alveolar structure and function. An overexuberant response can to lead to a possible disruption of the thin epithelial-endothelial barrier, leading to transudation of serum components into the airspace and severe, if not lethal, impairment of gaseous exchange. Therefore, the immune response within this region of the lung must carefully balance pro and anti-inflammatory responses without compromising host defences.

One of the most fundamental homeostatic substances in the lung is pulmonary surfactant, so named for its well-defined ability to lower surface tension at the interface of alveolar gas and the liquid hypophase that covers the epithelium of the lung. Pulmonary surfactant is a complex, multifunctional material produced by type II alveolar epithelial cells, consisting of approximately 90% lipids and 10% proteins by weight (Wright and Clements, 1987). Although its surface-active properties have been known for some time, more recently antibacterial and anti-inflammatory activities have been associated with surfactant. There is evidence indicating pulmonary surfactant lipids and surfactant proteins (SP-A, B, C and D) alter the bactericidal activity of the alveolar macrophage (Milleron *et al*, 1991; Hayakawa *et al*, 1989). Recent reports suggest surfactant proteins (SP-A and SP-D) regulate a variety of immune cell functions *in vitro* including enhanced chemotaxis and phagocytosis and alterations in the production of ROIs and cytokines (Weissbach *et al*, 1994). *In vivo* and *in vitro* studies

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have suggested that phagocyte function in particular production of ROIs and inflammatory cell mediators may be responsible, in part, for aspects of lung injury (Tate and Repine, 1983). The excessive and inappropriate production of ROIs can lead to local tissue damage associated with inflammatory conditions including acute (adult) respiratory distress syndrome (ARDS). This may be related to changes in surfactant components including phospholipid composition (Hoffman *et al*, 1989). Other reports have also demonstrated the inhibitory effect of pulmonary surfactant on ROI generation in neutrophils (Ahuja *et al*, 1996), peripheral blood monocytes (Geertsma *et al*, 1994) and alveolar macrophages (Yasuda *et al*, 1994). However, these and other studies have provided variable and conflicting reports on the bactericidal promoting activity of surfactant and surfactant components on phagocytic cells, possibly due to differences in experimental designs (Speer *et al*, 1991; Webb and Jeska, 1986). Most importantly, the mechanisms of respiratory burst inhibition by surfactant lipids have not been well characterised in terms of the active surfactant component(s) and mechanisms of action.

In the light of these previous studies, it seems logical to suggest that pulmonary surfactant and in particular its lipid content may indeed play a significant role in the immunoregulation of the lung. The interests of this research lie in better defining the effects of surfactant components on different facets of the activation responses in inflammatory cells. The potential benefits of such a function are considerable since it would facilitate the protection of the delicate gas exchanging regions of the lung and the type II epithelial cells from the adverse effects of undesirable immune reactions to inhaled particles.

Potential benefits from this study include improved characterisation of the effect of different pulmonary surfactant lipids on macrophage function that may suggest more than one type of surfactant preparation could be used clinically. For example, the preparations of surfactant currently being used clinically were developed to essentially address the surfactant deficiency in infant respiratory distress syndrome. Their efficacy and safety could be different in other disease states such as ARDS or pneumonia. It has also been suggested that surfactants may be useful in asthma (Wright *et al*, 2000). Since this complex disease has an underlying inflammatory component, anti-inflammatory agents could be useful components of engineered human surfactants designed for use in asthma. These are only obvious areas in which alternative surfactant preparations could be considered. As research in the field progresses, a variety of surfactants based on different compositions (including lipids, proteins and synthetic peptides such as 'KL$_4$') will be developed for different diseases. The KL$_4$ peptide is a 21 residue peptide containing repeated stretches of one lysine and 4 leucines. This artificial peptide is physiologically active when recombined with dipalmitoyl phosphatidycholine (DPPC). In addition surfactant liposomes may prove to be an important method of dispersion of antibiotic and other drugs in the airways. Surfactant lipid uptake studies may suggest the feasibility to facilitate to facilitate gene therapy for cystic fibrosis or familial SP-B deficiency using surfactant liposomes as a vehicle.

1.1   IMMUNOLOGY OF THE LUNG

The body is in direct contact with its external environment through the air inhaled during breathing. Each day the tracheobronchial tree and terminal respiratory units are exposed to more than 7,000 litres of ambient air that may contain infectious micro-organisms and hazardous dusts (Moffett *et al*, 1993). As a result of airborne exposure, a wide variety of pulmonary infections, inflammatory lung disorders and malignancies may be seen. For the most part, the lungs are able to prevent the development of disease by a variety of non-specific defence mechanisms including clearance and secretory pathways. However, in addition to anti-microbial substances that are naturally present in the secretions lining the respiratory tract, a variety of cells participates in the pulmonary immune response.

1.1.1   STRUCTURE OF THE RESPIRATORY SYSTEM

The chest or thoracic cavity is a compartment bounded by the rib cage and closed by a sheet of skeletal muscle called the diaphragm. The interior of the thorax, including each lung is lined with a sheet of epithelial tissue, the pleura. Each lung is enclosed in a separate pleural cavity. The left lung is smaller than the right and consists of two lobes, whereas the right lung has three lobes. The lungs are ventilated with atmospheric air by way of a treelike airway. The nose, pharynx, larynx, trachea and the right and left primary bronchi are structures that lie outside the lungs. Within the lung, each bronchus branches extensively (Figure 1.1).

A

Left lung

Pulmonary vessels

Right lung

Bronchi

Ribs

Heart

Terminal Bronchioles

Intercostal muscles

B

Terminal bronchiole

Acinus

Alveolar duct with alveoli
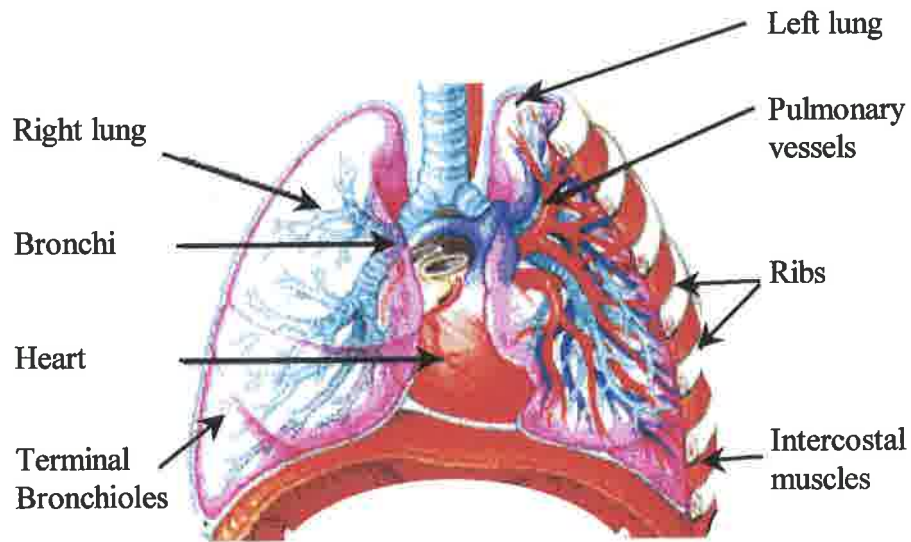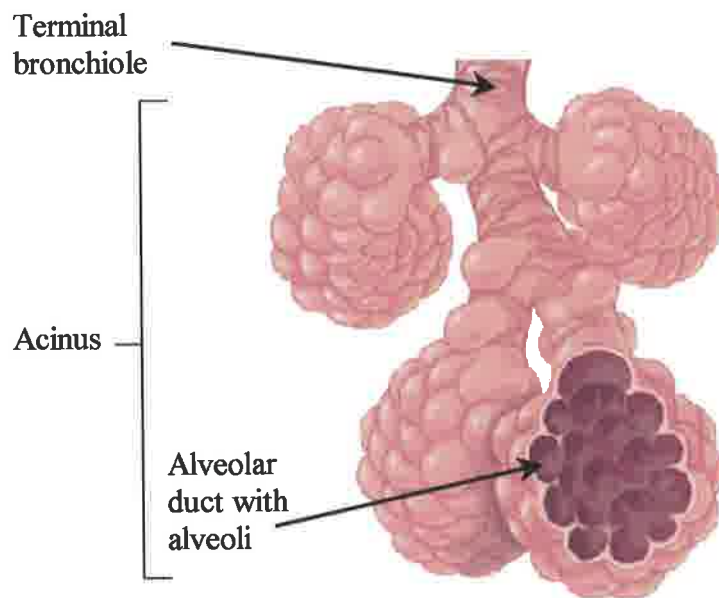
Figure 1.1 – **(A)** Structure of the respiratory airway. **(B)** Pulmonary acinus arises from a single terminal bronchiole, which branches into alveolar ducts, which in turn terminates in alveoli. Modified from Moffett *et al*, 1993.

Subsequent branching results in about 150,000 smaller bronchioles termed terminal bronchioles (Moffett *et al*, 1993). These are surrounded only by multi-unit smooth cells. The absence of cartilage in these terminal bronchioles makes them susceptible to collapse if enough external pressure is applied across their walls. Bronchioles with diameters smaller than 0.5 mm are referred to as respiratory bronchioles. These contain a few alveoli in their walls and are the beginning of gaseous exchange. The respiratory bronchioles eventually terminate in the gas exchange zone of the lung, the alveolar ducts and sacs (Figure 1.1). These structures give rise to numerous spherical alveoli. However, only alveoli located in the alveolar sacs provide most of the surface area for gaseous exchange.

1.1.2   CELLS INVOLVED IN PULMONARY HOST DEFENCE

Varieties of cells participate in the immune response, and the relative contribution of different cell types varies with different diseases and the degree of inflammation. This topic is only briefly discussed here, with emphasis on the cells affected by surfactant. Nearly a century ago Metchnikoff argued that phagocytosis is an important defence mechanism against the constant assault on the body by micro-organisms (Metchnikoff, 1905). His conclusions have been amply confirmed and much is known about the cell types involved and the factors that regulate their production, how phagocytes identify and interact with infectious agents and how they "engulf" and kill micro-organisms.

There are three types of "professional phagocytes" capable of ingesting and digesting exogenous antigens such as whole micro-organisms, insoluble particles, injured and dead host cells, cellular debris and clotting factors. Two types circulate in the blood

stream (polymorphonuclear leukocytes and monocytes), and one type resides in the tissues throughout the body. The circulating phagocytes may be encountered in tissues throughout the body in small numbers, including the interstitial and alveolar spaces of the lungs. A dramatic increase in number of these cells in certain tissues constitutes one of the histologic hallmarks of differing kinds and duration of inflammatory processes. The neutrophil is one of the major cells implicated in a variety of non-infectious diseases including idiopathic pulmonary fibrosis (Schaaf *et al*, 2000), asthma (Amin *et al*, 2000), and emphysema (Pinot *et al*, 1999; Janoff *et al*, 1979) as well as a variety of infectious disorders (Amin *et al*, 2000; Tate and Repine, 1983). Neutrophils normally comprise 1 – 3 % of cells recovered in bronchoalveolar lavage fluid (BALF) and it has been suggested that neutrophils are important in the etiology of acute (adult) respiratory distress syndrome (ARDS) as these cell types are the major cells recovered in BALF of ARDS patients (Nakos *et al*, 1998).

The mononuclear phagocyte system consists of circulating monocytes in the blood and lymphatics and macrophages in the tissues. During haematopoiesis in the bone marrow, the colony forming unit, granulocyte-macrophage (CFU-GM) progenitor cells differentiate into pro-monocytes, which leave the bone marrow and enter the blood, where they further differentiate into mature monocytes (Figure 1.2). Their production and release from the bone marrow is increased during infection. Monocytes circulate in the bloodstream where they have a half-life in man of about 70 hours, during which time they enlarge and migrate into the tissue and differentiate into tissue macrophage.

CFU-GM →

Neutrophils

Monoblast   Promonocyte   Monocytes   Monocytes   Macrophage

Bone Marrow   **Blood**   **Tissue**
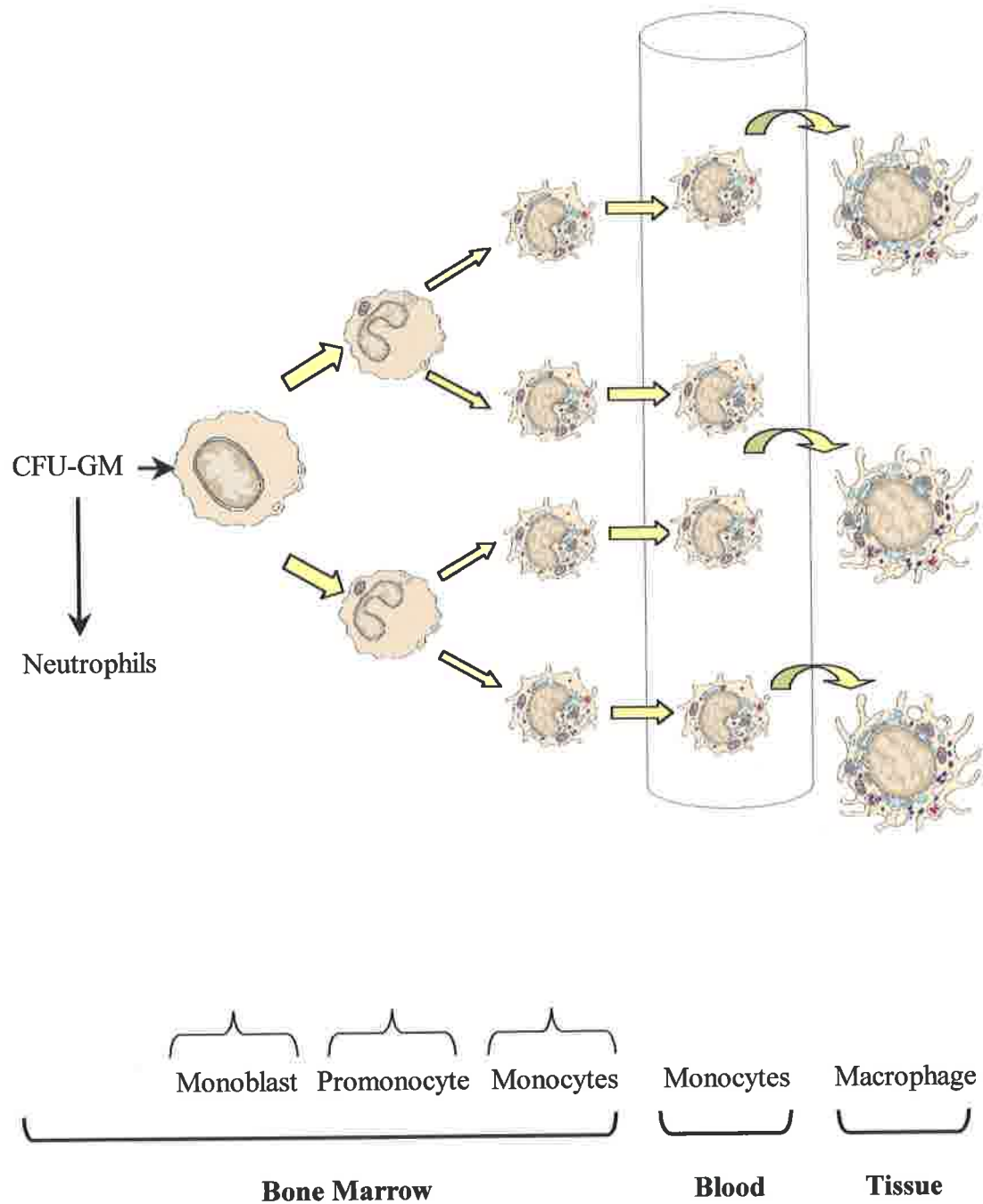
Figure 1.2 – The mononuclear phagocyte system, showing the origin of macrophages in the bone marrow.   CFU-GM; colony forming unit – granulocyte macrophage.

Typical locations of macrophages include the lungs (alveolar macrophages), liver (Kupffer cells), central nervous system (microglial cells) and skin to name a few (Zielasek and Hartung, 1996). The tissue macrophage is the most differentiated cell and most functionally active cell of the mononuclear phagocyte system. They play an important role as a phagocytic cell, antigen presentation to T-cells and the production of a number of inflammatory mediators including lipid derived mediators, ROIs and cytokines (Jackson, 1997). Examples of secreted cytokines include tumour necrosis factor alpha (TNF-α) and interleukin (IL) 1 and 6 which function in a bactericidal capacity as well as aiding in the regulation of the immunological response (Xie *et al*, 1997).

1.1.3   ALVEOLAR MACROPHAGES

Macrophages can be isolated from the alveolar space by bronchoalveolar lavage (BAL) (Bates *et al*, 1997). In a normal, non-smoking subject, this technique yields approximately 1×10^9 cells with 90 - 95% of this number alveolar macrophages (Fels and Cohn, 1986). However, isolation of macrophages by this technique may yield activated macrophages. In addition, obtaining "normal" volunteers to undergo lavage can prove to be difficult as it is an invasive and painful procedure. Pulmonary or alveolar macrophages are found in the alveoli, small and large airways, interstitium of the lung, and lining of the pulmonary vessels. These macrophages play a critical role in protecting the lung from infection by the enormous quantity of inhaled bacteria or other particles, some of which may gain access to the lower airways (Goldstein *et al*, 1974). Neutrophils may assist the pulmonary macrophage especially when this first line of defence is overwhelmed. The sources of pulmonary macrophages are generally

peripheral blood monocytes that have migrated into the pulmonary environment or local mononuclear phagocytes that replicate in the alveolus or interstitium. Their size ranges from approximately 9 - 40 μm in diameter when first harvested (Cohen and Cline, 1971). Alveolar macrophages are representative of large tissue macrophages with a well-developed vacuolar apparatus and more than the usual number of mitochondria (Cohen and Cline, 1971). The dense granule or secondary lysosome is the prominent cytoplasmic organelle (Figure 1.3). The cytoplasm contains scattered strands of rough endoplasmic reticulum and a well developed Golgi complex. The most characteristic ultrastructural feature of alveolar macrophages is the abundance of membrane bound cytoplasmic inclusions containing proteolytic enzymes. The surfaces of macrophages are covered in microvilli, ruffles and small surface blebs. In the alveolus, the cells are found in a surfactant rich alveolar fluid composed of approximately 90% phospholipids, few other lipids and 5 - 10% surfactant specific proteins, each genetically distinct (Notter and Finkelstein, 1984). Human pulmonary alveolar macrophages generate greater quantities of ROIs than do monocytes upon stimulation. This difference is reflected in their superior capacity to kill *Pseudomonas aeruginosa* and *Listeria monocytogenes* (Kemmerich *et al*, 1987). However, under the influence of 'priming' agents such as interferon gamma (IFNγ), the respiratory burst is enhanced in monocytes but not alveolar macrophages. The cause of this reduced activity is not entirely understood but exposure to high oxygen levels and / or surfactant, in particular surfactant lipids, has been postulated to suppress some macrophage functions.
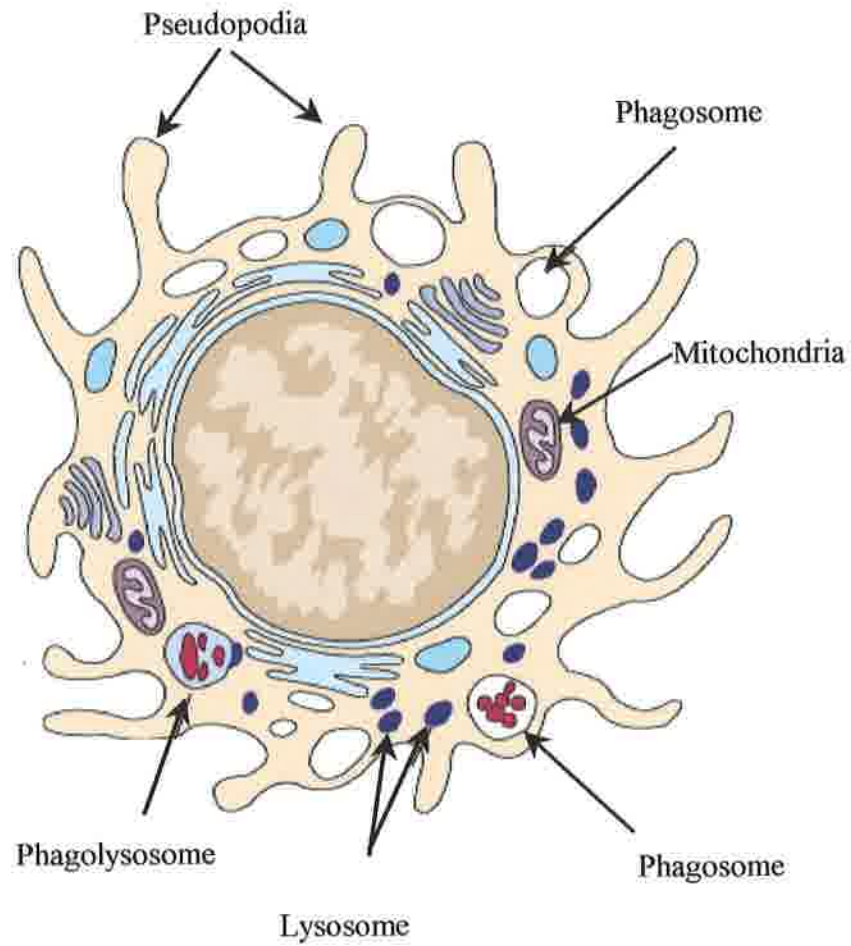
Figure 1.3 – Illustration showing typical morphology of a macrophage. Macrophages are 5 – 10 fold larger than monocytes and contain more organelles, especially lysosomes. Modified from Kuby.J., 1997.

1.2   SURFACTANT

It was surfactant's ability to reduce surface tension and thereby increase compliance (elasticity) of the excised lung that led to its initial discovery by von Neergaard in the 1920s (reviewed in Veldhuizen *et al*, 1998). Little was known about surfactant until the mid- 1950s when revived interest by Pattle (Pattle, 1955) and Clements (Clements, 1957) led to its rediscovery. They not only recognized the ability of surfactant to prevent lung collapse at the end of expiration by spreading rapidly to form an insoluble surface film, but also demonstrated that reductions in surface area resulted in a corresponding fall in surface tension. The clinical manifestation of a surfactant deficiency is seen most clearly in the premature newborn infant who is unable to produce adequate amounts of surfactant. Laboured breathing and inadequate oxygenation characterize the resulting disease, infant respiratory distress syndrome (IRDS). In ARDS surfactant is often inactivated or unable to effectively reduce surface tension (Nakos *et al*, 1998). By the early- 1960s the ability of lipids to act as surface tension reducing agents was well recognized. The phospholipid phosphatidylcholine (PC; lethicin) had been discovered as a component of egg yolk in 1847. The presence of the disaturated lecithin, 1,2-dipalmitoyl phosphatidylcholine (DPPC) in lung tissue had been reported in 1946 (Thannhauser *et al*, 1946). Further studies soon established that phospholipids comprised the major surface tension reducing component and DPPC was a major constituent (Brown, 1964). Since then there have been a number of insights into the uses of surfactant (reviewed in Brown and Pattishall, 1993). In the late 1980s and early 90s, the role of surfactant in modulating the immune functions of leukocytes within the lung was of great interest. Research led to a better understanding

of the composition of surfactant, metabolism, biophysical properties and immunological activity of surfactant.

1.2.1 PHOSPHOLIPID BIOCHEMISTRY

1.2.1.1 PHOSPHOLIPID STRUCTURE

Most phospholipids in the body are synthesised endogenously as esterification of two fatty acids onto the glycerol-3-phosphate backbone. Phospholipids are usually characterised by a hydrophobic tail and a hydrophilic head group (Figure 1.4). Phospholipids are said to be amphipathic due to their hydrophobic and hydrophilic properties (Christie and Hunter, 1985).

The glycerol backbone has three positions available for binding of organic molecules. At the *sn*-1 and *sn*-2 positions, fatty acids are esterified to the backbone. The grouping at the *sn*-3 position can take the form of choline, ethanolamine or serine for example (Figure 1.5).
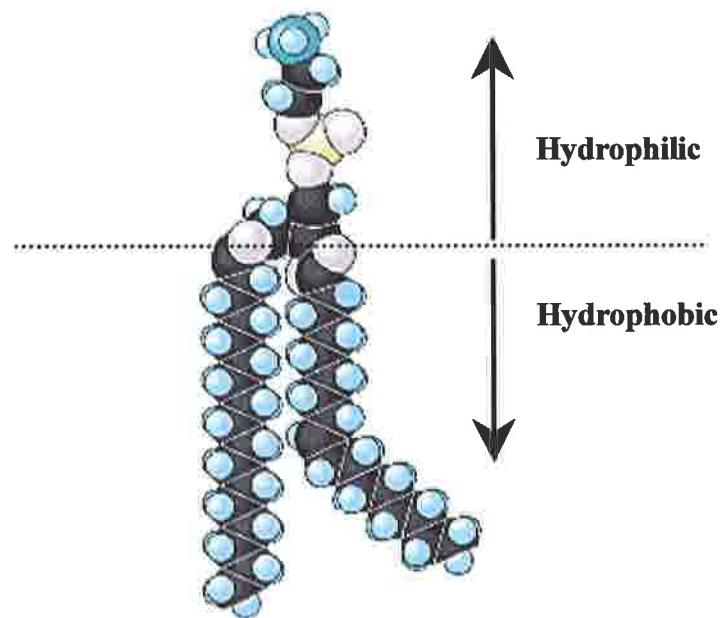
Figure 1.4 – General phospholipid structure. The hydrocarbon tails confer hydrophobicity to this structure while the head group (e.g. choline, ethanolamine) will confer the hydrophilic property.

(A) Phosphatidylethanolamine

(B) Phosphatidylcholine

(C) Phosphatidylserine

Figure 1.5 – Examples of common phospholipids. The hydrophobic R groups are indicated in yellow and the very hydrophilic head groups in blue. The positions *sn*-1, *sn*-2 and *sn*-3 are represented by *, **, *** respectively.
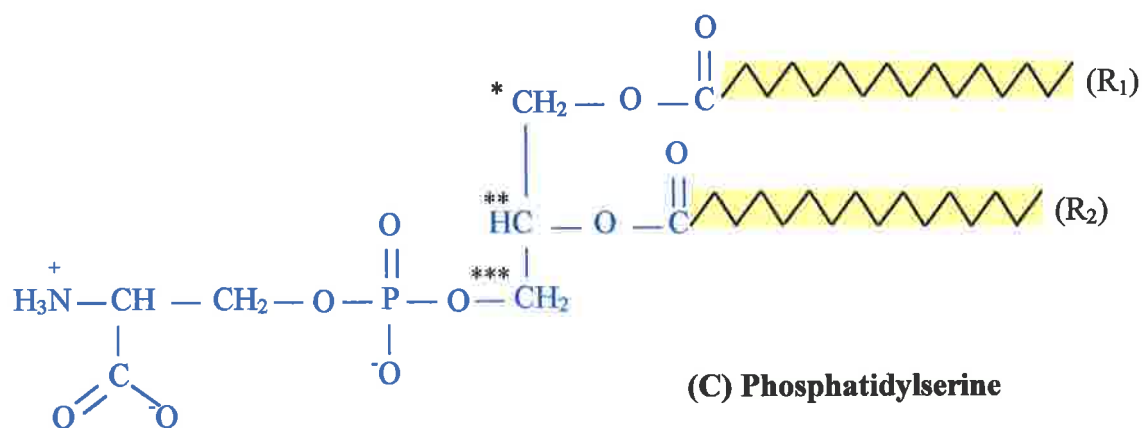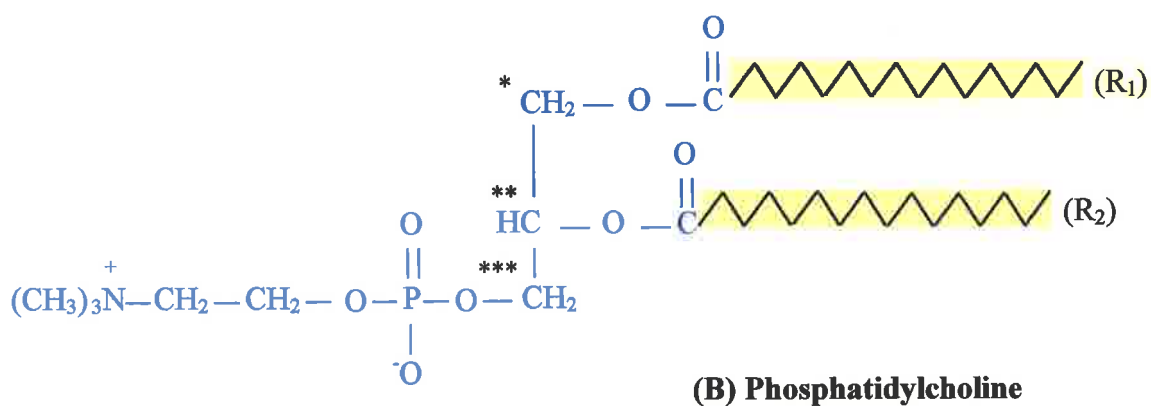
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The western diet generally includes at least 30 - 40% of calories as fat of which 90% is in the form of triacylglyceride fatty acids (Laposata, 1995). Hydrolysis of triacylglycerol forms fatty acids (carbon chains with a carboxyl and methyl terminus). The carbon at the carboxy terminus is conventionally numbered 1 and so on until the final carbon in the terminal methyl group. The fatty acid palmitic acid for example is a 16 carbon fatty acid and may be represented as C16:0 or

$$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2\overset{\displaystyle O}{\overset{\displaystyle \|}{C}}\text{-OH}$$

The "C16:0" denotes 16 carbons and zero double bonds i.e. the fatty acid is saturated as the valencey of carbon is full. In contrast "C18:2Δ9,12" has 18 carbons with 2 double bonds at positions 9 and 12 and the familiar name is linoleic acid. The more double bonds in the fatty acid chain, the more unsaturated the moiety; if there are two or more double bonds, the fatty acid is termed polyunsaturated. There is an alternative classification for lipids and is known as the Omega (ω) classification for monounsaturates and is determined by the number of carbons between the methyl and nearest double bond. For example the n-3 (or ω3) fatty acids are commonly found in fish oils and n-6 (or ω6) are essential fatty acids found in vegetable oils (Laposata, 1995).

1.2.1.2   LIPID FUNCTION

Lipids play roles both in energy metabolism and in aspects of biological structure and function. Phospholipids are very useful as biological detergents, exemplified as surfactant in the lung (see section 1.2.4). Phospholipids also form the basic building blocks of cellular membranes. Cell membranes are composed of lipids and proteins which forms a semi-permeable barrier arranged in a bilayer configuration: parallel sheets of phospholipids with the polar head orientated towards the water interface and the fatty acyl chains interacting with the hydrophobic core (Figure 1.6A). The membrane bilayer configuration allows selective transport while enclosing the cell and its contents. This cell membrane model was proposed by Singer and Nicolson in 1972 and was thought to be a dynamic or fluid membrane contributing to cell regulation (Singer and Nicolson, 1972) as opposed to a static lipid-protein matrix (Figure 1.6B). Lateral diffusion of transmembrane proteins is strongly dependent on the fluidity and composition of the membrane and relatively independent of the chemical nature of diffusing species (Homan and Pownall, 1988). The degree of motion of transmembrane proteins, that in turn can have a dramatic effect upon the cell, is dependent on the composition of the acyl chains and their chain length. In addition, the headgroup charge, determined by the ionic composition of the aqueous environment, will play a role in protein diffusion in and across the membrane. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) provide the constitutive structural framework and are normally distributed asymmetrically. PC, PS and PI provide for charged membrane surfaces, allowing water and / or ions to bind to their polar headgroups while PE is found in the poorly hydrated regions of the membrane (Boesze-Battaglia and Schimmel, 1997).

A

Bilayer

B

Phospholipids

Transmembrane
proteins
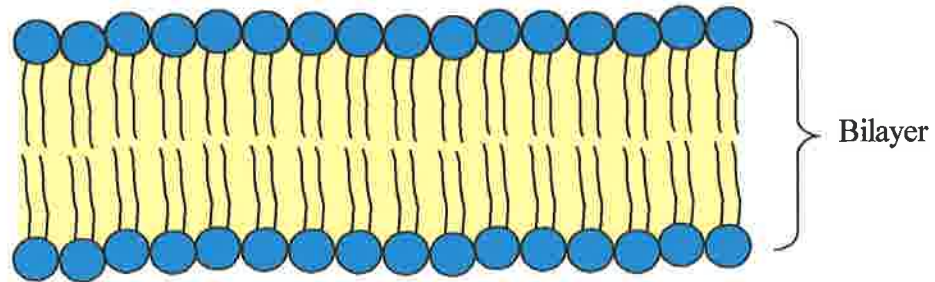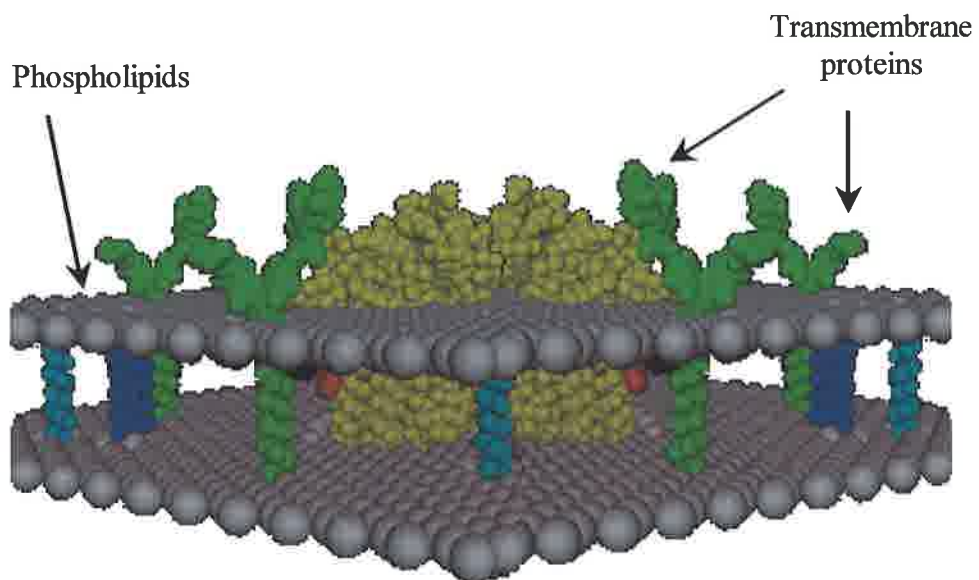
Figure 1.6 – **(A)** The multiple tails on membrane-forming lipids make the molecules cylindrical, so that planar bilayer sheets can be formed. **(B)** Schematic representation of the lipid bilayer of eukaryotic cells.

PC and sphingomyelin are found preferentially in the outer leaflet of the membrane bilayer. The aminophospholipids PE and PS are localised in abundance in the inner leaflet (Fadok *et al*, 1998). Cholesterol is the next most abundant lipid after phospholipids and functions to alter the fluidity of the membrane providing structural rigidity. Acyl chain length, the degree of saturation, governs the temperature for transition of isolated lipids from gels to a fluid state (Hamilton, 1989). In summary, alterations in membrane phospholipids will most likely affect membrane fluidity.

1.2.2   SURFACTANT LIPID COMPOSITION

Pulmonary surfactant is a complex mixture of phospholipids, few other lipids and four genetically distinct surfactant-specific proteins (Wright, 1997). The overall lipid and phospholipid compositions of surfactants isolated from a number of experimentally used species (mouse, rat, rabbit, and sheep), of bovine surfactant, and of human surfactant are listed in Table 1.1. Surfactant composition is conserved over a range of animal species suggesting surfactant plays an important role in mammalian physiology. The most abundant phospholipid in all species listed including *Homo sapiens* is phosphatidylcholine (PC) which accounts for approximately 80% of the total phospholipids. Of this 80% almost half of the PC is in its disaturated form, DPPC. These levels of phospholipid are consistent with those reported by other authors (Ahuja *et al*, 1996; Barrow, 1990). Palmitic acid is usually esterified to PC. Thus, about 40 – 50% of the total phospholipid in mammalian surfactant is composed of DPPC.

Species	Phospholipid composition (% total *w/w*)						Reference
	PC	PG	PI	PS	PE	SM	
Mouse	72.3	18.1	-	-	1.9	3.3	(Langman *et al*, 1996)
Rat	82.3	7.5	1.8	0.1	5.1	0.8	(Daniels *et al*, 1995)
Rabbit	80.6	7.2	4.0	1.9	4.4	1.5	(Harwood, 1987)
Ovine	81.0	7.9	2.6	-	4.8	1.7	(Possmayer *et al*, 1984)
Bovine	79.2	11.3	1.8	-	3.5	-	(Yu *et al*, 1983)
Human	70.0	8.0	3.0	3.0	6.0	6.0	(Harwood, 1987)

Chol, cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

Table 1.1 – Lipid composition of lung surfactant in selected mammalian species.

The phospholipid content of pulmonary surfactant has been estimated to be between 100 and 300 µg / ml of alveolar lining fluid (Miles *et al*, 1999; Hayakawa *et al*, 1989). The definitive analysis of surfactant lipid concentrations in lung lavage fluid involves the use of a marker of dilution to correct for variable recovery of alveolar lining fluid. However, the question of the most appropriate dilutional marker remains unresolved. It has been demonstrated that urea, but not protein (albumin) is a valid dilutional marker with which to estimate alveolar lavage fluid recovery during small volume lung lavage (Dargaville *et al*, 1999). Subsequently, the amount of phospholipid in the alveolar lavage fluid preparations used in the study by Hayakawa *et al* 1989, was determined by estimating the amount of phospholipid phosphorous following perchloric acid digestion by a colorimetric assay. DPPC has been reported to be the major disaturated phospholipid in lung surfactant accounting for greater than 90% of all disaturated phospholipids in mammals (Daniels *et al*, 1998). The remaining PC in surfactant is composed primarily of molecular species containing monoenoic and dienoic fatty acids at the *sn*-2 position, with only minor amounts of short chains or polyunsaturated acyl groups (Veldhuizen *et al*, 1998). The acidic phospholipids, phosphatidyglycerol (PG) and phosphatidylinositol (PI), account for approximately 3 - 15% of the total surfactant phospholipid pool with most species. The remaining phospholipids appear in small amounts. In particular, phosphatidylserine (PS) is present in low concentrations and is not surprising in view of the known role of PS in triggering apoptosis when exposed on the outer leaflet of cells (Blankenberg *et al*, 1999; Fadok *et al*, 1998). Cholesterol is a lipid that can be isolated from lung surfactant but only accounts for approximately 4% of the total lipids within lung surfactant of adult humans (Harwood *et al*, 1975).

1.2.3   SURFACTANT PROTEIN COMPOSITION

The presence of two types of surfactant specific proteins were initially identified by King and co-workers in 1973 (King *et al*, 1973). Since then a number of studies have provided descriptions of the structure and functions of these proteins, the structure and sequences of the genes encoding them, their cellular origin, processing and regulation. Surfactant-associated proteins make up only 5 - 10% of total surfactant by weight (Notter and Finkelstein, 1984). To date there are only four surfactant proteins identified, each genetically different but may be classified into two groups. The classification of surfactant proteins is based on the fact that they are found in lavage fluid, synthesised by alveolar type II cells and specifically localised to the lung. The hydrophobic proteins are surfactant proteins B and C (SP-B and SP-C, respectively) and the hydrophilic surfactant proteins are designated surfactant proteins A and D (SP-A and SP-D, respectively). The surfactant proteins (except SP-C) are also synthesised by the clara cell of the airway (Wong *et al*, 1996).

The most abundant surfactant protein is SP-A. The SP-A monomer is a glycoprotein of approximately 32 kDa with three distinct structural domains. A long stretched collagenous domain is connected via a linking region to a globular region. This region contains a calcium-dependent carbohydrate recognition domain, which is able to bind both lipids and type II cells, as well as other structures such as micro-organisms. A complex oligosaccharide is also attached to this region of the SP-A molecule. These molecules are termed collectins because of their mixed collagen like and globular structure. The fully processed and secreted form of SP-A consists of 18 SP-A monomers (octadecamer or six trimers), organised by means of covalent disulphide

bridges and non-covalent interactions. In addition to its similarity to collagen structure, SP-A has a distinct structural homology to C1q, a member of the complement pathway. This homology with C1q prompted Tenner (Tenner *et al*, 1989) to investigate the ability of SP-A to activate macrophages to phagocytose opsonised sheep erythrocytes. In Tenner's study, SP-A was a potent activator. SP-A as well as SP-B has roles in the conversion of endogenous surfactant into tubular myelin. SP-A accelerates the adsorption of surfactant phospholipids at the air-water interface, stimulates the defence system which depends on macrophages, and possibly plays a role in the regulation of surfactant homeostasis (McIntosh *et al*, 1996). Experiments with mice carrying a null allele for SP-A (-/-) have confirmed and extended previous findings that SP-A is a molecule of the innate immune system (Lawson and Reid, 2000). However, the phospholipid composition, secretion and clearance, and incorporation of phospholipid precursors are normal in the SP-A (-/-) mice. SP-D, also a collectin, consists of 12 SP-D monomers (each monomer is approximately 43 kDa), three of which are joined to form a trimer. Four trimers form a cross-shaped molecule. This molecule is able to bind to LPS and to cell surfaces forming larger networks of cells or bacteria. The preceise role of SP-D in the innate immune system is, at present, uncertain. In contrast to SP-A, the phenotype of null SP-D mouse shares features of alveolar proteinosis disease along with an accumulation of activated macrophages and an increase in lamellar bodies (Botas *et al*, 1998).

The proteins most directly involved with pulmonary surfactant functions in terms of lowering surface tension at the air-water interface are SP-B and SP-C. The hydrophobic SP-B molecule is approximately 8 kDa in weight and is found mainly in dimer form,

with two SP-B monomers linked to each other via disulphide bonds. The main function of SP-B is to accelerate the formation of a surface-active film composed of phospholipids at the air water interface by means of increasing the adsorption rate. SP-C whose main function is to maintain the biophysical surface activity of surfactant lipids is 4 kDa in weight and has two palmitoyl groups attached by covalent bonding.

1.2.4 SURFACTANT METABOLISM

All components of surfactant are synthesised and secreted in the microsomal fraction of the alveolar type II pneumocytes (Wright, 1997; Chevalier and Collet, 1972). The surfactant components are synthesised in the endoplasmic reticulum, transported to the Golgi apparatus, and then packaged and stored in the lamellar inclusion bodies (Figure 1.7) (Massaro and Massaro, 1972). The lamellar body, which contains all the surfactant components except SP-D is secreted into the liquid hypophase that covers the alveoli. In the liquid lining, the secreted material may take the form of tubular myelin, a lattice like structure and a monomolecular film that is responsible for reducing surface tension within the lung. Surfactant is commonly separated by differential centrifugation into only two fractions, deemed heavy and light subtypes. The heavy sub-type fraction consists of tubular myelin aggregates and lamellar bodies while the light subtype fraction consists mainly of unilammellar vesicles (Magoon et al, 1983).

AIR

Monolayer

Surface-associated phase

Lipids

Removal

Degradation

Small unilamellar
aggregates

Tubular myelin

Large
aggregates

Reuptake

Alveolar
macrophage

Type I cell

Lamellar body

Type II cell

Synthesis

Capillary

Substrates
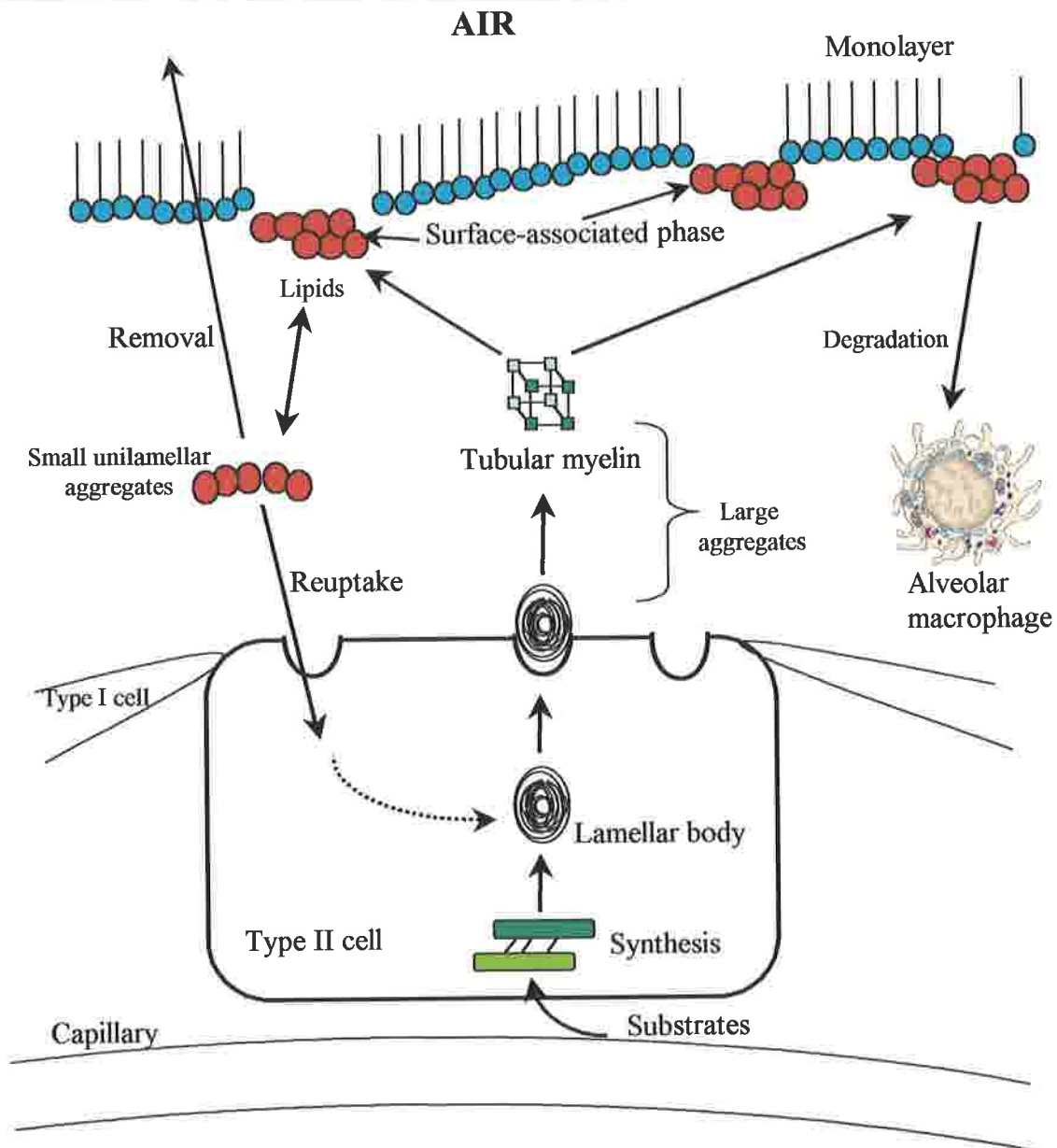
Figure 1.7 – Surfactant composition and 'life' cycle. Type II cells release lamellar
bodies, which are transformed into tubular myelin (large aggregates).
The tubular myelin provide the lipids which adsorb to the air-fluid
interface to form the surface film. With surface area expansion and
compression, small unilamellar vesicles (small aggregates) are generated
and re-enter the hypophase. Modified from Ikegami and Jobe, 1998.

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This thin film depends on the interactions of lipids and proteins in this environment (Williams and Benson, 1981). Eventually there is collapse of the film into the subphase. The majority of surfactant clearance appears to take place by uptake of surfactant by the type II alveolar epithelial cell, which both recycles and degrades followed by reutilization of surfactant components. A small proportion of surfactant is cleared by the alveolar macrophage and trace amounts of surfactant material seems to be cleared by the airways (Figure 1.7) (Wright, 1997). The alveolar type II cell is not a "traditional" immune cell, but several studies suggest that type II cells may have immunoregulatory functions (Simon and Paine, 1995). Since the type II cell is a major source of surfactant proteins and lipids, any factor that affects type II cells or their ability to secrete surfactant components may have an effect on the immune status of the lung.

1.2.5 THE EFFECT OF SURFACTANT LIPIDS ON INFLAMMATORY LEUKOCYTES

It is well established that surfactant plays a major role in preventing lung collapse at the end of expiration, however a role in the regulation of immune function is not well characterised. A number of studies have indicated that there is a functional relationship between pulmonary surfactant and the alveolar macrophage. There is limited information about the effects of natural surfactant phospholipids on human pulmonary monocytes and alveolar macrophages. The effect of surfactant on monocytes has not been addressed extensively but is relevant since monocytes are precursors of alveolar macrophages. It is becoming increasingly recognised that pulmonary surfactant or surfactant components may play a role in the modulation of inflammatory cell function.

Laforce and co-workers speculated that surfactant lipids might promote the bactericidal activity of alveolar macrophages (LaForce *et al*, 1973). In 1976, Juers provided evidence of alveolar lining material enhancing bactericidal killing which is specifically attributed to the lipid content (Juers *et al*, 1976). Subsequent studies have provided variable and conflicting reports on the bactericidal activity of surfactant and surfactant components, possibly due to differences in experimental designs. Numerous animal models (porcine, bovine, rabbit), different methodologies for measuring superoxide production and differing isolation techniques of phospholipids from surfactant or bronchoalveolar lavage fluid (BALF) preparations have been used, which may be responsible for conflicting reports.

Surfactant preparations obtained by BALF have been shown to exert suppressive effects on lymphocyte transformation to mitogen. This property was dose dependent and could be demonstrated in a number of mammalian species (Wilsher *et al*, 1988) and can be related to the presence of interstitial lung disease (Jones *et al*, 1991). Lung diseases such as ARDS and pulmonary alveolar proteinosis (PAP) possibly provide further evidence of the importance of surfactant in immunoregulatory function. Biochemical analysis of BALF from patients with PAP has revealed that the materials accumulated in the alveolar are mainly derived from lung surfactant (Hoffman *et al*, 1989). Incubating normal macrophages with PAP lavage material can induce inhibition of phagocytosis by alveolar macrophages (Milleron *et al*, 1991).

In addition Hayakawa *et al* (Hayakawa *et al*, 1992; Hayakawa *et al*, 1989), found natural surfactant or synthetic phospholipids inhibited the 'priming' of macrophages in a luminol enhanced chemiluminescence (LCL) system. However, Speer *et al*, 1991, found surfactant phospholipids without effect on a similar LCL system induced with PMA or opsonised zymosan in peripheral blood monocytes. Webb and Jeska, 1986 found an increase in the monocyte oxidative response when incubated with alveolar lining material and provided evidence that unsaturated lipids were responsible. The role of lipid components of the lung lining material evidently plays an immunomodulatory role. The precise inhibitory or stimulatory effect depends on the intricate interplay of many cells and chemical factors that may be difficult to model *in vitro*. The modification of phagocyte function by dietary supplementation with polyunsaturated fatty acids (PUFAs) has been demonstrated in several studies. The literature on the effect of dietary supplementation with ω-3 PUFA is more substantial than dietary supplementation with ω-6. Diet enhancement with fish oil (ω-3) did not result in modification of the neutrophil oxidative burst but did show profound alteration in neutrophil fatty acid composition (Guarini *et al*, 1998). This study indicates that the dietary alteration of membrane composition to modify the respiratory burst is not easily achieved.

There are also varieties of exogenous surfactants that are currently being investigated for potential clinical roles. Surfactant replacement therapy, common in neonates with respiratory distress syndrome is important to preserve lung function with respect to continual oxygenation of tissues. However, the role of exogenous surfactant in immunomodulation is not well understood.

1.2.6 The effect of surfactant proteins on inflammatory leukocytes

SP-A is the most abundant of the surfactant proteins and is involved in numerous aspects of surfactant spreading, secretion, metabolism and recycling (McIntosh et al, 1996). However, surfactant lipids spread and function adequately in the absence of SP-A and SP-D. Neither protein is included in the currently available surfactant replacement therapies. SP-A and SP-D affect the ability of the alveolar macrophages to perform host defence functions, such as phagocytosis (Tenner et al, 1989), generation of ROIs (Pasula et al, 1999), and production of cytokines such as TNF-α (Kremlev and Phelps, 1994). In addition a number of authors have provided evidence of the ability of SP-A and SP-D to promote host-defence functions (McIntosh et al, 1996; Weissbach et al, 1994). McIntosh et al, 1996, have shown that SP-A modulates the activity of LPS-activated alveolar macrophages, at least in part by decreasing TNF-α activity. However, the mechanisms by which this is accomplished are not well defined. Weissbach et al, 1994, have shown an increase in ROI production in response to zymosan coated with SP-A. These results are comparable with the complement component C1q, which has structural homology with SP-A. This suggests that SP-A could substitute for serum components in the pulmonary environment by acting as an opsonin. The immunomodulation of host defence by SP-A by reducing mediator production, thereby promoting host defence could potentially limit injury and promote normal healing. This would be of particular benefit in those individuals who suffer with ARDS or bronchopulmonary dysplasia (BPD) where both lung development and repair take place in the setting of significant lung inflammation. There is little evidence regarding the effects of SP-B and SP-C on immunomodulation within the lung. These

proteins are mainly involved with lowering surface tension at the air-water interface. While, SP-A and SP-D are integral components of the innate defence system of the lungs, the effects of these proteins appear to contrast those effects observed with surfactant lipids. Further, any requirement for SP-A or SP-D is even more uncertain, as neither of these proteins has been included in the formulation of any commercially available therapeutic surfactant.

1.2.7 OTHER FUNCTIONS OF SURFACTANT

Surfactant is not only found in the alveoli, but also in the bronchioles and small airways (Morgenroth and Bolz, 1985). Extra-alveolar surfactant may have a similar role to alveolar surfactant, stabilizing small noncartilagenous airways at low airway pressures. Thus, surfactant could prevent the collapse of small airways and air trapping.

Patency of airways can be reduced not only by collapse but also by accumulation of fluid. It has been shown that surfactant disperses fluid, eliminating fluid-caused obstruction in small rigid tubes (Liu *et al*, 1991). The presence of surfactant shifts fluid from the alveolus toward the interstitium, stabilizing the fluid balance in the alveolus, and preventing pulmonary oedema.

Surfactant may also affect airway function by changing mucus properties. Schlimmer *et al*, 1983, proposed that surfactant emulsifies mucus globules, preventing agglomeration and reducing adhesion. Mucus treated with surfactant obtained by BAL from pigs showed increased mucociliary transport when compared with mucus treated with saline (Ashbaugh *et al*, 1967). Mucociliary transport is not only determined by the properties

of mucus, but also by the movement of ciliated epithelium. It has been suggested that the sol phase in bronchial mucosa is regulated by surfactant and thereby influences ciliary motility (Morgenroth and Bolz, 1985).

Any condition characterised by mucus abnormality, mucociliary transport deficiency, airway obstruction or bronchoalveolar collapse could potentially benefit from exogenous surfactant therapy. These conditions would include asthma, pneumonia, cystic fibrosis, chronic bronchitis, mucus hypersecretion in acute quadriplegia, pneumoconiosis and chronic smoking (Brown and Pattishall, 1993).

1.3   MACROPHAGE FUNCTIONS

The alveolar macrophage provides the first resistance to immunological challenge within the lung. The ability of phagocytic cells to become activated in response to an immunological challenge is central to the innate response against bacterial invasion (Gebreselassie *et al*, 1996). Early experiments by Mackaness in the 1960s described their role in cellular immunity to intracellular parasites (Mackaness, 1970). The capacity of macrophages to destroy microbes and cellular debris has become well established. There is an increasing interest in the alveolar macrophage with respect to lung inflammation due to their production of inflammatory mediators and their bactericidal as well as phagocytic role. Bacterial endotoxin, (also known as lipopolysaccharide, LPS) interacts mainly with monocytes and macrophages (Pajkrt and Van Deventer, 1996) and is of great interest because of the role of LPS in sepsis. However, the role of the alveolar macrophage in lung inflammation is not well

characterised. This may be due to the difficulties of obtaining alveolar macrophages by BAL without causing a state of activation. The alveolar macrophage participates in innate immunity by recognising and removing non-self particles, in part using a variety of pattern-recognition receptors. One example of these receptors includes the mammalian Toll-like receptors (TLRs). These are expressed in various tissues including the lung and have the capacity to mediate cellular responses to microbial antigens such as LPS (Yang *et al*, 1999; Yoshimura *et al*, 1999; Zhang *et al*, 1999; Kirschning *et al*, 1998). Macrophages are versatile in their bactericidal mechanisms due to their phagocytic and extensive secretory capability. These cell types are known to have greater than 50 secretory products including cytokines, biologically active lipids, ROIs, complement components and free fatty acids (du Bois, 1985). All these mediators have a potential for antibacterial activity.

1.3.1   PHAGOCYTOSIS

The mucociliary action of the airways is of less importance in maintaining sterility of deep tissues compared with the ability of the macrophage to phagocytose. The removal of micro-organisms, particles and senescent cells occurs by receptor-mediated endocytosis or more commonly by phagocytosis. The alveolar macrophage population is capable of phagocytosing very large numbers of organisms. The mechanism of phagocytosis is a dynamic process in which bacteria are attached to the macrophage membrane in preparation for ingestion. Macrophages move toward micro-organisms down a density gradient of chemotactic molecules. The attachment of phagocytes to bacteria occurs by two broad mechanisms. Firstly, this may be directly with pili or adhesins. Secondly, indirectly by binding host components (Rest, 1995). This

attachment step is mediated by specific macrophage receptors and is dependent upon the nature of the bacterial surface. Many micro-organisms may activate the complement cascade and thus generate complement components. The production of opsonins through the complement cascade is the single most important factor in promoting phagocytosis by alveolar macrophages. Normally, surfactant protein A (SP-A) promotes binding, opsonisation and phagocytosis of bacteria and viruses by alveolar macrophages including *Staphylococcus aureus* (McNeely and Coonrod, 1993) and *Herpes simplex* virus (Van Iwaarden, 1991). Non-opsonic phagocytosis may also occur. The involvement of receptors in non-opsonic phagocytosis has been described and perhaps the best characterised is the mannosyl / fucosyl receptor (Lewis and O'D.McGee, 1992). Internalisation begins with pseudopodia surrounding regions of organic or inorganic particles recognised with opsonins e.g. immunoglobulin G (IgG) and complement, C3, C3b and C3bi (Fels and Cohn, 1986). Fusion of pseudopodia encloses the material within a membrane-bound structure called a phagosome, which then enters the endocytic-processing pathway. The phagosome fuses with a lysosome to form a phagolysosome (Hirsch, 1962). The contents of this structure are digested by the release of inflammatory mediators from the lysosome and the digest products are then removed by a process termed exocytosis (Figure 1.8). After the bacteria causing the lesion have been destroyed (or after the insult by heat, chemicals etc) there is usually reversal of the inflammatory changes. The vessel walls regain their normal permeability. Most of the emigrated neutrophils probably die (by apoptosis) and the fragments phagocytosed by macrophages. If the bacteria are not completely eliminated or the tissue injury continues, chronic inflammation can occur which is seen frequently in tuberculosis caused by *Mycobacterium tuberculosis*.

A

B
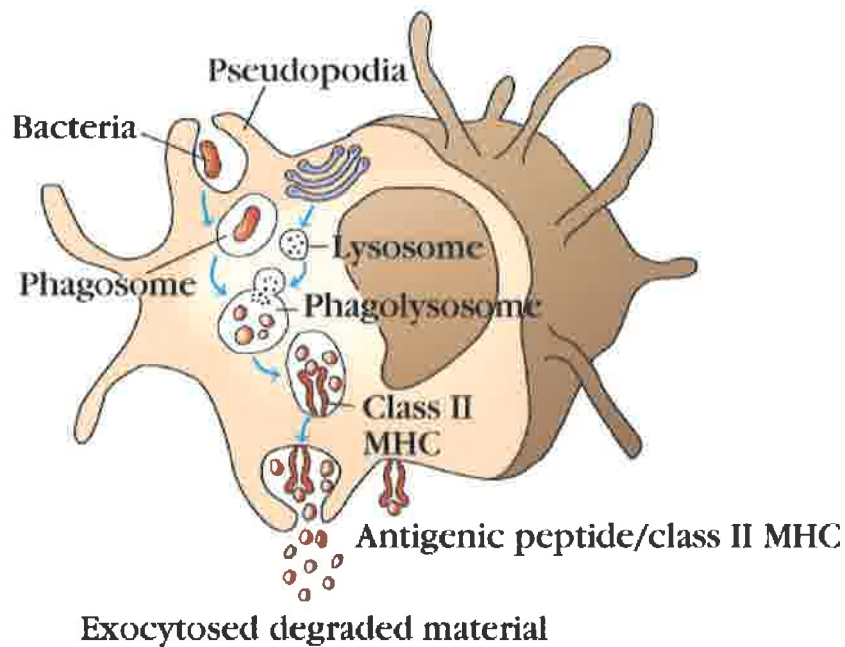
Figure 1.8 – The process of phagocytosis (reprinted with permission from Kuby.J., 1997). **(A)** Scanning electron micrograph of a macrophage making contact with bacterial cells, an early step in phagocytosis. **(B)** Phagocytosis and the processing of exogenous antigen by macrophages.

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1.3.2   ENZYME PRODUCTION AND SECRETION

A number of anti-microbial and cytotoxic substances produced by activated macrophages are responsible for the destruction of phagocytosed micro-organisms. These include a diverse range of enzymes that can be classified into three major groups (Fels and Cohn, 1986).

1. Lysosomal acid hydrolases

2. Neutral proteases

3. Lysozyme

All of these can be secreted into the extracellular environment, although the targets of many of these enzymes are intracellular. Lysozyme is a major secretory product of macrophages accounting for approximately 25% of all extracellularly released proteins (Osserman, 1975). This enzyme cleaves the $\beta1 - 4$ linked units of repeating disaccharides in bacterial cell walls. However, the efficacy of this enzyme as an antibacterial agent is uncertain as relatively few bacteria are susceptible to hydrolysis by lysozyme. Many Gram negative bacteria have LPS that protects the bacteria from the action of this enzyme, by blocking access to the repeating disaccharides (Janoff *et al*, 1979). Alveolar macrophages synthesise and secrete lysozyme constitutively and are hypothesised to be the chief source of this enzyme in both lung tissue and bronchial secretions (Cohn and Benson, 1965). The principal role of lysozyme in antibacterial defence is not well established. It may well be that it plays a digestive rather than anti-microbial role.

Plasminogen activator, elastase, collagenase, RNAse, DNAse and other enzymes are capable of lysing bacteria once they are delivered to the site of ingested bacteria upon phagosome – lysosome fusion. Although these enzymes are able to lyse bacteria and to digest bacterial cell walls, a direct role for these enzymes in antibacterial defence has not been established clearly (Nathan, 2000). Some neutral proteases play a central role in the degradation of connective tissue of the lung and these are postulated to be an important factor of certain chronic lung diseases such ARDS (Murakami *et al*, 2000; Janoff *et al*, 1979).

1.3.3   CYTOKINE PRODUCTION

Cytokines are regulatory proteins that control the survival, growth, differentiation and effector function of tissue cells. Cytokines encompass those families of regulators known as growth factors, colony stimulating factors, interleukins, monokines, lymphokines and interferon. Macrophages produce and secrete a variety of pro- and anti- inflammatory cytokines when activated or stimulated by other leukocyte products (Table 1.2). Some of these cytokines influence the ability of the host to respond to infections (Pascual *et al*, 1997). Interleukin-1 (IL-1) is an endogenous pyrogen capable of raising body temperature by inducing prostaglandin production. The increase in body temperature may be correlated with various modulatory aspects of the immune system including the acute phase response (Martin *et al*, 1999). For example IL-1 acts on T-helper (T_H) cells and provides a co-stimulatory signal for activation following antigen recognition. Further IL-1 may act on B-cells for enhanced proliferation and antibody production.

Cytokine	Stimuli for production	Biological action
GM-CSF	LPS, IL-1, TNF-α	Protection from bacterial and parasitic infections; enhances neutrophil functions; PGE$_2$, IL-1 and TNF-α induction
IFN-α	Viruses and bacteria	Antiviral, anti-mitotic; decreased c-*myc* expression
IL-1	Microbial products, TNF-α, GM-CSF, IL-2, antigen presentation	Induction of PGE$_2$ and IL-2, 4 and 6; fever; hypotension; acute phase protein response
IL-6	IL-1, TNF-α, PDGF	Proliferation of myeloma cell lines, haemopoietic cells; induces Ig production in B cells; fever
IL-10	LPS, TNF-α, Microbial products	Downregulates MHC class II expression; inhibits cytokine and radical production; inhibits macrophage adherence
M-CSF	LPS, IL-1	Macrophage colonies, antiviral, induces PGE$_2$, IL, TNF-α production
TNF-α	LPS, IL-2, IL-1, GM-CSF	Granulocyte colonies, terminal differentiation of myeloid cells, enhancement of neutrophil function

Note: The above are only a selection of the activities associated with individual cytokines.
CSF, colony-stimulating factor; G, granulocyte; IFN, interferon; Ig, Immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; M, macrophage; PDGF, platelet derived growth factor; PGE$_2$, prostaglandin E$_2$; TNF-α, tumour necrosis factor alpha.

Table 1.2 – Selection of cytokines secreted by macrophages.

A number of other important cytokines are secreted by activated macrophages. Tumour necrosis factor alpha (TNF-α) is a protein initially characterised by its ability to cause lysis of tumour cells (Old, 1985) and like IL-1 it is induced by LPS. It is a multifunctional cytokine, capable of influencing growth and differentiation and is known to 'prime' and activate phagocytes (Moore *et al*, 1991). TNF-α is one of the mediators of shock in Gram-negative bacterial septicaemia and its presence in the blood of patients with meningococcal septicaemia is associated with a fatal outcome (Hatherill *et al*, 2000; Wheeler and Bernard, 1999).

1.3.4   LIPID MEDIATOR PRODUCTION

When the inflammatory cascade is activated, the enzyme phospholipase A_2 (PLA_2) metabolises the membrane phospholipids of inflammatory cells to produce platelet activating factor (PAF) and arachidonic acid. When the macrophage is activated by an inflammatory stimulus, $25 - 40\%$ of its membrane lipid content is mobilised to produce arachidonic acid (Freeman and Lynn, 1980). Cyclo-oxygenases or 5' lipoxygenases are able to further metabolise arachidonic acid to produce a number of prostaglandins and leukotrienes, which have potent inflammatory effects (Bulger and Maier, 2000). Prostaglandins and other eicosanoids have potent local effects in the manifestation of inflammation. Prostaglandin E_2 (PGE_2) is important for its anti-inflammatory effects. It has been shown to regulate the cytokine response of macrophages (Fink, 1998). In addition to the important extracellular effects of lipid mediators during the inflammatory response, lipid molecules derived from the plasma membrane can also function as intracellular messengers effecting a variety of cellular responses (Jackson, 1997).

1.3.5   FREE RADICAL PRODUCTION

A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons. The presence of one or more unpaired electrons induces a magnetic moment which interacts in a magnetic field (a property termed paramagnetism), and makes the species highly reactive. This broad definition encompasses many biological and chemical species. For example, the oxygen we breathe is a paramagnetic diradical species. Radicals can be formed by the loss of a single electron from a non-radical, or by the gain of a single electron by a non-radical. If a single electron is added to the ground state oxygen molecule (O_2), then superoxide (O_2^-) is formed. The production of superoxide is associated with macrophage and monocytic phagocytosis. In addition to production of this free radical, there is an increase in oxygen consumption and glucose catabolism via the hexose monophosphate shunt (HMP). This dramatic change in oxidative metabolism is usually referred to as the respiratory burst (synonymous with oxidative burst) and is a very important mechanism of bacterial cell cytotoxicity due to the production of superoxide and its subsequent ROIs (see section 1.4).

Nitric oxide (NO·) is a short-lived free radical gas generated by lightning and pollution but is also secreted by mammalian cells (Nathan and Xie, 1994). In living organisms, NO· is produced from L-arginine by oxidation of a guanido nitrogen, with L-citrulline as a coproduct. The reaction is catalysed by NO synthases (NOS). One very important isoenzyme is inducible NOS (iNOS). Macrophage NO· inhibits the mitochondrial respiration of tumour target cells and pathogenic fungal organisms and is therefore thought to play an important role in host defence (Morris and Billiar, 1994). Murine

macrophages are capable of producing large amounts of NO· under the proper inflammatory conditions and are one of the cells in which the inducible NO synthase (iNOS, NOS-2) was first described (Nathan, 1992). Alveolar macrophages can be induced to produce NO·via iNOS. Inducible NOS is a cytoplasmic enzyme that serves as a primary defence against pathogens that have invaded the intracellular environment of many somatic cells, including cells not specialised for host defence. Some investigators for example have demonstrated that rat alveolar macrophages produce NO· in response to lipopolysaccharide (LPS) and / or IFN-γ (Lorsbach *et al*, 1993). NO·and superoxide produced by activated alveolar macrophages can react with each other to form the strong oxidant, peroxynitrite (ONOO⁻). However, it is controversial whether or not human neutrophils produce NO; and as a consequence peroxynitrite.

1.4 THE RESPIRATORY BURST

In 1933 Baldridge and Gerard made the discovery of the respiratory burst, a term used for the collection of biochemical changes that occur during phagocytic activation (Baldridge and Gerard, 1933). This chain of biochemical and cytophysiological events occurs at the site of inflammation where the most effective stimulants are present e.g. bacteria and complement components, C5a. The respiratory burst can be induced by a variety of stimuli including membrane-perturbing agents such as phorbol myristate acetate (PMA), high concentrations of fluoride ion, certain small peptides (N-formylmethionylleucylphenylalanine and concanavalin (a lectin) (Liles *et al*, 1995; DeChatelet *et al*, 1976). Hence, activation of the respiratory burst does not require the act of phagocytosis itself. The association of bacterial engulfment with the enormous

consumption of oxygen by activated neutrophils was described in detail in 1956 (Stähelin *et al*, 1956). The respiratory burst was associated with increased glucose catabolism via the hexose monophosphate shunt (HMP), and found to be independent of mitochondrial electron transport as demonstrated with the use of azide and cyanide inhibitors. Soon after, the association of bacterial killing with the products of the respiratory burst, superoxide, hydrogen peroxide and other ROIs was made (Andre *et al*, 1988; Channon *et al*, 1987). The enzyme responsible for the one electron reduction of oxygen to superoxide is a unique complex to granulocytes and other phagocytic cells and is termed nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The respiratory burst continues throughout the process of phagocytosis and the oxidants formed are released extracellularly and reduced to water by host detoxification systems such as SOD and catalase. The release of these oxidants has been correlated with an enhanced ability to kill bacteria (Robinson *et al*, 1984), parasites and tumour cells (Tan *et al*, 1991; Gridley *et al*, 1991).

Oxidative metabolism is highly variable, depending on the cell type, the organ or compartment from which the cells are obtained, the animal species, the type of stimulant and the activation state of the cell. The alveolar macrophages only release trace quantities of oxygen metabolites during phagocytosis (Fels and Cohn, 1986). The magnitude of respiratory burst decreases markedly when monocytes mature into alveolar macrophages (Lewis and O'D.McGee, 1992). This may in part be due to the lipid environment in which the monocyte has entered. Apart from the role of host defence, extracellular releases of excess amounts of ROIs may have an untoward side effect of promoting tissue damage in the host.

1.4.1 GENERATION OF ROIs

ROIs can be generated from chemical modifications of superoxide by myeloperoxidase (MPO) -dependent and independent mechanisms. Macrophages as opposed to polymorphonuclear cells and peripheral blood monocytes have very low levels of MPO and therefore rely upon MPO -independent mechanisms. The MPO -dependent pathway is employed by both polymorphonuclear cells and monocytes (Kettle and Winterbourn, 1997; Albrecht and Jungi, 1993).

Normally, superoxide (O_2^-) is short lived and undergoes chemical modifications either by superoxide dismutase (SOD) or spontaneously at physiological pH to form hydrogen peroxide (H_2O_2) (Equation 1.1) (Jones, 1994). In the MPO -independent pathway, the highly reactive oxygen intermediates, the hydroxyl radical (OH) and singlet oxygen ($^1O_2$) are produced. The hydroxyl radical is the most reactive intermediate and is formed by combination of superoxide with hydrogen peroxide, via the metal cation-catalysed Haber-Weiss reaction (Equations 1.2 – 1.4).

$$O_2^- \ + \ O_2^- \ + \ 2H^+ \longrightarrow H_2O_2 \ + \ O_2 \qquad \textbf{(Equation 1.1)}$$

$$Fe^{2+} \ + \ H_2O_2 \longrightarrow Fe^{3+} \ + \ OH\cdot \ + \ OH^- \qquad \textbf{(Equation 1.2)}$$

$$Fe^{3+} \ + \ O_2^- \longrightarrow Fe^{2+} \ + \ O_2 \qquad \textbf{(Equation 1.3)}$$

$$\textbf{Net:} \quad O_2^- \ + \ H_2O_2 \xrightarrow{\text{metal catalyst}} OH\cdot \ + \ OH^- \ + \ O_2 \qquad \textbf{(Equation 1.4)}$$
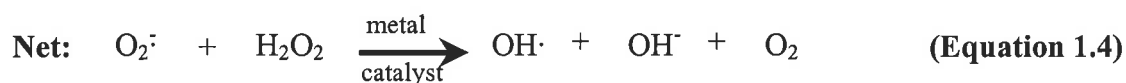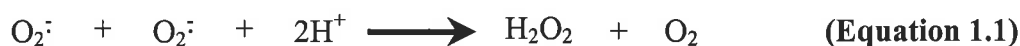
In the MPO –dependent system, hypochlorous acid (HOCl) believed to be the strongest oxidant produced by neutrophils in appreciable amounts is formed from the enzyme catalysed reaction of chloride with hydrogen peroxide (Kettle and Winterbourn, 1997). As shown in Equation 1.1, superoxide dismutates to form hydrogen peroxide, which then serves as a substrate for MPO, a haem enzyme that produces oxidised halogens. The initial step is haloperoxidation to produce the hypochlorite anion (OCl$^-$) (Equation 1.5).

$$H_2O_2 \ + \ Cl^- \longrightarrow H_2O \ + \ OCl^- \qquad \textbf{(Equation 1.5)}$$

An appreciable amount of hypochlorous is released into the extracellular space. In this pH environment, you can get hypochlorous acid due to protonation. The products of halide oxidation can react with additional peroxide in the following reaction (Equation 1.6) from which singlet oxygen ($^1O_2$) is derived.

$$OCl^- \ + \ H_2O_2 \longrightarrow Cl^- \ + \ H_2O \ + \ {}^1O_2 \qquad \textbf{(Equation 1.6)}$$
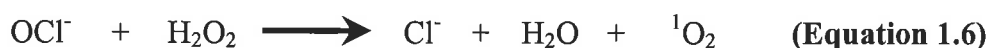
Iodide or bromide can substitute for chloride in these reactions. However, chloride is the favoured reactant because of its presence within the phagolysosome. Most of the hypochlorite or chlorine is consumed on various biologic substrates, immediately after production. Hypochlorite is probably highly bactericidal because of its oxidising capacity, particularly it ability to react with low molecular weight amines to yield

chloroamines. Despite this bactericidal potential, patients with MPO deficiency do not appear to be at increased risk for serious infections.

1.4.2   BIOLOGIC EFFECTS OF THE RESPIRATORY BURST

There are varieties of targets for oxidant attack including unicellular organisms, parasites (toxoplasma) and fungi. Small bacteria are engulfed and attacked by oxidants in the phagosome, however fungal hypha are too large to be ingested. It has been estimated that stimulated phagocytes produce somewhere in the order of $0.01 - 10$ nM of superoxide / minute / mg cells (Barber $et\ al$, 1995) or 1.03 nmol / 10^7 leukocytes per 15 minutes (Babior $et\ al$, 1973). Superoxide is relatively benign and hydrogen peroxide (H_2O_2) tends to be bactericidal at high concentrations (DeLeo $et\ al$, 1998). Further, hydrogen peroxide has the capability to transverse the plasma and nuclear membranes, thereby contributing to DNA adduct formation. It is also possible that hydrogen peroxide can inactivate a few enzymes by oxidation of essential thiol groups. One example of this is the enzyme glyceraldehyde-3-phosphate dehydrogenase, an enzyme of the glycolytic pathway. As discussed in section 1.4.1, superoxide and hydrogen peroxide are precursors for the more toxic ROIs of which several forms exist.

During phagocytosis, little oxidants escape the phagosome. However, if the production of ROIs exceeds the capacity of tissues to catabolise them, then the ROIs may become harmful leading to disease and possibly death. Fortunately, the body is well equipped with detoxification systems (see section 1.4.3). The production of ROIs can damage nucleic acids and inactivate enzymes by oxidation of their thiol groups (Nathan $et\ al$, 1980). Most importantly, ROIs can cause lipid peroxidation by attacking

polyunsaturated fatty acids within the membrane bilayer, thereby initiating lipid peroxidative chain reactions. Lipid peroxidation may be broadly defined as the oxidative deterioration of polyunsaturated lipids. Hydroxyl radicals are capable of extracting a hydrogen atom from a methylene (CH_2) group whereas superoxide is not. In addition, it is unlikely for superoxide to enter the cell due to its charged nature. Removal of a hydrogen atom results in an unpaired electron on the carbon. The carbon rearranges itself to form a conjugated diene, which can undergo various reactions. One of which is to combine with oxygen, which gives rises to a peroxy radical ($ROO\cdot$). These radicals are capable of abstracting hydrogen from another lipid molecule hence continue the chain reaction of lipid peroxidation. A consequence of lipid peroxidation is the alteration of membrane fluidity. The Singer-Nicholson fluid mosaic concept proposes that the lipid bilayer functions as a neutral two-dimensional solvent, having little influence on membrane protein function. However, it is becoming increasingly clear that lipids exist in several phases in model lipid bilayers, including gel, liquid-ordered and liquid-disordered states, in order of increasing fluidity (see section 1.5.1). Polyunsaturated fatty acids are essential for normal cell membrane functioning because many membrane properties, such as fluidity and permeability, are closely related to the presence of unsaturated and polyunsaturated side chains. Lipid peroxidation results in loss of membrane polyunsaturated fatty acids and oxidized phospholipids as polar species contributing to increased membrane rigidity. This may alter recruitment of membrane proteins such as the glycosylphosphatidylinositol (GPI)-linked CD14 receptor (Bruch and Thayer, 1983) to a new micro-envrionment, where the phosphorylation state can be modified by local kinases.

Oxidant damage to the host is best documented in the lung. Phagocyte generated oxidants have been implicated in pathogenesis of both acute and chronic disease. In acute lung disease, pulmonary oedema in ARDS and shock lung may occur. One explanation for this is the release of C5a that may cause neutrophils to aggregate and produce superoxide, which is trapped in the lung and consequently destroys capillaries and permits extrusion to plasma. In chronic lung conditions, neutrophils can accumulate especially when subjected to chronic irritation. This follows the release of elastase that may digest fibres of lung (Yuan *et al*, 2000). The existences of natural elastase inhibitors provide a protective measure but the production of hypochlorous acid inhibits these elastase inhibitors and hence digestion of the fibres may occur.

1.4.3 HOST DETOXIFICATION OF ROIs

Hydrogen peroxide is damaging in living organisms because it can give rise to the formation of hydroxyl radicals. It is therefore biologically imperative to control the amount of hydrogen peroxide produced. Two types of enzymes exist that can remove hydrogen peroxide from inside the cells. These include the catalases and the peroxidases. This enzyme can be located in the peroxisomes of the liver and kidney and to a lesser extent in microperoxisomes. This enzyme typically consists of four sub-units, each of which contains a haem group bound to its active site. Each subunit contains one molecule of NADPH bound to it, which helps to stabilise the enzyme. Glutathione peroxidase, discovered in animal tissue in 1957, can also be found in plants and some bacteria. This enzyme is found in high activity in the lung (Chiu *et al*, 1976) and its substrate is the low molecular weight thiol compound glutathione. Hydrogen peroxide undergoes two electron reductions to water by catalase and glutathione

peroxidase thereby bypassing the formation of the hydroxyl radical. Electrons from glutathione are shuttled to hydrogen peroxide, yielding water. Glutathione is then regenerated by glutathione reductase using electrons from NADPH.

Superoxide is a precursor of hydrogen peroxide, therefore superoxide dismutase (SOD) is an important enzyme in host detoxification. Several forms of SOD exist. In neutrophils, the highly stable copper-zinc superoxide dismutase enzymes (CuZnSODs) may be found in the cytosol and maganese-SOD in the mitochondria, but these enzymes cannot offer protection against externally-generated superoxide. The SOD enzymes can also react with singlet oxygen and hydroxyl radicals because they often contain tryptophan and methionine residues, which react with this species.

Besides enzyme detoxification, host protection mechanisms against oxidant damage include the biological activity of some small molecules. Ascorbic acid (vitamin C) or ascorbate can act as a reducing agent. Ascorbate reacts rapidly with superoxide and even more rapidly with hydroxyl radical to give semihydroascorbate which can be further oxidised to dehydroascorbate which is eventually broken down to oxalic and L-threonic acids (Halliwell, 1999). Ascorbate also scavenges singlet oxygen, and combines quickly with hypochlorous acid. Hence ascorbate may well help to protect against oxygen derived species. Vitamin E or (α-tocopherol) is a fat-soluble molecule that tends to concentrate in the interior of membranes. This molecule quenches and reacts with singlet oxygen and could therefore protect the membrane against this species. Vitamin E is also oxidised by superoxide generating systems and will react with hydroxyl radicals. However, tocopherols major antioxidant role in biological

membranes under most conditions is to react with lipid peroxy and alkoxy radicals, donating labile hydrogen to them and so terminating the chain reaction of peroxidation by scavenging chain-propagating radicals. Other protection mechanisms include sequestration of metal ions and the ability to repair damage due to radicals, for example, peroxidised lipids may be excised out of membranes (Saran and Bors, 1990). Thus protection against ROI toxicity is a very important mechanism that operates in conjunction with many enzymes, proteins and molecules.

1.4.4 NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADPH) OXIDASE

The enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the enzyme responsible for the transport of electrons across the plasma membrane to form superoxide in the interior of the phagocytic vacuole (Segal, 1996; Holmes *et al*, 1967). This multi-component enzyme catalyses the reaction between oxygen and NADPH (Figure 1.9). Oxygen gains an electron in a process termed oxidation, while NADPH is reduced to $NADP^+$ as it loses an electron. Due to an increase in the rate of formation of $NADP^+$, there is an increase in glucose catabolism via the hexose monophosphate shunt (HMP). The limiting factor of HMP metabolism is the availability of $NADP^+$. The purpose of increased oxidation of glucose through the HMP is to regenerate NADPH. This is achieved in the cytosol or via NADPH linked hydrogenases in the mitochondria. For each glucose molecule metabolised in the HMP, two molecules of $NADP^+$ are reduced to NADPH (Babior, 1984). $NADP^+$ production increases during the respiratory burst through the actions of NADPH oxidase and the glutathione-dependent system, using NADPH to detoxify hydrogen peroxide.
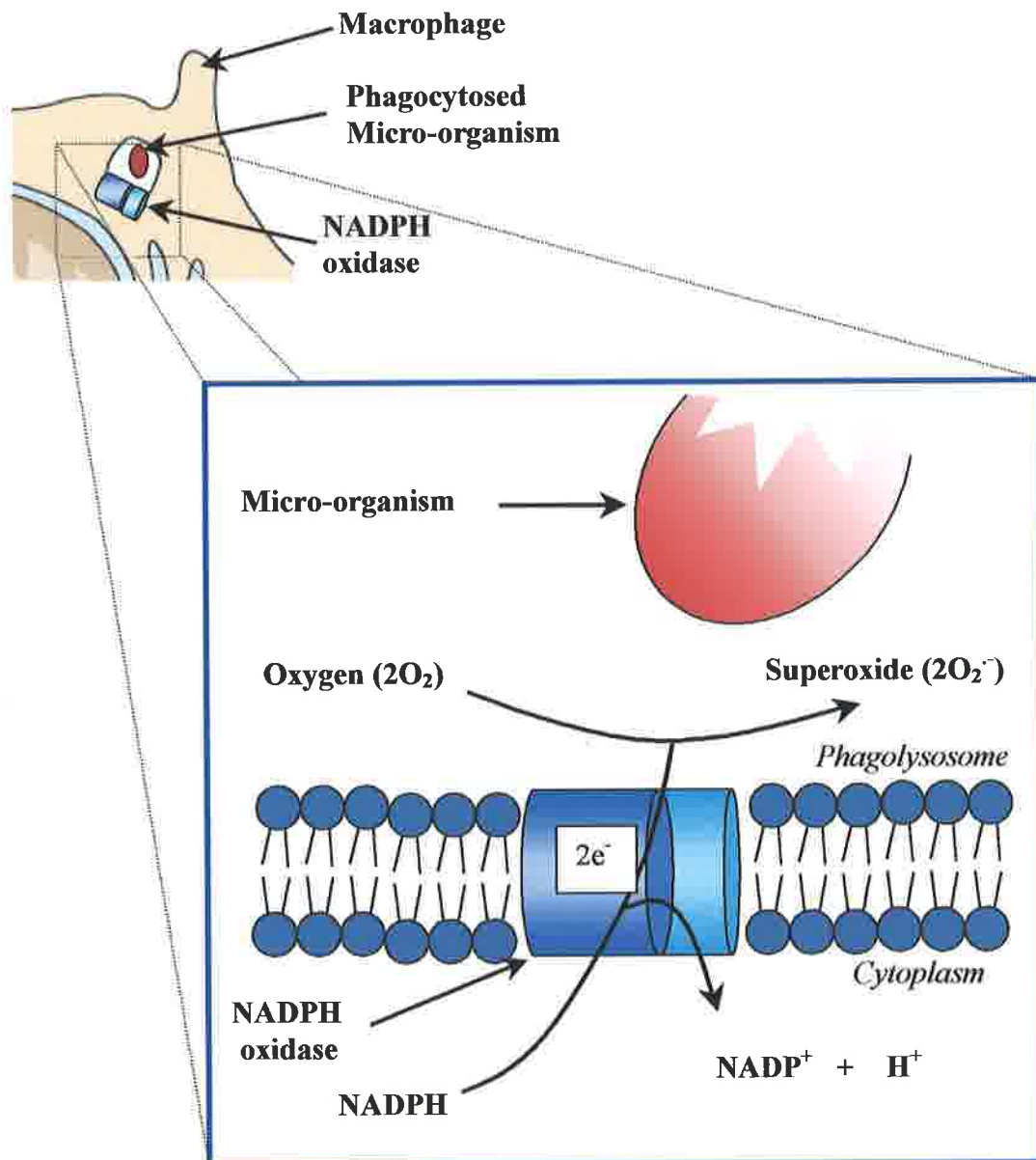
Figure 1.9 – NADPH oxidase is activated upon phagocytosis of bacteria. This enzyme is responsible for the transport of electrons across the plasma membrane and the subsequent production of ROIs.

The NADPH oxidase is a multicomponent system and the state of activation depends on the nature, composition and distribution of the individual components of the oxidase. NADPH oxidase becomes activated through a series of phosphorylation events of one or more components. This occurs as a result of receptor dependent and independent mechanisms. The inactive NADPH oxidase is segregated into membrane and cytoplasmic locations. During activation the cytoplasmic components, p47^{phox}, p67^{phox}, p40^{phox} and Rac2 must translocate to the membrane where they combine with gp91^{phox} and p22^{phox}. These latter two components comprise flavocytochrome b_{558}, a short electron transport chain capable of transporting electrons across the membrane (Clark *et al*, 1990; Royer-Pokora *et al*, 1986). This process is highly regulated and is becoming better understood. It is important that this complex is only active when the micro-organism is engulfed and that the process occurs within the phagolysosomal vacuole as the ROIs produced are potentially toxic to the host (see section 1.4.2).

Chronic granulomatous disease (CGD) has led to a greater understanding of the respiratory burst. Patients with CGD have a defect in the NADPH oxidase complex leading to a predisposition to frequent and often severe infections, caused by what are common pyogenic organisms such as *Staphylococcus aureus* and *Serratia marscescens* (Odell and Segal, 1991). Neutrophils from these patients are incapable of killing bacteria and thus demonstrate the pivotal role of NADPH oxidase in maintaining the sterility of tissues protected by inflammatory leukocytes.

1.4.5   FLAVOCYTOCHROME B

The important membrane bound component is flavocytochrome *b*. Flavocytochrome *b* has the lowest midpoint potential, -245mV, of any cytochrome in mammalian cells and is also referred as flavocytochrome b$_{-245}$ (Jones, 1994; Abo *et al*, 1992). The oxidation-reduction midpoint potential of flavocytochrome b$_{-245}$ permits it to participate efficiently in the oxidation of molecular oxygen to superoxide. The flavocytochrome may also be denoted by b$_{558}$ for it's α-band absorption maximum of 558-559 nm (Segal and Jones, 1978). Flavocytochrome *b* is a heterodimeric transmembrane protein identified by Allisons group (Segal *et al*, 1978). It is composed of two sub units or *phox* proteins (phagocyte oxidase) in a 1:1 ratio; a small sub unit, molecular weight of 22 kDa (p22^{phox}) and a larger 91 kDa (gp91^{phox}) sub unit. The latter protein is heavily glycosylated (denoted by g) and contains binding sites for both flavin adenine dinucleotide (FAD) and NADPH (Segal *et al*, 1992). FAD is a transducer molecule that accepts electrons for transduction to 2 haems attached to one or both subunits within the cytochrome *b* (Segal, 1996). In resting neutrophils, flavocytochrome *b* is located in specific granules (Babior, 1992) and in the plasma membrane (approximately 90% and 10% respectively). When the neutrophil is activated the flavocytochrome *b* component is translocated and fused with the phagolysosome or plasma membrane (Chanock *et al*, 1994). In addition to the translocation of flavocytochrome *b* the cytoplasmic components also translocate to the plasma membrane (Royer-Pokora *et al*, 1986). Flavocytochrome *b* appears to be the central component of the NADPH oxidase, containing the components for electron transport across the membrane. It is likely that the other components of this oxidase are required for regulating the enzyme (DeLeo and Quinn, 1996; Jones, 1994).

1.4.6 CYTOPLASMIC COMPONENTS

Generation of superoxide by the NADPH oxidase requires the involvement of a number of cytoplasmic proteins that attach to the flavocytochrome at the plasma membrane (Figure 1.10). These cytoplasmic components include p47^{phox} (Segal *et al*, 1985), p67^{phox} (Leto *et al*, 1990) and p40^{phox} (Wientjes *et al*, 1993). These proteins have an apparent molecular mass of 47 kDa, 67 kDa and 40 kDa respectively. The phosphorylation of p47^{phox} possibly results in a conformational change of this protein leading to the exposure of a Src homology 3 domain (SH3). This protein translocates with p67^{phox} and p40^{phox} as a 240 kDa complex in equimolar amounts (Park *et al*, 1992). Association of this complex with the flavocytochrome *b* may occur via the SH3 domains which interact with proline rich residues on other proteins (Segal *et al*, 1985). A proline rich domain has been identified on p22^{phox} (Parkos *et al*, 1988). On activation, this entire complex as well as a free form of p47^{phox} translocates to flavocytochrome *b* where p47^{phox} is the first protein to interact with flavocytochrome *b* (Cross and Curnutte, 1995). This latter process is required for the translocation and binding of p67^{phox}.
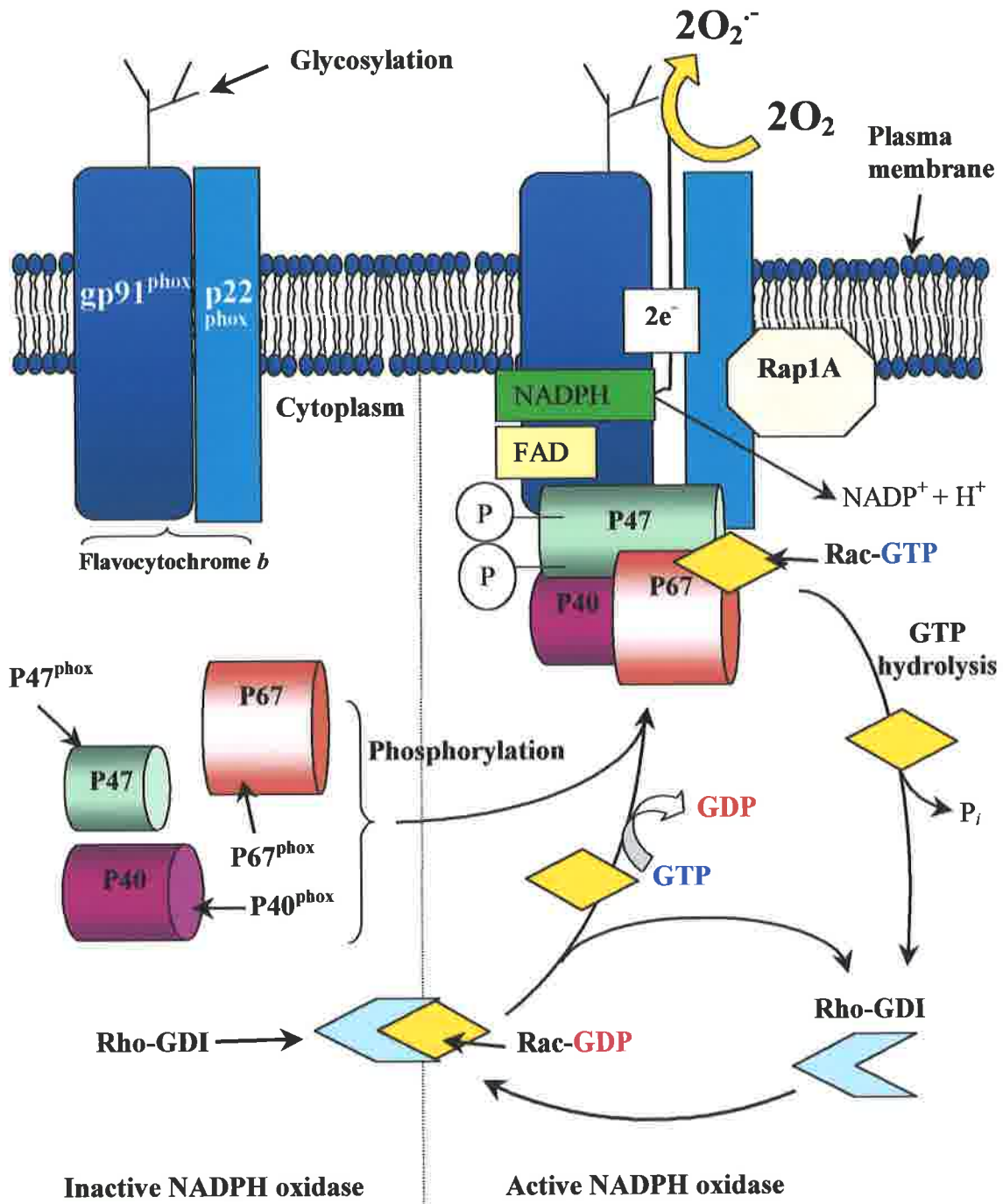
Figure 1.10 – Schematic diagram illustrating the interaction of the cytoplasmic components of the NADPH oxidase with the membrane bound flavocytochrome *b*. Activation requires dissociation of GTP dissociation inhibitor (GDI) from Rac 2 (Rac-GDP) followed by exchange of guanine diphosphate (GDP) for guanine triphosphate (GTP). Activation also requires phosphorylation of at least one unit (p47^{phox}). Hydrolysis of GTP from Rac leads to inactivation.

Abo and Pick identified a heterodimeric protein complex in guinea pig macrophages that is essential for optimal superoxide production (Abo and Pick, 1991). The two proteins in this complex were identified as Rac, a 22 kDa low molecular weight mass guanosine triphosphate (GTP) binding protein and a guanosine diphosphate (GDP) dissociation inhibition factor, rho GDI. This latter protein is thought to keep Rac in a GDP bound soluble form in resting neutrophils (Abo *et al*, 1991). Simultaneously, Rac 2, homologous with Rac, was identified in the cytosol of human neutrophils (Knaus *et al*, 1991). Rac 2 and GDI are held together via a lipid tail on Rac 2 that binds to the hydrophobic region of GDI. Following activation of the neutrophil, Rac 2 dissociates from the GDI and translocates to the membrane as Rac 2-GTP, independently of cytoplasmic factors (Dorseuil *et al*, 1995). It is likely that Rac 2 serves to modulate the functions of one or more of the proteins of the NADPH oxidase. The hydrolysis of GTP from Rac 2 leads to inactivation of the NADPH oxidase. Another low molecular weight GTP-binding protein associated with the NADPH oxidase is Rap1A (Babior, 1992). This protein is physically associated with flavocytochrome *b*. Certain Rap1A mutants inhibit superoxide production in EBV-transformed B lymphocytes (Maly *et al*, 1994), suggesting a role for Rap1A in NAPDH oxidase activation.

In summary, the essential elements for electron transport by the respiratory burst oxidase are $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the two subunits of the flavocytochrome b, all assembled into an oligomer that contains these components in equimolar amounts. Assembly is assisted and regulated by Rac and Rap1A. The formation of the oligomer complex allows an NADPH binding component to transfer electrons from NADPH to

FAD and then to oxygen, using the haem of the flavocytochrome as an intermediate electron carrier.

1.4.7   ASSAYS OF NADPH OXIDASE ACTIVITY

The activity of NADPH oxidase can be assayed utilising numerous techniques that qualify or quantify ROIs produced as a result of the chemical modifications of oxygen. Colorimetric assays include the superoxide dismutase-inhibitable rate of cytochrome c reduction in which superoxide released from cells reacts with cytochrome c (Bhatnagar *et al*, 1981). This method requires the interaction of cytochrome c with superoxide before it dismutates to hydrogen peroxide. Therefore, the concentration of cytochrome c must be kept high as well as the addition of catalase to overcome hydrogen peroxide interference (Jones and Hancock, 1994). Alternatively the nitro blue tetrazolium (NBT) slide test (Vowells *et al*, 1995) detects the reduction of this yellow dye by superoxide to form a diformazan (blue / black) precipitate on and within cells. The disadvantages of this assay include loss of non-adherent cells, and although sensitivity is comparable with cytochrome c reduction, it is not as sensitive as other currently available methods (Webb and Jeska, 1986).

Several reagents are available which react with the products of the NADPH oxidase to provide a fluorometric measurement. The use of the fluorescent probe scopolectin provides a quick and easy measurement of hydrogen peroxide. However, the reaction system must be free of phenol red, which is commonly present in cell culture media. Further, the oxidation of scopolectin may be inhibited by some contaminants of cell preparation. One of the most common and increasingly used fluorescent techniques to

measure some components of the respiratory burst is flow cytometry (Carulli, 1998). Measurement of superoxide and hydrogen peroxide by flow cytometry is a rapid and sensitive technique and is mainly used to assess intracellular production of oxygen radicals, whereas the extracellular production is measured by other assays, particularly chemiluminescence.

Measuring chemiluminescence resulting from the production of reactive oxygen intermediates is an easy, extremely sensitive and cost effective method for determining NADPH oxidase activity (Allen, 1986). Much of the current interest in chemiluminescence arises from the many advantages of analysis based on this technology (see section 1.8).

1.5   SIGNAL TRANSDUCTION MECHANISMS INVOLVED IN NADPH OXIDASE ACTIVATION

Phagocyte activation may be defined as the enhanced capability of these cells to disable or destroy a variety of intracellular pathogens and cellular targets. The release of ROIs is an example of one such bactericidal mechanism. As previously introduced (section 1.4.4), the enzyme responsible for the production of ROIs is the membrane bound NADPH oxidase. The NADPH oxidase complex within cells of myeloid lineage and other leukocytes is dormant until cells are activated; at which time the NADPH oxidase translocates and associates with the plasma membrane. The activation of NADPH oxidase may involve many different signalling mechanisms. These mechanisms depend on the agonist and the signalling pathways that lead to the assembly of the various components of the NADPH oxidase. Due to the complexities of the signalling pathways triggered in the intact cell and the probability that redundant pathways exists, it has not been possible to completely define the pathways leading to NADPH oxidase activation. Phagocytes respond to a number of stimulants including particle (zymosan) and lipid soluble activators (phorbol esters, PMA) that leads to the activation of NADPH oxidase. However, the mechanism of NADPH oxidase activation is not completely elucidated. The signal transduction events leading to NADPH oxidase activation can be categorized into (1) receptors and ligands, (2) signal molecules and (3) transcription factors.

1.5.1   CELL MEMBRANE, LIPID RAFTS AND CELL SURFACE RECEPTORS

The generation of cellular signals begins with an initial interaction between a ligand and receptor. Indeed, ligand-mediated clustering of cellular receptors is often the first step in the mechanisms of numerous signal transduction pathways. Macrophages bear around 50 ligand receptors (Adams and Hamilton, 1984). These receptors have traditionally been grouped according to structural and functional characteristics. Traditionally it is the opsonic receptors, including FcγR receptors and complement receptors that have been studied most intensively (Allen and Aderem, 1996). Ultimately the aim of all classes of receptors is to activate genes involved in a cell's response to stimuli. The agonist-receptor interaction is the first event in signal transduction, and the complex must persist to sustain a response.

It is now becoming clear that lipid-microenvironments on the cell surface, known as lipid rafts, also take part in the signalling processes. The lipid raft hypothesis, formulated more than ten years ago, were thought to consist of dynamic assemblies of cholesterol and sphingolipids in the exoplasmic leaflet of the bilayer. Most biological phospholipids have low acyl chain melting points (T_m) and because of this property, cellular membranes are generally thought to exist in a fluid, liquid crystalline phase (L_c). The preponderance of saturated hydrocarbon chains in cell sphingolipids allows for cholesterol to be tightly intercalated. The inner leaflet is probably rich in phospholipids with saturated fatty acids and cholesterol but its characterization is still incomplete. The membrane surrounding lipid rafts is more fluid, as it consists mostly of phospholipids with unsaturated, and therefore kinked, fatty acyl chains and cholesterol.

In other words, lipid rafts form distinct liquid-ordered phases in the lipid bilayer, dispersed in a liquid-disordered matrix of unsaturated glycerolipids.

One of the most important properties of lipid rafts is that they contain a given set of proteins that can change their size and composition in response to stimuli. This favours the specific protein-protein interactions, resulting in the activation of signalling cascades. The lipid rafts recruit proteins to a microenvironment, where the phosphorylation state can be modified by local kinases and phosphatases, resulting in down stream signalling. In the case of tyrosine kinase signalling, adaptors, scaffolds and enzymes are recruited to the cytoplasmic side of the plasma membrane as a result of ligand activation (Hunter, 2000). In addition lipid rafts may also exclude non-raft-associated enzymes such as membrane phosphatases that could affect the signalling process. Proteins with raft affinity include glycosylphosphatidylinositol (GPI) – anchored proteins such as CD14, doubly acylated proteins such as Src-family kinases or the α subunits of heterotrimeric G proteins. A striking feature of GPI-anchored proteins is that their ligation on the cell surface by suitable antibodies results in signal transduction that is characterized by transient elevation of calcium; tyrosine phosphorylation of cellular substrates and initiation of effector function such as the oxidative burst. Such signalling is surprising considering that these proteins lack a cytoplasmic domain and thus no direct contact with the interior of the cells (Horejsi *et al*, 1999).

Lipids may play a role in signal transduction process by maintaining association between GPI-anchored protein and intracellular tyrosine kinases (Benson, 1993). These GPI anchored protein complexes exhibit a particular lipid composition enriched in cholesterol and sphingolipids. Stulnig *et al*, 1997, demonstrated that inhibiting cholesterol synthesis suppressed the calcium response via GPI anchored proteins. This suggests that cellular cholesterol is an important prerequisite for signal transduction via CD14 in the LPS model. The raft concept has been controversial, largely because it has been difficult to prove definitively that rafts exists in living cells. One reason why it has been so difficult to prove that rafts exists in cells is that they are too small to be resolved by standard light microscopy. The formulation of the raft hypothesis was influenced by the discovery that, on entering the Golgi, some proteins form large complexes with lipids, which resist solubilization by non-ionic detergents (Brown and Rose, 1992). Detergent-resistant membrane complexes float to low density during sucrose gradient centrifugation and are enriched in raft proteins and lipids, providing a simple means of identifying raft components. One useful approach in raft research has been the manipulation of raft lipid constituents. This treatment leads to the dissociation of proteins from rafts, which can be readily detected by common methods used to analyse raft association e.g. FACS analysis. Feeding cells with polyunsaturated fatty acids leads to the replacement of saturated fatty acids with unsaturated ones in acylated proteins, causing these proteins to dissociate from rafts (Webb *et al*, 2000). It may therefore possible that other lipid moieties may play a similar role.

1.5.2   SIGNALLING MOLECULES AND PHOSPHOLIPASES

Most extracellular agonists exert their effects on cells by activating or inhibiting transmembrane signalling systems that control the production of second messengers. Most receptors for agonists of phagocytic cells initiate a well-studied pathway of signal transduction. Upon receptor-ligand interaction and coupling to a guanine nucleotide binding protein (G protein) the activation of one more phospholipases occur, leading to an increase in various intracellular messengers. Lipid derived messengers affect a large number of enzymatic reactions of lipid metabolisms *in vivo* and *in vitro*. Nearly all activators of the respiratory burst induce the liberation of arachidonic acid from membrane lipids most likely by activating membrane phospholipases such as PLA_2 or PLC, which are enzymes dependent on calcium (Ca^{2+}). Lipid derived messengers occur due to the interactions of many phospholipases with their phospholipid target. The action of the phospholipases PLC, PLA_2 and PLD on phospholipids results in the formation of products, some of which may have second messenger function (Figure 1.11). Situations leading to a rise in the level of free fatty acids in membranes are potentially capable of affecting the respiratory burst.
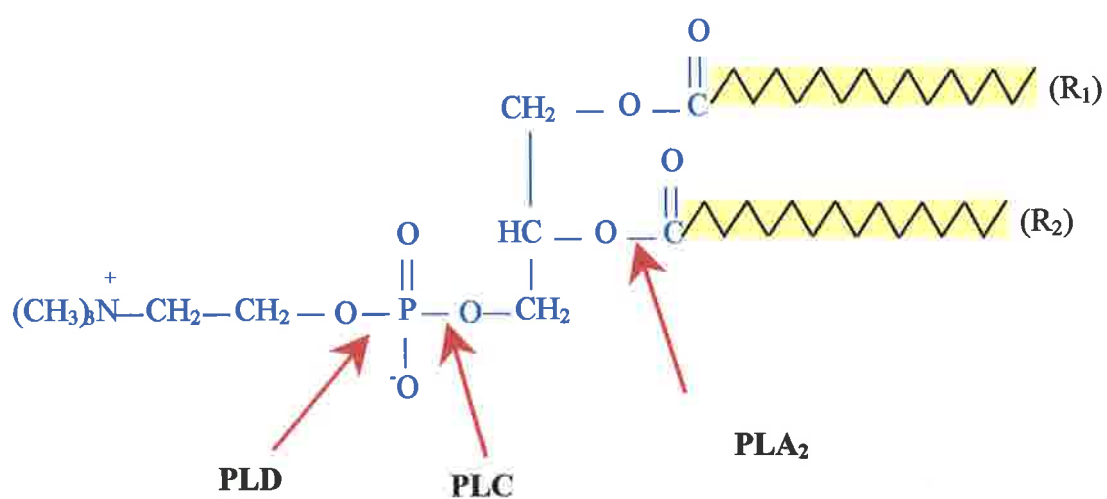
Figure 1.11 – Enzyme cleavage of phosphatidylcholine. The action of the phospholipases PLC, PLA$_2$ and PLD on phospholipids results in the generation of products, some of which have second messenger function.

1.5.2.1   PLD AND ACTIVATION OF NADPH OXIDASE

In recent years, PLD activation has emerged as a central step in the triggering of the respiratory burst. It is known known that most physiological agonists induce or enhance PLD activation in neutrophils, monocytes and macrophages (McPhail *et al*, 1999). PLD cleaves phosphatidylcholine and generates phosphatidic acid and an inactive choline molecule. The former product is a potential second messenger that may be involved in the activation of the NADPH oxidase. Phosphatidic acid is subsequently dephosphorylated by phosphatidate phosphohydrolase to diacylglycerol (DAG), the physiological activator of protein kinase C. Diacylglycerol produced by this pathway is slow, but protracted compared with that resulting from hydrolysis of phosphatidylinositol (Nishizuka, 1992). Phosphatidic acid activates numerous enzymes, including PLCγ and PLA$_2$. A cell-free system has been developed in which the activation of NADPH oxidase is induced by the addition of PA. Characterization of this system revealed that a multi-functional cytosolic protein kinase was a target for PA, and that two NADPH oxidase components were substrates for the enzyme (Waite *et al*, 1997). Partial purification of the PA-activated protein kinase separated the enzyme from known protein kinase targets of PA. The partially purified enzyme was selectively activated by PA, compared to other phospholipids, and phosphorylated the oxidase component p47^{phox} on both serine and tyrosine residues. In the presence of primary alcohols, PLD catalyses a transphosphatidylation reaction, and the incorporation of radiolabelled ethanol into phosphatidylethanolamine has been widely used to monitor stimulus dependent activation of PLD (McPhail, 1993). Inhibition of PLD-mediated phosphatidic acid production prevents neutrophil fMLP-induced superoxide release. During phagocytosis PLD-dependent diacylglycerol formation sustained protein kinase

C activity. However, PLD activation appears not to be mediated by protein kinase C phosphorylation (Kessels et al, 1993).

1.5.2.2 PLC AND ACTIVATION OF NADPH OXIDASE

Several isoforms of PLC have been purified and several sequences have been deduced from cloned cDNAs. The nomenclature of these isoforms utilises Greek letters to designate enzymes with different primary structure and Arabic numerals to designate isozymes arising from proteolytic processing or alternative splicing. Although all isozymes catalyse phosphatidylinositol hydrolysis, they have only limited structural similarity. PLCβ and PLCγ have similar catalytic activity, but differ in their mode of activation. Both types hydrolyse plasma membrane phosphoinositol bis-phosphate (PIP$_2$) to form inositol triphosphate (IP$_3$) and DAG. In the case of PLCβ, G proteins activate the quiescent enzyme. Contrary to PLCβ, PLCγ is independent of G proteins, and is activated by receptors that are themselves tyrosine kinases or are associated with such kinases. Regardless of the type of PI-specific PLC activated, stimulation results in similar cascade events. The release of IP$_3$ promotes the release of Ca^{2+} from intracellular stores. DAG and Ca^{2+} serve to activate several kinases e.g. (Protein Kinase C; PKC) via tyrosine phosphorylation, which is a likely kinase that phosphorylates p47^{phox}, a key component of the NADPH oxidase complex. Secretion of superoxide is dependent on these events to the extent that decreased free cytosolic calcium or stimulation in the absence of extracellular calcium reduced superoxide generation (Pozzan et al, 1983). Considerable amounts of DAG arise from the hydrolysis of PC by another activation-dependent PLC (Billah and Anthes, 1990). This PLC will target the

bond between the phosphate group and glycerol (Figure 1.11). This phospholipase, however, is activated more slowly than the PI-specific enzyme.

1.5.2.3 PLA$_2$ AND ACTIVATION OF NADPH OXIDASE

In many cells the most common substrate for PLA$_2$ is phosphatidylcholine (Pick and Bromberg, 1982). PLA$_2$ will cleave the fatty acyl chain esterified at the *sn*-2 position from the glycerol backbone (Figure 1.11). PLA$_2$ is a family of enzymes that catalyse the hydrolysis of phospholipids and 1-alkylphospholipids (PC and / or PE), generating two main precursor molecules, arachidonic acid and 1-*O*-alkyl-*sn*-glycero-3-phosphorylcholine (lyso-PAF) (Billah and Anthes, 1990). While free arachidonic acid is oxidised by cyclo-oxygenases or lipoxygenases to prostanoids and leukotrienes, respectively (Powell and Gravelle, 1990). The activity of PLA$_2$ is enhanced by phosphorylation, and its product, arachidonic acid stimulates PKCα, thus fuelling a positive feedback loop that is regulated by Ca^{2+} dependence of both enzymes (insel, 1991). Three general types of PLA$_2$ have been described which can be categorized as calcium dependent and independent. The calcium dependent PLA$_2$s can be in turn subdivided into secretory and membrane-bound forms.

1.5.3 PROTEIN KINASES

PKC is a ubiquitous phosphorylating enzyme that exists as a family of closely related isoforms with differential mechanisms of activation, playing an important role in cell functions, secretion, proliferation and differentiation (Nishizuka, 1992). Activation of PKC involves the formation of a quartenary complex between PKC, calcium, phospholipid and DAG. A role for protein kinase C and protein phosphorylations is

suggested by the stimulatory effects of phorbol esters and DAGs and the inhibition by staurospine and other PKC inhibitors (Dewald *et al*, 1988). The involvement of calcium ions and phospholipid dependent isoforms of PKC in LPS-mediated signal transduction remains controversial (Thelen and Wirthmueller, 1994; Mauduit *et al*, 1989). PKC activation by LPS in human monocytes has been documented and implicated in the production of inflammatory mediators (Sweet and Hume, 1996). However, a variety of LPS responses may occur independently of phorbol ester sensitive PKC. Ultimately, the level of LPS and state of activation of cells studied may alter the response to PMA and perhaps the relative importance of PKC pathways in LPS responsiveness.

Signal transduction leading to the activation of mitogen activated protein kinase (MAPK) by receptor kinase is well established (Davis, 1993). One potential signalling pathway activated by LPS in macrophages is the MAPK pathway identified as a downstream event of Ras proteins. Many different ligands can activate a pathway that activates intrinsic or extrinsic protein tyrosine kinase (PTK) activity (Sweet and Hume, 1996). Tyrosine phosphorylation of specific proteins may play an important role in activation of the respiratory burst. Tyrosine phosphorylation kinase activation is responsible for the activation of PLD and Cy2, two processes involved in the transmembrane signalling for the activation of the neutrophil respiratory burst.

1.5.4 TRANSCRIPTION FACTORS

The co-ordinated switching on and off genes is mediated by transcription factor proteins. The diversity of transcription factors reflects the complexity of genes required for organismal processes. For example, the development of the immune response requires NF-κB family of transcription factors. The timing and specificity of transcription factor activity is often regulated by associated proteins, which may be activators or inactivators of gene transcription. NF-κB is known to be translocated to the nucleus in LPS-stimulated macrophages, and appears to play an important role in the generation of pro-inflammatory cytokines by these cells (Chow *et al*, 1995). NF-κB was originally identified as a B cell-specific nuclear protein that bound an enhancer region, the κB motif, in the immunoglobulin κ light chain gene (Sen and Baltimore, 1986). Subsequently, NF-κB is one of the transcription factors that are activated and upregulates and induces expression of a variety of genes and proteins that induce acute and chronic inflammation. As an inactive form, it is bound to an inhibitor, IκB, residing in the cytoplasm. In response to LPS or TNF-α activation, it is phosphorylated and allowed to release NF-κB for translocation from the cytoplasm into the nucleus. Inflammation results in production of ROIs, which are activators of NF-κB and TNF-α. TNF-α transduces signals through several possible pathways that may involve G-proteins, PLA_2, and free radicals as effector molecules.

1.5.5 'PRIMING' OF THE NADPH OXIDASE

Some cells particularly neutrophils, monocytes and macrophages can respond to an activating stimulus with a greater capacity for mediator production if they are first 'primed' with an agonist, by a mechanism that is not fully elucidated. 'Priming' of phagocytes for enhanced NADPH oxidase activity upon exposure to a second stimulus such as opsonised particles is an important mechanism for enhanced bactericidal activity (Hughes *et al*, 1997). A variety of substances, both physiological and pharmacological has been shown to act as 'priming' agents. Examples of these agents include LPS, cytokines (TNF-α), IFNγ and the bacterial cell wall component muramyl dipeptide (Condliffe *et al*, 1998). The key concept regarding 'priming' is that these agents do not elicit the effector functions on their own (although they may do so when applied at high concentrations). 'Priming' also requires the presentation of a 'priming' agent to the cell for a variable period before the cell is exposed to an activating stimulus. Hence the neutrophil and monocytic respiratory burst that occurs in response to an agonist may be enhanced by upto 10 fold by prior exposure of the cells to a 'priming' agent (Zughaier *et al*, 1999).

Many 'priming' agents have clear biological relevance *in vivo* and are released in response to infection, haemorrhage and trauma. For example, circulating LPS has been associated with the development of ARDS (Parsons *et al*, 1989), and persistent high levels of plasma TNF-α and IL-6 have been linked to poor outcome in septic shock (Pinsky *et al*, 1993).

There is a large body of evidence concerning the signalling pathways involved in 'priming', reviewed in Condliffe *et al*, 1998; Hallett and Lloyds, 1995, however, there is considerable controversy and the pathways involved remain unclear. Certain common themes have emerged including the modulation of agonist receptors, heterotrimeric GTP-binding proteins, phospholipase A_2, C and D activation and more recently protein phosphorylation. Full activation of the respiratory burst in phagocytes results from translocation and assembly of the cytosolic components of the NADPH oxidase enzyme system ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac; as discussed in section 1.4.6) with the membrane associated flavocytochrome. Phosphorylation is the essential event in activation of the NADPH oxidase. This translocation involves multiple $p47^{phox}$ – directed phosphorylation events (El Benna *et al*, 1994). It has been shown that mitogen activated protein kinases (MAPK) may participate in the regulation of oxidant production by phosphorylation of $p47^{phox}$ (Partrick *et al*, 2000; El Benna *et al*, 1996).. Further evidence suggests tyrosine phosphorylation may be required to activate PKC, which may also be the kinase involved in the extensive phosphorylation of $p47^{phox}$ (Dusi *et al*, 1994). Phosphorylation of $p47^{phox}$ is reversed once cells are inactivated, indicating a dephosphorylation in turning off the oxidase and hence the involvement of phosphatase enzymes. Further, studies investigating the time-course of tyrosine phosphorylation was consistent with a role in 'priming', and manipulation of tyrosine phosphorylation levels using tyrosine phosphatase or tyrosine kinase inhibitors resulted in 'priming' and inhibition of 'priming' respectively (Lloyds and Hallett, 1994). It therefore seems likely that tyrosine phosphorylation plays an important, if as yet uncharacterised, role in 'priming'.

1.6 LIPOPOLYSACCHARIDE AND MACROPHAGE ACTIVATION

Monocyte and macrophage activation can be defined as the acquistion of the ability to perform and complete a complex function e.g. chemotaxis, phagocytosis. Activation of these cells is not normally permanment due to inductive and suppressive signals including phosphorylation and dephosphorylation events. Cell activation can occur in response to both particulate and soluble agents. The mechanism of signal transduction however, varies according to the type of agent used. Some cells, particularly monocytes and macrophages, can respond to an activating stimulus with a greater capacity for mediator production if they are first 'primed' with an agonist, by a mechanism that is not fully elucidated (section 1.5.5). One of the most potent inducers of monocyte and macrophage activation is lipopolysaccharide (LPS).

1.6.1 LIPOPOLYSACCHARIDE

LPS can enhance the immune response and elicit symptoms from chills and fever to circulatory shock. In the 1870's Robert Koch identified the ability of an infectious microbe to cause disease. Within a decade of this discovery, it was shown that bacteria often make people sick by producing proteins (exotoxins). Then, in 1892, Richard Pfeiffer (one of Koch's students) found that disintegration of *Vibro cholerae* resulted in the release of an exotoxin and endotoxin. This latter product was named by Pfeiffer since it was a heat stable molecule, sequestered within the microbe and characteristic of all gram negative species. Endotoxins are released when cells die or when growth is rapid (Rietschel and Brade, 1992). The structure of endotoxin was further characterised

in the 1930's and 40's by André Boivin, Walter Morgan and Goebel whom identified polysaccharide, lipid and protein components of endotoxin. The toxic element was confined to the phosphorylated lipopolysaccharide component by investigations in the 1940's and endotoxin was renamed by Shear to LPS, emphasising its chemistry (Rietschel and Brade, 1992). Monocytes / macrophages play a central role in mediating the response to LPS. These cells can respond to picomolar concentrations of LPS by releasing mediators that act as autocrine and paracrine signals. LPS released from bacteria can elicit various cellular effects *in vivo* and *in vitro*. These can range from moderate and adequate immune response to massive induction of cytokine release, leading ultimately to septic shock and death (Ulevitch and Tobias, 1995).

1.6.2   THE STRUCTURE OF LIPOPOLYSACCHARIDE

LPS is a complex glycolipid and an essential cellular constituent of gram negative bacteria (Ziegler-Heitbrock, 1995). There are two regions of LPS, a hydrophilic and hydrophobic domain (Figure 1.12A). The lipophilic component has typically six acyl chains linked to a pair of phosphorylhexosamines (Ziegler-Heitbrock, 1995). The lipophilic component resides in the outer membrane and is termed lipid A or diphosphoryl lipid A (DPLA). It is well established and agreed that the biologically active endotoxic structure within the complex glycolipid that constitutes LPS is lipid A. This component is a diphosphorylated, glucosamine disaccharide linked by a β1 - 6 oxygen bridge, with usually six fatty acids consisting of 14 carbons (or medium to long chain fatty acids) (Figure 1.12B).

A

Outer core   Inner core

| O-Specific chain | | Core | | Lipid A |

| Polysaccharide | | Lipid |

⬡ Monosaccharide   ⊙ Phosphate   ∿∿∿∿ Fatty acid
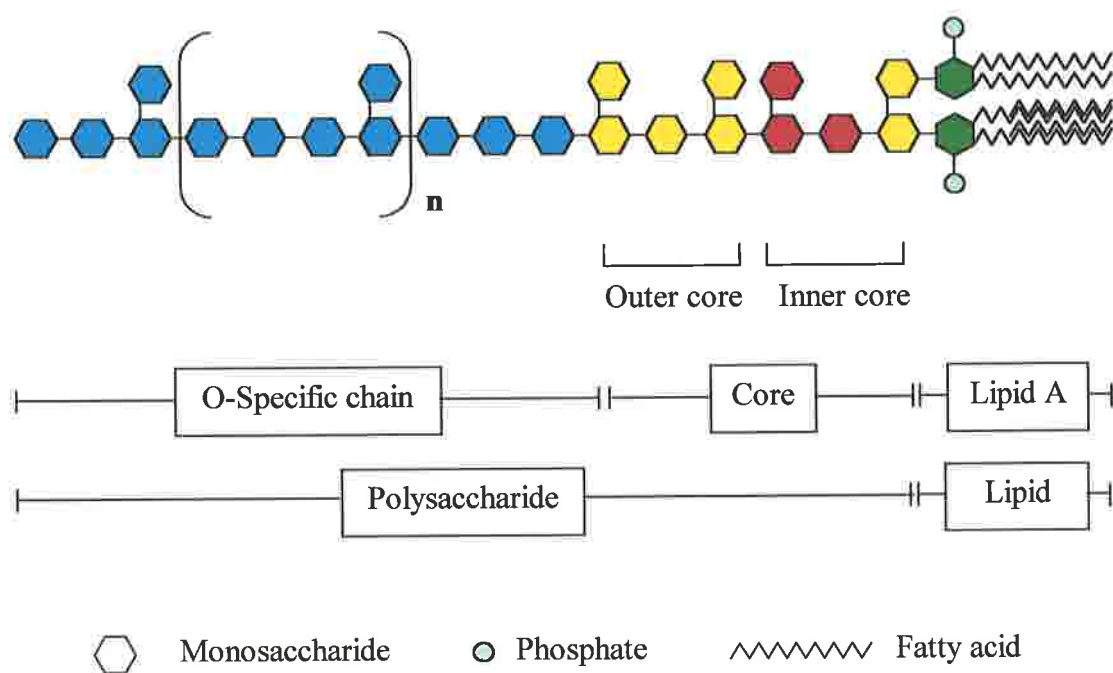
B
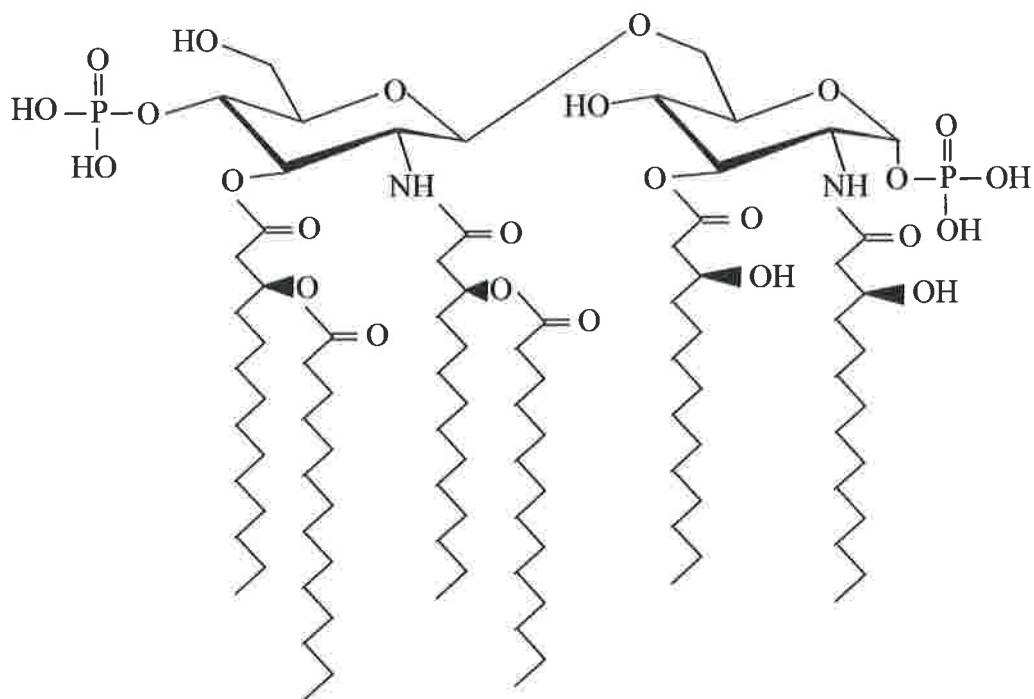
Figure 1.12 – **(A)** Schematic structure of *Escherichia coli* LPS. **(B)** Chemical structure of Lipid A.

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In addition to the lipid component there is a polysaccharide compartment consisting of two domains, a core polysaccharide and an O - specific chain which is the most variable and evokes an immune response. This latter domain typically consists of 20 - 40 repeating units consisting of 3 - 8 sugar residues. The distal sugars tend to be specific in different species (Rietschel *et al*, 1994). The inner core bears at least two unusual sugars, one of which is heptose. The other sugar is kdo (3 deoxy-D-mann-2-octulosonic acid) which is found in all endotoxins and links the polysaccharide to the lipid (Figure 1.12). The LPS molecule by virtue of its amphipathic nature may bind by non-specific interactions to the cell membrane (Chaby and Girard, 1993). However there is ample evidence that LPS interacts with specific binding sites, one of which is the LPS receptor CD14 (section 1.6.4) (Ingalls *et al*, 1998; Ziegler-Heitbrock and Ulevitch, 1993).

1.6.3   LIPOPOLYSACCHARIDE AND BIOLOGICAL ACTIVITY

Activation of monocytes and macrophages by LPS occurs through both complement pathways by either polysaccharide or lipid A activation. LPS also acts on a variety of cells including CD14 positive and CD14 negative cells, lymphocytes (Hammerling *et al*, 1976), endothelial cells (Schleimer and Rutledge, 1986) and macrophages (Doe and Henson, 1978) for example. An essential host defence mechanism is the ability of phagocytes to engulf and digest foreign particles. Evidence from a number of studies has been provided on the role of LPS in suppressing phagocytosis and binding of particles via Fcγ receptors and complement receptors (CR3) (Wonderling *et al*, 1996; Sundaram *et al*, 1993; Coleman *et al*, 1985). LPS can 'prime' phagocytes for degranulation and phagocytosis. This mobilisation of inflammatory leukocytes is an

important early event in host defence against invading Gram negative bacteria since several of their granule constituents such as bactericidal permeability-increasing protein bind avidly to LPS and exhibit direct or indirect antibacterial activity (Ulevitch and Tobias, 1999). LPS suppression activity may therefore serve as a pathogenic mechanism of Gram negative micro-organisms for evading phagocytosis and hence further proliferation of infection leading to sepsis and circulatory shock. Circulatory shock reflects an acute impairment of cellular oxygen uptake and oxygen supply. The maldistribution of blood flow and hence maldistribution of oxygen is the primary mechanism for circulatory shock. Both oxygen uptake and oxygen supply is increased in sepsis. In septic shock, the defect lies in the altered oxygen utilisation in relation to the high oxygen requirements of an active immune system.

LPS stimulates macrophages to produce and release an array of inflammatory mediators (section 1.3). These mediators may act independently or together to provide a beneficial or sometimes harmful effect. The low level of production of these mediators provides an immune response that is controllable and not detrimental to the defence of the invading agent. However, high levels or inappropriate secretion of these inflammatory mediators may lead to an excessive immune activation leading to high fever, hypotension and lethal shock. In addition, the LPS stimulated ROI production may also contribute to tissue damage in inflammation.

1.6.4　MODE OF ACTION OF LPS

The molecular basis of the recognition of LPS by serum proteins and or / cells has only recently been elucidated. The seminal discovery by Tobias *et al*, 1986, of LPS binding protein (LBP) and their elucidation of its structure and function (Wright *et al*, 1989), followed by their identification of CD14 as an LPS receptor (Wright *et al*, 1990) has provided the basis for our current detailed understanding of this important pathway of innate immunity.

Originally it was thought that LPS may activate cells of the myeloid lineage, directly or in complex with a serum protein, LBP, mediated through the cell surface receptor CD14 (Wright *et al*, 1990). High LPS concentrations are capable of triggering the macrophage responses directly, without interaction with LBP. The monocyte / macrophage is able to respond to less than 10 ng / ml of LPS but only in the presence of LBP (Wright *et al*, 1990). The titre of LPS in human serum is nearly always below 1 ng / ml during sepsis. Thus, the effect of LBP in LPS stimulation of monocytes and macrophages is very important. LBP is synthesised in the hepatocytes as a 55 kDa protein and is further glycosylated and released into the circulation as a 60 kDa glycoprotein (Chaby and Girard, 1993). As demonstrated by Wright *et al*, 1990, LBP plays a role in LPS signal transduction acting as a LPS transfer protein that moves LPS monomers from aggregates or bacterial membranes to a binding site on CD14. LBP also has the ability to act as a phospholipid transfer protein. Wang *et al*, 1998, showed that phosphatidylinositides in serum can inhibit both LPS-mCD14 binding and LPS-induced responses in monocytes. These observations suggest a mechanistic role of lipid modulation through mCD14 receptor binding. LPS can also bind to several other cell

surface molecules in mammalian cells including CD11b / CD18, and the oxy LDL scavenger receptor (Ingalls *et al*, 1998).

CD14, is a 55 kDa glycosyl-phosphatidylinositol (GPI) linked membrane protein expressed on the surface of monocytes and macrophages and to a lesser extent on granulocytes. The alveolar macrophage is the predominant cell expressing CD14 within the air spaces (Striz *et al*, 1995). Although these macrophages express less CD14 receptors than circulating monocytes, they are still able to respond via the LPS-CD14 pathway. CD14 is not expressed on B cells or by the immature myeloid cell line U937. CD14 may be membrane bound (mCD14) or in a soluble form (sCD14; which may be present within surfactant). Membrane bound CD14 lacks a cytoplasmic domain (Lund-Johansen *et al*, 1990) suggesting this receptor does not communicate with the cell interior directly following interaction with LPS, or the LPS-LBP complex. Therefore, it is possible that binding to CD14 activates a second messenger leading to cellular activation. Many workers have hypothesised that one or more additional transmembrane proteins act in concert with LPS-CD14 complexes to initiate the signalling processes leading to cell activation. Toll like receptors (TLR) are one class of receptors that fit the requirement for CD14 co-receptor signalling (Brightbill and Modlin, 2000; Beutler, 2000). Toll is a transmembrane protein that was discovered as a necessary player in the establishment of dorsal-ventral polarity in *Drosophilia* embryos (Anderson, 1998). Several mammalian homologues of Toll have been discovered, and recent studies had shown that TLR4 can initiate signalling steps similar to those seen in response to LPS (Medzhitov *et al*, 1997). A constitutively active TLR4 construct drives Nuclear Factor κB (NF-κB) activation and cytokine production in transfected cells

(Medzhitov *et al*, 1997). Additionally TLR uses MyD88 to activate NF-κB (Medzhitov *et al*, 1998). NF-κB activation plays a major role in response to LPS, and these data suggest that activation of a receptor such as TLR4 may be sufficient to explain the transcriptional responses to LPS.

1.6.5   TREATMENT OF LPS INDUCED DISEASE

Bacterial infections have become the major nosocomial infectious disease problem in patients hospitalised in the United States and Many European countries (Young *et al*, 1982). In the United States alone the annual incidence of sepsis in hospitalised patients is estimated to be 400,000, leading to 200,000 cases of septic shock and 100,000 deaths (Parrillo *et al*, 1990) with similar figures estimated for Europe. There are currently no drugs available to reduce this number despite the enormous efforts being undertaken. There are a variety of methods that are available to attempt to treat this condition including (Tanamoto, 1997):-

(1) direct inhibition of LPS

(2) control of mediator production

(3) inhibition of signal transduction pathways using inhibitors of protein kinases and

(4) use of endotoxin antagonists

1.7 MONOCYTE MODEL USING MONOMAC-6 (MM6)

Monocytes, the circulating precursors of macrophages, play an essential role in maintaining the sterility of tissues by the production of a range of mediators including cytokines and the release of ROIs and vasoactive lipids. The micro-environment in which monocytes mature into alveolar macrophages maybe important in determining their functional characteristics. Cultured human monocytes, which present functional similarities with alveolar macrophages, have been extensively used as a model system to study the biology of alveolar macrophages and in particular their response to pulmonary surfactant (Walti *et al*, 1997; Geertsma *et al*, 1993).

Cell lines established from a variety of transformed tissues provide an excellent tool for studying various questions related to the normal cellular counterpart as well as tumour physiology. Some of the best-characterised human cell lines of the monocyte lineage includes U937 and THP-1, representative cells of early stage monocytic differentiation. However, these cells must be induced to differentiate in order to develop properties attributed to mature monocytes.

The human monocytic cell line, MonoMac-6 (MM6) was established in the late- 1980s, offering many advantages over the other available cell lines (Ziegler-Heitbrock *et al*, 1988). In 1981, a myeloid metaplasia was diagnosed in a 60-year-old male based on his bone marrow biopsy. In 1985 he was further diagnosed as having a monoblastic leukaemia due to the fact that his white blood cell count was greater 35 x 10^9 / L and 70% of these were leukaemic cells. The leukaemic cells were positive for sodium fluoride sensitive non-specific esterase (Ziegler-Heitbrock *et al*, 1988). Transverse

electron microscopy showed that MM6 contained compartments for protein export and for endocytosis such as lysosomes and endocytic vesicles, features not reported for U937 and THP-1. From this patient, two clones were produced MM6 and MM1. Both these clones expressed sodium fluoride sensitive non-specific esterase, produce ROIs and stain with the monoclonal antibody My4. The THP-1 and U937 are negative for such markers. MM6 in addition constitutively exhibits phagocytosis of antibody coated erythrocytes in 80% of cells and reacts with a monoclonal antibody panel specific for monocytes i.e. M42, LeuM3 (Chan, 1995). Seventy five percent of MM6 express My4 staining which is indicative of CD14 receptors. MM6 appears to be the only cell line studied to express phenotypic and functional features of mature monocytes (Ziegler-Heitbrock *et al*, 1988). This cell line has proven extremely useful because to varying degrees they constitutively or after stimulus express *in vitro* many of the properties manifested by their *in vivo* counterparts. Continuous cell lines provide additional advantages beyond mere conveniences including availability of virtually unlimited numbers of homogenous cell populations of phenotypic interest. In addition, cell lines provide the capability to clonally retain or exclude certain phenotypic traits or to permit studies in synchronous systems.

1.8 CHEMILUMINESCENCE

Chemiluminescence was originally detected using liquid scintillation. Nowadays, the use of modified photomultiplier tubes, which quantitates the reaction rate over a period, provides a more accurate assessment. This type of luminometer provides instantaneous results in forms of digital displays or charts. High-sensitivity photon counters designed for measurement of chemiluminescence has the potential for data collected and processed via desktops. In addition, several luminometers from instrument manufacturers include reagent mixing and injection, temperature control and various other functions further improving the ease at which chemiluminescence assay performs.

Chemiluminescence is the study of light emitted chemical reactions and can be used to assess the microbe-humoral-phagocytic interaction (Kopprasch *et al*, 1996; McCapra, 1990; Allen, 1986). The interest of bioluminescence and chemiluminescence has risen since the elucidation of the chemical structures luciferins and dioxetanes, responsible for light emitting reactions which were first elucidated in the late sixties. Light emitting technology is based on the transition of an electron from the ground state to an excited state induced by absorption of UV-vis radiation (Rongen *et al*, 1994). The return to the ground state occurs with emission of radiation termed fluorescence if the molecule is in a singlet state or phosphorescence if the molecule is in triplet state. However, in chemiluminescence the organic molecule is in an electronically excited state without absorption of radiation. The exploitation of the fluorescent properties of excited molecules provides the basis for chemiluminescence. Native chemiluminescence is the relaxation of biologically excited molecules to ground state by photon emission.

Chemiluminescent efficiency is measured by quantum yield (Φ_{CL}) defined as the fraction of molecule emitting a photon on return to ground state i.e. photons emitted per number of oxygenation events (Allen *et al*, 1986). The Φ_{CL} is the product of three ratios:-

$$\Phi_{CL} = \Phi_c : \Phi_e : \Phi_f$$

Where Φ_c is the fraction of molecules yielding an excitable molecule, Φ_e is the fraction of molecules in an electronically excited state and Φ_f is the fraction of these molecules emitting a photon. An increase in the efficiency of a chemiluminescence system can be attained with an efficient fluorophore or chemiluminogenic probe where energy is transferred to such a molecule.

Chemiluminescence measurements may be applied to intact cell systems, cell fractions, cell-free systems and whole blood. However, the chemiluminescent measurement of oxidant production in whole blood is limited by the presence of haemoglobin due to possible photon absorption and the scavenging potential of erythrocytes (Lindena *et al*, 1987). The exquisite sensitivity of chemiluminescence allows dilution of whole blood to overcome interference and to obtain an accurate assessment of oxidant production. The application of native chemiluminescence in the assessment of the microbe-humoral-phagocytic interaction is problematic but is overcome with the use of chemiluminogenic probes. Chemiluminescence techniques are reliable, non-destructive, low cost yet sensitive methods for studying ROIs from phagocytic cells.

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1.8.1   CHEMILUMINOGENIC PROBES

Native chemiluminescence (without enhancing probes) assessment is limited by two technical considerations. Firstly, the substrate presented for oxygenation is, for the most part, defined by the microbe phagocytosed and typically of poor quantum yield (Allen, 1986). Consequently at least 10^6 phagocytes per test would be required in addition to acquiring increased performance detection systems. Secondly, the variety of substrates presented for oxygenation would provide different chemiluminescence quantum yields, since the molecular composition of microbes varies greatly (Allen *et al*, 1986). This does not easily allow intercomparison of chemiluminescent responses from different microbial species. The technical considerations associated with native chemiluminescent measurements are overcome with the addition of a chemiluminogenic probe. A chemiluminogenic probe may be defined as an exogenous substrate whose oxygenation is associated with a high yield of electronically excited product. These chemiluminogenic probes must possess a high quantum yield, be chemically reactive with oxygenating agents, non-toxic to cells and non destructive to the process of phagocytosis (Albrecht and Jungi, 1993; Allen *et al*, 1986). The use of probes offers a great increase in sensitivity and specificity and hence a reduction in the number of phagocytes per test. For example, the use of luminol requires greater than 10^4 phagocytes per test (Lundqvist and Dahlgren, 1996). The luminescence measured by the use of probes effectively eliminates substrate variability with regard to quantum yield allowing intercomparison of data obtained using different microbes.

The fluorescence of chemiluminogenic probes depends on the distinct products of phagocyte oxidative metabolism and the dioxygenation mechanism (Stevens *et al*, 1994). Probes that are susceptible to oxygenation include the cyclic hydrazide luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) and lucigenin (10,10'-dimethyl-9,9'biacridinium dinitrate, DBA) (Faulkner and Fridovich, 1993; Allen, 1986). Luminol and lucigenin have a similar chemiluminescence quantum yield but have very different reactivities. These probes can be considered as the substrate of the individual oxidative burst as they selectively react with its products. Lucigenin is a reductive dioxygenation, dependent on NADPH oxidase while the dioxygenation of luminol is mostly but not exclusively dependent on oxidase-driven haloperoxidase activity (Stevens *et al*, 1994). In both scenarios of probe utilisation, light production depends on the formation of an unstable endoperoxide or dioxetane which decomposes to an electronically excited product, which releases a photon as it falls to ground state (Faulkner and Fridovich, 1993).

Probe concentration and number of phagocytic cells will affect luminol or lucigenin chemiluminescence. In addition, experimental considerations to soluble or particulate activators must be accounted for. The chemiluminescent response will only reflect phagocytic activity if probe and stimulus are not rate limiting. There must be a need to compromise between physical conditions and optimal conditions for chemiluminescence. This is particularly so with luminol with respect to pH. Luminol is optimal at pH 8.5 while lucigenin is optimal at physiological pH (Hosker *et al*, 1989). Although the assay is easy to perform, NADPH activity is difficult to quantify but very sensitive with respect to qualitative work.

1.8.2   LUMINOL

Luminol chemiluminescence was first reported on in 1928 by Albrecht. Since then the chemiluminescence of luminol (Brestel, 1985), isoluminol and its derivatives have been extensively studied (Lundqvist and Dahlgren, 1996). Luminol is a cyclic hydrazide, 177.2 Daltons in weight and has the ability to cross biological membranes, as it is a small lipophilic molecule. It is therefore oxidised intracellularly and / or extracellularly although this has been a matter of debate and remains unclear (Lundqvist and Dahlgren, 1996; Briheim *et al*, 1984). Albrecht and Jungi, 1993, provided evidence that luminol chemiluminescence emanating from stimulated monocytes is taking place exclusively in the extracellular compartment and requires exocytosis of myeloperoxidase (MPO). It has been shown that MPO deficient neutrophils exhibit low levels of luminol chemiluminescence as compared to controls (Stevens *et al*, 1978). Normally, neutrophils rich in MPO produce the majority of their oxidant species via the MPO-hydrogen peroxide system. To this extent, luminol is a good indicator of oxidative metabolism in PMNs.

In alkaline dimethyl sulphoxide (DMSO), luminol autooxidises with intense luminescence and a quantum yield of approximately 5% (Van Dyke, 1985). The univalent oxidation of luminol yields a luminol radical that can both reduce oxygen to superoxide and can react with superoxide yielding an unstable endoperoxide. In the case of activated neutrophils, the univalent oxidant is MPO-hydrogen peroxide. The unstable endoperoxide releases nitrogen while converting to an aminopthallate (Faulkner and Fridovich, 1993). The aminopthallate is the light emitting species when it drops to a ground state (Figure 1.13).
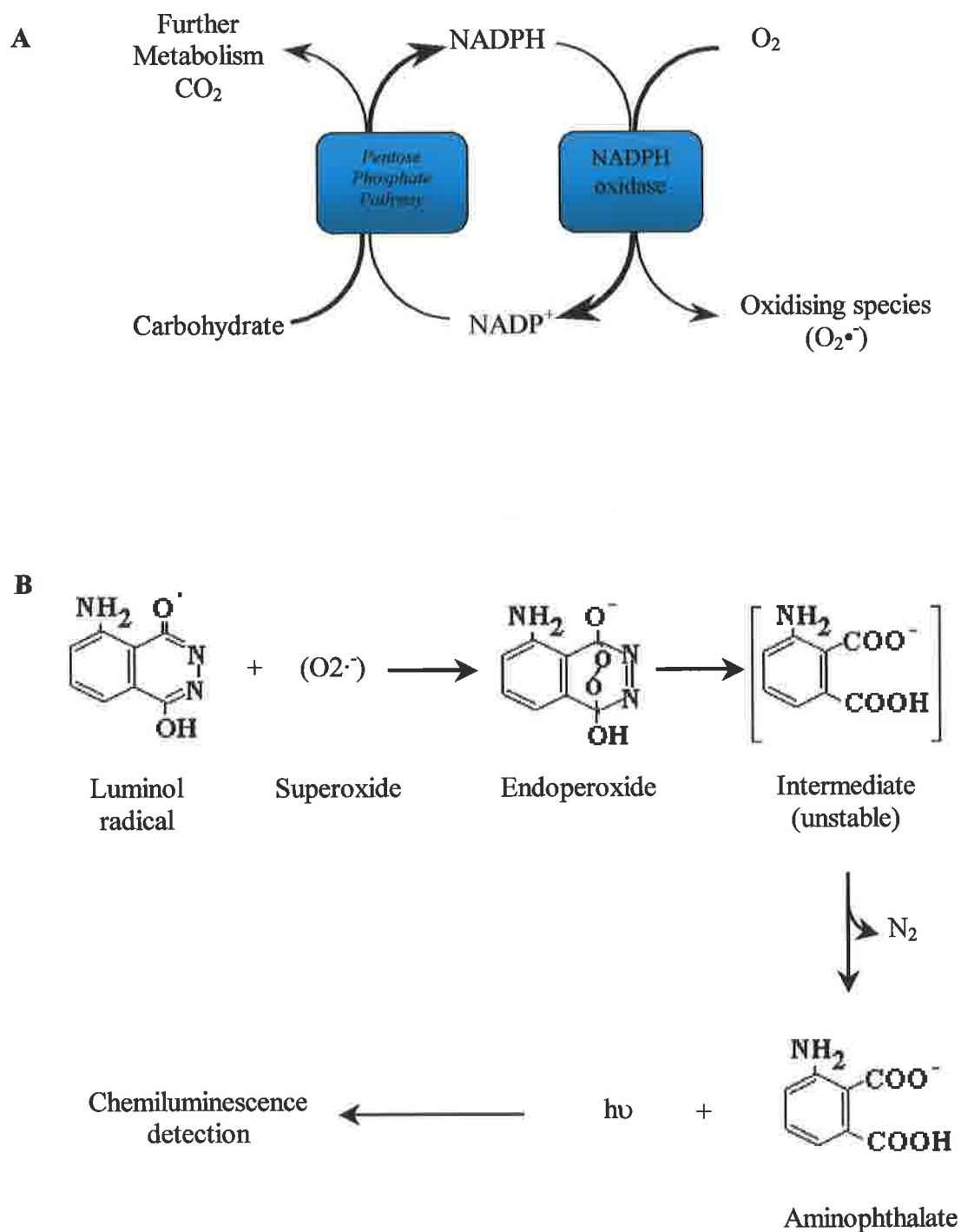
Figure 1.13 – Luminol determination of NADPH oxidase activity. **(A)** Superoxide and other oxidising species are produced via the action of NADPH oxidase. **(B)** Reaction of superoxide involved in the oxidation of luminol.

Brestel, 1985, suggested that luminol chemiluminescence reflects the co-oxidation of luminol by MPO and hydrogen peroxide which react in the presence of chloride ions to produce hypochlorous acid (Brestel, 1985). A disadvantage of luminol chemiluminescence is that light emission cannot be correlated with a single type of oxygen metabolite. However, luminol chemiluminescence appears to be more reliable than lucigenin chemiluminescence. Further, Barber *et al*, 1995 and Tsukamoto *et al*, 1998 both suggested that interfering species, possibly phospholipids or products of lipid peroxidation may play a role in non superoxide lucigenin chemiluminescence in biological tissues.

Many investigators have employed luminol enhanced chemiluminescence of isolated PMNs to measure their oxygenation capacity (Albrecht and Jungi, 1993). The type of stimulus (Jones and Hancock, 1994), amount and exocytosis of MPO (Hosker *et al*, 1989) and the production of H_2O_2 (Bhatnagar *et al*, 1981) determine the magnitude and time course initiated by diffusion of luminol (Briheim *et al*, 1984). Soluble activation through the action of a phorbol ester (e.g. PMA) results in complete degranulation and full activation of the redox metabolism whereas particulate activation (e.g. zymosan) causes specific and azurophilic degranulation. Particulate challenge with zymosan leads to the formation of a phagolysosome, providing the optimal environment for MPO activity. However, luminol may not be suited to living systems because of auto-oxidation to generate superoxide and the poor quantum yield at neutral pH (Faulkner and Fridovich, 1993).

1.9   AIMS AND OBJECTIVES

Pulmonary surfactant is a complex mixture of phospholipids, lipids and proteins that line the alveolar regions of the lungs. Although its major function is to prevent alveolar collapse by lowering surface tension forces, lung surfactant is also known to have effects on inflammatory leukocytes including alveolar macrophages. However, the mechanism of these effects is poorly understood. Monocytes, the circulating precursors of macrophages play an essential role in maintaining the sterility of tissues by the production of a range of mediators including cytokines and the release of ROIs and vasoactive lipids. The micro-environment in which monocytes mature into alveolar macrophages maybe important in determining their functional characteristics. Surfactant is part of the normal environment for alveolar macrophages *in vivo*. Since alveolar macrophages represent a major source of mediator production during inflammatory processes and because surfactant can affect some responses of the cells, it seems logical to suggest that pulmonary surfactant and in particular its lipid content may indeed play a significant role in the immunoregulation of the lung.

The purpose of this study is to investigate the hypothesis that exogenous surfactant lipids can exert an immunoregulatory effect on the mechanisms involved in cell killing by monocytes and macrophages. Of particular interest is the production of reactive oxygen intermediates (ROIs) during the respiratory burst because of their tumoricidal as well as bactericidal activities.

The main objectives of the study are:

(1) To determine the classes of surfactant lipids that up or down-regulate the respiratory burst. For these lipids, what concentrations are required and do they need to be internalised for their effects? This will provide important information regarding what component(s) of pulmonary surfactant are responsible for modulating the respiratory burst.

(2) What are the mechanisms for the action of the lipids that modulate the respiratory burst? The underlying mechanisms of surfactant inhibition on ROI production have not been well characterised especially in terms of signalling events.

(3) What is the cell-specificity of the action of these regulating lipids? In addition, can these lipids modulate other aspects of phagocyte function?

The results of this investigation will not only improve our understanding of the cell biology and regulation of the respiratory burst but may also allow the development of therapies aimed at regulating oxidant production.

CHAPTER 2: MATERIALS AND

METHODS

2.1 GENERAL CHEMICALS

All chemicals used were of Analar grade unless stated otherwise. Acetic acid, chloroform, diethylaminoethyl (DEAE) cellulose (Whatmann® DE52), dimethyl sulphoxide (DMSO), ethanol, 1M Hepes, 1M HCl, methanol (HPLC grade), orthophosphoric acid and 3mm Whatmann filter were purchased from Fisher Scientific U.K. (Leicestershire, U.K.). Aprotinin, Coomassie blue G-250, deoxycholate, endotoxin-free albumin, endotoxin free water, ethylene glycol, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, guanosine 5'-O-(3-thiotriphosphate) (GTPγS), leupeptin, β-mercaptoethanol, naphthylethylenediamine chloride, nicotinamide adenine dinucleotide phosphate (NADPH), nitrite reductase, phenylmethylsulphonylfluoride (PMSF), sodium borohydride (NaBH$_4$), sodium dodecyl sulphate (SDS), sodium nitrite, sodium orthovanadate, sulphanilamide, tergitol-NP40 (NP40), triethylamine, Tris-base, Triton X-100, Trypan blue and Tween-20, were purchased from Sigma-Aldrich Company Ltd. (Dorset, U.K.). All gases and liquid nitrogen were purchased from BOC Gases (Surrey, U.K.). Phosphate buffered saline (PBS tablets without calcium and magnesium) was purchased from Oxoid (Basingstoke, U.K.). Calcium chloride (CaCl$_2$), di-sodium hydrogen orthophosphate (Na$_2$HPO$_4$), ethylene glycol-bis-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), glucose, magnesium chloride (MgCl$_2$), naphthylenediamine chloride, potassium chloride (KCl), potassium dihydrogen orthophosphate (KH$_2$PO$_4$), sodium azide (NaN$_3$), sodium chloride (NaCl), sodium periodate, sucrose, sulphanilamide and trypan blue were purchased from BDH (Dorset, U.K.). Recombinant interferon gamma (IFNγ) was purchased from Genzyme Diagnostic (West Malling, U.K.).

2.2 CELL CULTURE

2.2.1 CELL LINES

The human monocytic cell line MonoMac-6 (MM6) was obtained from the German Collection of Microorganisms and Cell Cultures (DSM ACC 124, Braunschweig, Germany). The rat alveolar macrophage cell line NR8383 and the mouse macrophage cell line RAW 264.7, were obtained from the European Collection of Animal Cell Cultures (ECACC, Wiltshire U.K.). All cells under all experimental conditions were cultured at $37^{\circ}C$ in a humidified atmosphere with 5% carbon dioxide (CO_2) unless otherwise stated.

2.2.2 PREPARATION OF SUPPLEMENTED MEDIA

Roswell Park Memorial Institute (RPMI) 1640 medium without L-glutamine and Dulbecco's modified eagle medium (DMEM) were supplemented (*v/v*) with 1% bovine insulin (10 µg / ml), 10% heat inactivated foetal bovine serum (FBS), 1% 2 mM L-glutamine, 1% non-essential amino acids, 1% penicillin (50 IU / ml) / streptomycin (100 µg / ml) and 1% sodium pyruvate, purchased from Gibco, Paisley, U.K.

F-12K medium (Kaign's modification) with 2 mM L-glutamine purchased from Gibco, Paisley, U.K. was supplemented as for RPMI media but with the inclusion of 15% FBS (*v/v*) and the omission of L-glutamine.

UltraCULTURE™, a serum-free medium, was purchased from BioWhittaker U.K. Ltd., Wokingham, U.K. and supplemented as for RPMI 1640 media without FBS.

2.2.3   SUB-CULTURING OF MM6 CELLS

MM6 cells were harvested and sub-cultured (passaged) when growth became confluent i.e. sub-culturing of MM6 cells was performed after 2 to 4 days of culture when the cell density reached approximately 1×10^6 cells / ml. The use of this cell line was limited to less than 25 passages. It has been reported that prolonged culture may result in the loss of particular phenotypes (Ziegler-Heitbrock *et al*, 1988). MM6 cells are a non-adherent suspension culture in which cells were harvested by centrifugation at $500 \times g$ for 5 minutes and re-suspended in supplemented RPMI medium by gentle pipetting. MM6 cells were seeded at 4×10^5 cells / ml and incubated in a humidified atmosphere.

2.2.4   SERUM FREE CULTURE OF MM6 CELLS

The conversion of MM6 cells from growth in serum-containing medium to serum-free medium was achieved through the 'weaning process'. Cells were harvested, sub-cultured and seeded as described in section 2.2.3. However, at each passage the cells were re-suspended in supplemented UltraCULTURE™ media containing a lower fraction of FBS (i.e. 5%, 2%, 1%, 0.5% and 0.1% *v/v*). The weaning process was repeated until serum was eliminated from the culture.

2.2.5 SUB-CULTURING OF NR8383 CELLS

NR8383 cells exist as a co-culture of two cell populations and phenotypes, one of which are adherent and the other floating in suspension. The floating population is the reproducing phenotype and thus sub-culturing requires the removal of this cell type alone. The adherent phenotype was used for experimental purposes. Cells were harvested and sub-cultured every seven days. The F12-K media and suspended floating cells were removed by decanting. The remaining adherent population was removed from the culture vessel by three lightly agitated washings with supplemented F12-K media accompanied with mild scraping. The washing media were recovered and centrifuged at 200 × g for 5 minutes. The culture reproduced optimally when the floating cells existed at cell densities of 2 - 4 × 10^5 cells / ml. Differentiation to the adherent phenotype approximately preserves this cell density. Cells were sub-cultured in 10 ml of supplemented F12-K media. Volumes should be preserved as larger volumes may results in a greater number of cells remaining in suspension.

2.2.6 SUB-CULTURING OF RAW 264.7 CELLS

RAW 264.7 cells were sub-cultured in supplemented DMEM. RAW 264.7 cells were harvested and sub-cultured when growth became confluent i.e. after 4 days of culture when the cell density reached approximately 1 × 10^6 cells / cm^2. Due to the adherent nature of RAW 264.7, cells were harvested mechanically (scraping), centrifuged at 500 × g for 5 minutes and suspended in supplemented DMEM at 4 × 10^5 cells / cm^2.

2.2.7 ISOLATION AND CULTURING OF HUMAN PERIPHERAL BLOOD MONOCYTES

Human peripheral blood monocytes were isolated, purified, and cultured as previously described (Pinot *et al*, 1999). Briefly, monocytes were isolated from buffy coats from normal donors whose blood were collected in heparinised test tubes. Buffy coats were mixed with Hanks Balanced Salt Solution (HBSS) at 1:1 ratio followed by Ficoll-hypaque centrifugation, 600 × g for 20 minutes at RT (Sigma-Aldrich Company Ltd, Dorset, U.K.). The leukocyte-rich fraction (settled at the Ficoll-hypaque surface) was removed, washed twice in HBSS and finally resuspended at 1×10^7 cells / ml in supplemented RPMI medium. Suspensions of 1 ml were plated in 35 mm Petri dishes (Falcon, BD, U.K.). Monocytes were purified by washing off the non-adherent cells after incubation for 90 minutes, at 37°C in a 5% CO_2 humidified atmosphere.

2.2.8 DETERMINATION OF CELL NUMBER

A haemocytometer with improved Neubauer ruling, a glass slide containing two chambers and a flat cover slip was used to estimate cell number. Each chamber consists of a grid of known volume (0.1 mm^3 or 0.1 µl) etched on glass. Briefly, 15 µl of an aliquot of evenly suspended cells was applied to both chambers. Using a light microscope cells were counted in each grid, a mean cell count was taken and multiplied by 10^4 to obtain a count of cells / ml. The cell count was performed in duplicate.

2.2.9 FREEZING OF CELLS

MM6 cells were suspended at approximately 50×10^6 cells / ml in 4% DMSO (v/v) in FBS. NR8383 or RAW 264.7 cells were suspended at approximately 6×10^6 cells / ml in 9% DMS0 (v/v) in FBS. The cells were transferred to a sterile cryotube and initially frozen at a slow rate in an insulated freezing box at -70°C for 24 hours. The cryotube was transferred to liquid nitrogen for long term storage.

2.2.10 THAWING OF CELLS

Cryogenic ampoules containing the cell line of interest were removed from liquid nitrogen storage and thawed rapidly in a pre-warmed water bath at 37°C. Thawed cells were washed in 10 ml of pre-warmed non-supplemented culture media to remove the cryo protective DMSO. A small aliquot of cells was removed to determine cell number and cell viability. The cells were centrifuged at $500 \times$ g for 5 minutes followed by a further washing and centrifugation. MM6 or RAW 264.7 cells were suspended in supplemented RPMI 1640 medium at a density of 4×10^5 cells / ml. NR8383 rat alveolar macrophages were suspended in supplemented F12-K at a density of 3×10^5 cells / ml. Once the growth of the cells was established, sub-culturing was performed.

2.3 PREPARATION OF SURFACTANT AND LIPID MEDIA

2.3.1 PREPARATION OF SILANISED GLASSWARE

Bijou vials were silanised in order to improve the recovery of lipid materials reconstituted in these vessels. Glass Bijou vials purchased from Laboratory Sales Ltd. (Rochdale, U.K.) were washed overnight in 1M HCl. Following acid washing, the Bijou vials were rinsed and washed overnight in distilled water. The vials were dried at room temperature (RT) for 2 - 3 days. The interior of each vial was silanised with RepelCote (BDH, Dorset, U.K.), capped and autoclaved by heating to 121°C at 105 kPa (15 psi).

2.3.2 PREPARATION OF LIPID MEDIA

The following products were all purchased from Sigma-Aldrich Company Ltd (Dorset, U.K.) and derived from egg yolk unless otherwise stated. Further each product was stated to be at least 99% pure: Phosphatidylcholine (tPC; type XVI-E) and phosphatidylethanolamine (tPE; type III) are natural products for which the fatty acid composition will vary; 1,2-dipalmitoyl phosphatidylcholine (DPPC); 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC); phosphatidylinositol (PI; from bovine liver); phosphatidylserine (PS; from bovine brain); sphingomyelin (SM) and cholesterol (Chol). Phosphatidylglycerol (PG) was derived from egg yolk tPC by the action of cabbage phospholipase D in the presence of glycerol.

The physiological concentrations of PC within the lung have been previously estimated to be between 100 – 300 µg / ml (Hayakawa *et al*, 1989). The concentrations of phospholipids utilised were approximately those encountered by monocytes or alveolar macrophages within the lung. The desired amounts of lipids were dissolved and / or diluted in the silanised vials in chloroform. These dilutions were dried as a thin film under nitrogen on ice and stored in the dark at -70°C. Lipid preparations were hydrated in supplemented medium and sonicated (Sanyo, Soniprep) on ice to minimise potential oxidation and breakdown of the lipid.

Non-surfactant or "in house" lipid mixture was prepared by mixing the following lipids as a percentage of 10, 100 or 500 µg / ml in a silanised vial. The lipids in artificial surfactant included 69% tPC, 5% PE, 7% PG, 3% PI, 3% PS, 6% SM and 7% cholesterol. These ratios have been previously reported to occur in natural surfactant (Harwood, 1987). This artificial lipid surfactant was then dried as a thin film under nitrogen on ice and stored in the dark at -70°C. Lipid preparations were hydrated as above.

2.3.4 PREPARATION OF COMMERCIAL SURFACTANTS

Survanta® purchased from Abbott Laboratories Kent, U.K., is a sterile, non-pyrogenic bovine lung extract containing DPPC, palmitic acid and tripalmitin. The solution contains phospholipids (25 mg / ml), neutral lipids (1.15 mg / ml), fatty acids (2.4 mg / ml) and surfactant associated proteins (<1.0 mg / ml). Curosurf® purchased from Serono Pharmaceuticals Limited, Middlesex U.K. consists of a sterile suspension containing phospholipids (80 mg / ml) from porcine lung. This natural modified

surfactant contains 99% polar lipids with > 40% DPPC and 1% hydrophobic associated proteins (SP-B, SP-C). The extraction procedure excludes the hydrophilic SP-A (Pinot *et al*, 1999). Exosurf Neonatal™ (Exosurf) obtained from GlaxoWellcome, Hertfordshire, U.K. was supplied as a vial of white sterile freeze-dried powder containing colfosceril palmitate (125 mg / ml of chemically modified DPPC). Exosurf was reconstituted with preservative and endotoxin-free sterile water. The desired dilutions of each surfactant were made with supplemented RPMI media and used within 8 hours of reconstitution.

2.4   DETERMINATION OF CELL VIABILITY

Determination of cell viability was performed prior to sub-culturing of cells and prior to and following experimental investigation. Cell viability was assessed by the exclusion of the dye trypan blue and the CellTiter 96® AQ$_{ueous}$ one solution proliferation assay purchased from Promega, U.K. The CellTiter 96® AQ$_{ueous}$ one solution proliferation assay is a colorimetric assay for the determination of living cells in culture. The reaction involves the bio-reduction of the novel tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), to a coloured formazan product accomplished by dehydrogenase enzymes found in metabolically active cells.

2.4.1   TRYPAN BLUE DYE EXCLUSION

Equal volume of cell suspension and 1% trypan blue (0.085g NaCl and 0.1g trypan blue in 10 ml distilled water) were incubated together for 15 minutes at RT before

microscopic observation. The percentage of cells remaining unstained correlated to the percentage of viable cells within the culture.

2.4.2   CELLTITER 96® AQ~ueous~ ONE SOLUTION PROLIFERATION ASSAY

Each culturing condition was tested for cell viability with this method, in triplicate. Briefly, 20µl of CellTiter 96® AQ~ueous~ one solution reagent was added to each well of a 96 well assay plate containing samples (5×10^3 cells) in 100µl of culture medium. The plate was incubated for 1 - 4 hours at 37°C in a humidified 5% CO_2 atmosphere. Optical densities measured at 490 nm were proportional to the quantity of formazan product and directly proportional to the number of living cells in culture. Results are reported as a mean percentage of control.

2.5   PREPARATION OF STIMULANTS

Zymosan A (cell wall extract from *Saccharomyces cerevisiae*), phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) from *E. coli* (serotype O111:B4) were purchased from Sigma-Aldrich Company Ltd. (Dorset, U.K.). PMA was dissolved in DMSO at a stock concentration of 1 mg / ml and working dilutions of 100 ng / ml were made in PBS. LPS was dissolved in endotoxin-free water at a stock concentration of 20 µg / ml.

An opsonised zymosan (OpZ) suspension was prepared according to a modified method as previously reported by (Allen, 1986). Zymosan A (500 mg) was suspended in 200 ml of sterile saline. The suspension was boiled for 20 minutes followed by cooling to 22°C. The suspension was centrifuged at $500 \times g$ for 15 minutes and the supernatant

decanted. The zymosan pellet was re-suspended with 200 ml of heat inactivated fresh or fresh-frozen pooled human sera (Welsh Blood Transfusion Service). The suspension was gently agitated at 22°C for 30 minutes followed by centrifugation at 500 × g for 15 minutes. The supernatant was discarded and the pellet re-suspended with an additional 200 ml of fresh sera and incubated as previously described. The opsonised zymosan pellet was washed with 200 ml of sterile saline and centrifuged at 500 × g for 15 minutes. The supernatant was discarded and the pellet was resuspended in sterile saline (2.5 mg / ml) and stored frozen at -70°C.

2.6   LIMULUS AMOEBOCYTE LYSATE (LAL) ASSAY FOR ENDOTOXIN

All reagents including surfactant and lipid preparations were tested for contamination levels of endotoxin by the kinetic LAL assay (KQCL), purchased from Biowhittaker U.K. Ltd. (Wokingham, U.K.). Care was taken at every stage of this assay to avoid contamination with endotoxin, i.e. endotoxin-free materials were used. The assay was performed according to the manufacturer's instruction. The priciple of the assay involves a pro-enzyme isolated from LAL which reacts with endotoxin and subsequently the pro-enzymes becomes activated. The active enzyme releases p-nitroaniline (pNA) from a synthetic substrate producing a yellow colour. Briefly, endotoxin standard was reconstituted in pyrogen-free water and adjusted to 50 endotoxin units (EU) / ml, where 1 EU approximates to 100 pg of endotoxin. This suspension was vortexed for 5 minutes prior to use. The standard was serially diluted to give standard values of 0.005, 0.05 and 0.5 EU / ml. Each standard and test (50 μl)

were performed in duplicate; pipetted into wells of a microtiter plate. The plate was placed in the KQCL reader and set to warm up to 37°C. After warm up, the LAL reagent was reconstituted by adding 2.6 ml of pyrogen-free water. To each well 50 μl of reconstituted LAL reagent was added and readings were taken for 100 minutes. Unknowns were determined from the standard curve.

2.7   LUMINOL ENHANCED CHEMILUMINESCENCE (LCL)

LCL according to the method of Allen *et al*, 1986, with minor modifications was used to quantitate the release of reactive oxygen intermediates (ROIs). LCL was detected at RT using a standard luminometer. Experiments were performed in quadruplicate and each experiment was repeated three times. Following treatment, the cells were washed twice with phosphate buffered saline (PBS) and suspended in standard buffer (endotoxin free water containing 4.58 mM KH_2PO_4, 8.03 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.45 mM $CaCl_2$ and (*w/v*) 0.76% NaCl, 0.033% KCl, 0.1% glucose, 0.1% endotoxin-free albumin 0.1%, pH 7.3) at 2×10^6 cells / ml. To every ml of cell suspension (2×10^6 cells / ml) 100 μl of a 3.5 mM solution of luminol (Sigma-Aldrich Company Ltd., Dorset, U.K.) was added. Following gentle mixing and a 10 minute adaptation period in the dark, 150 μl cell / luminol suspension were transferred to a 96 well plate (FluoroNunc-PolySorp, Gibco, Paisley, U.K.). LCL was initiated by the addition of 50 μl of OpZ (125 μg / ml) or PMA (100 ng / ml) in appropriate wells. Temporal traces of evolving chemiluminescent reactions were recorded. There was no appreciable light emission in this system in the absence of cells or stimulants. LCL results are expressed as relative light units (RLUs) or as in equation 3.1:

$$\% \text{ of control } = \frac{\text{Peak LCL response of experimental group}}{\text{Peak LCL response of control group}} \times 100 \qquad \textbf{(Equation 3.1)}$$

2.7.1 OPTIMISATION OF LCL IN MM6 CELLS

The respiratory burst activity of MM6 cells vary according to the conditions of cell culture. A series of investigations were performed to provide optimal conditions for the production of ROIs by MM6 cells and hence the subsequent LCL. Lipopolysaccharide (LPS) was used as a 'priming' agent for respiratory burst activity and OpZ or PMA were the agents employed to stimulate NADPH oxidase, the enzyme responsible for the production of superoxide.

2.7.1.1 'PRIMING' OF MM6 CELLS WITH LPS FOR ROI PRODUCTION

MM6 cells at a density of 1×10^6 cells / ml were incubated with 100 ng / ml LPS for 0, 1, 3, 6, 12 or 18 hours. Alternatively, MM6 cells were incubated with 0, 0.01, 0.1, 1, 10, 100 or 1000 ng / ml LPS for 18 hours. LCL was performed as described in section 2.7.

2.7.1.2 STIMULATION OF THE RESPIRATORY BURST WITH OpZ OR PMA

MM6 cells at a density of 1×10^6 cells / ml were 'primed' with 100 ng / ml LPS for 18 hours at 37°C in a humidified atmosphere with 5% CO_2. LCL was performed as described in section 2.7 but stimulation of the respiratory burst occurred with 0, 39, 78, 156, 312 or 625 µg / ml of OpZ or 0, 16, 31, 62.5, 125, 250 ng / ml of PMA.

2.7.2   QUENCHING EFFECT OF SURFACTANT OR PHOSPHOLIPIDS ON LCL

Chemiluminescence was performed on MM6 cells suspended in standard buffer containing phospholipids in order to determine whether phospholipids interfere / quench LCL. MM6 cells (1×10^6 cells / ml) were 'primed' with 100 ng / ml LPS for 18 hours at 37°C in a humidified atmosphere with 5% CO_2. Following 'priming', cells were washed in PBS ($\times 3$) and re-suspended in standard buffer containing 100 or 1000 µg / ml of tPC, DPPC, PAPC, Survanta®, Curosurf®, or cholesterol. Controls were obtained from MM6 cells treated in an identical manner with LPS but suspended in standard buffer in the absence of lipid. LCL was elicited with OpZ as in section 2.7.

2.7.3   EFFECT OF SURFACTANT ON ROI PRODUCTION IN MM6 CELLS

To test the effect of surfactant on the production of ROIs in monocytes, MM6 cells (1×10^6 cells / ml) were incubated with 0, 20, 100 or 500 µg / ml of Survanta®, Curosurf®, or Exosurf Neonatal™ in supplemented RPMI media for 2 hours. Following incubation the cells were washed in PBS ($\times 3$) followed by suspension in RPMI media and incubation with 100 ng / ml LPS for 18 hours. LCL was assayed as described in section 2.7.

2.7.4   EFFECT OF NON-SURFACTANT LIPIDS ON ROI PRODUCTION IN MM6 CELLS

MM6 cells (1×10^6 cells / ml) were incubated with 0, 20, 100 or 500 µg / ml of in-house lipid mixture in supplemented RPMI media for 2 hours. Following incubation the cells were washed in PBS ($\times 3$) followed by suspension in RPMI media and incubation with 100 ng / ml LPS for 18 hours. LCL was assayed as described in section 2.7.

2.7.5   **EFFECT OF PHOSHOPHOLIPIDS ON ROI PRODUCTION IN MM6 CELLS**

To determine and characterise the source of any modulatory effect of surfactant phospholipids, MM6 cells at 1×10^6 cells / ml were incubated with 0, 10, 125, 250 or 500 μg / ml of tPC, tPE, cholesterol, SM or PG for 2 hours in supplemented RPMI media. Following phospholipid or lipid incubation the cells were washed in PBS (×3) followed by suspension in media and incubation with LPS (100 ng / ml) for 18 hours. LCL was assayed as described in section 2.7 using OpZ as the stimulus.

2.7.6   **EFFECT OF PC ON ROI PRODUCTION IN MM6 CELLS CULTURED IN SERUM-FREE CONDITIONS**

The effects of exogenous supplied phospholipids on NADPH oxidase activity in MM6 cells cultured under serum-free conditions was determined since unidentified serum lipid component(s) may also contribute to the modulation of this enzyme. Under serum-free conditions, dose and time course studies of exogenous lipid effects on ROI production were performed. MM6 cells at 1×10^6 cells / ml were incubated with 125 μg / ml tPC, DPPC or PAPC for 0, 0.25, 0.5, 1, 3, 6, 12, 18 or 24 hours. MM6 cells at 1×10^6 cells / ml were incubated with 0, 1, 10, 62, 125, 250 or 500 μg / ml of tPC, DPPC or PAPC for 2 hours. Following lipid incubation the cells were washed in PBS (×3), suspended in UltraCULTURE™ media at 1×10^6 cells / ml and incubated with 100 ng / ml LPS for 18 hours. LCL was elicited with OpZ as in section 2.7. MM6 cells at 1×10^6 cells / ml were incubated with 125 μg / ml of tPC, DPPC and PAPC for 2 hours. LCL was assayed as described in section 2.7 but stimulated with PMA only.

2.7.7   DURATION OF THE EFFECT OF PC ON ROI PRODUCTION

To see if the effects of PC on ROI production were reversible, MM6 at 1×10^6 cells / ml were incubated with 100 μg / ml of DPPC or tPC or 125 μg / ml PAPC for 2 hours. These concentrations of lipid and the time of incubation were previosuly found to be optimal for inhibiting or enhancing ROI production. Following lipid incubations, cells were washed in PBS (×3) and suspended in fresh RPMI media for a further 24 or 36 hours. Finally, cells were washed once in PBS and 'primed' with 100 ng / ml LPS for 18 hours. LCL was assayed as described in section 2.7.

2.7.8   EFFECT OF LONG TERM DPPC EXPOSURE ON MM6 CELLS

Since surfactant is present all the time and alveolar macrophages are constantly bathed in this fluid, the long term culturing of MM6 cells with DPPC was tested. MM6 cells at 0.5×10^6 cells / ml were incubated with 100 μg / ml of DPPC for 3, 6 or 9 days. Every three days the cells were passaged as described in section 2.2.3 into fresh media containing 100 μg / ml of DPPC. Following lipid incubations, cells were washed in PBS (×3) and suspended in RPMI media (1×10^6 cells / ml) and 'primed' with 100 ng / ml LPS for 18 hours. LCL was assayed as described in section 2.7.

2.7.9   EFFECT OF DPPC ON ROI PRODUCTION IN OTHER CELL SYSTEMS

To validate the effects of DPPC in other inflammatory cells, studies on the effect of DPPC on ROI production were extended to include human peripheral blood monocytes, mouse macrophages and rat alveolar macrophages. Human peripheral blood monocytes were isolated from normal blood donors (section 2.2.7), Raw 264.7 and NR8338

macrophages were incubated with 10, 100 or 500 μg / ml DPPC for 2 hours at 37°C in a humidified atmosphere with 5% CO_2. LCL was assayed as described in section 2.7.

2.7.10 EFFECT OF PC ON ROI PRODUCTION FOLLOWING LPS 'PRIMING'

To determine whether DPPC modulates ROI production following LPS treatment, LCL studies were repeated as before except that DPPC was added to the cells after they were 'primed' with LPS. MM6 cells at 1×10^6 cells / ml were 'primed' with 100 ng / ml LPS for 18 hours. Cells were washed in PBS (×3) and incubated with 100 μg / ml of DPPC for 2 hours. LCL was assayed as described in section 2.7.

2.8 PREPARATION OF [^{3}H] LPS

In order to test whether DPPC could prevent the binding of LPS to MM6 cells, a radioassay for the determination of tritiated LPS binding to MM6 cells was performed. The assay was initially optimised prior to testing the effects of DPPC on LPS binding. LPS from *E. coli* (serotype O111:B4) was biosynthetically radiolabelled with tritium [^{3}H] according to the method of Haeffner-Cavaillon *et al*, 1982. 10 mg of purified LPS was dissolved in 1ml of 70 mM (14.9 mg / ml) sodium periodate (oxidising agent), and incubated for 2.5 hours at RT. LPS was precipitated by the addition of 20 ml of ice-cold ethanol and the mixture was left for 30 minutes at 4°C. The precipitated LPS was collected by ultracentrifugation at 20,000 × g for 15 min at 4°C. The LPS pellet was re-suspended in 1 ml of ice cold water mixed with 40 μl of ethylene glycol (anti-freeze agent). The LPS mixture was treated with 5 mCi of tritiated sodium borohydride (NaB^{3}H$_4$) (Dupont NEN, Hertfordshire, U.K.) which was diluted with 500 μl of the

reducing agent $NaBH_4$ (4 mg / ml). The mixture was then incubated at 4^oC overnight. A further 500 µl of $NaBH_4$ (6 mg / ml) was added to the mixture and left for a further 30 min at 0^oC. Two drops of acetic acid was added to inactivate the excess sodium borohydride. To remove boric and acetic acid, 4 ml of methanol was added to the mixture and then removed by evaporation under nitrogen gas. 2.5 ml amounts of radiolabelled LPS solution were desalted using a Sepharose PD-10 column (50 × 15mm) (Pharmacia Biotech, Hertfordshire U.K.). The radiolabelled LPS had a specific activity of 1 µCi / mg LPS and an LPS concentration of 50 µg / ml.

2.8.1 BINDING OF RADIOLABELLED LPS TO MM6

1×10^6 MM6 cells were washed in PBS and re-suspended in 200 µl of supplemented RPMI media. The cells were transferred to 1.5 ml Eppendorf tubes and incubated with various concentrations of [^{3}H]LPS (0 - 10 µg / ml; prepared as above). The tubes were gently mixed and incubated at 4^oC for different times (0 - 3 hours). Following three washes in ice-cold PBS to remove unbound LPS, the tubes were spun at 500 × g for 5 minutes. The cell pellet was suspended in 200 µl PBS and transferred into 5 ml of scintillation fluid and the bound radiolabelled LPS was measured using a rack-beta scintillation counter (LKB, Wallac, Sweden).

2.8.2   CALCULATION OF LPS BINDING TO MM6 CELLS

The number of LPS molecules bound per cell (Equation 3.2) was calculated from the specific activity of the [^{3}H]LPS, assuming an average molecular mass of 4 kDa for LPS (Kitchens *et al*, 1992). Measurements were made on triplicate samples.

$$Z = \frac{\text{Specific Activity} \times \text{Measured CPM} \times 6.23 \times 10^{23}}{4000 \times (10^6) \times (10^9)} \quad \textbf{(Equation 3.2)}$$

Where:

- Z is the number of LPS molecules bound per cell
- Specific Activity of [^{3}H]LPS represents LPS concentration (ng / ml) divided by total counts per minute (CPM).
- Measured CPM is total CPM of sample.
- The cell count was 10^6 and 10^9 equate to grams of LPS.
- The assumed average molecular mass of LPS is 4 kDa.
- 6.23×10^{23} is Avogadro's constant.

2.8.3   EFFECT OF ANTI-CD14 AND UNLABELLED LPS ON BINDING OF RADIOLABELLED LPS TO MM6 CELLS

MM6 cells at 1×10^6 cells / ml were incubated with 10 µg / ml *E. coli* LPS or Anti-CD14 antibodies (clones UCHM-1, Sigma-Aldrich Company Ltd, Dorset, U.K. or LeuM3, Becton-Dickinson, U.K.) for 1 hour at 4°C. These conditions were previously determined to be optimal for LPS binding to MM6 cells. Following incubation, cells were washed in PBS (×2). [^{3}H]LPS binding was assessed as described in section 2.8.1.

2.8.4   **EFFECT OF DPPC ON BINDING OF RADIOLABELLED LPS TO MM6 CELLS**

MM6 cells at 1×10^6 cells / ml were incubated with 0, 10, 100 or 500 µg / ml of DPPC for 2 hours. Following lipid incubations, cells were washed in PBS (×3). LPS binding was assessed with 10 µg / ml tritiated *E. coli* LPS as in section 2.8.1. This concentration of LPS was chosen as it gave the optimal binding of LPS to MM6 cells under these conditions.

2.9   PHENOTYPIC QUANTIFICATION OF Fcγ, COMPLEMENT AND CD14 RECEPTORS ON MM6 CELLS BY FLOW CYTOMETRY

Fcγ, complement and CD14 receptor expression were investigated to determine whether DPPC inhibits the production of ROIs by reducing the expression of one or more of these receptors that are important for bactericidal functions for monocytes (Allen and Aderem, 1996). MM6 cells were labelled with monoclonal antibodies to human cell surface receptors using a modification of a method described earlier (Van Weyenbergh *et al*, 1998). Purified mouse anti-human monoclonal antibody to CD14 (clone UCHM-1), and R-Phycoerythrin (PE) conjugated goat anti-mouse IgG were purchased from Sigma-Aldrich Company Ltd (Dorset, U.K.). Purified mouse anti-human monoclonal antibodies to CD11b/MAC-1 (clone ICRF44), CD64 (clone 10.1) and CD16 (3G8) were purchased from Pharmingen, Becton Dickinson, U.K.

2.9.1 OPTIMISATION OF PRIMARY ANTIBODY BINDING

The monoclonal antibodies to CD11b/MAC-1 (receptor for iC3b), CD14 (the LPS receptor), CD16 (FcγIII) and CD64 (FcγI) were incubated (separately) with 0.5×10^6 MM6 cells in a titre of 1 part antibody to 1 part wash buffer (PBS with 1% endotoxin free BSA) i.e. 1:1 for 1 hour on ice. The following titres were also set up:- 1:2, 1:3, 1:10, 1:20, 1:100 or 1:200. The cells were washed and resuspended in 100 µl of ice cold washing buffer. Detection of unconjugated bound monoclonal antibodies were detected by staining with 1:10 diluted R-PE conjugated secondary antibody for 30 minutes on ice in the dark. Following incubation cells were washed in buffer and analysed on immediately on the flow cytometer (section 2.9.4).

2.9.2 OPTIMISATION OF CONJUGATED ANTIBODY BINDING

The optimal titre of each monoclonal antibody tested in 2.9.1 were incubated with 0.5×10^6 MM6 cells in wash buffer for 1 hour on ice. The cells were washed with ice cold washing buffer and re-suspended in 100 µl of ice cold washing buffer. Detection of unconjugated bound monoclonal anti-human antibody was detected by staining with 1:10, 1:20, 1:33 or 1:100 of R-PE conjugated secondary antibody for 30 minutes on ice in the dark. Control cells of secondary antibody at each titre were also performed. Following incubation cells were washed in buffer and analysed on the flow cytometer immediately (section 2.9.4).

2.9.3   **EXPRESSION OF FCγ, COMPLEMENT AND CD14 RECEPTORS FOLLOWING DPPC INCUBATION**

MM6 cells at 1×10^6 cells / ml were incubated with 0, 10, 100 or 500 µg / ml DPPC for 2 hours at 37°C with 5% CO_2. Following lipid incubation, cells were washed with PBS (×3) and 0.5×10^6 MM6 cells were suspended in supplemented RPMI. Saturating amounts of monoclonal antibodies to CD11b / MAC-1, CD14, CD16, or CD64 as determined in section 2.9.1 were added for 1 hour on ice. The cells were washed with ice cold washing buffer (PBS with 1% endotoxin free BSA) and re-suspended in 100 µl of ice cold washing buffer. Detection of monoclonal antibodies was carried out by staining with 1:10 titre (as determined in section 2.9.2) R-PE conjugated secondary antibody for 30 minutes on ice in the dark. Control cells of secondary antibody were also performed. Following incubation cells were washed in buffer and analysed immediately on the flow cytometer (section 2.9.4).

2.9.4   **FLOW CYTOMETRIC MEASURMENT AND ANALYSIS**

The measurements were performed with a FACScan single laser at 488 nm (Becton Dickinson, U.K.). Fluorescence parameters from single cells were collected using a logarithmic amplifier. Red fluorescence from R-PE conjugated goat anti-mouse IgG was collected through the FL2 channel. 10,000 cells were analysed per sample and data acquired in acquisition to analysis mode processed using CellQuest (Becton Dickinson, U.K.). Flow cytometric data analysis was performed using WinMDI version 2.8 (Joseph Trotter, Scipps Institute, U.S.A.). The fluorescence distribution was displayed as a single histogram. Results are expressed as median fluorescence intensity (MFI ±

1SD). The secondary antibody was used to set the background fluorescence levels. The experiments were performed in triplicate.

2.10 ELECTRON MICROSCOPY OF MM6 ULTRASTRUCTURE

The ultra structure of MM6 cells following DPPC treatment was investigated by electron microscopy. MM6 cells (1×10^6 / ml) were preincubated with DPPC at 10, 100 or 500 µg / ml for 2 hours at 37°C. Controls were incubated without DPPC. The cells were washed in PBS (×3) and processed for electron microscopy studies as follows. All processing was carried out at room temperature unless otherwise stated.

2.10.1 FIXATION

After respective treatments, cells in suspension of PBS (1×10^6 cells) were fixed in 2.5% (*v/v*) glutaraldehyde in 0.1M sodium phosphate buffer pH 7.4 (Agar Scientific, Stansted, U.K.) for 3 hours. The cells were washed for 10 minutes in double distilled water and centrifuged at 600 × g for 5 minutes. The washing process was repeated twice followed by incubation in 2% (*w/v*) osmium tetroxide in veronal acetate buffer pH 7.4 (Agar Scientific, Stansted, U.K.) for 2 hours. The cells were washed as previous and processed for dehydration and infiltration (Palade, 1952).

2.10.2 DEHYDRATION AND INFILTRATION

The cells were dehydrated in a graded series of ethanol. Cells were dehydrated in 30%, 50%, 70% and 90% ethanol for 10 minutes at each step. A final dehydration step of 100% ethanol for 15 minutes (×2) was performed. Following dehydration MM6 cells were incubated with 50% (v/v) LR White monomer in 100% ethanol (London Resin Company, reading U.K.) for 15 minutes followed by 30 minutes (×4) in LR White monomer.

2.10.3 EMBEDDING

Size O gelatin capsules were pre-cooled in an aluminium heat sink on ice. Embedding solution was prepared by adding 15 μl of LR White accelerator to 10 ml LR White monomer at 0°C and stirred for 30 seconds. Aliquots of embedding mixture (0.65 μl) were added to pre-cooled capsules. Samples were inserted, capsules sealed and the whole apparatus was placed in a fridge at 2°C overnight. Blocks were removed from the heat sink and cured for 2 hours at 50°C (Newman, 1989).

2.10.4 ULRAMICROTOMY AND STAINING

Ultra-thin sections (80 – 100 nm) were cut using a Reichert-Jung Ultracut E ultramicrotome (Reichert-Jung, Austria) and sections were collected on the shiny side of 300 mesh hexagonal copper grids (Agar Scientific, Stansted, U.K.). Grids were passed through 50 μl drops of staining and wash solutions as follows:- 2% (w/v) uranyl actetate in double distilled water for 3 minutes, washing twice with double distilled water for 30 seconds and staining in Reynold's lead citrate for 1 minute. The grids were

finally washed in double distilled water for 30 seconds (×3) and allowed to air dry (Reynolds, 1963).

2.10.5 ELECTRON MICROSCOPY

After drying, the grids were viewed at 80kV in a Phillips CM12 transmission electron microscope (Phillips Electron Optics, Cambridge U.K.). Negatives were digitised with an Astra 1200S scanner (UMAX Ltd., Willich Germany). The electron microscopy was kindly performed by the Medical Microscopy Sciences Department, University of Wales College of Medicine, Heath Park, Cardiff.

2.11 QUANTITATION OF UPTAKE OF PC INTO MM6 MEMBRANE BY HPLC

Quantitation of MM6 membrane lipids was kindly performed by Mr R.H.K. Morris, School of Applied Sciences, University of Wales Institute, Cardiff U.K. MM6 cells prepared at a density of 1×10^6 / ml were preincubated with 250 μg / ml DPPC for 2 hours at 37°C in a 5% CO_2 atmosphere. Cells were washed in PBS (×3) and membrane phospholipids were extracted using a modification to the method of Bligh and Dyer, 1959. The separation of membrane phospholipids was determined by high performance liquid chromatography (HPLC). All HPLC equipment including a GBC LC1150 gradient pump, a Rheodyne 10 μl injection loop and a data catcher unit were purchased from Polymers Laboratories, Cheshire, U.K. A 10 cm silica gel column (Jones Chromatography, Hengoed, Wales) was used as the stationary phase. The mobile phase

consisted of a methanolic ammonium hydroxide gradient in chloroform utilising a binary gradient that was composed of two solvents A and B:

Solvent A: chloroform / methanol / 30% ammonium hydroxide (80:19.5:0.5)

Solvent B: chloroform / methanol / water / 30% ammonium hydroxide (60:34:5:0.5:0.5)

Major phospholipid species (PG, PE and PC) were separated from MM6 cell membrane total lipids by HPLC and detected by a light scattering evaporating detector (EMD 960) as described previously (Becart *et al*, 1990). The uptake of DPPC into MM6 cell membranes was determined by comparing the ratio of PC:PE in MM6 cells incubated without lipid to the ratio of PC:PE MM6 cells incubated with DPPC.

2.12   MEASUREMENT OF NADPH OXIDASE ACTIVITY IN A CELL-FREE SYSTEM

To investigate if DPPC could directly inhibit the NADPH oxidase, a cell free system was used. Following fractionation of activated cells, the NADPH oxidase is found in the plasma membrane fraction (Bolscher *et al*, 1989). This cell-free system was used to investigate whether DPPC impairs the assembly of the active enzyme. NADPH oxidase activity was measured by electron paramagnetic resonance (EPR) oximetry, as the rate of oxygen consumption by the cytosolic and membranous components of the NADPH oxidase that were isolated from MM6 cells. In addition, chemiluminescence studies were also performed to support EPR data.

2.12.1 FRACTIONATION OF MM6 CELLS

MM6 cells (1×10^8) were fractionated according to Bolscher *et al*, 1989. Unstimulated cells were suspended in sonication buffer (0.34 M sucrose, 10 mM Hepes, 1 mM EGTA, 1 mM PMSF, 100 µM leupeptin in PBS, pH 7.0) at a concentration of 5×10^7 MM6 cells / ml and were sonicated for 3×15 seconds intervals using the Sanyo, Soniprep sonicator. The sonicated suspension was centrifuged for 10 minutes at 160 × g to remove unbroken cells and cell nuclei. The postnuclear supernatant was layered on a discontinuous gradient of 5 ml of 30% (*w/v*) sucrose resting on 10 ml of 50% (*w/v*) sucrose. The gradient was centrifuged in a Beckman SW28 rotor (High Wycombe, U.K.) at 90,000 × g for 2 hours at 4°C in an ultracentrifuge (Beckman L5-65, High Wycombe, U.K.). The application zone, containing soluble oxidase-component was stored at −70°C. The membranes were collected from the interface between the 50% and 30% sucrose layers and stored in small aliquots at −70°C. Both membranes and soluble fractions can be stored in this way for several months.

2.12.2 MEASUREMENT OF OXYGEN CONSUMPTION

EPR spectroscopy is the most sensitive and direct method of detecting and quantifying free radicals. In addition, much information can be gained by the use of stable paramagnetic molecules (probes) whose EPR linewidths are sensitive to molecular motions and the presence of other paramagnetic species. Oxygen, a di-radical, can alter the relaxation rates and hence the linewidths of certain oxygen-sensitive paramagnetic probes in a characteristic manner. EPR oximetry has been used extensively to measure oxygen concentrations *in vitro* and *in vivo* (Swartz *et al*, 1992). The neutral nitroxide,

4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-oxyl (^{15}N PDT; purchased from MSD Isotopes, St Louis, MO, USA), whose line shape and width are very sensitive to oxygen (James *et al*, 1995) was used in this study. Oxygen consumption rates were obtained by measuring oxygen concentration in a closed chamber over time and finding the slope of the resulting linear plot. MM6 membrane (10 μl) and cytoplasmic components (10 μl) (which were equivalent to 1×10^6 MM6 cells) were added to 400 μl of assay buffer (10 mM Hepes containing 0.17 M sucrose, 75 mM NaCl, 0.5 mM EGTA, 1 mM MgCl$_2$, 2 mM NaN$_3$ (pH7.0) and 10 mM guanosine 5'-O-(3-thiotriphosphate)(GTPγS). Assembly of the NADPH oxidase was initiated by the addition of SDS at a final concentration of 100 μM as described by (Bromberg and Pick, 1985). After 3 minutes, ^{15}N PDT and NADPH was added at a final concentration of 0.5 mM and 250 mM respectively. The assay mixture was drawn into a glass capillary tube and sealed at both ends avoiding entrapment of any air bubbles. The EPR linewidth was scanned repeatedly at 60 second intervals for 15 minutes at room temperature using a Varian E104B spectrometer (Palo Alto, USA) operating at 9.5 GHz. Data were acquired using 'In house' data acquisition software. Linewidth measurements were equated with an oxygen concentration (μM) obtained from a standard plot of the ^{15}N PDT probe at various oxygen concentrations between 100% nitrogen and air (210 μM oxygen). Each test was repeated in triplicate. Control samples (lacking SDS and NADPH) were assayed on each day of testing. Results were expressed as the consumption of oxygen with time for each experimental setting.

2.12.3   EFFECT OF DPPC ON OXYGEN CONSUMPTION IN A CELL-FREE SYSTEM

DPPC was added to the membrane and cytosol assay buffer (section 2.12.2) prior to the addition of SDS and NADPH to a final concentration of 100 µg / ml. SDS, ^{15}N PDT and NADPH were added to the mixture and oxygen consumption determined as in section 2.12.2.

2.12.4   EFFECT OF DPPC ON CHEMILUMINESCENCE IN A CELL-FREE SYSTEM

Superoxide production measured by chemiluminescence was used to confirm that DPPC did not affect the activity of NADPH oxidase in the cell-free system. The production of ROI in the cell-free system was significantly lower than the cellular system. Therefore, Diogenes™ a more sensitive, specific yet expensive probe than luminol was used to estimate superoxide production. DPPC was added to standard chemiluminescence buffer (section 2.7) containing the membrane and cytosol fractions to a final concentration of 100 µg / ml. SDS, Diogenes™ (as per manufacturers instruction) and NADPH were added to the mixture and chemiluminescence was performed at room temperature in standard luminometer as in section 2.7. Experiments were performed in quadruplicate and each experiment was repeated three times.

2.13   ANALYSIS OF P44 / P42 AND P38 MITOGEN ACTIVATED PROTEIN KINASES (MAPK)

Since activation of the NADPH oxidase is in part mediated through MAPK signalling, the effect of DPPC on these enzymes was determined. The active forms of MAPK were initially optimised in this study so that peak stimulation of MAPK could be studied. The phosphorylated forms of the p44 / p42 and p38 MAPKs were analysed by commercially available kits (Phosphoplus p44 / p42 MAPK and p38 MAPK Antibody kit; New England Biolabs U.K. Ltd, Hertfordshire, U.K.). Analysis of p42 / p44 and p38 MAPKs were performed according to the manufacturer's instructions with minor modifications.

2.13.1   PREPARATION OF PROTEIN SAMPLES

Following appropriate experimental treatment, 5×10^6 MM6 cells were washed twice in ice cold PBS and lysed for 15 minutes on ice in 100 µl of lysing buffer (100 mM NaCl containing 10 mM Tris-HCL (pH 7.2), 2 mM EDTA, 0.5% (w/v) deoxycholate, 1% (v/v) NP40, 10 mM MgCl$_2$, 1 mM PMSF and 100 µM sodium orthovanadate). Samples were sonicated (Sanyo, Soniprep) for 10 seconds on ice to shear DNA and reduce sample viscosity. Cell lysate samples were centrifuged at 12,000 × g for 5 minutes at 4°C and the lysate supernatant fraction was stored at −70°C. Protein concentration of the soluble cytosolic extract was estimated.

2.13.2   PROTEIN DETERMINATION FOLLOWING DETERGENT SOLUBILISATION

The Bio-Rad DC protein assay purchased from Bio-Rad Laboratories Ltd. Hertfordshire U.K., was used to colorimetrically determine protein concentration following detergent solubilisation. The reaction is a modification of the well-documented Lowry assay (Lowry *et al*, 1951). The assay is based on a reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. Briefly, "working reagent A" was prepared by adding 20 µl of reagent S to each ml of reagent A that would be required to complete the run. To a dry, clean microtiter plate 5 µl of standard (ranging from 0 – 1.5 mg / ml) or sample lysate was added to 25 µl of "working reagent A". This was performed in duplicate. To each well 200 µl reagent B was added followed by gentle mixing and incubation at RT for 15 minutes. Absorbencies were read at 750 nm within 1 hour.

2.13.3   SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (DENATURING SDS-PAGE)

Samples of lysate supernatants were subjected to electrophoresis to separate proteins following the method of Laemmli, 1970. Denaturing gel electrophoresis was performed using the NuPAGE™ electrophoresis system; lithium dodecyl sulphate (LDS) sample buffer, reducing agent, Multimark™ multicolored molecular weight standards and the XCell II™ Mini-Cell and PowerEase™ were obtained from NOVEX (Novel experimental technology, Frankfurt, Germany). This system is based upon a Bis-Tris-HCl buffered (pH 6.4) 10% polyacrylamide 1.0 mm gel run under reduced conditions with 3-(N-morpholino) propane sulphonic acid (MOPS) running buffer. Sample

supernatants, whose protein concentrations were pre-estimated, were adjusted to 5 µg / µl with LDS sample buffer (2 parts sample and 1 part LDS sample buffer), reducing agent and ultra pure water. Samples were vortexed and heated for 5 minutes at 95°C to fully denature the proteins, followed by centrifugation at 13,000 × g for 5 minutes to remove insoluble debris. To each well of the gel, 10 µl of heated sample supernatant was loaded using extra fine tips. Electrophoresis was carried out for 50 minutes at 200 volts (constant current) at RT. Pre-stained molecular weight markers (to verify electroblotting) and biotinylated protein markers were treated and loaded onto the gel according to manufacturers' instructions.

2.13.4   ELECTROBLOTTING OF PROTEINS

Electrophoretic transfer of proteins was performed following 10% (*w/v*) SDS-PAGE, using the XCell II™ blot module and reagents purchased from NOVEX, Frankfurt, Germany. Electrophoresed gels were removed from the bonded plastic casing. The gel / membrane sandwich was constructed according to the manufacturers' instructions and was orientated so the transfer of proteins from the gel to nitrocellulose membrane (0.45 µm pore) would occur in the direction of cathode to anode. The blotting module was placed in the Xcell II™ Mini-Cell tank, transfer buffer was poured into the blotting module, and proteins were electroblotted for 60 minutes with a constant 30 V at RT.

2.13.5   WESTERN IMMUNOBLOT DETECTION OF MAPK PROTEINS

Following, electrophoretic transfer, the nitrocellulose membrane was washed with 25 ml washing buffer (Tris-buffered saline / Tween; 2.4 g Tris base, 8 g NaCl to pH 7.6 with HCl supplemented with 0.1% (v/v) Tween-20,) for 5 minutes at RT. To block non-specific protein binding, the membrane was incubated with blocking buffer (Tris-buffered saline / Tween-20 supplemented with 5% (w/v) non-fat dried milk, Marvel) for 1 hour at RT. The membrane was incubated with a specific antibody to phosphorylated p44 / p42 (Thr202 / Tyr204) or phosphorylated p38 (Thr 180 / Tyr 182) anti-MAPK antibody (1:2000 or 1:1000 dilution in 10 ml blocking buffer respectively). Antibody incubations were performed overnight with gentle agitation at 4°C. The membrane was washed with wash buffer (3 × 5 minute washings in fresh changes of wash buffer). Followed by incubation with horse radish peroxidase (HRP)-conjugated secondary antibody (1:2000 dilution) and HRP-conjugated anti-biotin antibody (1:1000 dilution) in 10 ml of blocking buffer with gentle agitation for one hour at RT. The former antibody detects sample MAPK while the latter antibody is for detection of biotinylated molecular weight markers. The membrane was washed as described previously. Subsequent detection of MAPK was carried out by substrate development using LumiGLO® enhanced chemiluminescence (ECL). The membrane was incubated with 10 ml substrate for 1 minute at RT. The membrane was allowed to drip dry, wrapped in saran wrap and exposed to KODAK X-ray film (Amersham, Buckinghamshire, U.K.). The film was developed with 20% (v/v) developer and fixing agents (Sigma-Aldrich Company Ltd, Dorset, U.K.). The MAPK protein was identified by comparison with pre-stained molecular weight markers and MAPK control protein.

2.13.6   MM6 CELL CULTURE CONDITIONS FOR REDUCED BASAL LEVELS OF MAPK

MM6 cells were cultured under specific conditions in order to reduce basal levels of phosphorylated MAPKs. MM6 cells (0.5×10^6 cells / ml) were cultured in RPMI media supplemented with 0.5% FBS. To reduce basal levels of p44 / p42 MAPK, cultured cells remained in 0.5% FBS / RPMI for 2 days followed by re-suspension at 1×10^6 cells / ml in 0.05% FBS / RPMI for 2 hours at 37°C with 5% CO_2. To reduce basal levels of p38 MAPK, cultured cells remained in 0.5% FBS / RPMI for 1 day followed by re-suspension at 1×10^6 cells / ml in fresh 0.5 % FBS / RMPI for 2 hours at 37°C with 5% CO_2. MM6 cells were then treated appropriately for each investigation.

2.13.7   OPTIMISATION OF PHOSPHORYLATED MAPK EXPRESSION IN MM6 CELLS

2.13.7.1   ACTIVATION OF P44 / P42 MAPK WITH OPZ

MM6 cells (1×10^6 cells / ml) were 'primed' with 100 ng / ml LPS for 18 hours. Cells were washed in PBS (×3) and re-suspended at 1×10^6 / ml in 0% FBS / RPMI media. Phosphorylated p44 / p42 MAPK were activated with OpZ (125 µg / ml) for 0, 10, 20, 30, 40, 50 or 60 minutes. In an independent experiment MM6 cells, treated as above were stimulated with 0, 15, 62, 125, 250 µg / ml OpZ for 60 minutes. Controls samples were incubated without LPS and / or OpZ. Expression of phosphorylated p44 / p42 MAPK was detected as described in section 2.13.

2.13.7.2   ACTIVATION OF P44 / P42 MAPK WITH PMA

MM6 cells were treated as in section 2.12.3.1 but activated with PMA (100 ng / ml) for 0, 5, 10, 15, 30 or 60 minutes. In an independent experiment MM6 cells, treated as above were stimulated with 0, 1, 10, 100, 1000 ng / ml PMA for 10 minutes. Controls samples were incubated without LPS and / or PMA. Expression of phosphorylated p44 / p42 MAPK was detected as described in section 2.13.

2.13.8   EFFECT OF DPPC INCUBATION ON PHOSPHORYLATED MAPK ACTIVATION

2.13.8.1   DETERMINATION OF DPPC EFFECTS ON P44 / P42 MAPK ACTIVATION

MM6 cells (1×10^6 cells / ml) were incubated with 10, 100 or 500 µg / ml DPPC for 2 hours in 0.05% FBS / RPMI media. Following lipid incubation cells were washed ($\times 3$) in PBS and re-suspended in 0.05% FBS / RPMI media and 'primed' with 100 ng / ml LPS for 18 hours. Cells were washed in PBS ($\times 3$) and re-suspended at 1×10^6 / ml in 0.05% FBS / RPMI media. Phosphorylated p44 / p42 MAPK were activated with OpZ (125 µg / ml) for 60 minutes. In an independent experiment MM6 cells, treated as above were stimulated with 100 ng / ml PMA for 10 minutes (instead of OpZ). Controls samples were incubated without LPS and / or OpZ or PMA. Expression of phosphorylated p44 / p42 MAPK was detected as described in section 2.13.

2.13.8.2   DETERMINATION OF DPPC EFFECTS ON P38 MAPK ACTIVATION

MM6 cells (1×10^6 cells / ml) were incubated with 10, 100 or 500 µg / ml DPPC for 2 hours in 0.5% FBS / RPMI media. Following lipid incubation cells were washed (×3) in PBS and re-suspended in 0.5% FBS / RPMI media and 'primed' with 100 ng / ml LPS for 18 hours. Cells were washed in PBS (×3) and re-suspended at 1×10^6 / ml in 0.5% FBS / RPMI media. Phosphorylated p38 MAPK were activated with OpZ (125 µg / ml) for 60 minutes. In an independent experiment MM6 cells, treated as above were stimulated with 100 ng / ml PMA for 10 minutes (instead of OpZ). Controls samples were incubated without LPS and / or OpZ or PMA. Expression of phosphorylated p38 MAPK was detected as described in section 2.13.

2.14   QUANTITATION OF PKC ACTIVITY

To investigate the effects of DPPC on activation of PKC, activity of this enzyme was determined in PMA and OpZ stimulated cells pre-treated with DPPC. The activity of calcium- and phospholipid-dependent protein kinase C (PKC) was quantified using the SignaTECT™ Protein Kinase C (PKC) Assay System (Promega, U.K.). This system involves measuring the transfer of ^{32}P-labelled phosphate to the biotinylated peptide Neurogranin. The biotinylated ^{32}P-labelled Neurogranin is recovered from the reaction mix with the SAM²™ Biotin Capture Membrane, which is a novel streptavidin matrix. Scintillation counting allows for quantification. Analysis of PKC was performed according to the manufacturer's instructions.

2.14.1 PREPARATION OF CELL SAMPLES FOR PKC ASSAY

Following appropriate experimental treatment, 5×10^6 MM6 cells were washed twice in ice cold PBS and suspended in 500 μl ice cold extraction buffer (25 mM Tris-HCl (pH 7.4) containing 0.5 mM EDTA, 0.5mM EGTA, 0.05% Triton® X-100, 10 mM beta-mercaptoethanol, 0.5 mM PMSF, 1 μg / ml leupeptin and 1 μg / ml aprotinin). The suspended cells were sonicated on ice for 30 seconds (3×10 seconds intervals) using the Sanyo, Soniprep sonicator. The lysate was centrifuged at $14,000 \times g$ for 5 minutes at 4°C. The supernatant was passed over a 1 ml column of DEAE cellulose (Whatmann® DE52) that had been pre-equilibrated in extraction buffer. The column was washed with 5 ml of extraction buffer. The PKC containing fraction was eluted from the column using 5 ml of elution buffer (extraction buffer containing 200 mM NaCl). The PKC enzyme activities of all crude extracts were determined on day of sample preparation.

2.14.2 PROTEIN DETERMINATION OF PKC CRUDE EXTRACT

The Bradford protein assay is a simple procedure for determination of protein concentrations in solutions that depends upon the change in absorbance in Coomassie Blue G-250 upon binding of protein (Bradford, 1976). This colorimetric method relies upon binding of the dye to protonated amine groups within the polypeptides present. Briefly, a range of standards between 0 - 500 μg / ml of protein were prepared utilising BSA. The assay was performed in duplicate. 200 μl of sample, standard or blank were added to individual test tubes. To each tube 4 ml of working dye reagent (100 mg Coomassie Blue G-250 in 50 ml 95% ethanol, 100 ml 85% (*w/v*) orthophosphoric acid

made to one litre with distilled water) was added followed by incubation at room temperature for 15 minutes before measurement of absorbance at 620nm. The assay is non-linear at the low end of the standard curve but linear up to 1 mg / ml.

2.14.3 PKC ASSAY PROTOCOL

All reagents except [gamma-^{32}P]ATP were provided in the kit. The frozen reagents were thawed on ice and the PKC Activation 5X BUFFER was vortexed for 10 seconds prior to use. Wearing gloves, the required number of SAM^2™ Biotin Capture Membranes was cut from the pre-numbered sheet provided. A [gamma-^{32}P]ATP mix for 20 reactions was prepared by adding 1 μl of [gamma-^{32}P]ATP (3,000 Ci / mmol) 10 μCi / μl (purchased from Amersham, U.K.) to 100 μl of 0.5 mM ATP. PKC activity of each sample was tested in the presence of phospholipids (activated reaction) and a reaction in the absence of phospholipids (control reaction). The activated PKC reaction was prepared by combining 5 μl of PKC Coactivation 5X BUFFER, 5 μl PKC Activation 5X BUFFER, 5 μl PKC Biotinylated Peptide Substrate and 5 μl [gamma-^{32}P]ATP mix per sample. The control PKC reaction was prepared by combining 5 μl of PKC Coactivation 5X BUFFER, 5 μl 5X Control BUFFER, 5 μl PKC Biotinylated Peptide Substrate and 5 μl [gamma-32P]ATP mix per sample. The activated- and control- PKC reaction mixes for each sample were mixed gently and pre-incubated at 30°C for 5 minutes. The reactions were initiated by adding 5 μl of the crude enzyme extract to the activated and control PKC reactions (total volume per reaction was 25 μl). The reactions were incubated at 30°C for 5 minutes and terminated by the addition of 12.5 μl Termination BUFFER. 10 μl of terminated reaction was spotted onto individual pre-numbered SAM^2™ Biotin Capture Membrane squares. The SAM^2™ Biotin

Capture Membranes were placed into a washing container (following spotting of all samples) and washed for 30 seconds with 200 ml of 2M NaCl. The washing solution was disposed of in accordance with local regulations and a 3 × 2 minutes washing with 200 ml of 2M NaCl was performed. Following appropriate disposal of washing solutions 4 × 2 minutes washings in 200 ml of 2M NaCl containing 1% H_3PO_4 was performed. Brief washings in 100 ml of deionised water (2 × 30 seconds) and a final 15 second 95% ethanol wash was performed. The SAM^{2}TM Biotin Capture Membranes were dried on aluminium foil. The total counts for calculation of the specific activity of [gamma-^{32}P]ATP was determined by spotting 5 μl from any 2 reaction mixes onto individual 3 mm Whatmann filter paper squares. The filter papers were allowed to dry without washing. The SAM^{2}TM Biotin Capture Membranes and Whatmann filter papers were placed into individual scintillation vials containing 5 ml of scintillation fluid. Beta radiation was measured for one minute per sample using a rack-beta scintillation counter (LKB, Wallac, Sweden). A sample of Promega's PKC was diluted 12.5 fold in 0.1 mg / ml BSA and 0.05% Triton$^®$X-100 and included in each assay as a positive control. Each sample was tested in triplicate.

2.14.4   CALCULATION OF PKC ENZYME ACTIVITY

The enzymatic activity of PKC was determined by subtracting the activity of the enzyme in the absence of phospholipids (control reaction) from that of the enzyme in the presence of phospholipids (activation reaction) (Equation 3.3).

$$EA = \frac{(CPM_{\text{activation reaction}} - CPM_{\text{control reaction}}) \times 37.5}{(10) \times (time_{\text{min}}) \times (Y) \times (Z)} \quad \textbf{(Equation 3.3)}$$

Where:

- EA is the PKC enzyme activity in pmol / min / µg of protein.
- 37.5 is the sum of the reaction volume (25 µl) and the Termination BUFFER volume (12.5 µl).
- Sample applied to the SAM^2™ Biotin Capture membrane was 10 µl.
- Time is the length of incubation at 30°C.
- Y is the amount of protein in reaction in µg.
- Z is the specific activity of [gamma-^{32}P]ATP in cpm / pmol ATP calculated from section 2.14.4.1.

2.14.4.1   CALCULATION OF SPECIFIC ACTIVITY OF [GAMMA-^{32}P]ATP

The specific activity of [gamma-^{32}P]ATP in cpm / pmol ATP was calculated from equation 3.4.

$$\text{Specific activity of [gamma-}^{32}\text{P]ATP (cpm / pmol ATP)} = \frac{7.5 \times Z}{2500} \qquad \textbf{(Equation 3.4)}$$

Where:

- 7.5 is the sum of the reaction volume (25 µl) and the Termination BUFFER volume (12.5 µl) divided by the volume (5 µl) applied to 3 mm Whatmann filter paper.
- Z is the average counts / minute of the 5 µl samples applied to SAM^2™ Biotin Capture Membranes.
- 2,500 are the number of pmol of ATP in the reaction.

2.14.5   OPTIMISATION OF PKC ACTIVATION

2.14.5.1   STIMULATION OF PKC WITH PMA

MM6 cells (1×10^6 cells / ml) were incubated with 100 ng / ml PMA for 0, 5, 10, 15, 30 or 60 minutes. Control samples were incubated without PMA but with PBS for 60 minutes. PKC enzyme activity was quantified as described in section 2.14.

2.14.5.2   STIMULATION OF PKC WITH OpZ

MM6 cells (1×10^6 cells / ml) were incubated with 100 ng / ml LPS for 18 hours. MM6 cells were washed ($\times 3$) in PBS and suspended in fresh supplemented RPMI media. Following washing the cells were stimulated with 125 µg / ml OpZ for 0, 15, 30, 45 or 60 minutes. Controls samples were incubated without LPS and OpZ; with LPS but no OpZ and no LPS and OpZ. Equal volume of PBS was added where no LPS or OpZ was used. PKC enzyme activity was quantified as described in section 2.14.

2.14.5.3   EFFECT OF tPC, DPPC OR PAPC ON PKC ENZYME ACTIVITY IN PMA STIMULATED CELLS

MM6 cells (1×10^6 cells / ml) were incubated with 100 µg / ml tPC or DPPC and 125 µg / ml of PAPC for 2 hours. Following lipid incubations, MM6 cells were washed in PBS ($\times 3$) and suspended in fresh supplemented RPMI media. The cells were stimulated with 100 ng / ml PMA for 10 minutes (the optimum stimulation time with PMA as described in section 2.14.5.1). Control cells were incubated without lipids and / or PMA.

2.14.5.4   EFFECT OF tPC, DPPC OR PAPC ON PKC ENZYME ACTIVITY IN LPS

AND OpZ STIMULATED CELLS

MM6 cells (1×10^6 cells / ml) were incubated with 100 μg / ml tPC or DPPC and 125

μg / ml of PAPC for 2 hours. Following lipid incubations, MM6 cells were washed in

PBS (×3) and suspended in fresh supplemented RPMI media. Cells were stimulated

with 125 μg / ml OpZ for 30 minutes (the optimal stimulation time as described in

section 2.14.5.2). Control cells were incubated without lipids and / or PMA.

2.15   DETECTION OF NITRIC OXIDE BY THE GRIESS ASSAY

In living organisms nitric oxide (NO) is produced by oxidation of a guanidino nitrogen,

with L-citrulline as a co product. The reaction is catalysed by NO synthases and

requires NADPH and other cofactors. NO·release from mouse and rat macrophages is

implicated in tumor cell cytotoxicity and the killing of intracellular organisms

(Moncada *et al*, 1991). Evidence, however, suggests that human monocyte-derived

macrophages or myeloid leukemic cells differentiated along the monocytic lineage do

not consistently release NO· (Zembala *et al*, 1994). LPS and / or interferon gamma

(IFNγ) are potent stimulators of macrophage NO· production. NO· causes massive

oxidative damage via reactions of NO·with oxygen (forming strong oxidants including

nitrogen dioxide and peroxynitrites) (Anggard, 1994). DPPC at concentrations of 10,

100 or 500 μg / ml were incubated with 2×10^6 murine macrophages (RAW 264.7) for

2 hours in order to determine whether DPPC modulates the inflammatory actions of

macrophages by production of NO: Following lipid incubations cells were washed in

PBS (×3) and stimulated with LPS (100 ng / ml) and IFNγ (100 units / ml) for 1 hour.

The supernatants were aspirated and centrifuged at 400 ×g for 5 minutes in order to remove any cells.

The direct measurement of NO·production from cells has proved difficult because of its instability. NO·is quickly oxidised to nitrite in solution. It is then possible to correlate the amount of nitrite in solution to NO·already produced. The amount of nitrite in the tissue culture supernatants was measured by incubating 100 µl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine chloride in 5% phosphoric acid) with 100 µl of sample tissue culture supernatant that had previously been incubated for 40 minutes at RT with 10 µl NADPH (10 µM), 10 µl glucose 6-phosphate (5mM), 10 µl glucose 6-phosphate dehydrogenase (1.6 units / ml), 10 µl nitrate reductase (0.8 units / ml) and 10 µl sodium phosphate buffer (140 mM pH 7.4) for 10 minutes at RT in the dark. Following the incubation, the optical density of each sample was measured at 540 nm and compared to the standard curve of sodium nitrite (3.125 to 200 µM). The standards were double diluted in RPMI culture medium. Each sample was tested in triplicate.

2.16 DETERMINATION OF CYTOKINE RELEASE BY ELISA

Cytokines are regulatory proteins that control the survival, growth, differentiation and effector function of tissue cells. Monocytes and macrophages produce and secrete a variety of pro- and anti- inflammatory cytokines when activated or stimulated by other leucokyte products. Some of these cytokines influence the ability of the host to respond to infections (Pascual *et al*, 1997). MM6 cells were therefore incubated with DPPC in

order to determine whether this phospholipid modulates cytokine production in monocytes in response to LPS. The production of TNF-α, interleukin (IL)-1β, 6 and 10 were assayed using an enzyme linked immunosorbant assay (ELISA). The TNF-α, IL-1β and IL-6 cytokine ELISA assays utilised specific paired antibodies (R&D systems, Oxon, U.K.). The IL-10 cytokine assay was purchased from IDS Ltd. (Tyne and Wear, U.K.). These ELISAs are based on the sandwich principle, involving the adherence of the capture antibody to a Maxisorb 96 well-plate (Gibco, Paisley, U.K.). The plates were incubated overnight at RT followed by washing in wash buffer ($\times$3) (PBS / 0.05% Tween-20). The plate was blocked with blocking buffer (PBS / 1% BSA / 5% sucrose) at RT for 1 hour. This was to prevent non-specific binding sites. The plates were washed in wash buffer ($\times$3) followed by the addition of samples and standards. Each standard and sample were applied to the plate in duplicate and the position noted. The plate was incubated at RT for 2 hours followed by washing in wash buffer ($\times$3). A specific biotinylated antibody was added to the wells of the appropriate plate and incubated at RT for 2 hours. The plate was washed with wash buffer ($\times$3) and a 1 / 20,000 dilution of streptavidin-HRP conjugate was added to each well. The plate was incubated at RT for 20 minutes before washing ($\times$3) and addition of substrate, TMB (tetramethylbenzidine) substrate. The plate was incubated for a further 30 minutes at RT in the dark and the reaction was stopped by the addition of 0.5 M sulphuric acid. The absorbance of the solution in each well was determined at 450 nm. A standard curve was constructed and sample concentration determined. The determination of TNF-α in all experiments was performed by a colleague, R.H.K Morris, School of Applied Sciences, University Wales Institute, Cardiff.

2.16.1 DETERMINATION OF TNF-α, IL-1β, 6 AND 10 PRODUCTION IN LPS STIMULATED MM6 CELLS

The release of IL-1β, 6 and 10 from MM6 cells (1×10^6 cells / ml) was stimulated with or without LPS (100 ng / ml) for 10 minutes, 30 minutes, 1, 3, 6, 12, 18, 24 and 36 hours. However, the release of TNF-α was stimulated with 200 ng / ml LPS and 100 ng / ml PMA for 0 – 36 hours. Incubations were performed in triplicate. Following incubation, MM6 cells were centrifuged at $500 \times g$ for 5 minutes and aliquots of culture supernatant were removed and at stored at $-70^{\circ}C$ until required. TNF-α, IL-1β, 6 and 10 were determined as in section 2.16.

2.16.2 EFFECT OF PHOSPHOLIPIDS ON LPS STIMULATED CYTOKINE PRODUCTION

MM6 cells at 1×10^6 cells / ml were incubated with 0, 10, 100 or 500 μg / ml of tPC, DPPC or PAPC for 2 hours. Following lipid incubation, MM6 cells were washed in PBS (×3) and re-suspended in RPMI media. The cells were stimulated with 100 ng / ml LPS and incubated for 18 hours for IL-1β, 6 and 10 determination. For TNF-α determination, MM6 cells were stimulated with 200 ng / ml LPS and 100 ng / ml PMA for 4 hours. These stimulation times were determined to be optimal in previous experiments. Controls were incubated without lipid and / or LPS. All incubations were performed in triplicate. Following incubation, MM6 cells were centrifuged at $500 \times g$ for 5 minutes and aliquots of culture supernatant were removed and at stored at $-70^{\circ}C$ until required. TNF-α, IL-1β, 6 and 10 were determined as in section 2.16.

2.17   STATISTICS

Where appropriate the data were expressed as the mean $\pm$ 1 standard deviation. For multiple group comparisons, the data were subjected to one way analysis of variance (ANOVA) to determine overall difference between the group means and Tukey's honestly significant difference (HSD) for pairwise differences for within group comparisons. Between group comparisons were tested by Mann-Whitney's non-parametric test. Minitab software version 12.0 (Minitab Inc.) was used for all analyses.

CHAPTER 3: RESULTS

3.1   DETERMINATION OF THE VIABILITY OF CELLS TREATED WITH PHOSPHOLIPID

Trypan blue is a simple, cheap and rapid method for the estimation of cell culture viability. Using this method, cytotoxicity studies estimated the viability of MM6, NR8383, RAW 264.7 cells and human peripheral blood monocytes to be greater than 95% at all culture conditions tested. However, the trypan blue dye exclusion assay has previously been reported to significantly overestimate cell viability (Altman *et al*, 1993). Further, the possibility that phospholipids may effect this exclusion assay could not be discounted. Therefore, the estimation of cell viability was confirmed using the CellTiter 96® AQ$_{ueous}$ one solution proliferation assay. This is a colorimetric assay involving the bio-reduction of a novel tetrazolium salt (MTS) to its purple coloured formazan derivative by viable, metabolically active cells. There is a linear relationship between the formazan generated and the number of viable cells present (Mosmann, 1983). The viability of cells treated with surfactant, phospholipids or lipids were not significantly different from control cells with respect to reduction of MTS. Controls were MM6 cells incubated with culture media or PBS (the diluents of lipid and surfactant preparations) under identical conditions. Taken together these results indicate that the concentrations of surfactant and lipids used in this study were not cytotoxic and viability of cells remained greater than 90% in each instance.

3.2   DETERMINATION OF LPS IN REAGENTS

Depending on cell type and culture conditions, lipopolysaccharide (LPS) can have a variety of effects on cell function and growth. There is substantial evidence indicating that nanogram concentrations of LPS may affect experimental outcomes by triggering direct responses from cells including the release of bioactive lipids, cytokines and the 'priming' of cells for enhanced function (Ulevitch and Tobias, 1999). Further, repeated exposure of cells to LPS may lead to hyporesponsiveness or tolerance in which minimal or absent responses of a cell to a ligand or ligands may be seen (Ziegler-Heitbrock, 1995). Therefore cell culture media, sera, lipid preparations, water and all-purpose made reagents were assayed by the kinetic LAL assay to determine levels of LPS. No LPS contaminants could be detected down to sensitivities of less than 0.5 pg / ml.

3.3   DETECTION OF REACTIVE OXYGEN INTERMEDIATES (ROIs) BY LUMINOL ENHANCED CHEMILUMINESCENCE (LCL)

To investigate the hypothesis that phospholipids modulate NADPH oxidase activity in monocytes / macrophages, the activity of this enzyme was estimated by measuring the *in vitro* production of ROIs by the human monocytic cell MonoMac6 (MM6). The study evaluated the production of ROIs by LCL in MM6 cells activated with opsonised zymosan (OpZ) or phorbol 12-myristate 13-acetate (PMA) following LPS 'priming'. This model was initially optimised prior to testing the effects of phospholipids on ROI production.

3.3.1   OPTIMISATION OF ROI PRODUCTION IN MM6 CELLS

3.3.1.1   THE EFFECT OF 'PRIMING' MM6 CELLS WITH LPS FOR DIFFERENT TIMES ON ROI PRODUCTION

The effect of 'priming' MM6 cells with LPS (100 ng / ml) for various times on OpZ elicited ROI production was investigated. When MM6 cells (1×10^6 / ml) were challenged with OpZ (125 µg / ml) after 1, 3 or 6 hours incubation with LPS, LCL responses were not significantly different from MM6 cells incubated without LPS (Figure 3.1). However, LCL in MM6 cells cultured with LPS for 12 or 18 hours were significantly increased when compared to control cells incubated with PBS alone ($P<0.001$ analysed by Mann-Whitney). This enhanced effect of LCL and hence ROI production following incubation with LPS is termed 'priming' (Hayakawa *et al*, 1989). 'Priming' of MM6 cells with LPS for 18 hours results in a seven-fold increase in ROI production over control values (Figure 3.1). Not only the peak light emission was enhanced but the rate of formation of ROIs was also enhanced as the time of LPS incubation increased. There was no appreciable light emission in resting cells (no LPS and no OpZ) or from cells 'primed' with LPS but not stimulated with OpZ. From these results MM6 cells were routinely 'primed' by culturing for 18 hours to give optimal ROI production.
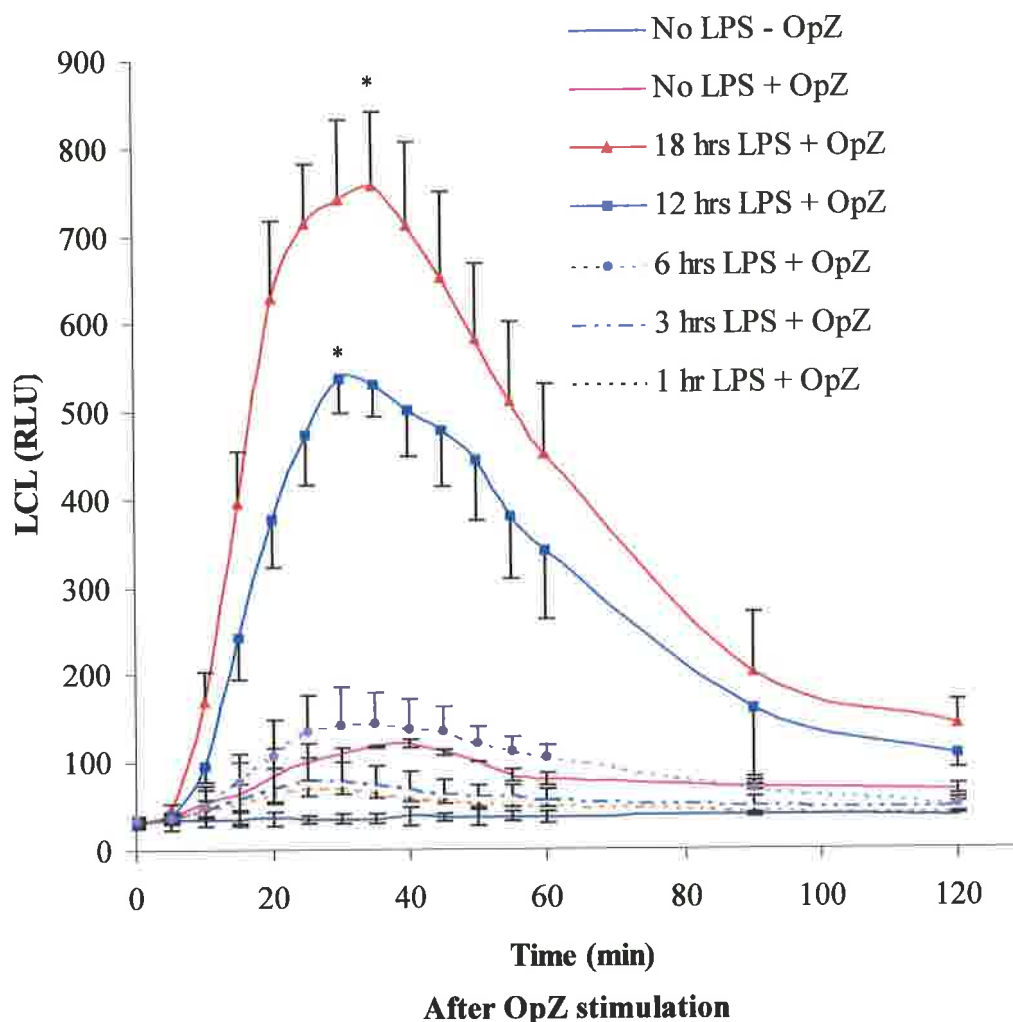
Figure 3.1: **Time course of LPS treatment on NADPH oxidase activity in MM6 cells stimulated with OpZ.** The production of ROIs in MM6 cells pre-incubated with LPS (100 ng / ml) for 0 – 18 hours was detected by LCL in OpZ (125 µg / ml) stimulated cells. Results are expressed as mean ± SD of 3 separate experiments. *P<0.001, analysed by Mann-Whitney (peak light emission of test was compared to peak light emission of control cells incubated in the absence of LPS).

3.3.1.2   DOSE-DEPENDENT EFFECTS OF LPS 'PRIMING' FOR ROI PRODUCTION IN MM6 CELLS

In another series of experiments to establish optimal conditions for NADPH oxidase activity, MM6 cells were 'primed' with differing LPS concentrations for 18 hours. MM6 cells treated with ≤ 1 µg / ml LPS showed a dose dependent enhancement of the oxidative burst as detected by OpZ-elicited LCL emission (Figure 3.2). As little as 0.1 ng / ml LPS can 'prime' MM6 cells for enhanced ROI production. MM6 cells produce the greatest amount of ROIs when 'primed' with LPS at doses equal to and above 10 ng / ml. LPS at 100 ng / ml was determined as an optimal concentration to 'prime' MM6 cells for ROI production. Therefore 100 ng / ml LPS was used in subsequent investigations where MM6 cells required 'priming' with LPS.

3.3.1.3   DOSE-DEPENDENCY OF OPSONISED ZYMOSAN (OPZ) OR PMA STIMULATION OF ROI PRODUCTION IN MM6 CELLS

Activation of the NADPH oxidase enzyme occurs in response to bacteria, bacterial antigens, zymosan or soluble stimulants such as PMA. The concentration of such stimulants required for activation of the NADPH oxidase was thus investigated. This final series of experiments for optimising LCL, investigated the stimulation of LPS-'primed' MM6 cells with varying concentrations of the yeast cell wall extract zymosan that had been previously opsonised with serum factors (OpZ) or the phorbol ester PMA. The optimal conditions for 'priming' MM6 cells with LPS in OpZ stimulated ROI production were used to assess the production of ROI in PMA stimulated MM6 cells.
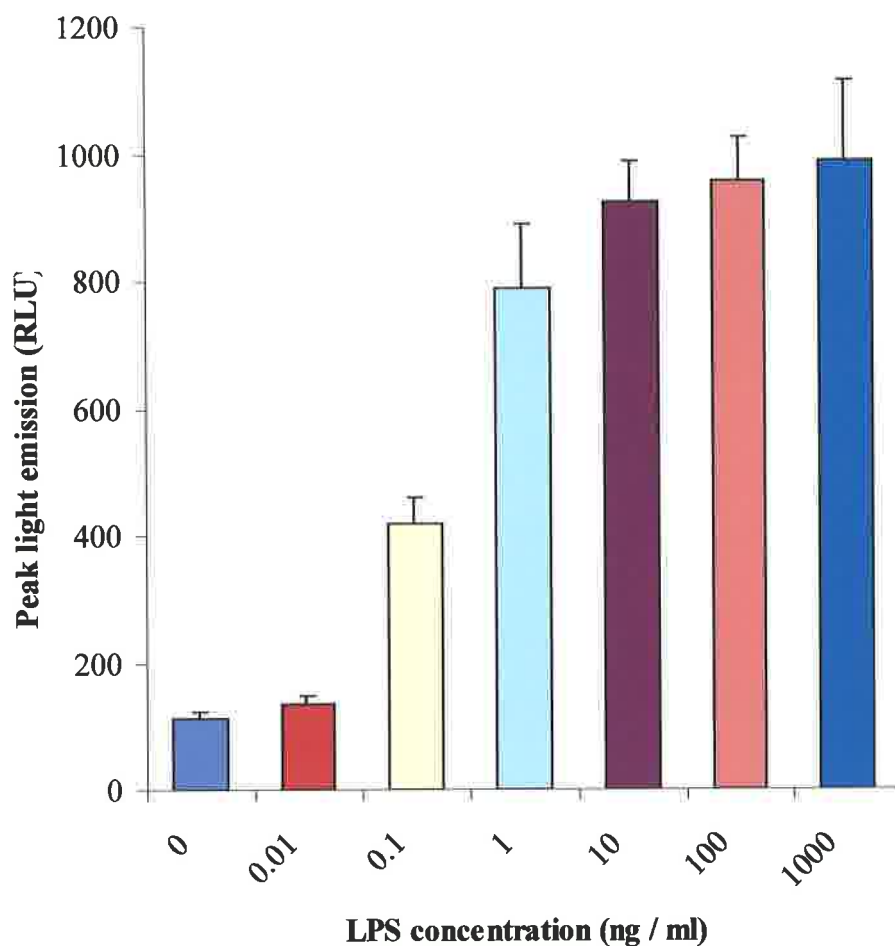
Figure 3.2: **Dose response of LPS treatment on NADPH oxidase activity in MM6 cells stimulated with OpZ.** The production of ROIs in MM6 cells (1×10^6 cells / ml) incubated with LPS ($0 - 1000$ ng / ml) for 18 hours was detected by LCL in OpZ (125 μg / ml) stimulated cells. Results are expressed as mean ± SD of peak light emitted in 3 individual experiments.

Both stimulants showed a dose-dependent increase in oxidative burst as measured by LCL. As the concentration of OpZ increased, the rate of formation of superoxide also increased. This may be due to the particulate nature of OpZ. Increasing the availability of OpZ to interact with MM6 cells increased the rate at which more ROIs were produced. However, at OpZ concentrations of 250 μg / ml and greater, the ratio of particles to cells is optimal and the peak ROI becomes saturated. PMA on the other hand is a lipid soluble stimulant and hence the rate of formation of ROIs remained unchanged as the stimulant by-passes the membrane-receptor-ligand activation pathway. OpZ and PMA induced reproducible and characteristic LCL traces (Figures 3.3 and 3.4 respectively). The kinetics of the LCL reaction on stimulation with OpZ differed from the PMA-induced response in that the time required for peak LCL generation varied. Luminescence generation with OpZ-stimulated MM6 cells peaked between 35 – 45 minutes after initial stimulus. PMA-stimulated MM6 cells peaked between 10 – 15 minutes. The PMA induced response had a rapid acceleration of photon emission (i.e. fast initial rate) and hence LCL. In contrast, OpZ was less accelerated (the initial rate of ROI production was low), lengthening the time required to reach peak LCL velocity (i.e. maximum rate of ROI production). In addition, OpZ induced LCL was preceded by a short lag period whereas an immediate LCL response was observed with MM6 cells stimulated with PMA. Without LPS 'priming', there is some LCL. However, these levels of LCL do not allow for sufficient discrimination between experimental assays, therefore 'priming' was routinely used. The optimal concentrations of OpZ (125 μg / ml) and PMA (100 ng / ml) were also used in subsequent experiments.
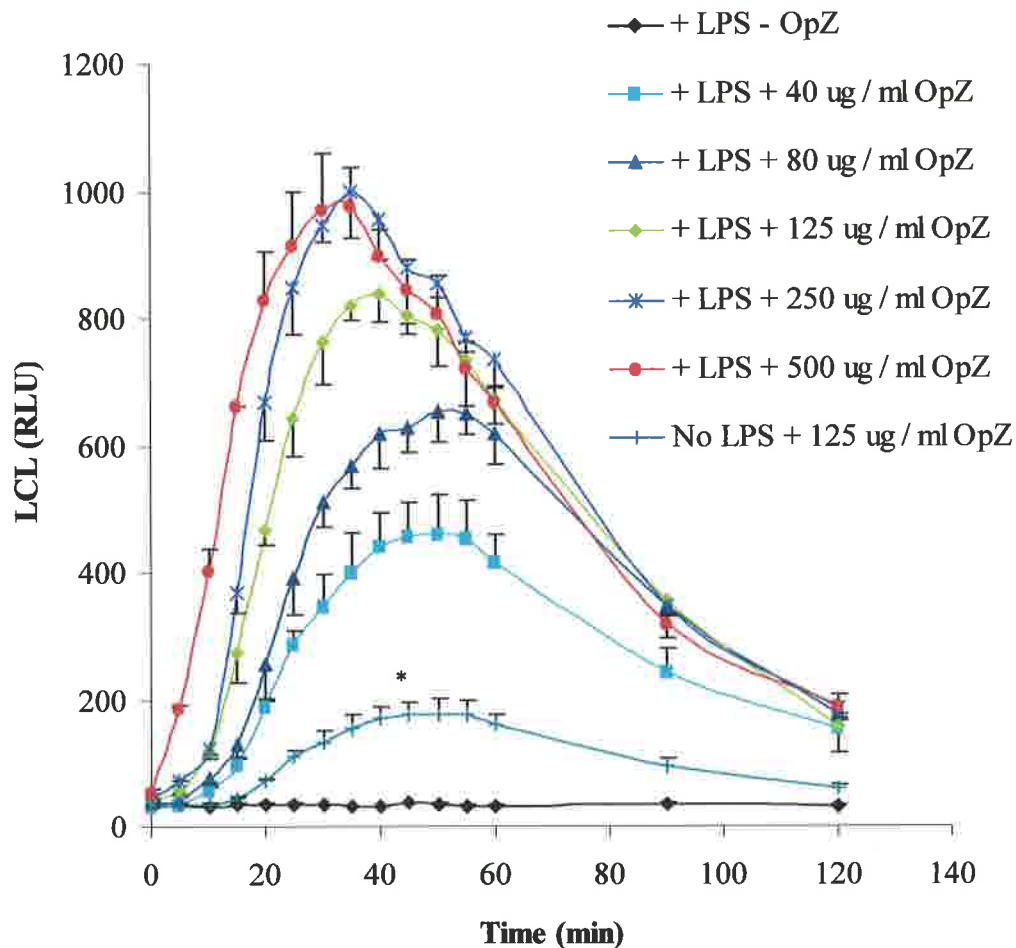
Figure 3.3: **OpZ stimulates 'primed' MM6 cells for NADPH oxidase activity in a dose dependent fashion**. The production of ROIs in MM6 cells (1×10^6 cells / ml) incubated with LPS (100 ng / ml) for 18 hours was detected by LCL when stimulated with different OpZ concentrations. Results are expressed as mean ± SD of 3 separate experiments. *P<0.05 analysed by Mann-Whitney compared to control cells (± LPS and No OpZ).

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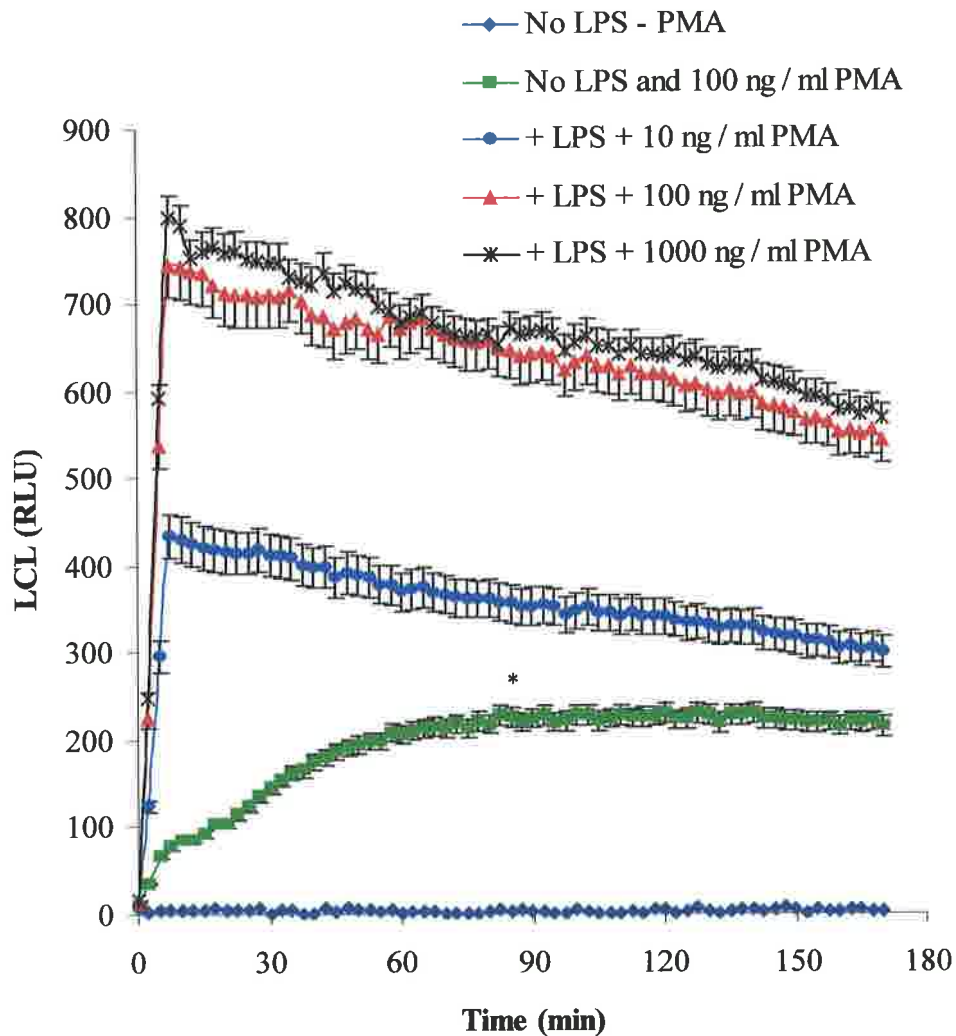
Figure 3.4: **PMA stimulates 'primed' MM6 cells for NADPH oxidase activity in a dose dependent fashion**. The production of ROIs in MM6 cells (1 × 10^6 cells / ml) incubated with LPS (100 ng / ml) for 18 hours was detected by LCL when stimulated with different PMA concentrations. Results are expressed as mean ± SD of 3 separate experiments. *P<0.01 when compared to control cells (± LPS and No PMA).

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In summary the results demonstrate that MM6 cells 'primed' with LPS release significant quantities of ROIs in cells stimulated with OpZ (125 µg / ml) or PMA (100 ng / ml). Stimulation of the NADPH oxidase with these agents release significant quantities of ROIs in cells not 'primed' with LPS. However, the levels of ROIs produced are significantly lower from that of LPS 'primed' cells. Therefore, in order to maximise any differences in ROI production due to incubation with surfactant lipids cells 'primed' with LPS prior to stimulation were used.

3.3.2 QUENCHING EFFECT OF PHOSPHOLIPIDS ON LCL

To verify that surfactant, phospholipids or other lipids to be used in this study did not interfere with LCL directly or act as direct scavengers of superoxide, the lipid preparations were added to standard buffer immediately prior to LCL. MM6 cells 'primed' with LPS were suspended in standard buffer at 2×10^6 cells / ml containing 0, 100 or 1000 µg / ml of Survanta®, Curosurf®, tPC, DPPC, PAPC, or cholesterol. OpZ (125 µg / ml) was used as the stimulus. The peak response of ROI production of cells suspended in standard buffer (with surfactant or lipid) was not significantly different from control cells suspended in standard buffer alone (Table 3.1). These results suggest that the addition of either preparation of surfactant or lipid had no direct effect on the LCL assay for ROI production.

Experimental Condition	Peak light emission (RLU ± 1SD; n=3)
No LPS and standard buffer alone	119 ± 13
MM6 cells primed with LPS (100 ng / ml for 18 hours) and suspended in standard buffer containing:-	
Standard buffer alone	825 ± 67
100 μg / ml Survanta®	856 ± 83[†]
1000 μg / ml Survanta®	876 ± 97[†]
100 μg / ml Curosurf®	901 ± 76[†]
1000 μg / ml Curosurf®	849 ± 58[†]
100 μg / ml tPC	804 ± 80[†]
1000 μg / ml tPC	850 ± 65[†]
100 μg / ml DPPC	823 ± 101[†]
1000 μg / ml DPPC	801 ± 78[†]
100 μg / ml PAPC	759 ± 54[†]
1000 μg / ml PAPC	828 ± 17[†]
100 μg / ml Cholesterol	859 ± 90[†]
1000 μg / ml Cholesterol	900 ± 67[†]

Table 3.1: **Surfactant or lipids do not interfere with LCL in MM6 cells.** MM6 cells 'primed' with LPS were suspended in a surfactant / phospholipid-rich standard buffer prior to OpZ stimulated LCL. The peak light emitted from the tests was not significantly different from cells suspended in standard buffer alone ([†]$P > 0.05$ not significant when compared to standard buffer alone by Mann-Whitney).

3.3.3   EFFECT OF SURFACTANT ON ROI PRODUCTION IN MM6 CELLS

To test the effect of surfactant on the production of ROIs in monocytes / macrophages, Survanta®, Curosurf® or Exosurf Neonatal™ were incubated with MM6 cells for 2 hours prior to LPS 'priming' and OpZ or PMA stimulation. The concentrations of surfactant used represented those levels that may be found within human pulmonary surfactant (Hayakawa *et al*, 1992; Thomassen *et al*, 1992; Speer *et al*, 1991). It was found that each surfactant at a concentration of 20 to 500 µg / ml markedly inhibited ROI production in OpZ or PMA elicited LCL responses (Figures 3.5, 3.6 and 3.7 respectively). Survanta®, a bovine lung extract containing phospholipids (>40% DPPC), neutral lipids, fatty acids and less than 4% surfactant associated proteins (SP-B and C) significantly inhibited ROI production in a dose dependent manner when stimulated with OpZ ($P < 0.0001$ by ANOVA). When Tukey's analysis was applied to this data, 100 and 500 µg / ml of Survanta® was found to contribute most to this significant inhibition (approximately 30% and 40% respectively; $P < 0.01$). Upon incubation with 20 µg / ml Survanta®, a significant decrease of 20% was found when compared to the control cells incubated without Survanta® ($P < 0.05$). However, when cells were stimulated with 100 ng / ml PMA, only incubation with 500 µg / ml of Survanta® could significantly reduce the production of ROI (by 25%) ($P < 0.01$ by ANOVA and Tukey's pairwise differences). Levels below 500 µg / ml had no significant effect when compared to control cells incubated without Survanta®.
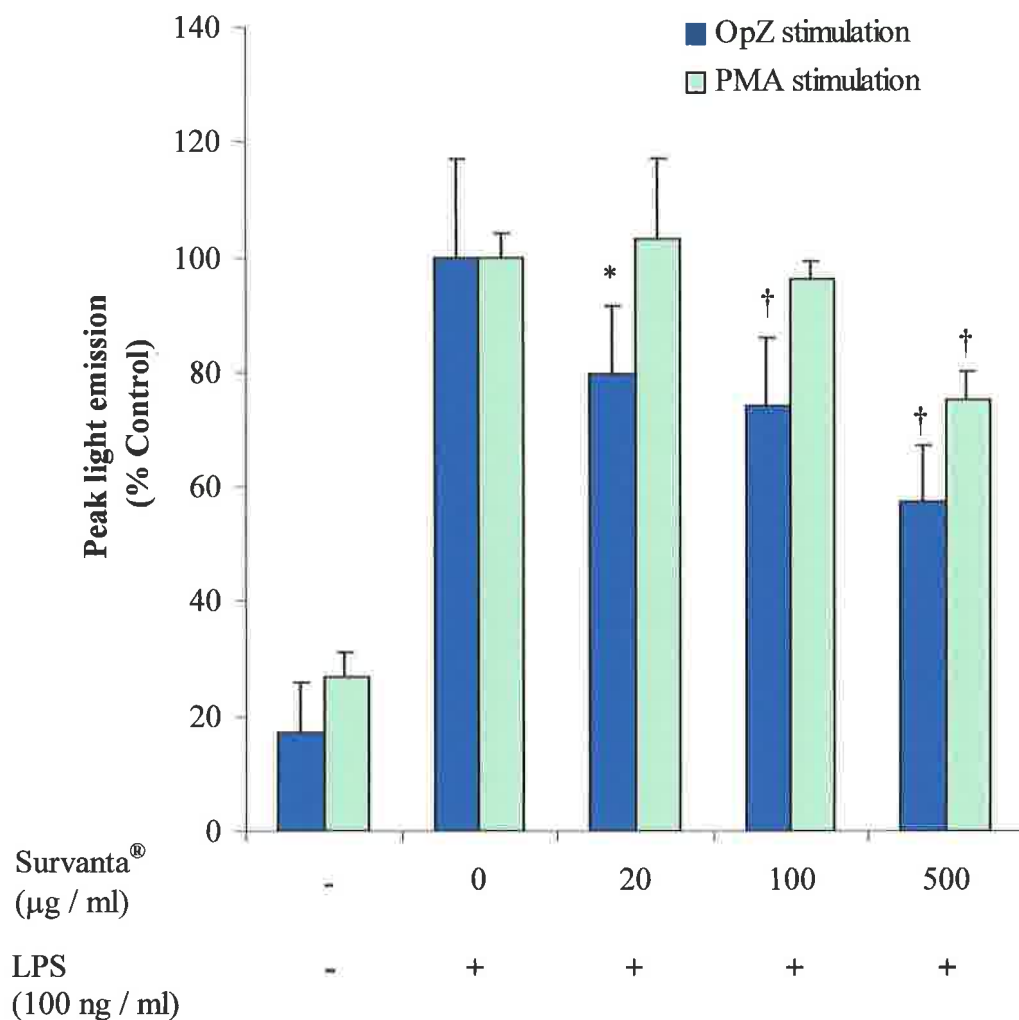
Figure 3.5: **Effect of Survanta® on the production of ROIs in MM6 cells stimulated with OPZ or PMA.** MM6 cells (1×10^6 cells / ml) were incubated with Survanta® for 2 hours, washed ($\times$3) in PBS and 'primed' with LPS (100 ng / ml) for 18 hours at 37°C. LCL was initiated with OpZ (125 µg / ml) or PMA (100 ng / ml). Results are expressed as mean ± SD of 3 separate experiments. *P<0.05, †P<0.01 analysed by ANOVA and Tukey's pairwise differences.

Curosurf® contains 99% polar lipids with greater than 40% DPPC and 1% hydrophobic associated proteins (SP-B, SP-C), however the extraction procedure excludes the hydrophilic SP-A. It can be seen in figure 3.6 that Curosurf® preparations inhibited OpZ-elicited responses in a dose-dependent fashion. This reduction was greatest when MM6 cells were incubated with 500 μg / ml Curosurf® for 2 hours (P<0.01 by Tukey's pairwise differences). However, a reduction of only 22% of ROI production compared to control MM6 cells was seen when incubated with this dose of surfactant. Further, PMA stimulation of ROI production was only significantly reduced compared to control when incubated with Curosurf® at 500 μg / ml (P<0.01 by Tukey's pairwise comparisons). This reduction was also approximately 20% of the control.

Finally, Exosurf Neonatal™ a synthetic surfactant containing chemically modified DPPC (Colfosceril palmitate), inhibited the production of ROIs in MM6 cells when treated for 2 hours (P<0.001 when analysed by ANOVA) (Figure 3.7). Tukey's pairwise comparisons indicated this significant reduction (P<0.01) to be attributed to Exosurf Neonatal™ doses of 100 and 500 μg / ml where a 30 - 40% reduction in ROI production from OpZ stimulated MM6 cells was respectively seen. The production of ROIs below 20 μg / ml was not significantly different from MM6 cells incubated without Exosurf Neonatal™. PMA elicited LCL responses were found only to produce a 30% inhibition of ROI production when MM6 cells were treated with 500 μg / ml of Exosurf Neonatal™. Treatment of cells with lower doses of this surfactant did not inhibit the production of ROIs when compared to controls.
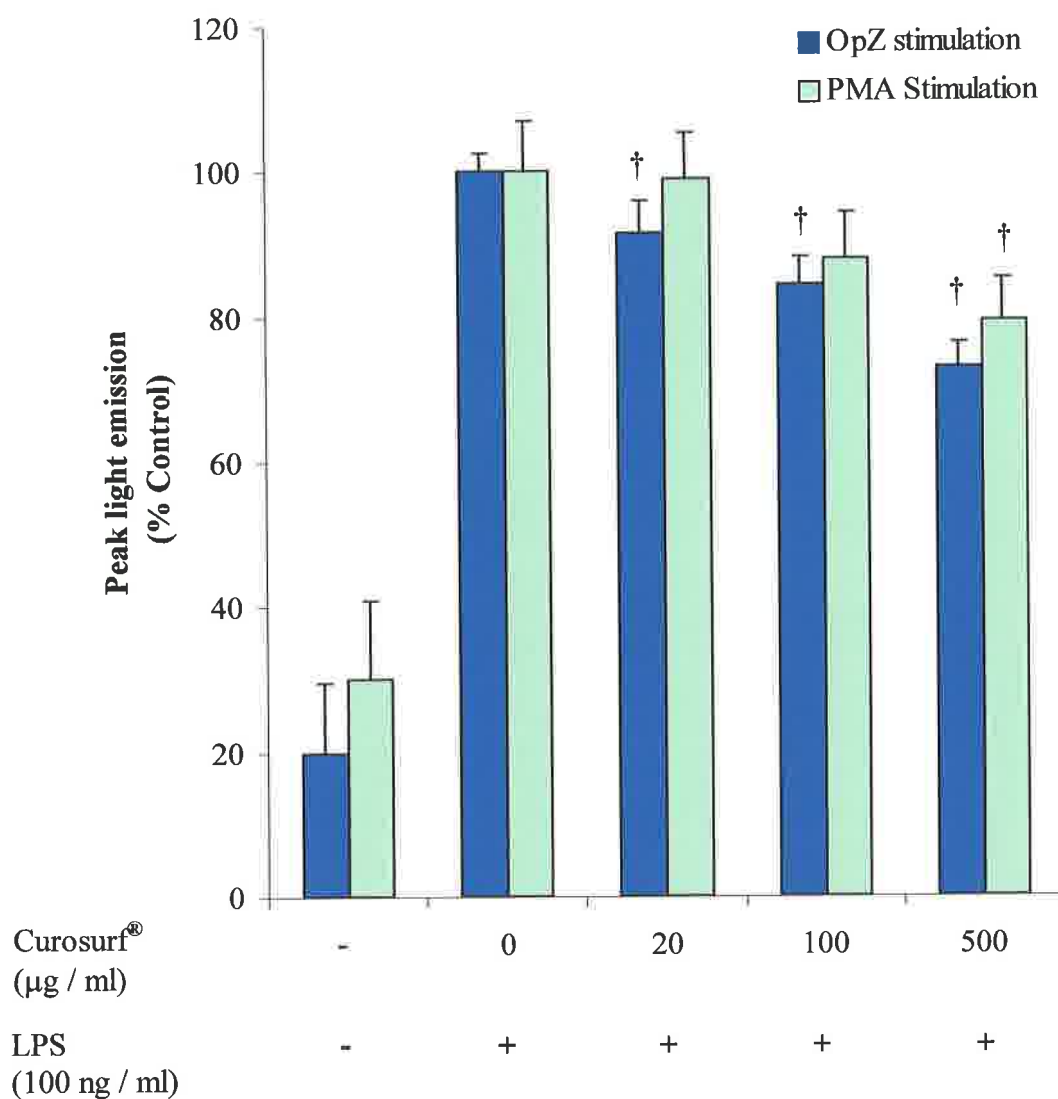
Figure 3.6: **Effect of Curosurf® on the production of ROIs in MM6 cells stimulated with OPZ or PMA.** MM6 cells (1×10^6 cells / ml) were incubated with Curosurf® for 2 hours, washed ($\times 3$) in PBS and 'primed' with LPS (100 ng / ml) for 18 hours at 37°C. LCL was initiated with OpZ (125 µg / ml) or PMA (100 ng / ml). Results are expressed as mean $\pm$ SD of 3 separate experiments. †P<0.01 analysed by ANOVA and Tukey's pairwise differences.

Figure 3.7: **Effect of Exosurf Neonatal™ on the production of ROIs in MM6 cells stimulated with OPZ or PMA.** MM6 cells (1×10^6 cells / ml) were incubated with Exosurf Neonatal™ for 2 hours, washed ($\times 3$) in PBS and 'primed' with LPS (100 ng / ml) for 18 hours at 37°C. LCL was initiated with OpZ (125 µg / ml) or PMA (100 ng / ml). Results are expressed as mean ± SD of 3 separate experiments. †P<0.01 analysed by ANOVA and Tukey's pairwise differences.

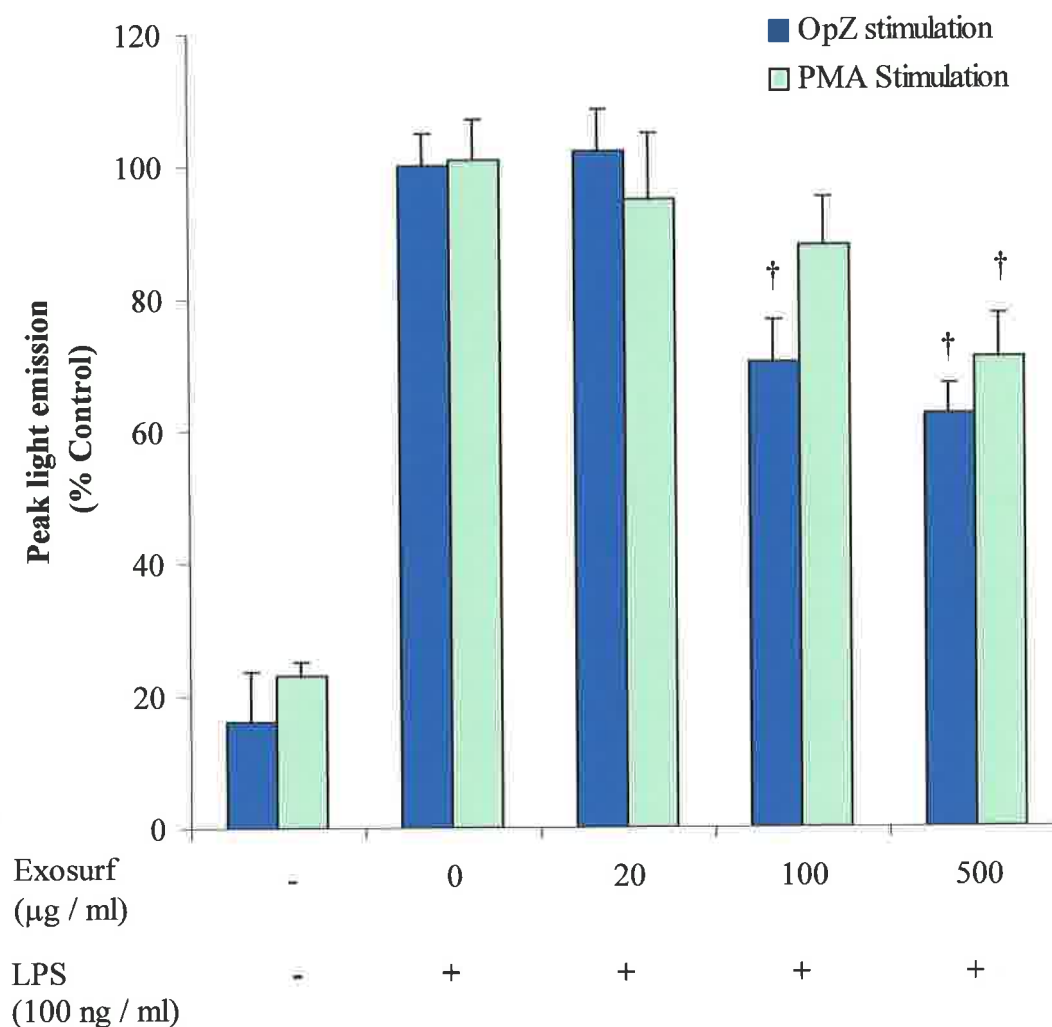Thus both natural (modified) and synthetic surfactants had statistically significant effects on LCL and hence the production of ROIs in OpZ stimulated MM6 cells. Similar inhibitory results were seen when the phorbol ester PMA was used as the stimulant, although there was a more marked inhibition of ROI production by surfactant when stimulated with OpZ. The greatest inhibition of ROI production in MM6 cells pre-cultured with surfactant was seen in cells pre-treated with Exosurf Neonatal™. Taken together these data suggest that the modulation of the production of ROIs and the magnitude of such modulation would depend on surfactant composition and the type of stimulus used. Common to each surfactant is the presence of surfactant phospholipids. In particular, Exosurf Neonatal™ is nearly all DPPC and excludes surfactant proteins. Since Exosurf Neonatal™ gave the most inhibition of ROI production, the study focuses on the effect of surfactant lipids alone on ROI production, especially DPPC since this is the major surfactant phospholipid.

3.3.4 EFFECT OF NON SURFACTANT LIPIDS ON ROI PRODUCTION IN MM6 CELLS

In this set of experiments, the study investigated the possible capacity of an "in house" non-surfactant lipid preparation to modulate LCL responses. This preparation was prepared by mixing 69% phosphatidylcholine (tPC), 5% phosphatidylethanolamine (tPE), 7% phosphatidylglceyrol (PG), 3% phosphatidylinositol (PI), 3% phosphatidylserine (PS), 6% sphingomyelin (SM) and 7% cholesterol as a percentage of 10, 100 or 500 μg / ml in a silanised vial. These ratios have been previously reported to occur in natural surfactant (Harwood, 1987). Accordingly, MM6 cells (1×10^6 cells / ml) were incubated with 10, 100 or 500 μg / ml of lipid for 2 hours, washed in PBS (×3)

and 'primed' with LPS. LCL responses were elicited with OpZ (125 µg / ml) or PMA (100 ng / ml). The non surfactant lipid preparation significantly inhibited ROI production in PMA or OpZ elicited LCL responses during a 2 hour incubation (P<0.01 when analysed by ANOVA) (Figure 3.8). Tukey's pairwise comparisons indicated this significant reduction (P<0.01) to be attributed to mixed lipid doses of 100 and 500 µg / ml where approximately a 20 and 40% reduction in ROI production from OpZ stimulated MM6 cells was respectively seen. Only a 25% reduction in ROI production was seen in PMA stimulated cells when incubated with 500 µg / ml of this lipid preparation (P<0.01 by ANOVA and Tukey's). Treatment of MM6 cells with 20 µg / ml of artificial surfactant did not significantly alter the production of ROIs when compared to control cells incubated without lipids (Figure 3.8).

3.3.5   EFFECT OF PHOSPHOLIPIDS ON ROI IN MM6 CELLS

To determine and characterise the source of any modulatory effect of surfactant phospholipids, the study investigated the effect of individually pre-incubating MM6 cells with the major phospholipid and neutral lipid classes found in surfactant on ROI production (see Table 1.1). These phospholipid and neutral lipids, commonly associated with human pulmonary surfactant, were incubated with MM6 cells for 2 hours at levels chosen to span those at physiological concentrations.
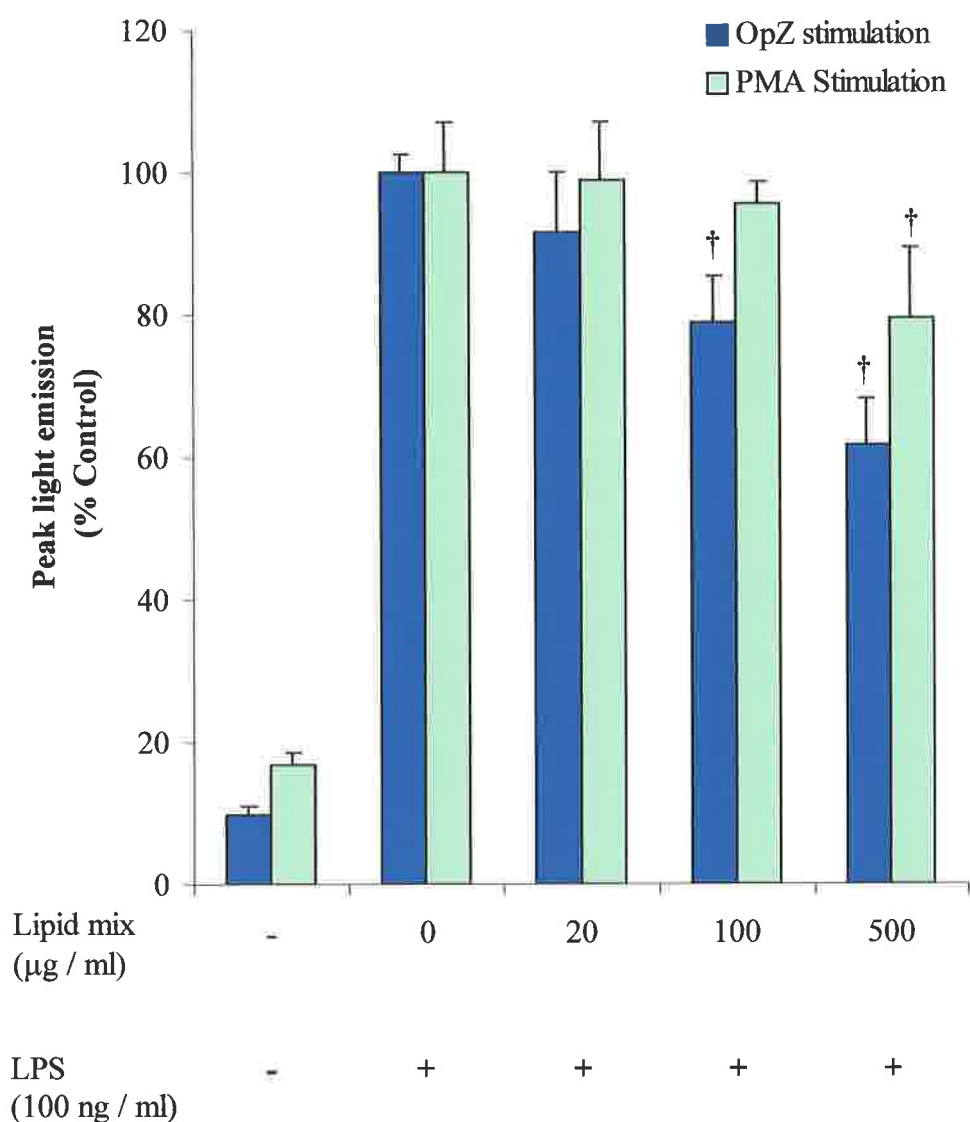
Figure 3.8: **Effect of non-surfactant "in house" lipid mix on the production of ROIs in MM6 cells stimulated with OPZ or PMA.** MM6 cells (1×10^6 cells / ml) were incubated with artificial surfactant for 2 hours, washed (×3) in PBS and 'primed' with LPS (100 ng / ml) for 18 hours at 37°C. LCL was initiated with OpZ (125 µg / ml) or PMA (100 ng / ml). Results are expressed as mean ± SD of 3 separate experiments. †P<0.01 analysed by ANOVA and Tukey's pairwise differences.

Each of the phospholipid and neutral lipids investigated produced a dose-dependent modulation of the respiratory burst (Figure 3.9). There were no significant differences in ROI production in MM6 cells treated with sphingomyelin (SM) at concentrations lower than 125 µg / ml when compared to cells treated without lipid. The concentrations of SM in natural surfactant are lower than 125 µg / ml. However, at 250 and 500 µg / ml of SM, there was a significant increase in ROI production compared to control cells (P<0.01 when analysed by ANOVA and Tukey's pairwise comparisons). Cholesterol and tPE also significantly enhanced the production of ROI in MM6 cells at concentrations greater than 125 µg / ml (P<0.01 analysed by ANOVA and Tukey's pairwise differences). TPE and PG are the second most abundant phospholipids in natural surfactant, but their levels account for less than 7% of the total phospholipids within surfactant. The most abundant phospholipid is tPC. The only phospholipids found to inhibit ROI production in this model were PG and tPC (Figure 3.9). These phospholipids significantly inhibited the production of ROIs (P<0.001 by ANOVA) with as little as 25 µg / ml of PG or tPC required, reducing ROI production by 20%. These inhibitory effects were greatest (40 - 70% reductions) at concentration of 250 - 500 µg / ml of PG or tPC respectively. PG is found in natural surfactant at low concentrations, approximately 15 – 30 µg / ml. In this study, there was a significant effect of PG on ROI production at this level. These results further establish that individual phospholipid components contribute a major effect of pulmonary surfactant on ROI production in MM6 cells. PG and tPC had an inhibitory role where as tPE, SM and cholesterol had a stimulatory role. The effects seen with the phospholipid tPC suggest a similar inhibitory role as seen in those surfactants tested in section 3.3.3. These surfactants contain mainly tPC (in the form of the disaturated species DPPC).
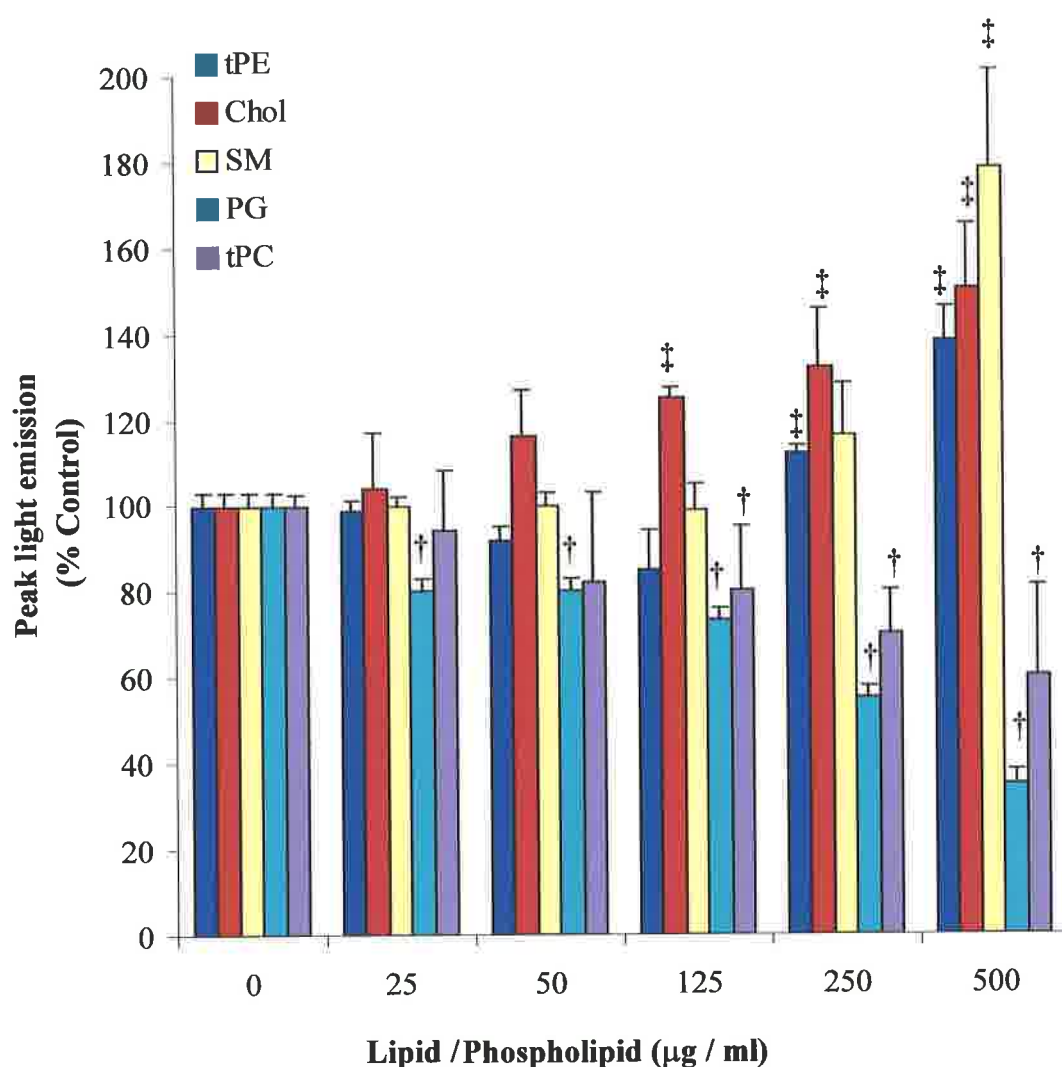
Figure 3.9: **Effect of phospholipids and lipids on the production of ROIs.** MM6 cells (1×10^6 cells / ml) were incubated with different lipids for 2 hours, washed ($\times 3$) in PBS and 'primed' with LPS (100 ng / ml) for 18 hours LCL was initiated with OpZ (125 µg / ml). Results are expressed as mean $\pm$ SD of 3 separate experiments. $\dagger P<0.001$ and $\ddagger P<0.01$ when analysed by ANOVA and Tukey's pairwise differences.

3.3.6   EFFECT OF PC SPECIES ON ROI PRODUCTION IN MM6 CELLS

3.3.6.1   EFFECT OF tPC, DPPC AND PAPC ON LCL (DOSE RESPONSE STUDY)

            IN CELLS CULTURED IN SERUM FREE MEDIA

The previous experiments showed that PC and PG had the greatest effect on ROI production in MM6 cells. To clearly define the effect PC species (e.g. DPPC, PAPC) may have on ROI production in MM6 cells, incubations were performed in serum-free media (UltraCULTURE™). This medium is unlikely to contain any exogenous lipids is will contain less lipid content than that of serum supplemented media. MM6 cells were carefully weaned onto this media and viability regularly checked at each passage, (cells were always greater than 90% viable). The study investigated the consequence of pre-incubating varying concentrations of tPC and the species DPPC or PAPC for 2 hours prior to LPS priming on the production of ROIs in OpZ (125 μg / ml) stimulated MM6 cells. PAPC was chosen as a subspecies to be tested because it has a longer acyl chain length (20 carbons) than that of the most common palmitate moiety. In addition the fatty acid moiety is unsaturated and contains four double bonds making this species distinct from DPPC. The time to peak ROI production and lag phase was not significantly different in MM6 cells pre-treated with tPC, DPPC or PAPC (Figures 3.10, 3.11 and 3.12 respectively). However, the LCL generated from MM6 cells pre-treated with tPC, DPPC or PAPC and LPS was significantly ($P<0.0001$ by ANOVA) different than MM6 cells treated without lipid (Figure 3.13). The modulation of peak light emitted was found to be dose-dependent in MM6 preincubated with tPC, DPPC or PAPC. Further analyses of this data using Tukey's pairwise comparison demonstrated a significant difference ($P<0.001$) amongst the means of peak ROI production as the concentration of the phospholipid increased to 63, 125, 250 and 500 μg / ml.
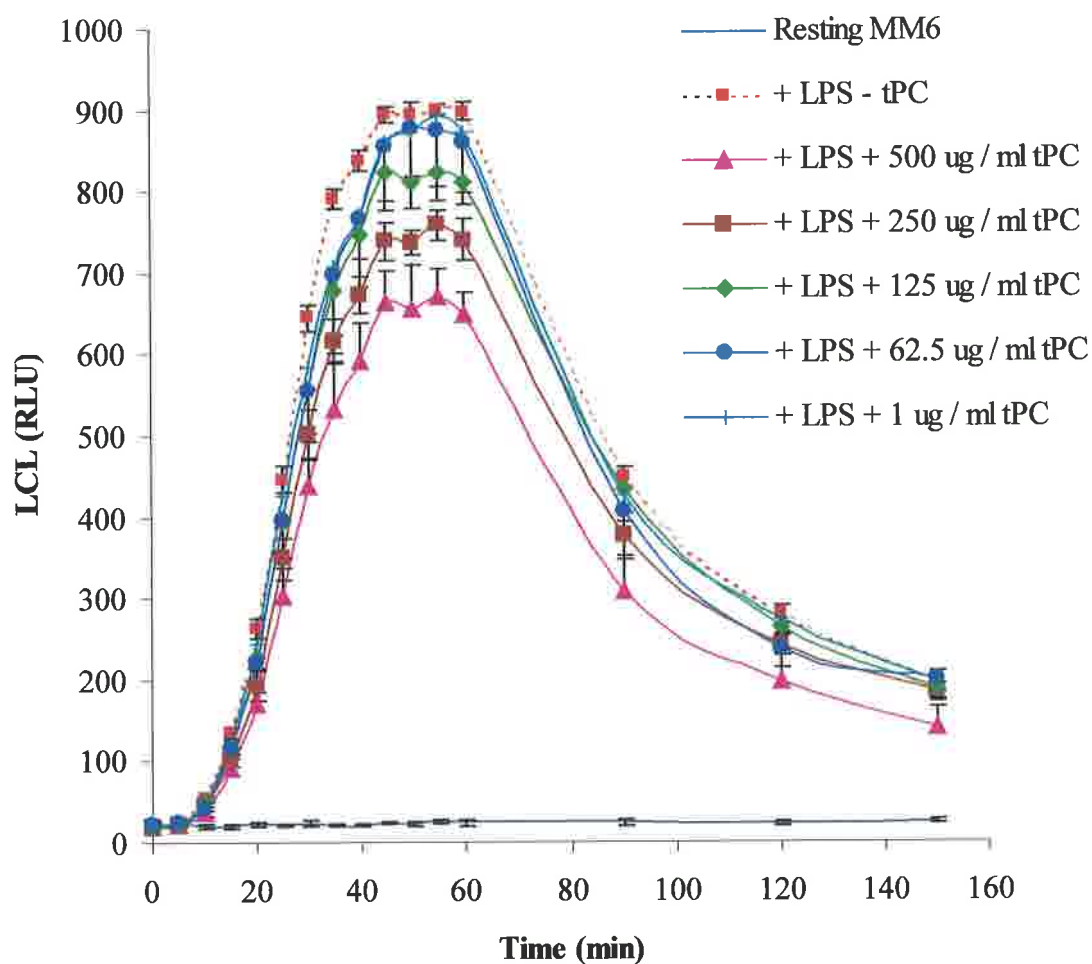
Figure 3.10: **The effect of tPC on ROI production in MM6 cells 'primed' with LPS and stimulated with OpZ.** MM6 cells (1×10^6 cells / ml) were incubated with tPC for 2 hours, washed ($\times$3) in PBS and 'primed' with LPS (100 ng / ml) for 18 hours in serum free media at 37°C. LCL was initiated with OpZ (125 µg / ml). Results are expressed as mean $\pm$ SD of 3 separate experiments.
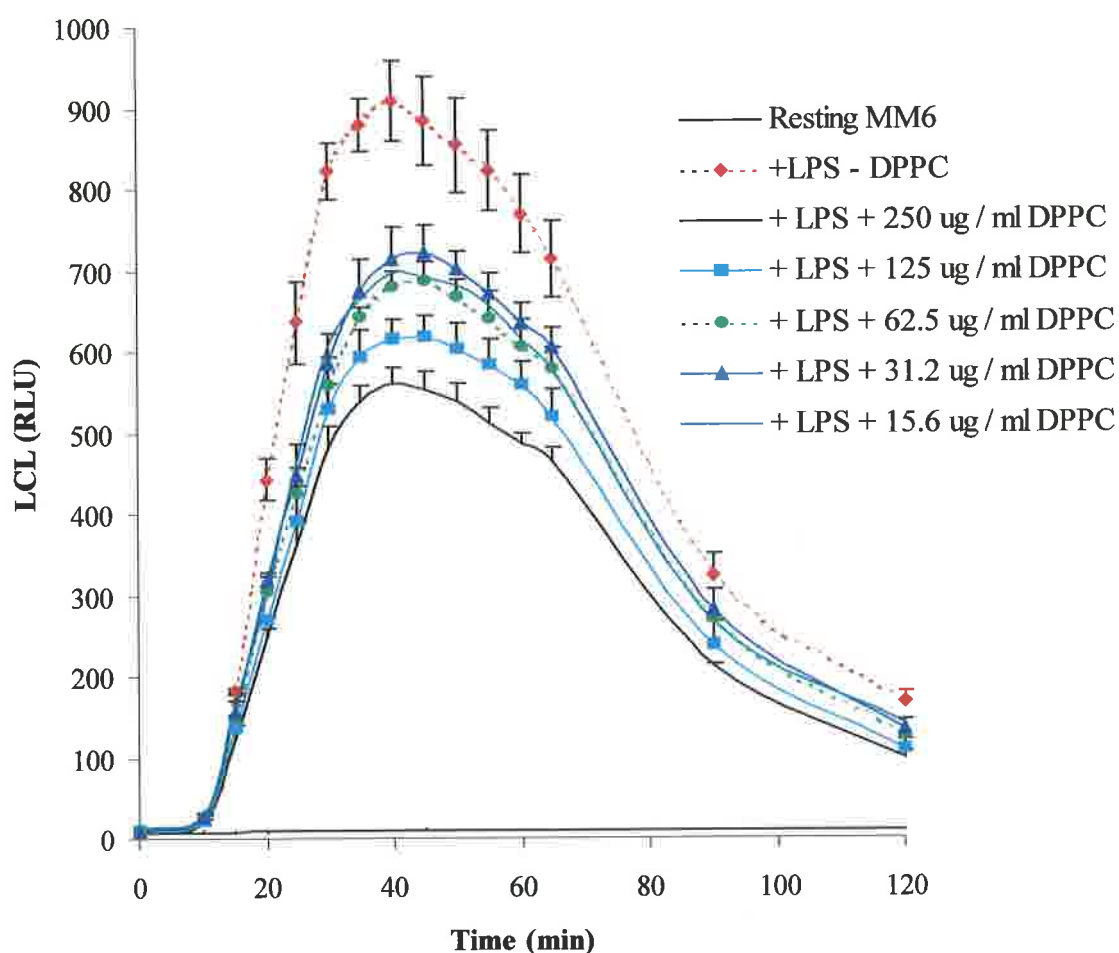
Figure 3.11: **The effect of DPPC on ROI production in MM6 cells 'primed' with**

LPS and stimulated with OpZ. MM6 cells (1×10^6 cells / ml) were

incubated with DPPC for 2 hours, washed ($\times 3$) in PBS and 'primed' with

LPS (100 ng / ml) for 18 hours in serum free media at 37°C. LCL was

initiated with OpZ (125 µg / ml). Results are expressed as mean ± SD of

3 separate experiments.
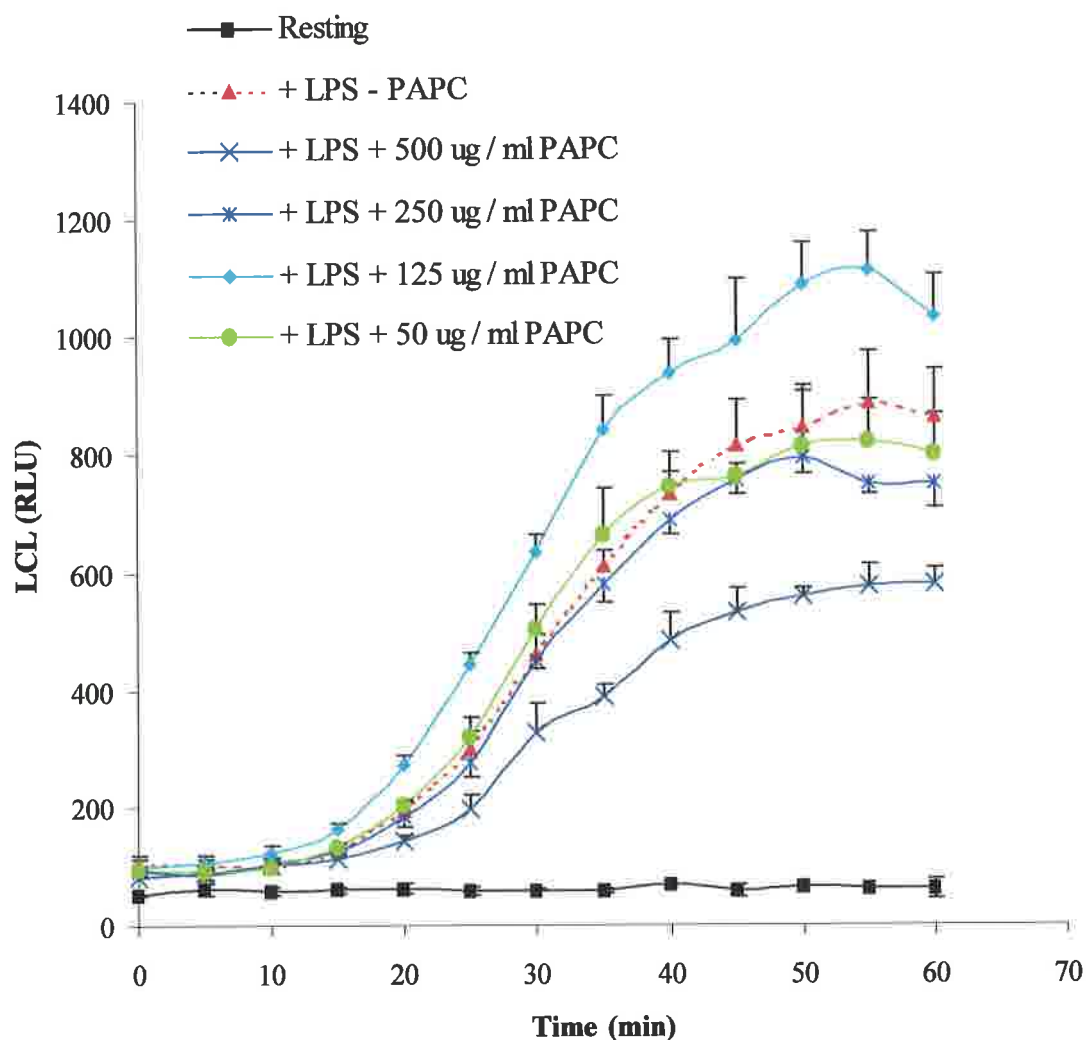
Figure 3.12: The effect of PAPC on ROI production in MM6 cells 'primed' with LPS and stimulated with OpZ. MM6 cells (1×10^6 cells / ml) were incubated with PAPC for 2 hours, washed ($\times 3$) in PBS and 'primed' with LPS (100 ng / ml) for 18 hours in serum free media at 37°C. LCL was initiated with OpZ (125 µg / ml). Results are expressed as mean ± SD of 3 separate experiments.
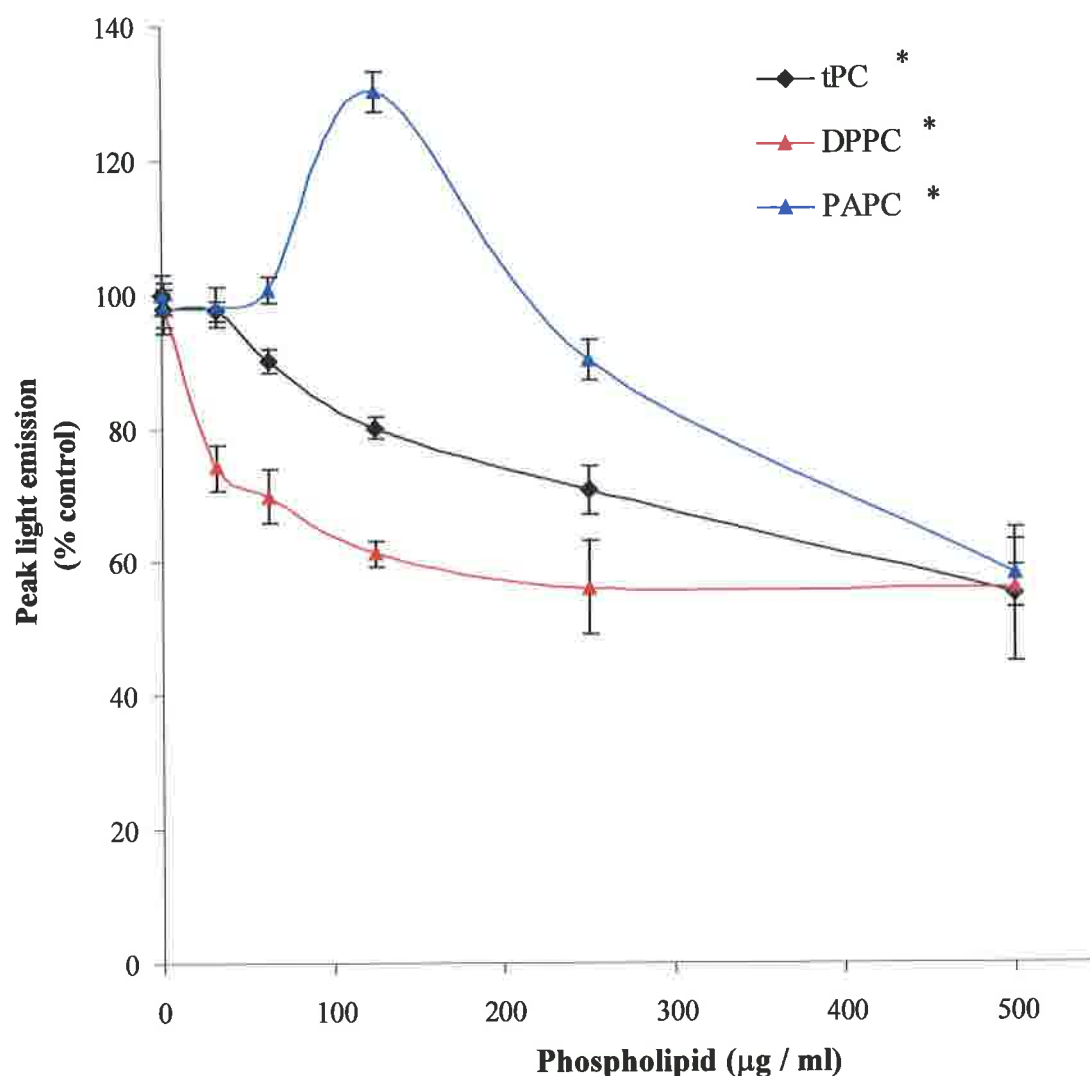
Figure 3.13: Dose response of phospholipid treated MM6 cells 'primed' for ROI production. MM6 cells (1 × 10^6 cells / ml) were incubated with phospholipid for 2 hours, washed (×3) in PBS and 'primed' with LPS (100 ng / ml) for 18 hours in serum free media at 37°C. LCL was initiated with OpZ (125 µg / ml). Controls (100%) were obtained from MM6 cells preincubated with LPS alone. Results are expressed as mean ± SD of 3 separate experiments. * P<0.001 using ANOVA.

However, the fatty acyl composition of the PC species was important to the modulation of ROI production. There were significant differences between tPC and PAPC when compared to DPPC ($P<0.01$) again using Tukey's pairwise comparisons. PC with saturated fatty acyl groups (DPPC) decreased ROI production while PC with unsaturated acyl groups (PAPC) increased ROI production.

Using PMA (100 ng / ml) as a stimulant, preincubation of each PC subspecies at 125 µg / ml for 2 hours followed by LPS 'priming' gave a significant reduction in the release of ROIs ($P<0.0001$ using ANOVA) (Figure 3.14). Further, a similar reduction in ROI production was seen in cells preincubated with lipid and not 'primed' with LPS. In both instances, Tukey's indicated that DPPC (125 µg / ml) was responsible for the significant reduction in ROI production.

These results clearly show that preincubation of MM6 cells with tPC in serum free media has a similar modulation of ROI production as seen in those cells incubated with tPC in the presence of serum. These results indicate that incubation of phospholipids in the presence or absence of serum with MM6 cells does not influence the effect of modulation on ROI production. Therefore, subsequent investigations were performed on MM6 cells cultured in RPMI serum supplemented media unless stated otherwise.
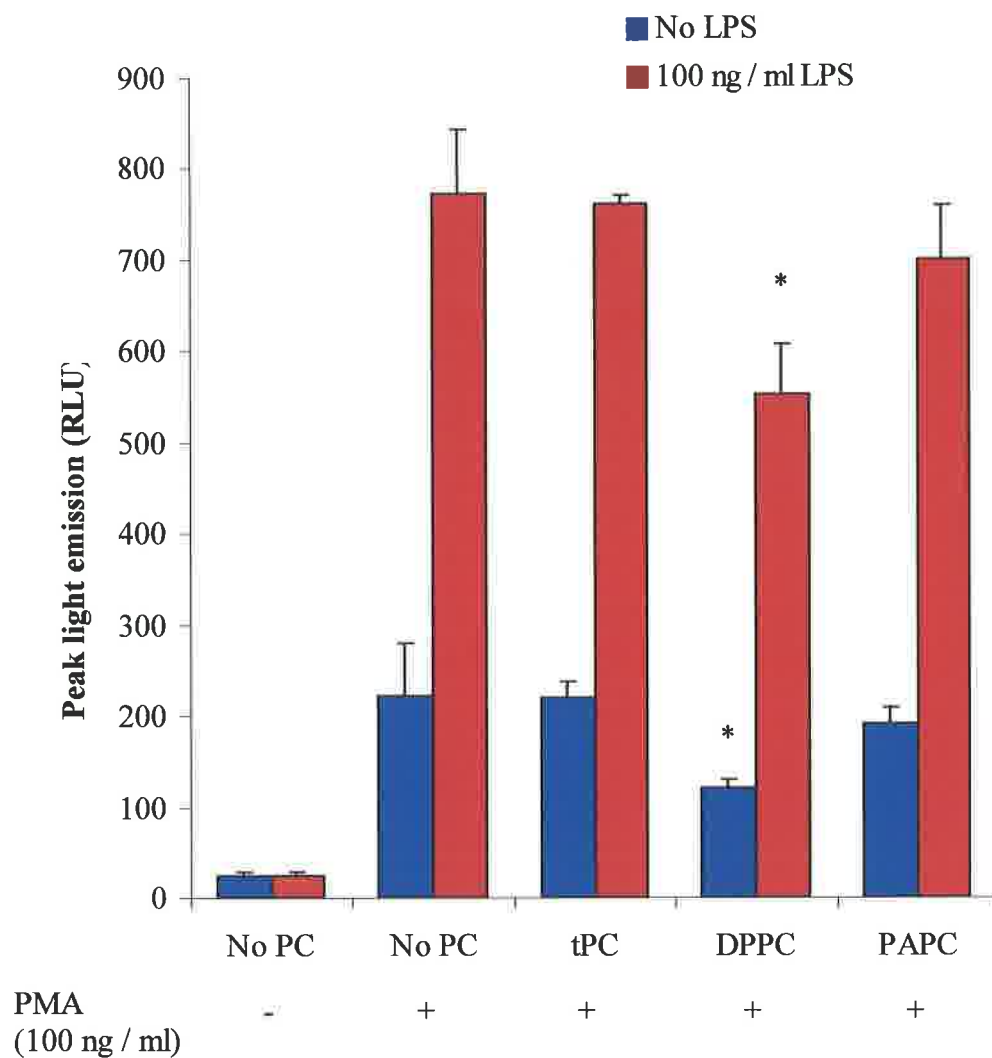
Figure 3.14: Phospholipids modulate ROI production in PMA elicited LCL responses. MM6 cells (1×10^6 cells / ml) were incubated with phospholipid (125 μg / ml) for 2 hours, washed (×3) in PBS and 'primed' with LPS (100 ng / ml) for 18 hours in serum free media at 37°C. LCL was initiated with PMA (100 ng / ml). Results are expressed as mean ± SD of 3 separate experiments. *P<0.01 using ANOVA and Tukey's multi-pairwise comparisons.

3.3.6.2   TIME COURSE STUDY OF PC EFFECTS ON LCL

Time courses of phospholipid modulated LCL responses were studied by pre-treating MM6 cells with tPC, DPPC or PAPC for different times. Each phospholipid (125 μg / ml) was incubated with MM6 cells for 15 or 30 minutes, 2, 6, 18 or 24 hours in serum free media at 37°C prior to LPS 'priming' and OpZ stimulation. Time course experiments showed that pre-treatment of MM6 cells for 2 and 6 hours with tPC significantly inhibited peak light emission compared with that of control cells incubated without tPC ($P<0.01$ analysed by Mann-Whitney) (Figure 3.15). The inhibitory effects of tPC were not seen if MM6 cells were incubated with this lipid for less than 2 hours. Further, the ability of tPC to modulate ROI production disappears after 18 hours pre-incubation with MM6 cells. However, MM6 cells pre-treated with DPPC induced a time dependent decrease in the production of ROI in OpZ elicited LCL. This significant reduction in ROI production ($P<0.001$ by Mann-Whitney compared to control) is evident within 30 minutes incubation with MM6 cells. At 24 hours incubation of DPPC, MM6 cells exhibited approximately 40% decrease in peak LCL generation as compared to that of the untreated group ($P<0.01$). There were no significant differences in MM6 preincubated with DPPC for 15 minutes when compared to the 100% control. In contrast, MM6 cells pre-treated with PAPC (125 μg / ml) for 15 or 30 minutes, 2, 6, 18 or 24 hours, showed a 20 – 40% *increase* in peak light emission compared to that of untreated MM6 cells ($P<0.001$). Further, the time of incubation for these lipids to take effect is critical, as is the dose of lipid used and the particular species present within the surfactant preparation. These results indicate DPPC, the major disaturated species within natural, modified-natural or synthetic surfactant, may account for approximately 40% inhibition of the production of ROIs in MM6 cells.
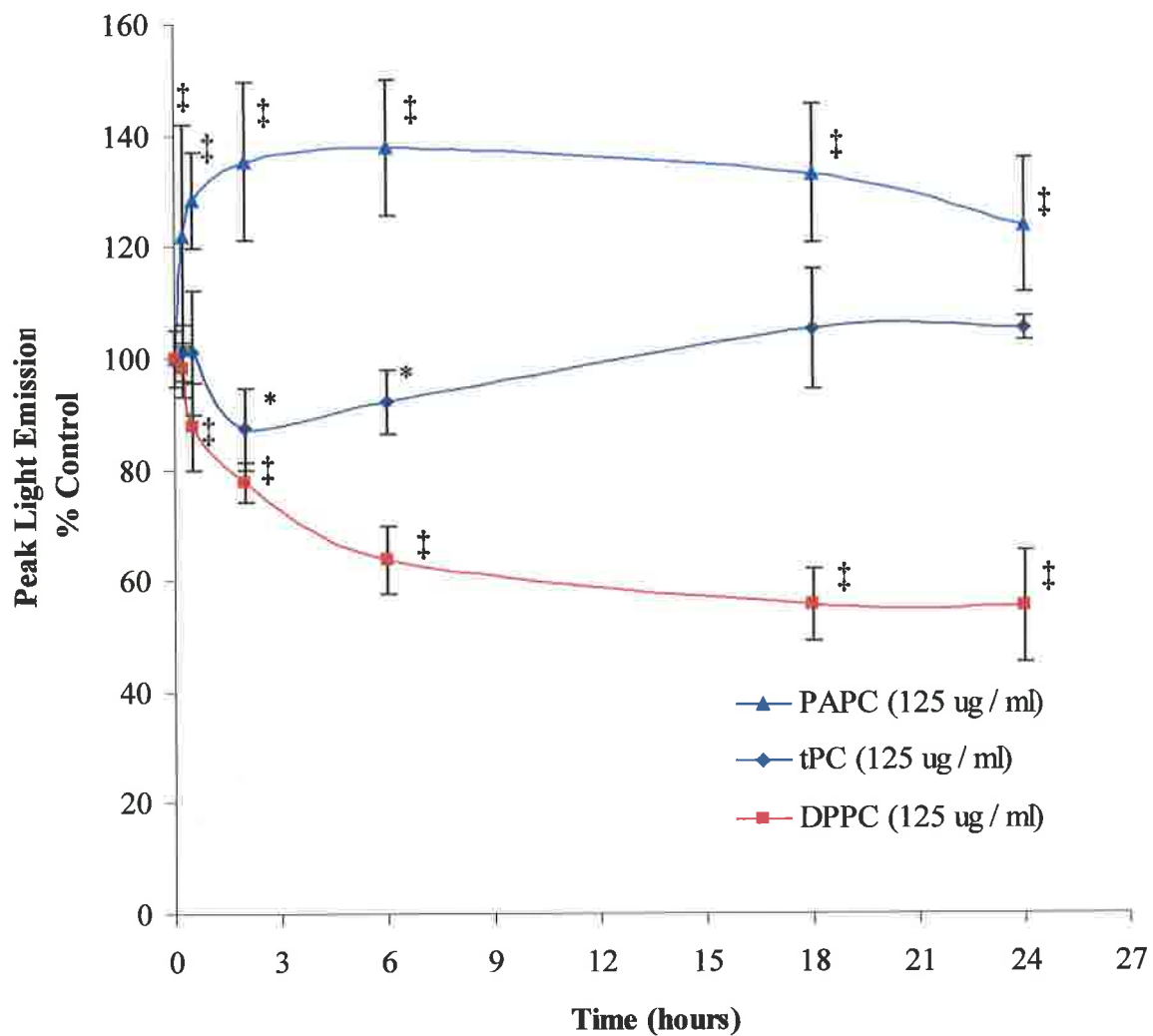
Figure 3.15: **Time course study of phospholipid treated MM6 cells 'primed' ROI production**. MM6 cells (1×10^6) were incubated with PC preparations, washed in PBS (x3) followed by incubation with LPS (100 ng / mL) for 18 hours at 37°C. Controls (100%) were obtained from MM6 incubated without phospholipid. LCL was elicited with OpZ (125 μg / mL). Results are expressed as mean (± SD) of 3 experiments. *P<0.01 and ‡P< 0.001 when compared to 100% control by Mann-Whitney.

3.3.7   EFFECTS OF LONG-TERM CULTURING OF MM6 CELLS IN DPPC ON ROI PRODUCTION

Pulmonary surfactant is the major constituent of the micro-environment surrounding alveolar macrophages.   These macrophages are constantly bathed in DPPC rich surfactant. The study has previously demonstrated that incubation of MM6 cells with DPPC for up to 24 hours inhibits ROI production in LPS 'primed' cells. However, since monocytes differentiate into alveolar macrophages in the constant presence of surfactant, the effect of long term culture in DPPC media on ROI production was tested. This part of the study investigated whether culturing of MM6 cells with DPPC over several passages would continue to inhibit the activity of the NADPH oxidase. MM6 cells were cultured as described in section 2.2.3 but with the addition of 500 µg / ml of DPPC to RPMI culture media. At each passage an aliquot of 5×10^6 cells / ml were washed in PBS ($\times 3$) and suspended in 5 mls of fresh RPMI media and 'primed' with LPS for chemiluminescent experiments as previously performed. The remaining cells were passaged with fresh media containing DPPC. Chemiluminescence studies of ROI production were performed at 1, 3, 6 and 9 days of culture. Incubation of MM6 cells with media containing DPPC (500 µg / ml) for 1 – 9 days displayed marked decreases in their OpZ and PMA elicited LCL responses (Figures 3.16 and 3.17 respectively). It is noteworthy that the OpZ-elicited LCL responses are inhibited by approximately 40% under these culture conditions as opposed to only 20% by PMA elicited responses. MM6 cells cultured for 1 day with DPPC exhibited approximately the same LCL response observed when MM6 cells were tested after 9 days of culture. Taken together, these results suggest that DPPC is an important component of surfactant that alters the oxidative function of monocytes or macrophages when in the lung.
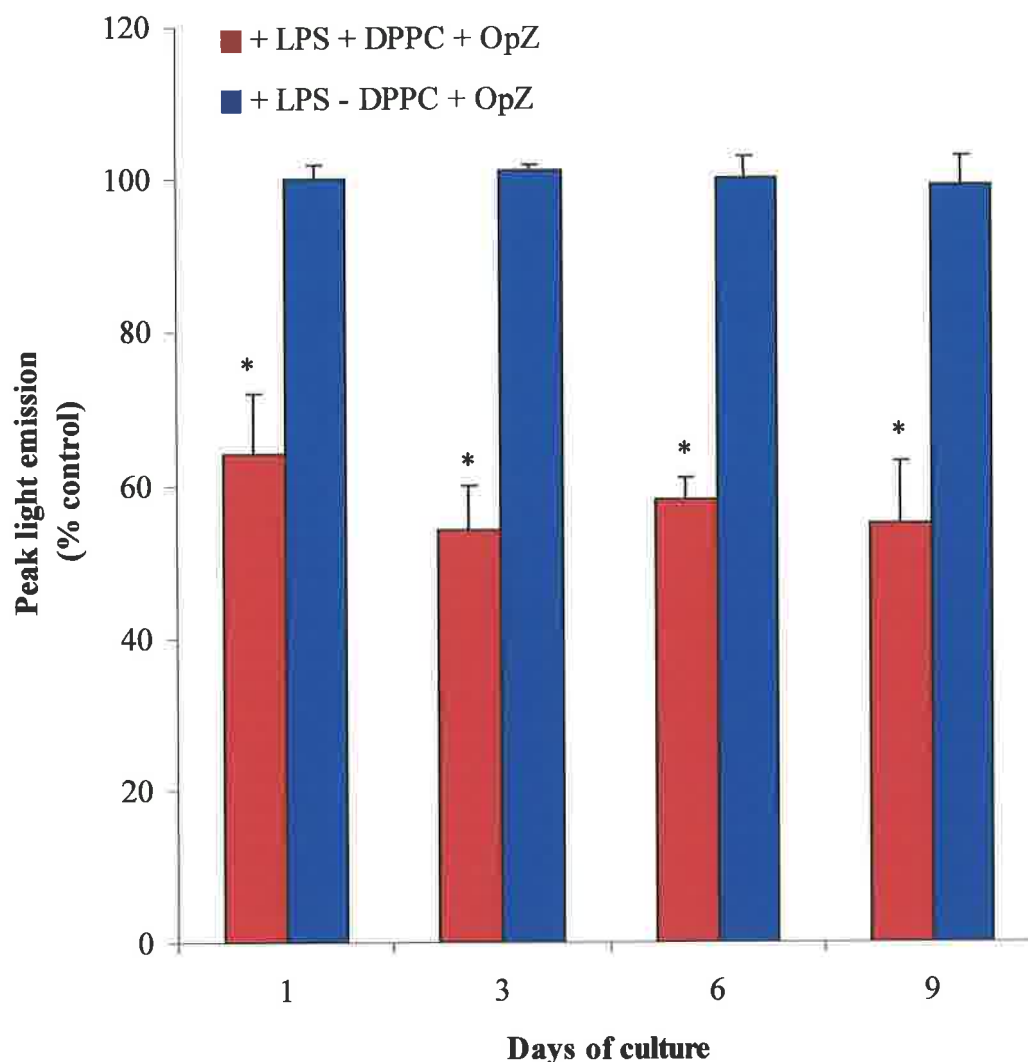
Figure 3.16: Effect of long-term culturing of MM6 cells with DPPC on OpZ elicited responses. MM6 cells were cultured with RPMI media containing DPPC (500 μg / ml) for upto 9 days. At each passage, 5×10^6 MM6 cells were washed in PBS (×3) and 'primed' for 18 hours with LPS. LCL was initiated with OpZ (125 μg / ml). Results are expressed as mean ± SD of 3 separate experiments. *P<0.05 (analysed by Mann-Whitney) when peak light emission of test was compared to peak light emission of control.
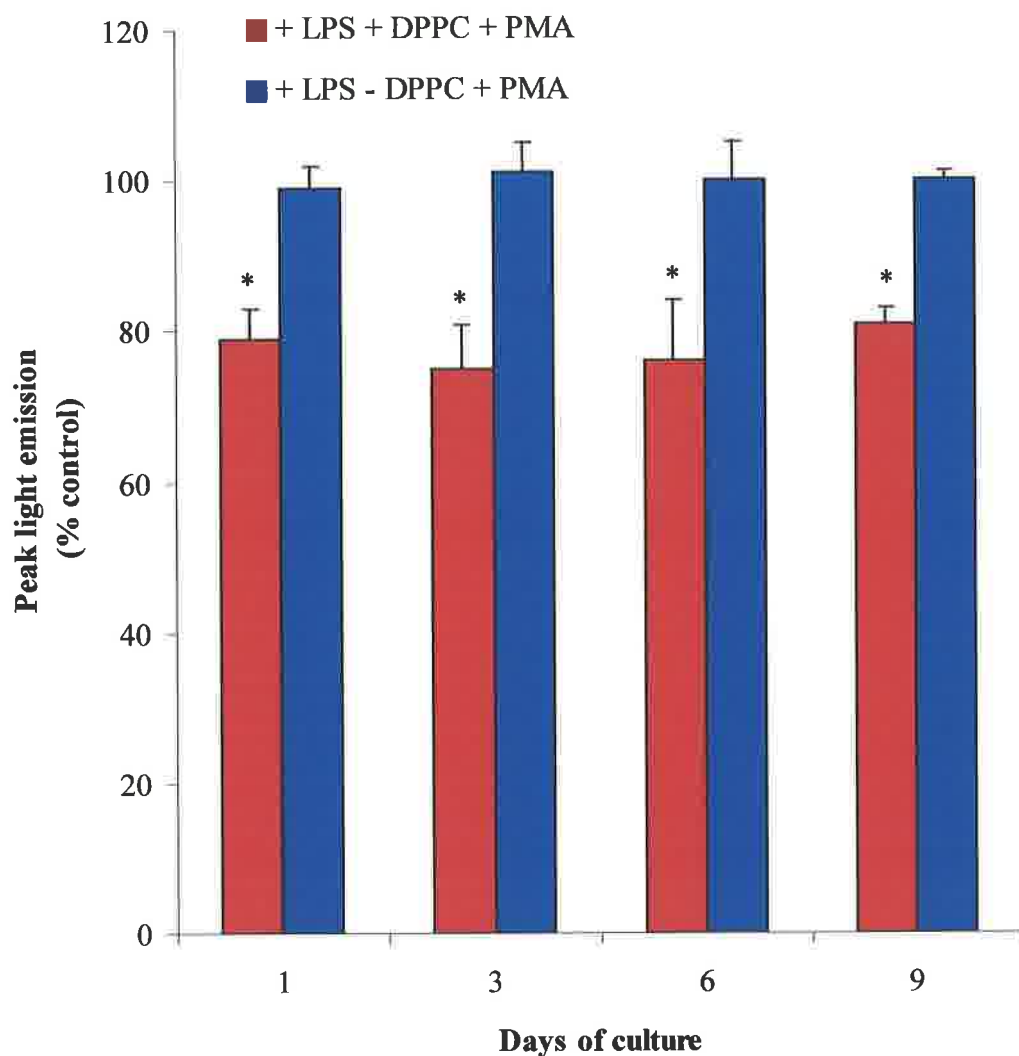
Figure 3.17: **Effect of long-term culturing of MM6 cells with DPPC on PMA elicited LCL responses.** MM6 cells were cultured with RPMI media containing DPPC (500 µg / ml) for upto 9 days. At each passage, 5×10^6 MM6 cells were washed in PBS (×3) and 'primed' for 18 hours with LPS. LCL was initiated with PMA (100 ng / ml). Results are expressed as mean ± SD of 3 separate experiments. *P<0.05 (analysed by Mann-Whitney) when peak light emission of test was compared to peak light emission of control.

3.3.8 ANALYSIS OF THE 'POST-PHOSPHOLIPID' EFFECT OF PC ON ROI PRODUCTION IN MM6 CELLS

These experiments were performed to investigate if the modulatory effect of PC species on MM6 ROI production was reversible or transient or required the continual presence of phospholipid. MM6 cells (1×10^6 cells / ml) were incubated with tPC (100 µg / ml), DPPC (100 µg / ml) or PAPC (125 µg / ml) for 2 hours. Following incubation, the phospholipids were removed from culture and MM6 cells were re-suspended in fresh RPMI media alone for a further 24 or 36 hours. Following LPS 'priming' and stimulation by OpZ (125 µg / ml) the LCL responses were recorded. When the cell preparations were washed free of lipid and incubated for a further 24 hours in media, prior to chemiluminescence, DPPC and PAPC exhibited the same degree of modulation as if the phospholipid were present (Figure 3.18). However, modulation of ROI production by DPPC and PAPC following 36 hours of PC removal is lost. TPC on the other hand had no effect on ROI production following either 24 or 36 hours removal of this phospholipid from the media. Taken together these results suggest the effects of phospholipid on the activity of NADPH oxidase is not permanent and is reversible after 36 hours.
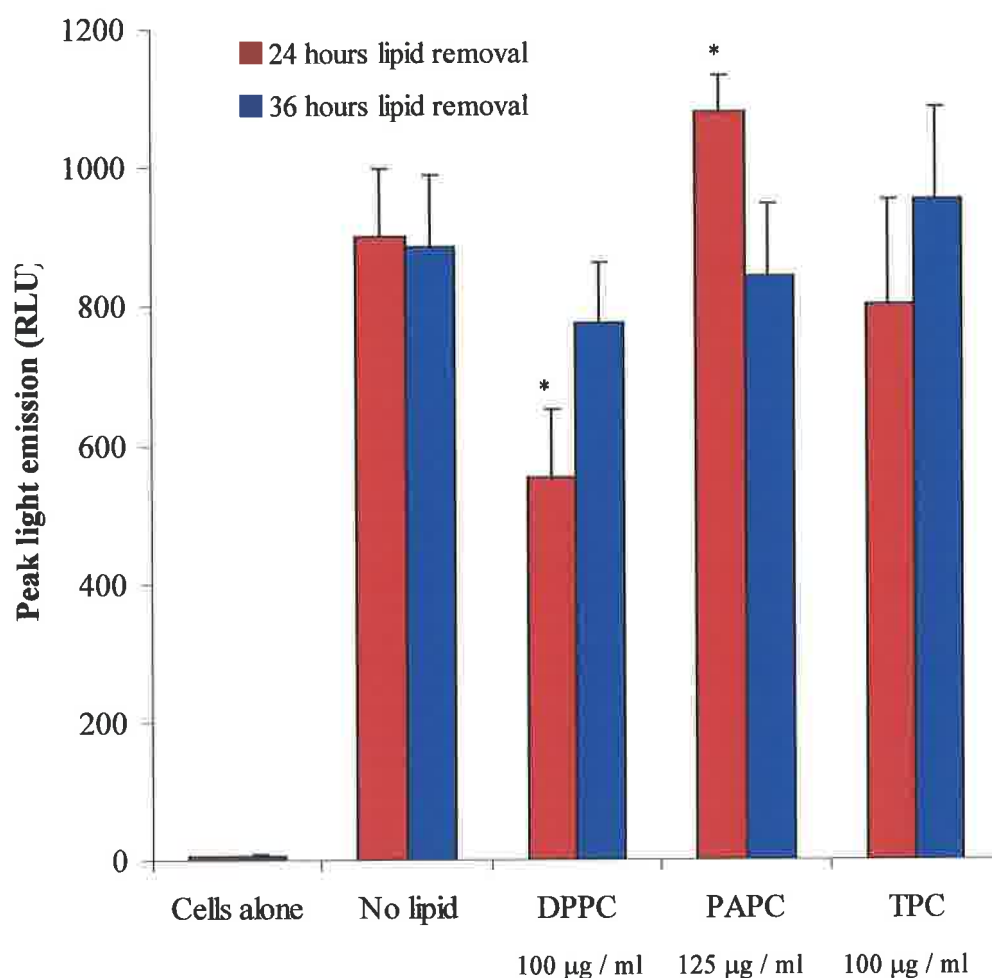
Figure 3.18: Modulation of ROI production after removal of PC from the media.
MM6 cells (1×10^6 cells / ml) were incubated with lipid for 2 hours, washed ($\times 3$) and suspended in RPMI media for 24 or 36 hours. Cells were 'primed' with LPS and LCL initiated with OpZ (125 µg / ml). Results are expressed as mean ± SD of 3 separate experiments. *P<0.05 (analysed ANOVA and Tukey's) when peak light emission of test was compared to peak light emission of control.

3.3.9 **EFFECT OF DPPC ON ROI PRODUCTION IN OTHER MONOCYTE / MACROPHAGE CELLS**

To support the study's finding that DPPC exerts a suppressive effect on the respiratory burst of monocyte / macrophages, studies were extended to include murine RAW 264.7 macrophages, rat alveolar macrophages (NR8383) and human peripheral blood monocytes.   Each cell type (1×10^6 cells / ml) was incubated with DPPC for 2 hours followed by washing in PBS and 'priming' with LPS (100 ng / ml) for 18 hours.   Cells were stimulated with OpZ (125 μg / ml).   Incubation of DPPC with the mouse macrophage RAW 264.7 demonstrated a dose-dependent decrease in ROI production in OpZ elicited LCL.   Peak light emission occurred at approximately 35 minutes following stimulation and LCL returned to basal levels after several hours (Figure 3.19).   When Raw 264.7 cells were incubated with 10, 100 or 500 μg / ml of DPPC a 5%, 31% and 60% respective decrease was seen in ROI production.   When comparing the peak light emission of each test by ANOVA the data demonstrated a significant decrease ($P<0.0001$) amongst the means of the treatments.   Tukey's pairwise comparisons indicated that the significant decrease was attributed to the cells treated with 100 or 500 μg / ml ($P<0.01$ respectively).
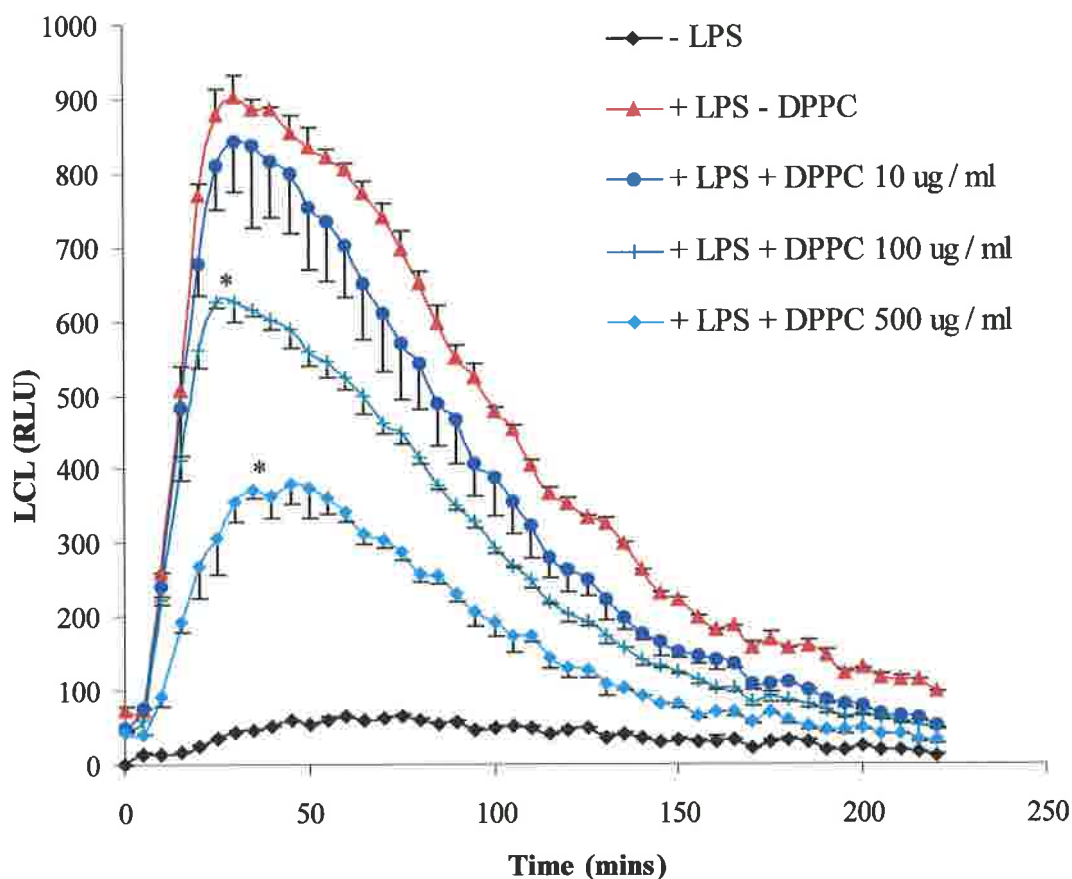
Figure 3.19: Modulation of ROI production by DPPC in RAW 264.7 cells. RAW 264.7 cells (1×10^6 cells / ml) were incubated with DPPC for 2 hours, washed ($\times 3$) and 'primed' with LPS (100 ng / ml) for 18 hours. LCL was initiated with OpZ (125 µg / ml). Results are expressed as mean ± SD of 3 separate experiments. *$P < 0.01$ (analysed ANOVA and Tukey's) when peak light emission of test was compared to peak light emission of control.

NR8383 cells are difficult to grow and maintain in culture. They are rat alveolar macrophages and exist in culture as two populations; an adherent and non-adherent population. The adherent population is the alveolar macrophage phenotype and consequently are very slow to grow to significant numbers for many investigations. Therefore these cells were only incubated with 100 µg / ml of DPPC, a concentration that has been suggested to represent *in vivo* conditions. NR8383 cells cultured with DPPC at this concentration have a reduced capacity to produce ROIs in response to OpZ in LPS 'primed' cells (Figure 3.20). This is a significant (20%) reduction in ROI release when compared to the control incubated without DPPC ($P<0.05$ analysed by Mann-Whitney). These results provide evidence that DPPC modulates the production of ROIs in macrophages, especially cells that normally reside in the alveoli.

Finally, DPPC was incubated with peripheral blood monocytes that were previously isolated by Ficoll-Hypaque® density gradient centrifugation. In this experiment, DPPC was able to inhibit the production of ROIs in LPS 'primed' monocytes in a dose dependent manner. No reduction in ROI production was seen at a concentration of 10 µg / ml when compared to the control cells incubated without DPPC. However as the concentration of DPPC increased to 100 and 500 µg / ml, a 20 and 50% reduction (respectively) in ROI production was seen (Figure 3.21).
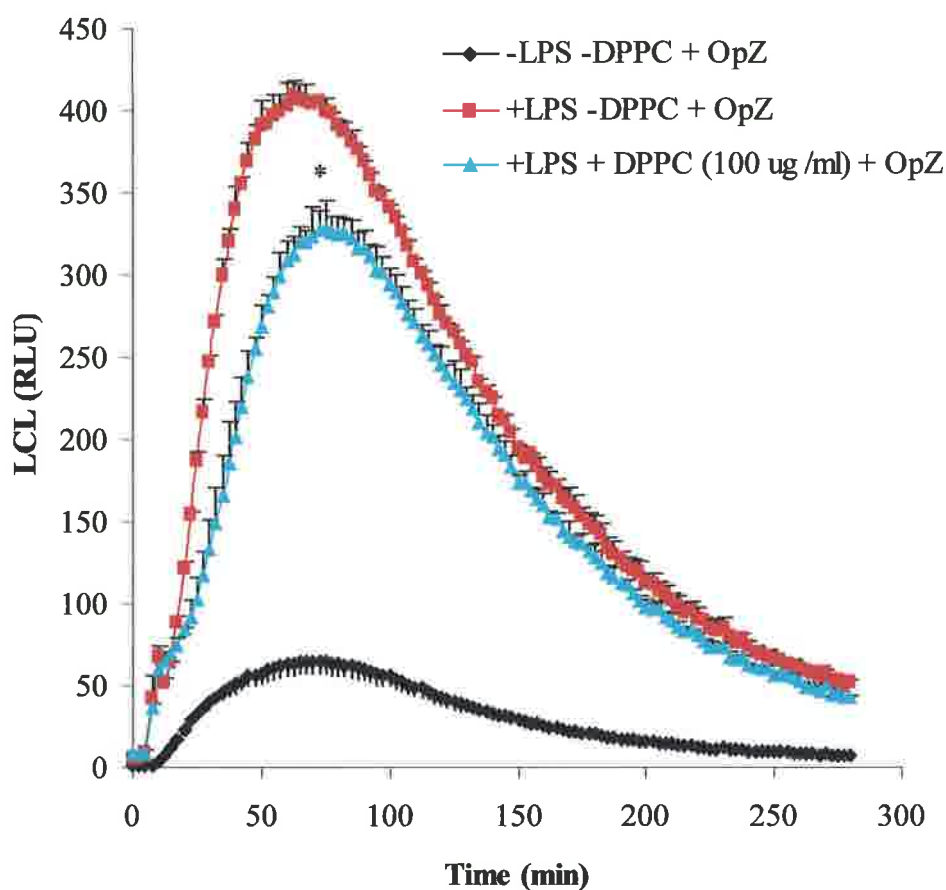
Figure 3.20: **Modulation of ROI production by DPPC in NR8383 alveolar macrophage cells.** NR8383 cells (1×10^6 cells / ml) were incubated with DPPC (100 µg / ml) for 2 hours, washed (×3) and 'primed' with LPS (100 ng / ml) for 18 hours. LCL was initiated with OpZ (125 µg / ml). Results are expressed as mean ± SD of 3 separate experiments. *P<0.05 (analysed by Mann-Whitney) when peak light emission of test was compared to peak light emission of control.
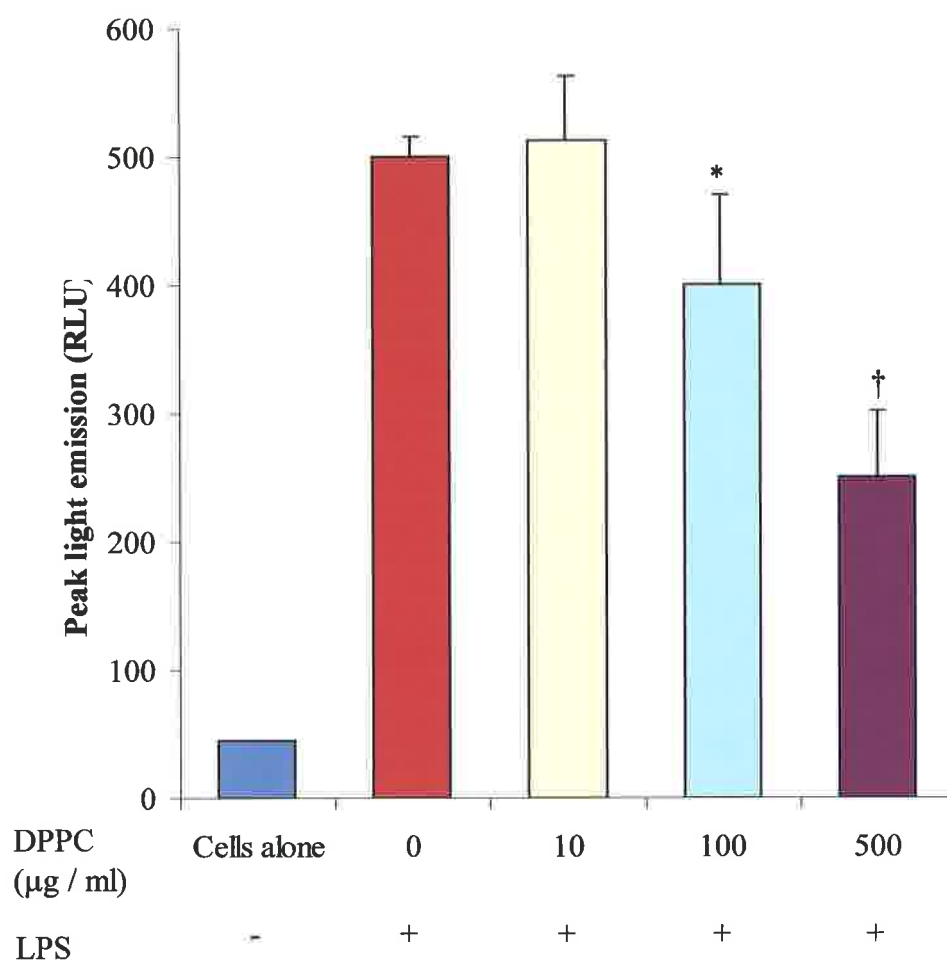
Figure 3.21: **Modulation of ROI production by DPPC in human peripheral blood monocytes.** Human peripheral blood monocytes (1×10^6 cells / ml) were incubated with DPPC for 2 hours, washed ($\times 3$) and 'primed' with LPS (100 ng / ml) for 18 hours. LCL was initiated with OpZ (125 µg / ml). Results are expressed as mean ± SD of 3 separate experiments. *P<0.05 and †P<0.01 (analysed ANOVA and Tukey's) when peak light emission of test was compared to peak light emission of control.

3.3.10 EFFECT OF DPPC ON ROI PRODUCTION AFTER LPS 'PRIMING'

The results of the previous sections have shown that surfactant lipids modulate ROI production in MM6 cells. In particular DPPC, the major phospholipid species of surfactant, down-regulates ROI production in LPS 'primed' cells. DPPC could be acting at various stages in the activation of the NADPH oxidase to produce these effects. For example DPPC could modulate the 'priming' effect of LPS, inhibited the binding of LPS or have direct effects on the NADPH oxidase or signalling pathways initiated by OpZ or PMA. Each of these possible effects were tested in the following sections.

The LCL studies were repeated as before except DPPC was added to the cells after they were 'primed' with LPS for 18 hours. Consistent with previous data, DPPC could still significantly inhibit the production of ROIs when compared to the control cells treated without DPPC (Figure 3.22). MM6 cells incubated with 10 μg / ml could significantly inhibit the production of ROIs by 20% (p<0.05 by ANOVA and Tukey's pairwise comparisons). Upon incubation of MM6 cells with higher doses of DPPC (100 or 500 μg / ml) added after LPS 'priming', a further reduction in ROI production is seen, approximately a 25% and 40% decrease respectively when compared to cells incubated without DPPC (Figure 3.22). Again, by Tukey's comparisons this data is highly significant (P<0.01). These results demonstrate the ability of DPPC to inhibit the production of ROIs in cells previously 'primed' with LPS for 18 hours. This indicates that the effect of DPPC is independent of the LPS 'priming' process i.e. independent of LPS binding and activation, since DPPC incubations did not occur until the 'priming' process was complete.
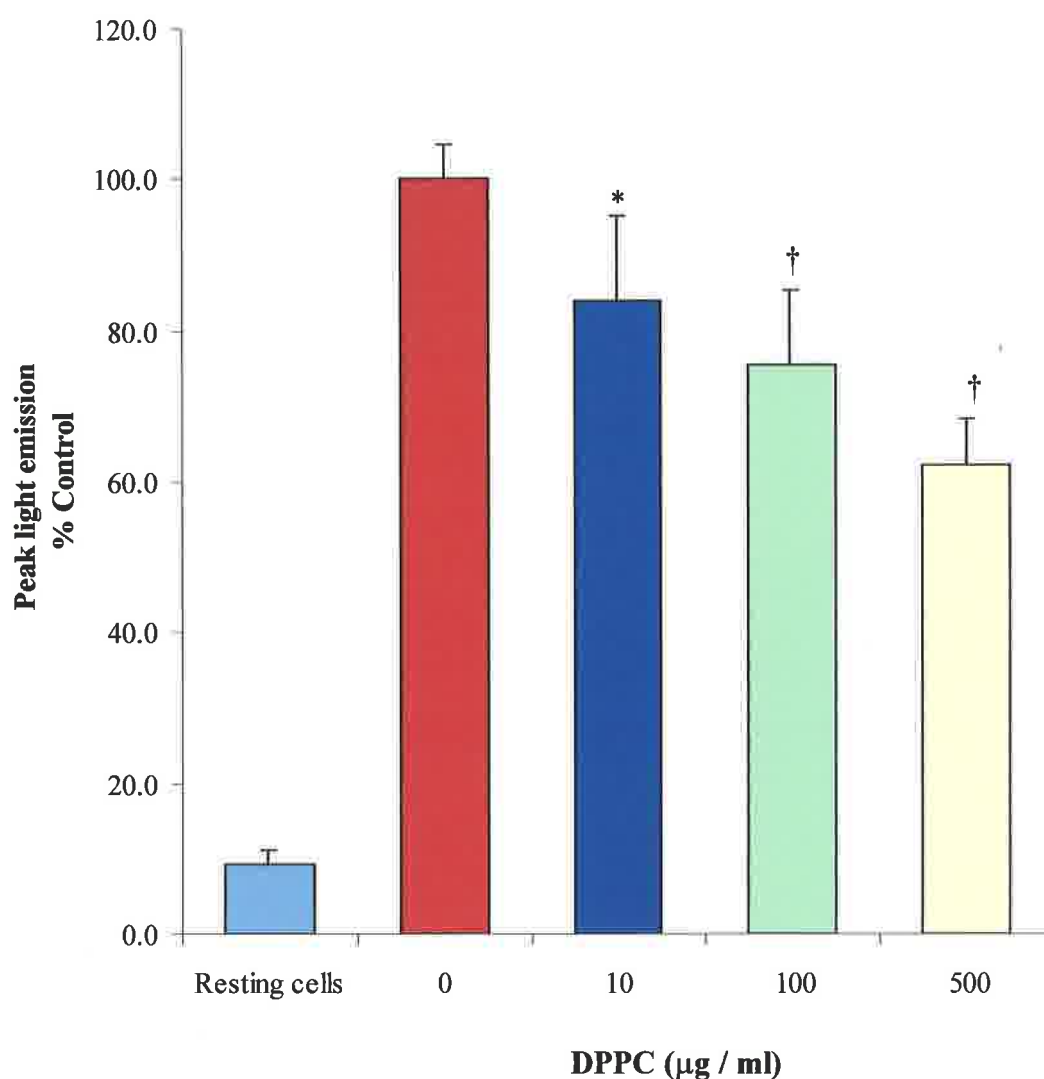
Figure 3.22: DPPC modulates ROI production in OpZ stimulated MM6 cells following LPS 'priming'. MM6 cells (1×10^6 cells / ml) were incubated with LPS (100 ng / ml) for 18 hours at 37°C. Cells were washed in PBS ($\times 3$) and incubated with DPPC for 2 hours. LCL was initiated with OpZ (125 µg / ml). Results are expressed as mean ± SD of 3 separate experiments. *P<0.05 and †P<0.01 using ANOVA and Tukey's multi-pairwise comparisons.

3.4 RADIOASSAY TO ASSESS THE BINDING OF LPS TO MM6 CELLS

Chemiluminescence experiments provided evidence that DPPC may exert its effect independently of LPS 'priming' (section 3.3.10). To provide conclusive evidence of such effects, radiolabelled LPS ($[^3H]$LPS) was used to test the hypothesis that DPPC does not prevent the binding of LPS to MM6 cells.

3.4.1 RADIOLABELLED LPS BINDS TO MM6 CELLS IN A TIME AND DOSE-DEPENDENT MANNER

The *E.coli* LPS that was radiolabelled with tritium had the same biological activity and LAL reactivity as unlabelled LPS. The time and dose required for $[^3H]$LPS to bind to MM6 cells was optimised in order to test the effects DPPC may have on binding of LPS ligand to its receptor(s) on cell surfaces. MM6 cells at 0.5×10^6 cells / 200 µl volumes were incubated with 10 µg / ml $[^3H]$LPS for $0 - 180$ minutes at 4°C. Scintillation counting provided counts of radiolabelled LPS bound to MM6 cells. The number of LPS molecules bound per cell was quantified using equation 3.2 in section 2.8.2. Approximately 70% of the $[^3H]$LPS was associated with the cell when incubated with MM6 cells for 30 minutes. The binding of radiolabelled LPS was maximal after a one-hour incubation (Figure 3.23). Further, the radiolabelled LPS remained cell associated for up to 3 hours incubation on ice. This indicates that LPS shedding did not occur during this period. In subsequent investigations, $[^3H]$LPS was incubated with MM6 cells for 1hour.
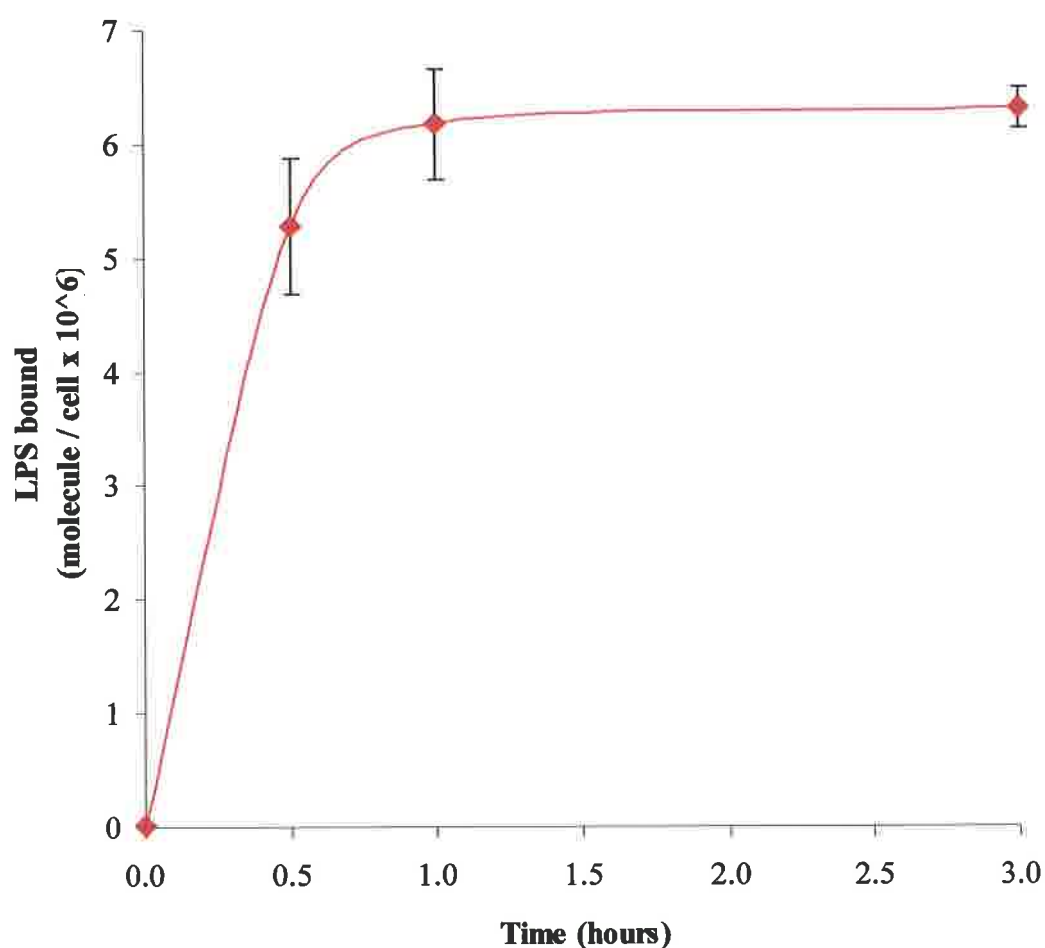
Figure 3.23: **Binding of radiolabelled LPS is dependent on time.** *E. coli* [^{3}H]LPS (10 μg / ml) was incubated on ice for 0.5, 1 or 3 hours with 0.5×10^6 MM6 cells / ml. Maximal LPS binding to MM6 cells was seen following a one-hour incubation on ice. Results are expressed as mean ± SD of 3 separate experiments.

The optimal concentration for saturated binding of [³H]LPS to MM6 cells was determined. MM6 cells were incubated with up to 10 μg / ml [³H]LPS at 4°C or 37°C. Performing the incubations on ice allows determination of cell-associated LPS that has not been internalised. Binding of [³H]LPS to MM6 cells is dose-dependent and markedly enhanced when incubated at 37°C (Figure 3.24). This difference in LPS binding reflects the ability of MM6 cells to internalise LPS thereby allowing the binding of further [³H]LPS molecules. However to investigate whether preincubation of DPPC with MM6 cells prevents the binding of *E. coli* LPS to MM6 cells, incubations with [³H]LPS were carried out at 4°C in order to prevent internalisation of LPS. The maximal amount of [³H]LPS binding to MM6 cells at 4°C was 10 μg / ml and was therefore used in subsequent studies. This concentration of LPS reflects both CD14 dependent and CD14 independent (i.e. non specific binding) mechanisms of LPS binding and uptake in cells.

3.4.2 EFFECT OF ANTI-CD14 ANTIBODIES AND UNLABELLED LPS ON BINDING OF [³H]LPS TO MM6 CELLS

The optimal concentration of LPS required to saturate the binding of this molecule on MM6 cells represents a large dose of endotoxin. At these levels, the possibility of non-specific binding of [³H]LPS could not be excluded. To investigate the specific binding of LPS via the CD14 receptor, MM6 (0.5×10^6 cells / ml) were treated with anti-CD14 antibody clones UCHM-1 or LeuM3 or with unlabelled LPS (10 μg / ml). These incubations were carried out for 1 hour at 4°C followed by washing in PBS. [³H]LPS binding was performed under optimal conditions already established.
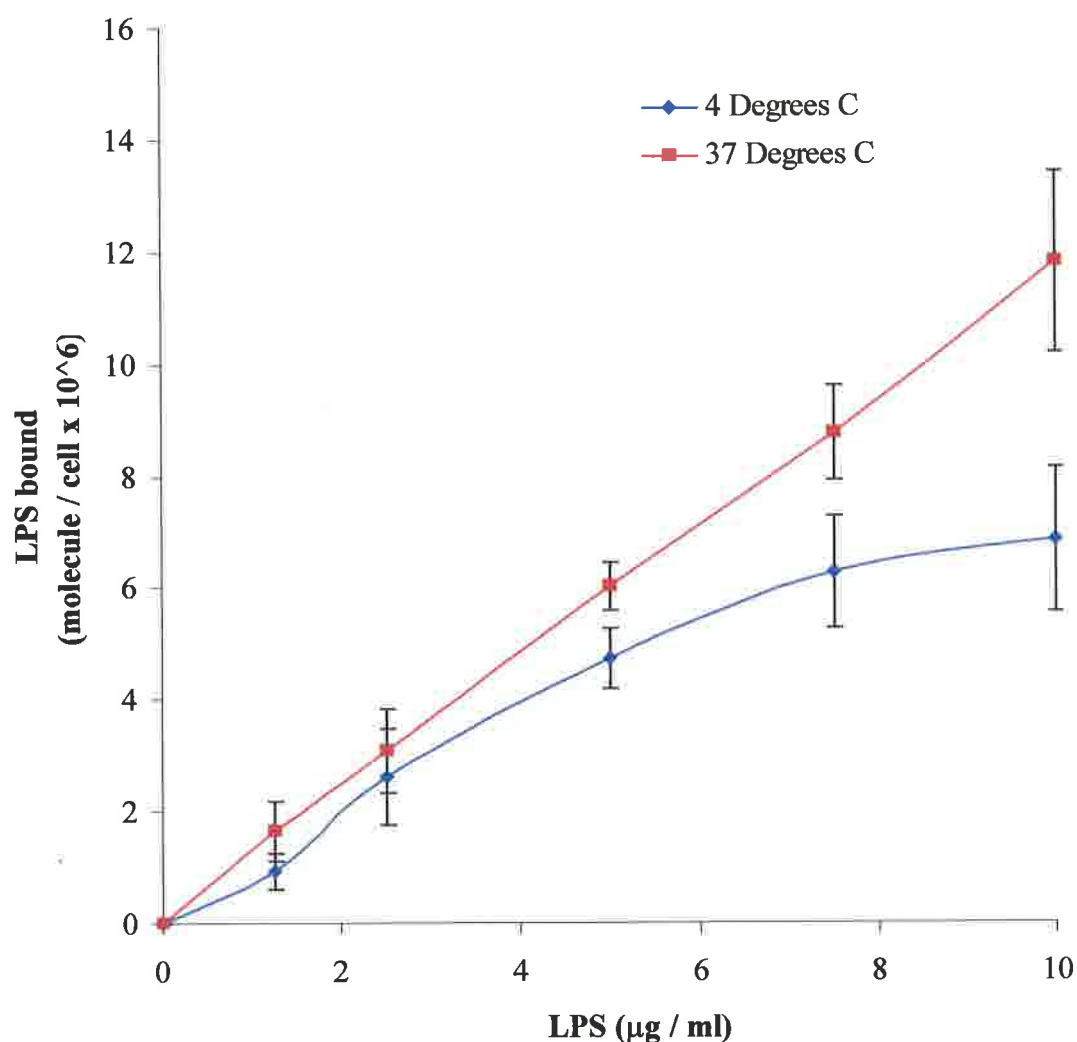
Figure 3.24: The binding of [³H]LPS is dependent on dose and temperature. Varying doses of [³H]LPS was incubated on ice or at $37^{o}C$ for 1 hour with 0.5×10^{6} MM6 cells. The binding of LPS was found to increase linearly with increased concentrations of LPS. Results are expressed as mean ± SD of 3 separate experiments.

The antibody LeuM3 is known to bind to the cell surface antigen CD14 and subsequently block CD14-dependent activation processes. Preincubation of MM6 cells with this antibody failed to block LPS binding to MM6 cells (Figure 3.25). However, the anti-CD14 antibody clone UCHM-1 significantly reduced the binding of [^{3}H]LPS by approximately 25% (P<0.01 by ANOVA and Tukey's pairwise comparisons). In addition, preincubation of MM6 cells with unlabelled LPS led to a significant reduction in the amount of [^{3}H]LPS binding to cells (P<0.01). These results suggest that approximately 25% binding of *E. coli* LPS to monocytes was attributed to the LPS-CD14 interaction. Inhibition was incomplete since there was substantial CD14 independent uptake of LPS during the 1 hour period.

3.4.3   DPPC DOES NOT PREVENT THE BINDING OF LPS TO MM6 CELLS

Using the optimal conditions previously established for saturable LPS binding to MM6 cells, the study investigated whether pre-incubation of DPPC (10, 100 or 500 µg / ml) with these cells could prevent the binding of [^{3}H]LPS. Binding of LPS in cells pre-incubated with DPPC was not significantly different from cells incubated in the absence of DPPC (Figure 3.26). However, a slight trend in increasing LPS bound was seen in cells incubated with increasing DPPC concentrations. This effect is not significant and cannot explain a decrease in ROI production in cells treated with DPPC. These results suggest that DPPC does not prevent the binding of LPS to MM6 cells thereby decreasing the ability of LPS to 'prime' MM6 cells for enhanced ROI production following stimulation with an agonist.
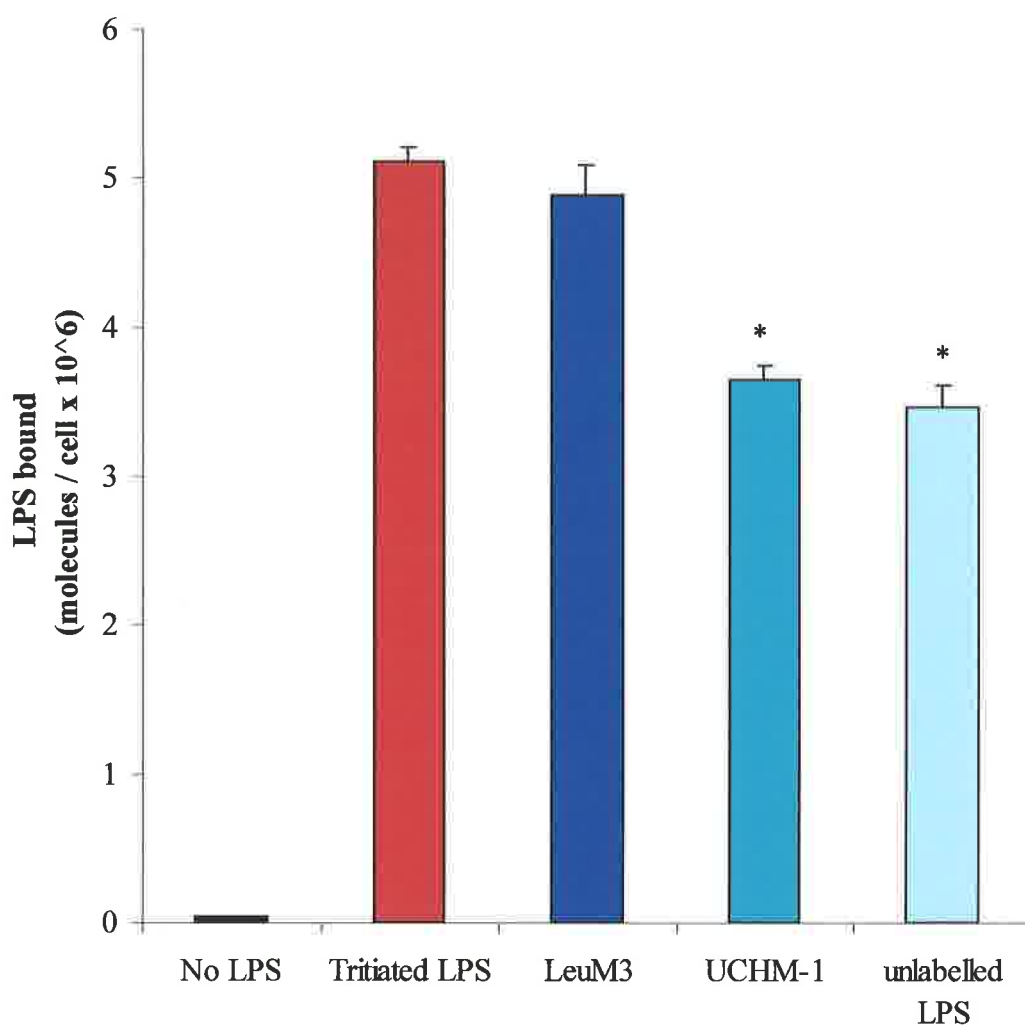
Figure 3.25: **The binding of [³H] LPS is inhibited by anti-CD14 ligands.** MM6 cells (0.5×10^6 / ml) were incubated with ligands to CD14 for 1 hour on ice. Cells were washed in PBS and incubated [³H]LPS (10 µg / ml) on ice for 1 hour. Results are expressed as mean ± SD of 3 separate experiments. *P<0.01 analysed ANOVA and Tukey's pairwise comparisons.
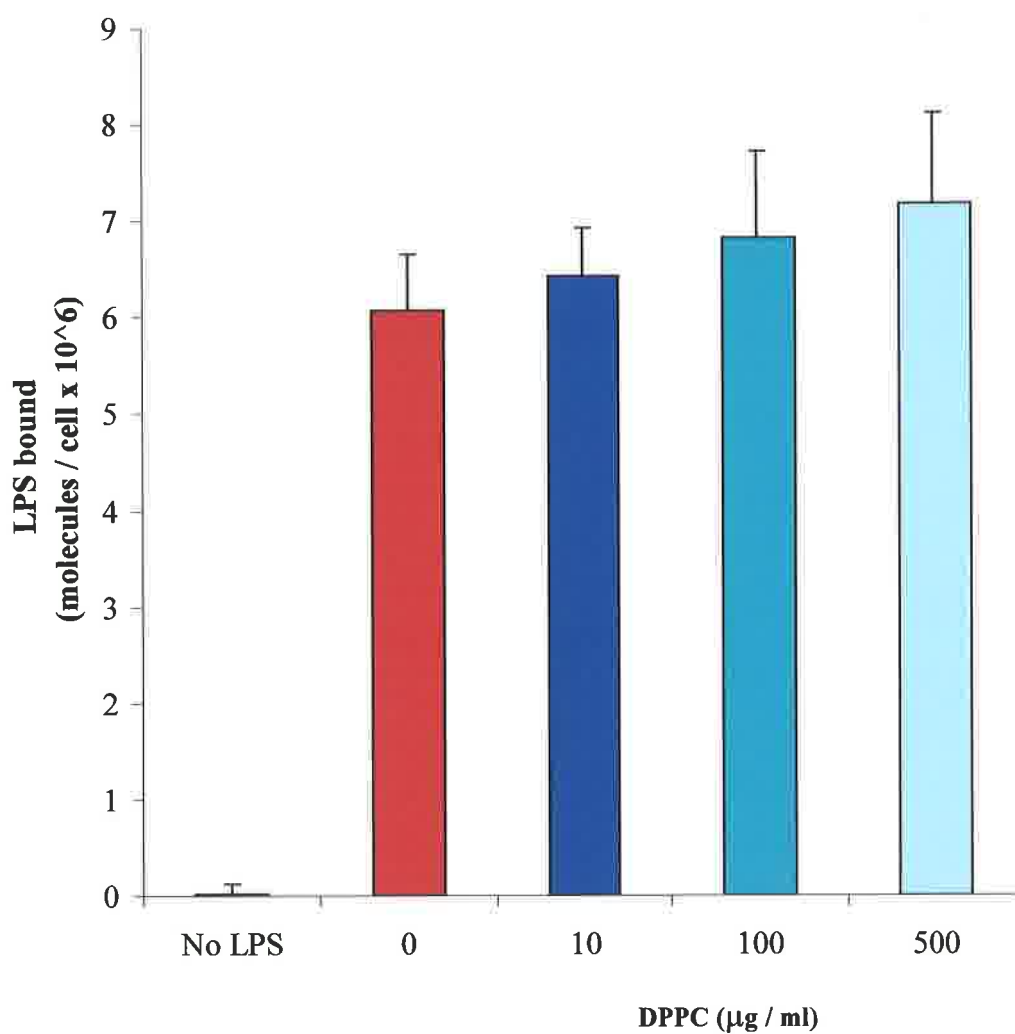
Figure 3.26: The binding of [^{3}H]LPS is not inhibited by DPPC. MM6 cells (0.5 × 10^6 / ml) were incubated with DPPC for 2 hours. Cells were washed in PBS and incubated [^{3}H]LPS (10 µg / ml) on ice for 1 hour. Results are expressed as mean ± SD of 3 separate.

3.5 PHENOTYPIC QUANTIFICATION OF CD14, FCγ AND COMPLEMENT RECEPTORS

Fcγ, complement and CD14 receptor expression were investigated to determine whether DPPC inhibits the production of ROIs by reducing the expression of one or more of these receptors that are important for bactericidal functions for monocytes (Allen and Aderem, 1996).

3.5.1 OPTIMAL BINDING OF PRIMARY ANTIBODIES TO CELL SURFACE RECEPTORS

In initial experiments, cell surface expression of CD11b/Mac1 (iC3b receptor), CD14 (LPS receptor), CD16 (FcγRIII receptor), or CD64 (FcγRI receptor) was optimised to determine the best working dilution. As this titre point is reached, saturation of binding sites maximises the intensity of the positive population before non-specific binding decreases the separation between negative and positive populations. The optimal concentration of antibodies to be used was determined by titration curves. This was performed by incubating MM6 cells (0.5×10^6 cells) with varying dilutions of each monoclonal antibody in equivalent volumes for each test (100 µl of buffer). The peak intensity for MM6 cells was determined at each dilution and the fluorescence intensity was plotted versus dilution (Figure 3.27). The optimal titre of antibody to use depends on the actual assay itself. The procedure used here was an indirect method with washing steps prior to fixation of MM6 cells. Therefore, the optimal dilution to use was when the maximum intensity plateaued (Owens and Loken, 1995). It can be observed

that maximal antibody-antigen binding of each cell surface antigen used was optimal when the titre of monoclonal antibody incubated with MM6 cells was a 1:10 dilution (Figure 3.27). This dilution is also maximal in terms of cost effectiveness, since ten times more tests can be used when compared to the maximal titre (neat solution), that does not provide any more sensitivity and does not improve on the signal to background ratio.

3.5.2 OPTIMAL DETECTION OF PRIMARY ANTIBODY USING PE CONJUGATED SECONDARY ANTIBODIES

To ensure that the detection of the monoclonal antibodies to CD11b / Mac-1, CD14, CD16 or CD64 was maximal, titration studies of secondary detection antibodies were performed. The optimal titre of each monoclonal antibody to a cell surface antigen was incubated for 1 hour on ice followed by washing in PBS. Varying dilutions of secondary antibody were incubated with the cells. The same titre of secondary antibody was incubated with MM6 cells incubated without the primary monoclonal antibody. This was to verify the non-specific binding of the secondary antibody. In each instance, a 1:10 titre of secondary antibody was sufficient to provide maximal detection of primary monoclonal antibodies while retaining low non-specific binding (Figure 3.28).
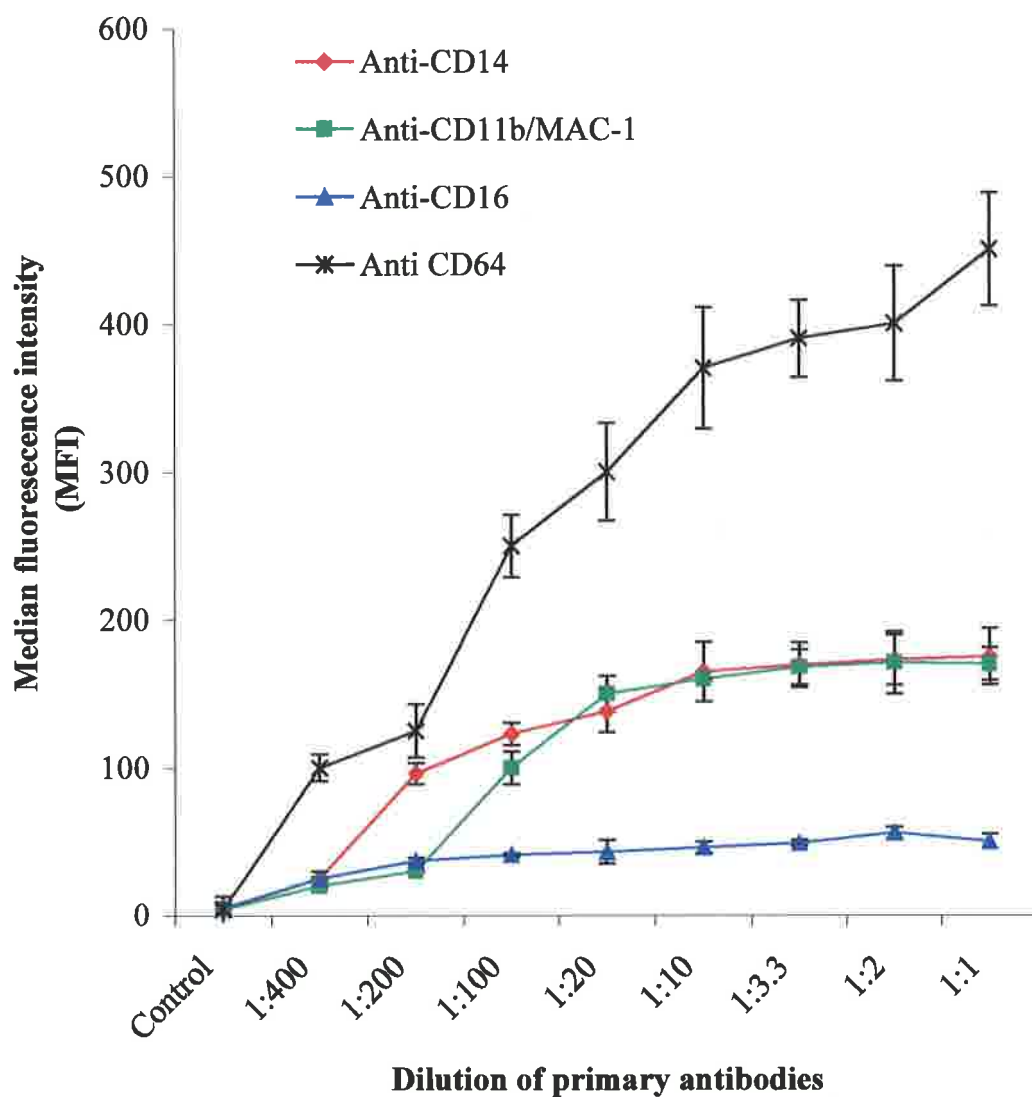
Figure 3.27: Titration of monoclonal antibodies detected by PE conjugated anti-mouse antibody. MM6 cells (0.5×10^6 / ml) were incubated with ligands to cell surface antigens for 1 hour on ice. Cells were washed in PBS and incubated with 1:10 of anti-mouse PE conjugate on ice for 1 hour. Results are expressed as mean ± SD of 3 separate experiments.
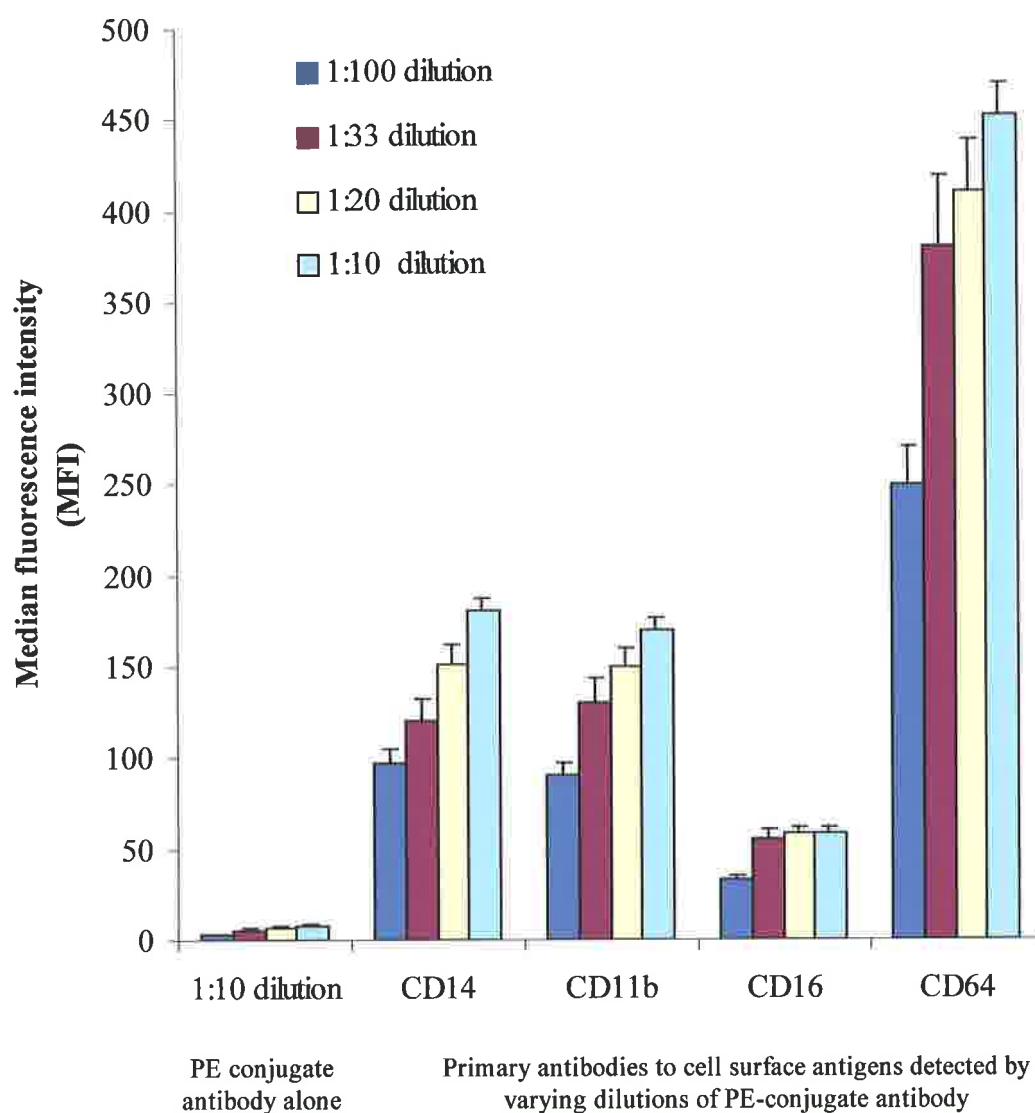
Figure 3.28: Titration of PE conjugate anti-mouse secondary antibody. MM6 cells (0.5×10^6 / ml) were incubated with optimal titres of monoclonal antibodies to cell surface antigens for 1 hour on ice. Cells were washed in PBS and incubated with varying titres of anti-mouse PE conjugate on ice for 1 hour. Results are expressed as mean ± SD of 3 separate experiments.

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3.5.3   EXPRESSION OF FCγ, COMPLEMENT OR CD14 RECEPTORS FOLLOWING DPPC INCUBATION

Optimal conditions for phenotypic expression of the cell surface receptors were established by FACS analysis (sections 3.5.1 and 3.5.2). These conditions were used to investigate the hypothesis, DPPC alters the expression of one or more of these important antigens with respect to phagocytosis, present on monocytes / macrophages. MM6 cells (0.5×10^6 cells / ml) were incubated with DPPC (10, 100 or 500 μg / ml) for 2 hours at 37°C. The cells were washed and immunostaining with primary (CD11b/MAC-1, CD14, CD16, or CD64) and secondary antibodies (anti-mouse R-PE) were performed. Antigen expression was quantified by flow cytometry. Typical FACS profiles are shown in Figure 3.29. The distribution of MM6 cells was displayed according to their forward scatter (FSC) and side scatter (SSC) properties (Figure 3.29A). In the analysis of cell-surface antigen expression, different fluorescence patterns were displayed. Figure 3.29B – 3.29D shows histograms of red fluorescence (FL2) due to MM6 cells that had bound monoclonal antibodies to its appropriate surface receptor followed by PE-conjugated anti-mouse bound to the monoclonal antibody. Fluorescence intensities were based on negative staining of cells by secondary antibody alone. Background staining was set to a median fluorescence intensity of (MFI) of less than 5. The complex could be quantified as the percent of fluorescing cells versus the total counted.

A

B

PE fluorescence (FL2)

■ Negative control
■ CD14

C

PE fluorescence (FL2)

■ Negative control
■ CD11b

D

PE fluorescence (FL2)

■ Negative control
■ CD16
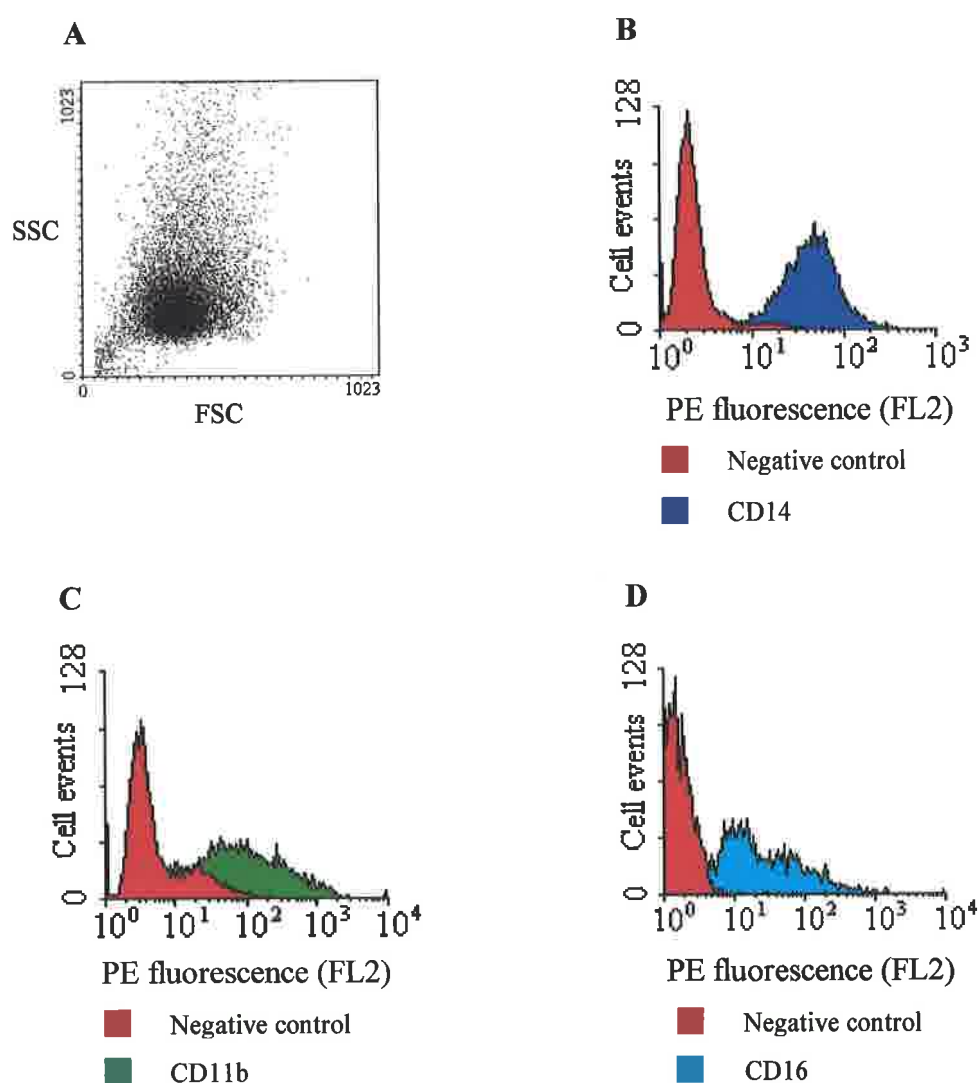
Figure 3.29: Typical FACS profiles of surface expression of CD14, complement (CD11b) and Fcγ (CD16) receptors. MM6 cells (0.5×10^6 / ml) were incubated with optimal titres of monoclonal antibodies to cell surface antigens for 1 hour on ice. Cells were washed in PBS and incubated with optimal anti-mouse PE conjugate titre on ice for 1 hour. Typical FACS profiles are shown. **(A)** Forward FSC / side scatter SSC plot; **(B - D)** Fluorescence histograms of cell surface antigen staining. Essentially identical results were obtained from 3 independent experiments.

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The expression of CD14, CD11b/MAC-1, CD16 and CD64 in cells pre-treated with 10, 100 or 500 μg / ml DPPC were not significantly different from cells treated without DPPC (Figures 3.30 – 3.33). Histograms with overlapping values of fluorescence were compared by the Kolmogorov-Smirnov statistics. This test provides a powerful tool for the objective statistical analysis of histograms that show unimodal distribution of values. Further the relative number (percentage) of cells that were positively immunostained were the same for DPPC treated and untreated for each marker. These results demonstrate that DPPC pre-incubation of levels of DPPC seen in pulmonary surfactant and those levels previously demonstrated to reduce ROI production do not alter the expression of the LPS receptor CD14. In addition complement and Fcγ receptor expression was also not significantly affected, suggesting DPPC does not reduce ROI production by impairing binding of stimulus to the membrane.
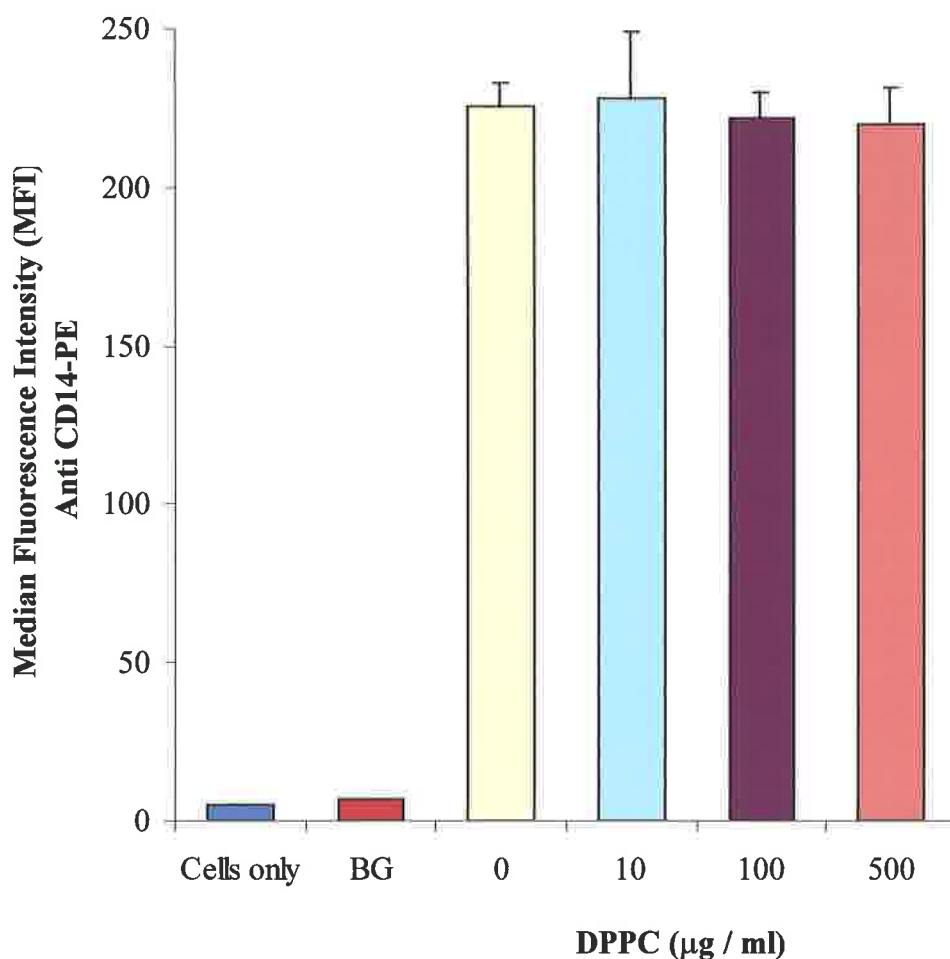
Figure 3.30: DPPC does not affect expression of the LPS receptor CD14. MM6 cells (1×10^6 / ml) were incubated with DPPC for 2 hours followed by washing in PBS ($\times 3$) and immunostaining for CD14. Antigen expression was quantified by flow cytometry. Results are expressed as mean ± SD of 3 separate experiments. BG; Background levels set by incubation with secondary PE conjugated anti-mouse antibody.
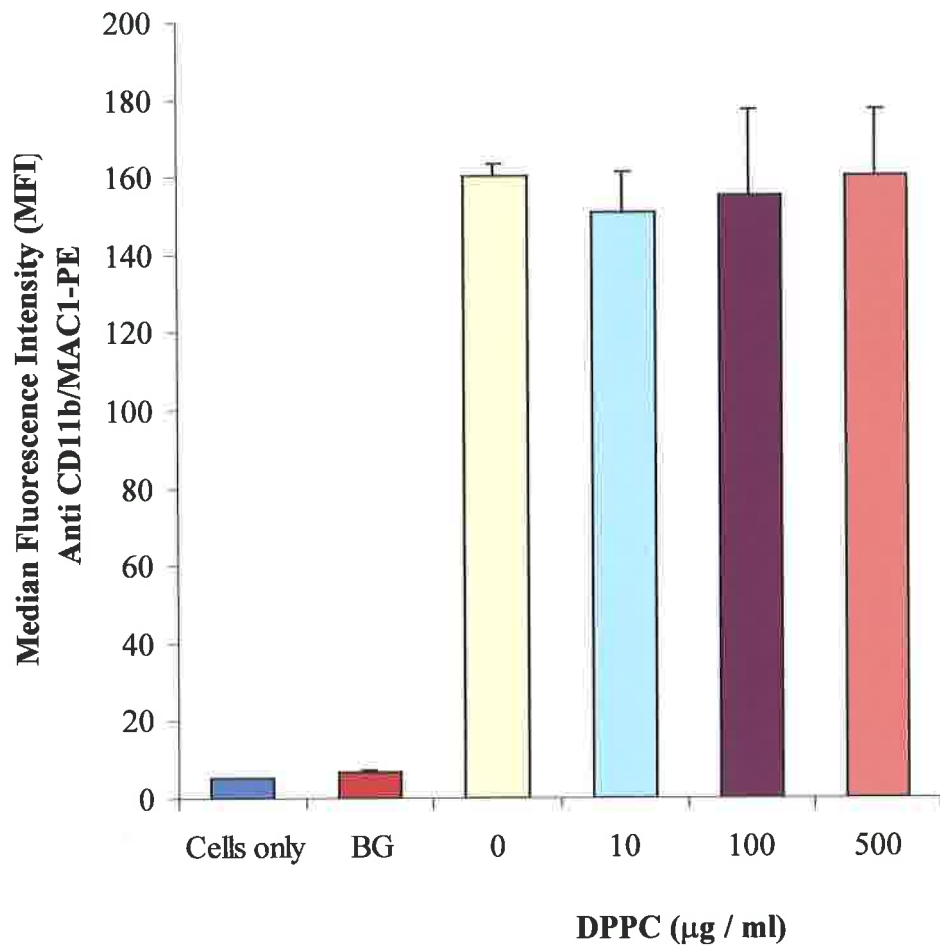
Figure 3.31: **DPPC does not affect expression of the iC3b receptor CD11b / MAC-1.** MM6 cells (1×10^6 / ml) were incubated with DPPC for 2 hours followed by washing in PBS (×3) and immunostaining for CD11b. Antigen expression was quantified by flow cytometry. Results are expressed as mean ± SD of 3 separate experiments. BG; Background levels set by incubation with secondary PE conjugated anti-mouse antibody.
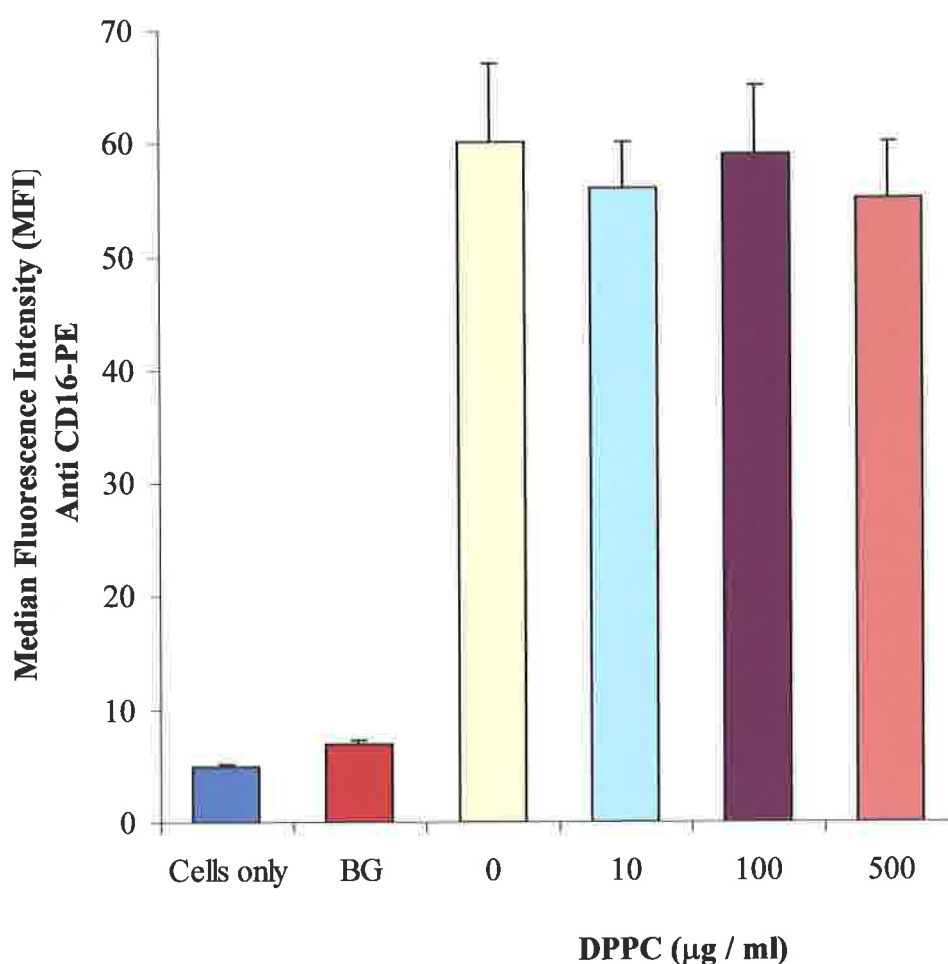
Figure 3.32: **DPPC does not affect expression of the FcγRIII receptor CD16.**
MM6 cells (1×10^6 / ml) were incubated with DPPC for 2 hours
followed by washing in PBS ($\times 3$) and immunostaining for CD16.
Antigen expression was quantified by flow cytometry. Results are
expressed as mean $\pm$ SD of 3 separate experiments. BG; Background
levels set by incubation with secondary PE conjugated anti-mouse
antibody.
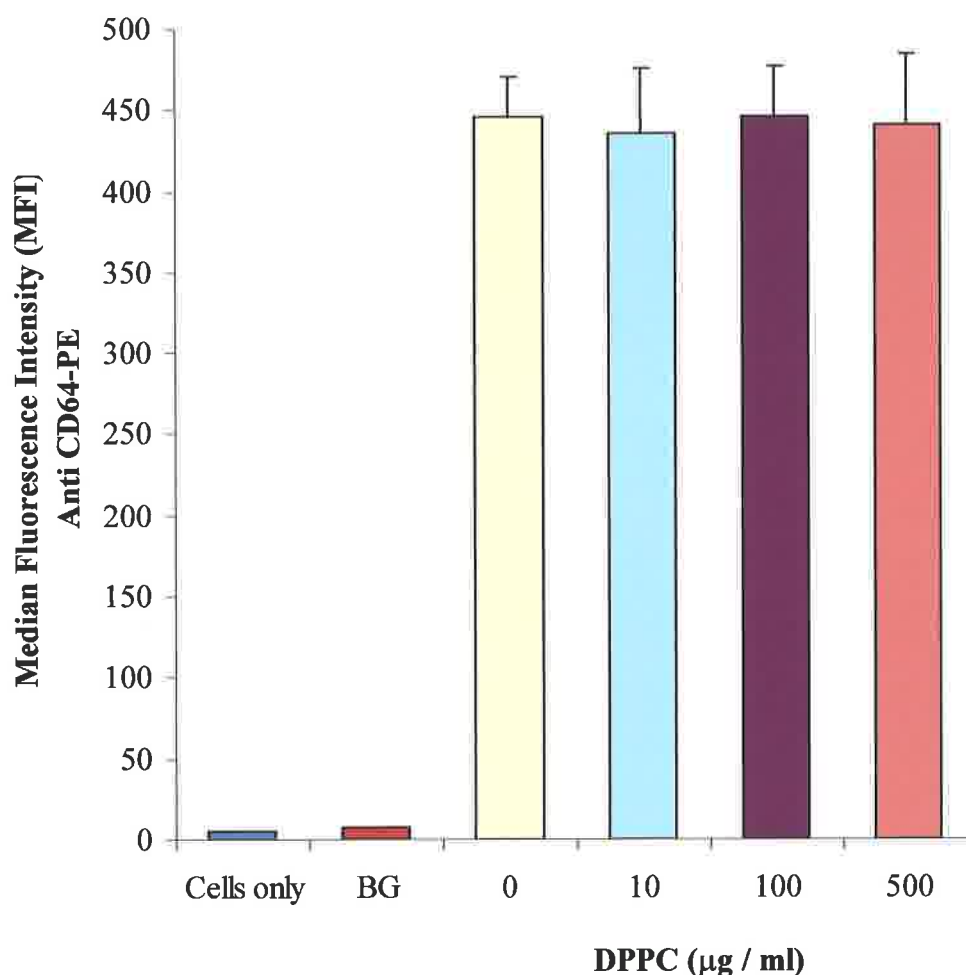
Figure 3.33: DPPC does not affect expression of the FcγRI receptor CD64. MM6 cells (1×10^6 / ml) were incubated with DPPC for 2 hours followed by washing in PBS ($\times 3$) and immunostaining for CD64. Antigen expression was quantified by flow cytometry. Results are expressed as mean ± SD of 3 separate experiments. BG; Background levels set by incubation with secondary PE conjugated anti-mouse antibody.

3.6 EFFECT OF DPPC ON MM6 CELL ULTRASTRUCTURE

DPPC uptake in MM6 cells was investigated by transmission electron microscopy (TEM) as described in section 2.10. Control monocytes presented with the presence of pseudopodes, numerous rough endoplasmic reticulum cysternae, a prominent Golgi complex, lysosomes, coated vesicles, endocytic vesicles, numerous mitochondria and lysosomal granules that appeared as electron dense structures of varying sizes. As opposed to the multilobed nucleus of granulocytes, the nuclei of this monocytic cell line presented as an indented or U-shaped nucleus with a prominent nucleolus and reticular appearing chromatin (Figures 3.34 and 3.35). To highlight the uptake of phospholipid, osmium tetroxide was used as a secondary fixative by reacting with lipids. Electron dense lipid structures such as lipid membrane structures can also be visualised in these samples. In MM6 cells incubated without DPPC, the vesicles present are clearly void of electron dense material typical of lipid-fixed material. MM6 cells incubated with DPPC showed an increase in the number of vesicles in the cytoplasm that appear to contain electron dense material indicative of lipid due to the nature of osmium tetroxide staining. Upon incubation with 10, 100 or 500 µg / ml of DPPC (Figures 3.36, 3.37 and 3.38 respectively), the number of lipid-containing vesicles increased in a dose dependent manner. This suggests that the content of these vesicles is likely to be DPPC or products of DPPC uptake and metabolism. In addition, MM6 cells pre-incubated with DPPC for 2 hours appeared to be more rounded and presented smoother cell membranes, suggesting that pre-incubation with DPPC resulted in membrane incorporation of DPPC and consequently, modifications in membrane morphology.

Lamellated inclusion bodies, an important feature seen in pulmonary surfactant, were detectable in the cytoplasm of some cells when MM6 cells were incubated 500 µg / ml of DPPC (Figures 3.38 and 3.39). The presence of a lamellar body indicates the uptake of DPPC as an intracellular storage granule. These events represent similar mechanisms encountered in human pulmonary surfactant composition and 'life' cycle within the pulmonary compartment (Wright, 1997). Taken together, these results suggest MM6 cells ingest DPPC in a dose-dependent manner. In addition, levels of DPPC that approximate to those seen in pulmonary surfactant may be taken up as lamellar bodies, events seen in normal pulmonary processes by alveolar macrophages.
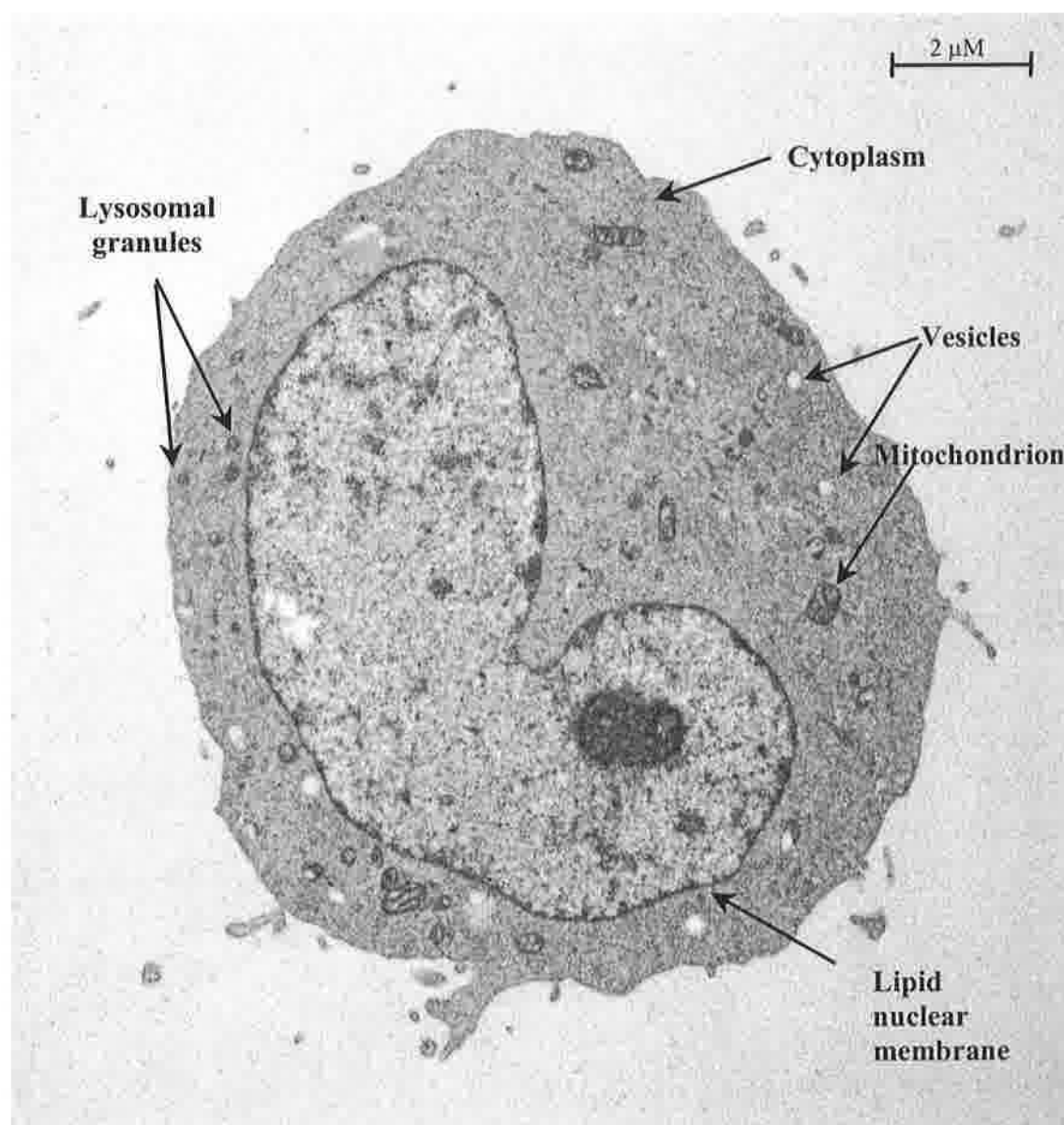
Figure 3.34: Ultra-structure of MM6 cells. MM6 cells incubated for 2 hours at 37°C (in the absence of DPPC) were processed for TEM and examined in a Phillips CM12 transmission electron microscope operating at 80 kV and magnification (× 3,810).
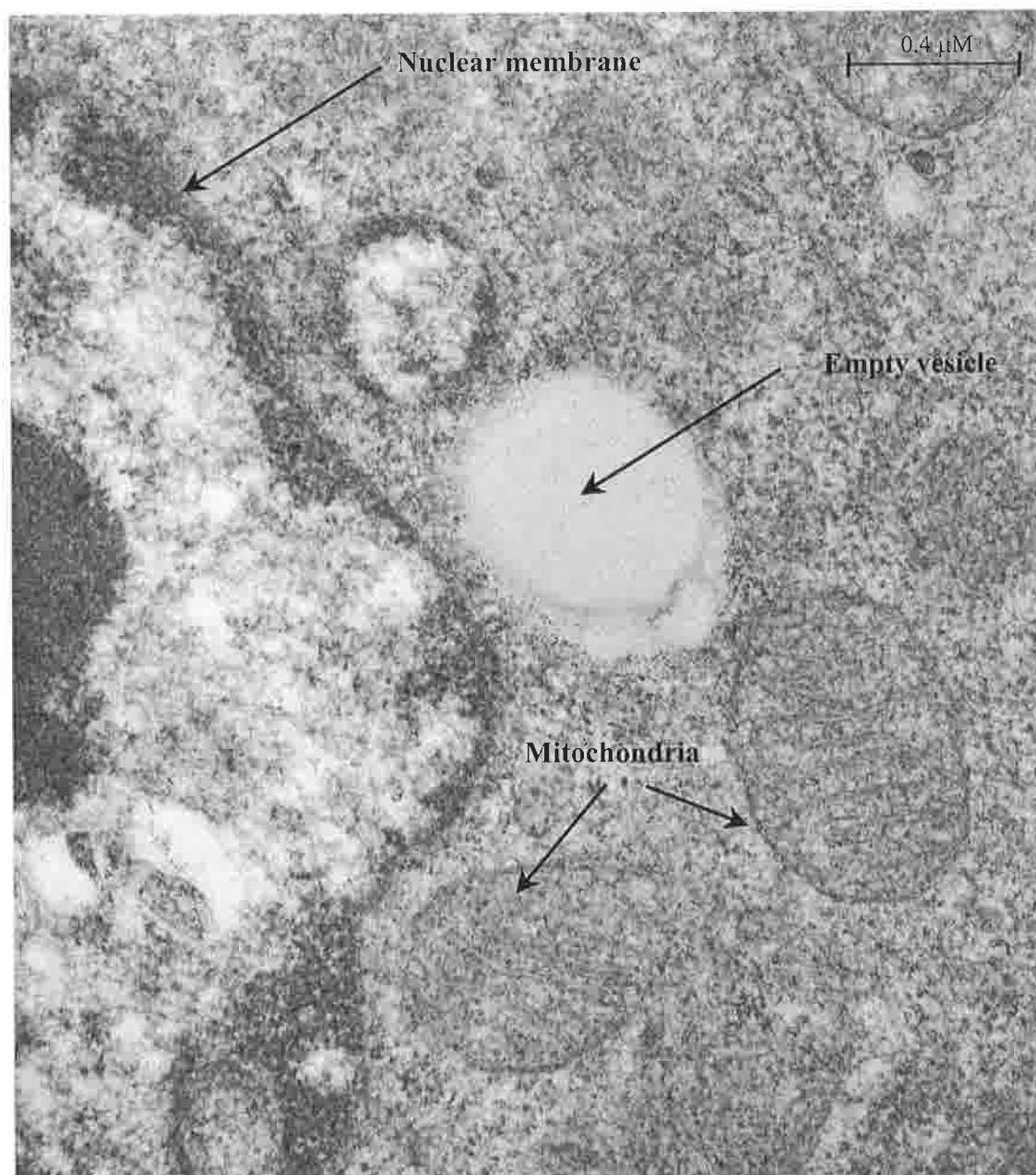
Figure 3.35: Ultrastructure of control MM6 cells at a higher power. A typical MM6 cell under incubated without DPPC was processed for TEM as described. This high power micrograph (×28,000) demonstrates numerous mitochondria and lipid stained structures. In the centre of the micrograph is the presence of a typical vesicle that does not contain electron dense material typical of lipid.

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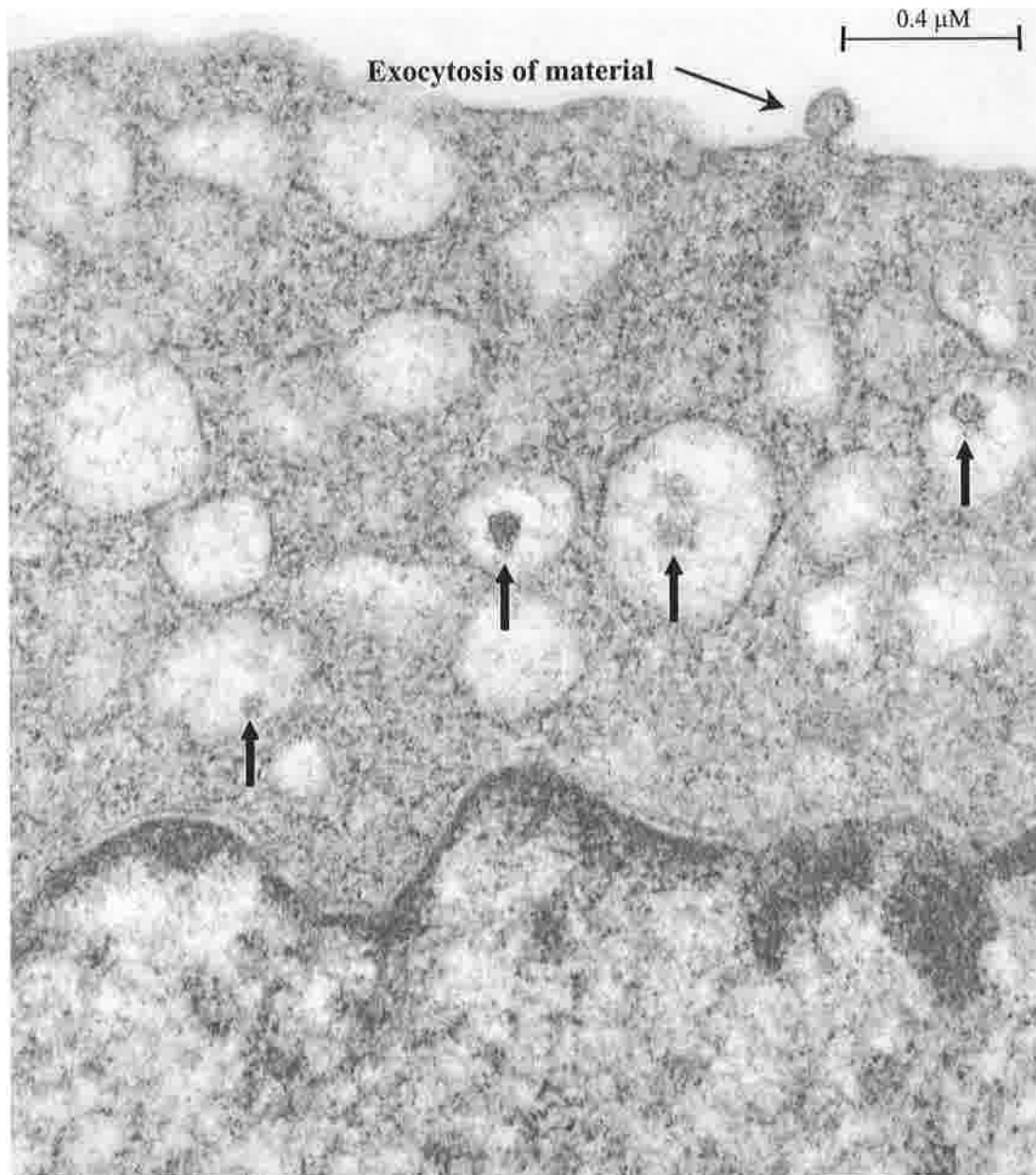
Figure 3.36: Ultrastructure of a typical MM6 cell incubated with 10 μg / ml DPPC for 2 hours. These cells processed for TEM were viewed under × 28,000 magnification. The vertical black arrows indicate typical vesicles containing electron dense material (lipid) stained with osmium tetroxide.
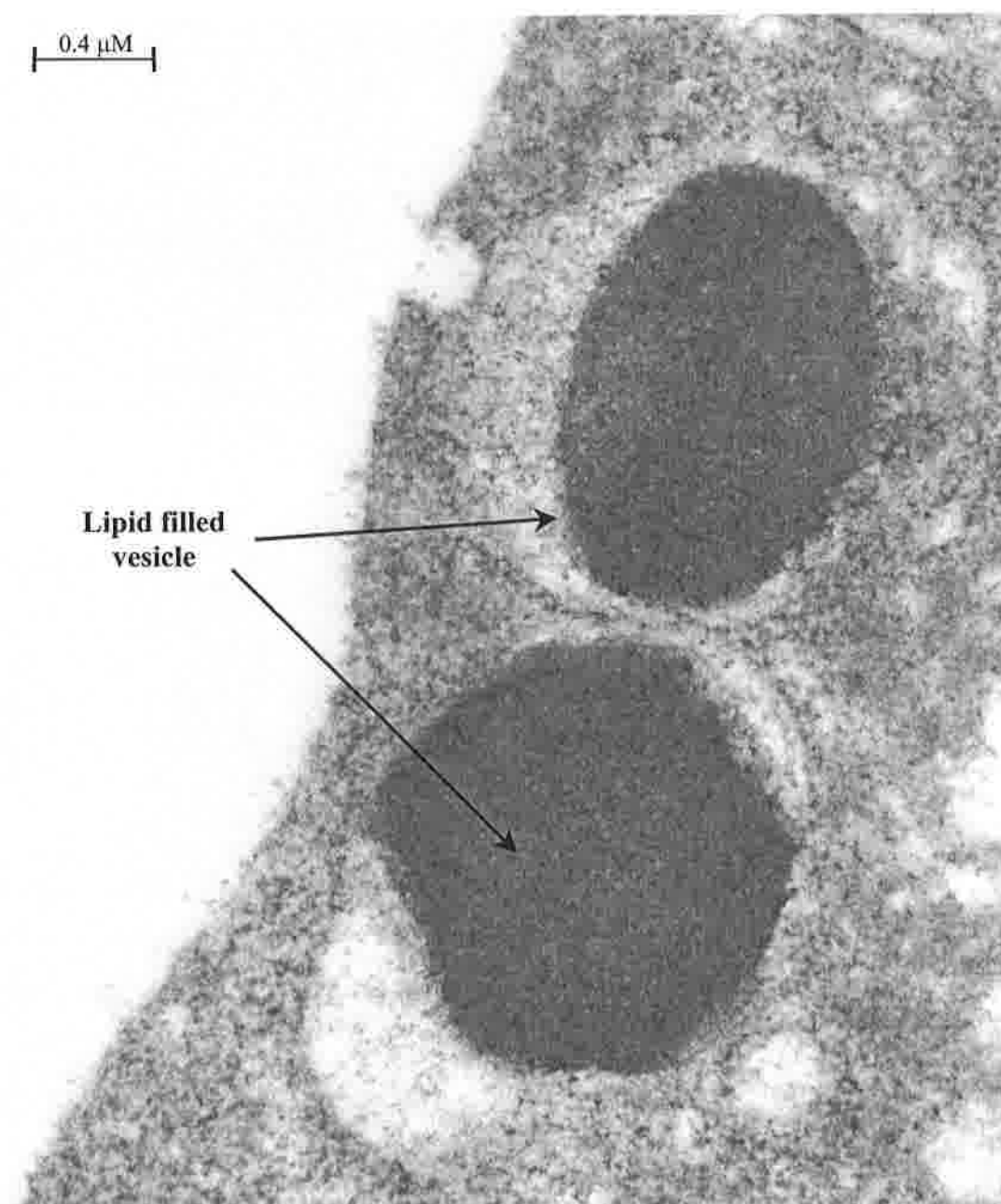
Figure 3.37: Ultrastructure of MM6 cells incubated with 100 µg / ml DPPC for 2 hours. A typical processed cell as viewed under × 28,000 magnification. Typical vesicles filled with an abundant amount of electron dense material indicative of lipid fixed material.
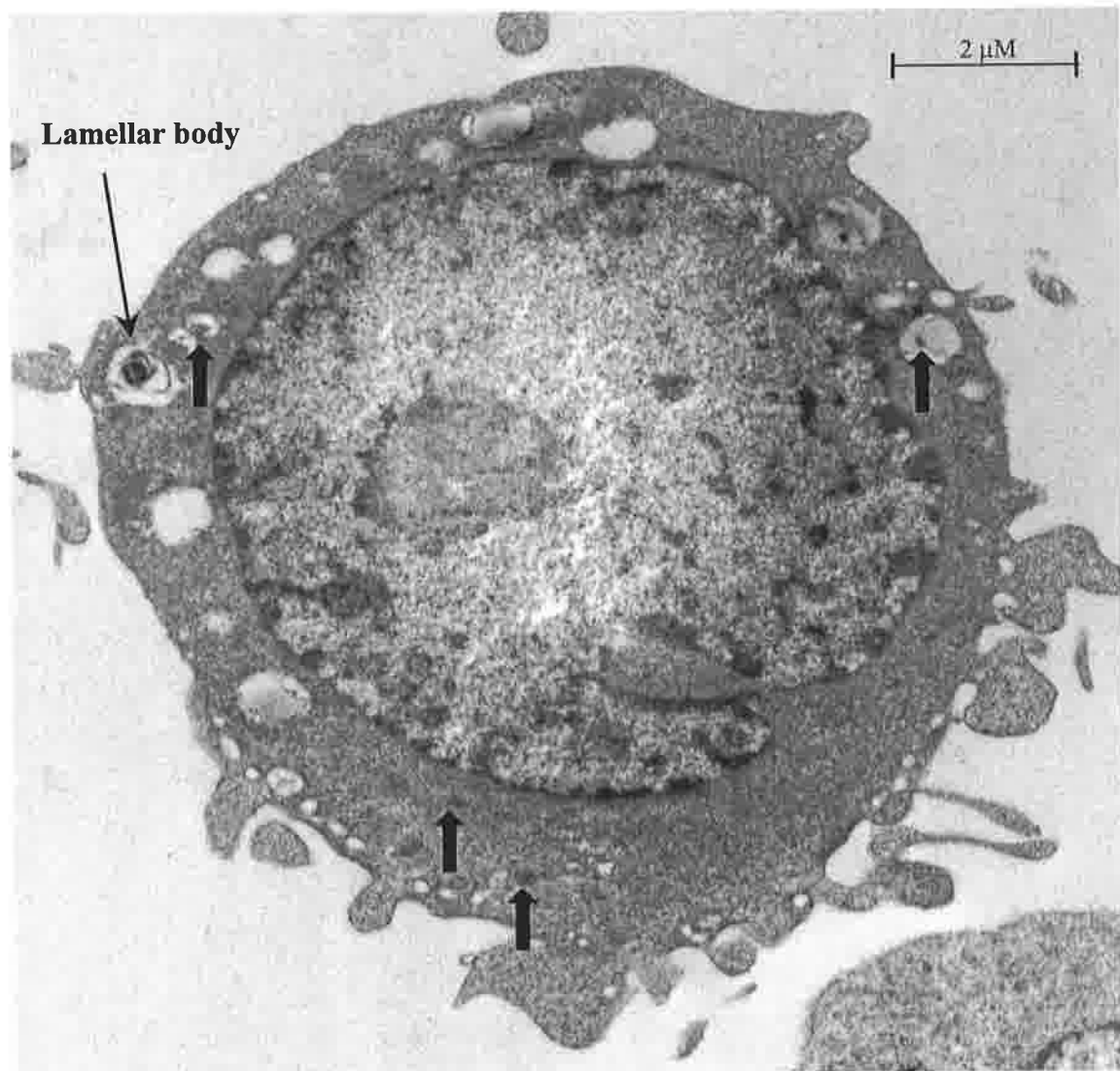
Figure 3.38: **Ultrastructure of MM6 cells incubated with 500 µg / ml DPPC for 2**

hours. TEM processed cells were viewed under × 3,800 magnification.

The black arrows indicate typical vesicles containing electron dense

material (lipid - stained with osmium tetroxide). Note the appearance of

a lamellar body.
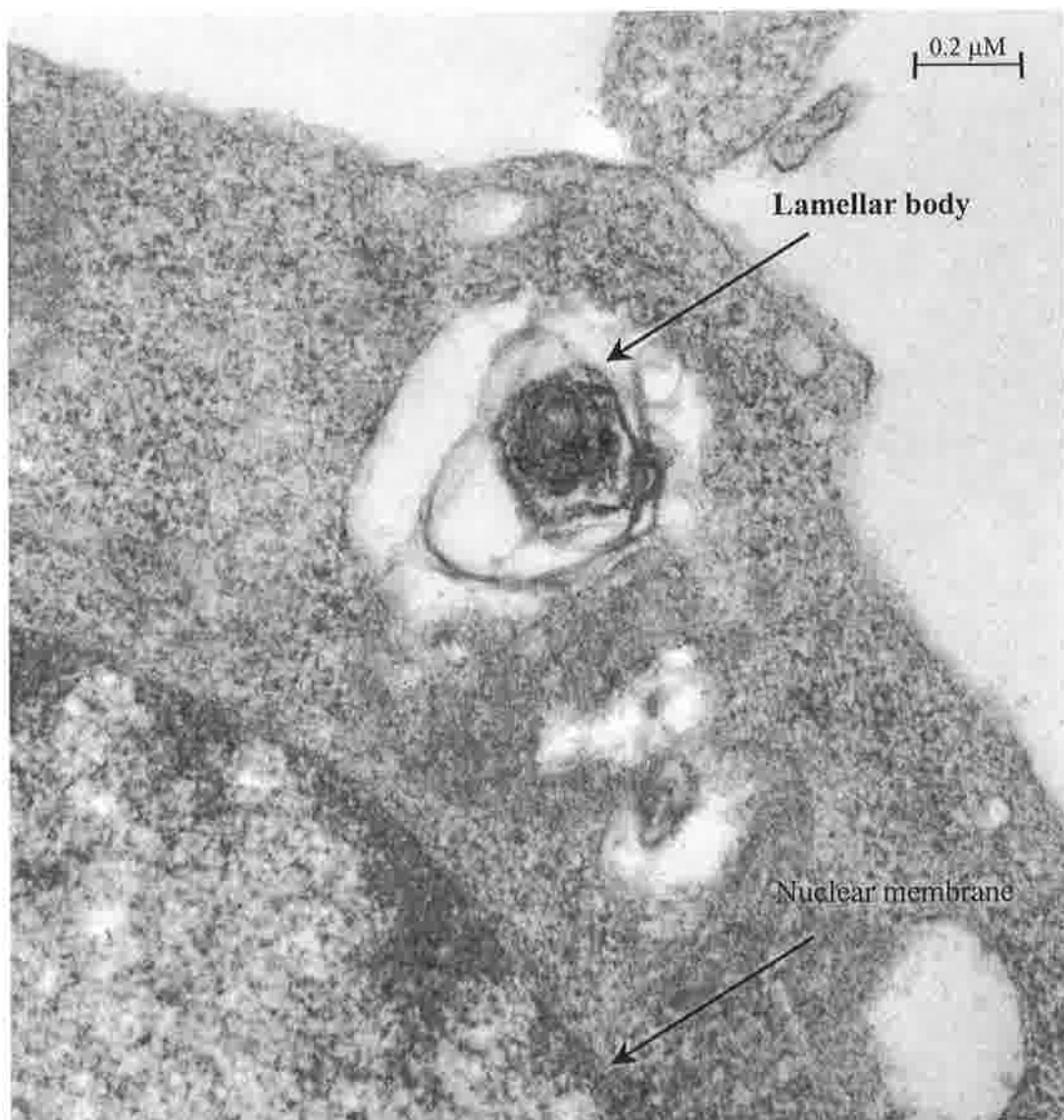
Figure 3.39: Ultrastructure of MM6 cells incubated with 500 µg / ml DPPC for 2 hours. A high power view of the lamellar body (× 28,000). This is a structure known to be taken up by alveolar macrophages within the pulmonary milieu.

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3.7   UPTAKE OF DPPC BY MM6 CELLS

The separation of the phospholipids PG, PE and PC within the membrane of MM6 cells preincubated with DPPC (250 µg / ml) was investigated by HPLC coupled with light scattering evaporative detection. Representative HPLC traces of MM6 cell membrane phospholipids clearly show an increase in PC in MM6 cells preincubated with DPPC when compared to control cells incubated without DPPC (Figure 3.40). Analysis of MM6 cell extracts demonstrated that total membrane PC was increased by 2.54 ± 0.21 fold (n=3) after 2 hours incubation with DPPC when monitoring the relative increase in the ratio of PC:PE in DPPC treated cells compared to untreated cells. No measurable increase in any other quantified phospholipids was seen (Table 3.2).

Peak	Name	R_T (min)	Height	Area	PC:PE (peak area ratio)
(A) Control					
1	PG	3.48	2.120	29.756	
2	PE	6.68	12.722	138.841	
3	PC	10.65	31.508	294.693	2.12
(B) DPPC 250 µg / ml					
4	PG	3.86	3.070	53.737	
5	PE	6.90	12.978	129.743	
6	PC	10.93	89.752	731.873	5.64

Table 3.2: Uptake of DPPC into MM6 cell membranes. Peak area ratios of PC:PE was increased by 2.54 fold in MM6 cells incubated with 250 µg / ml DPPC for 2 hours compared to untreated cells.
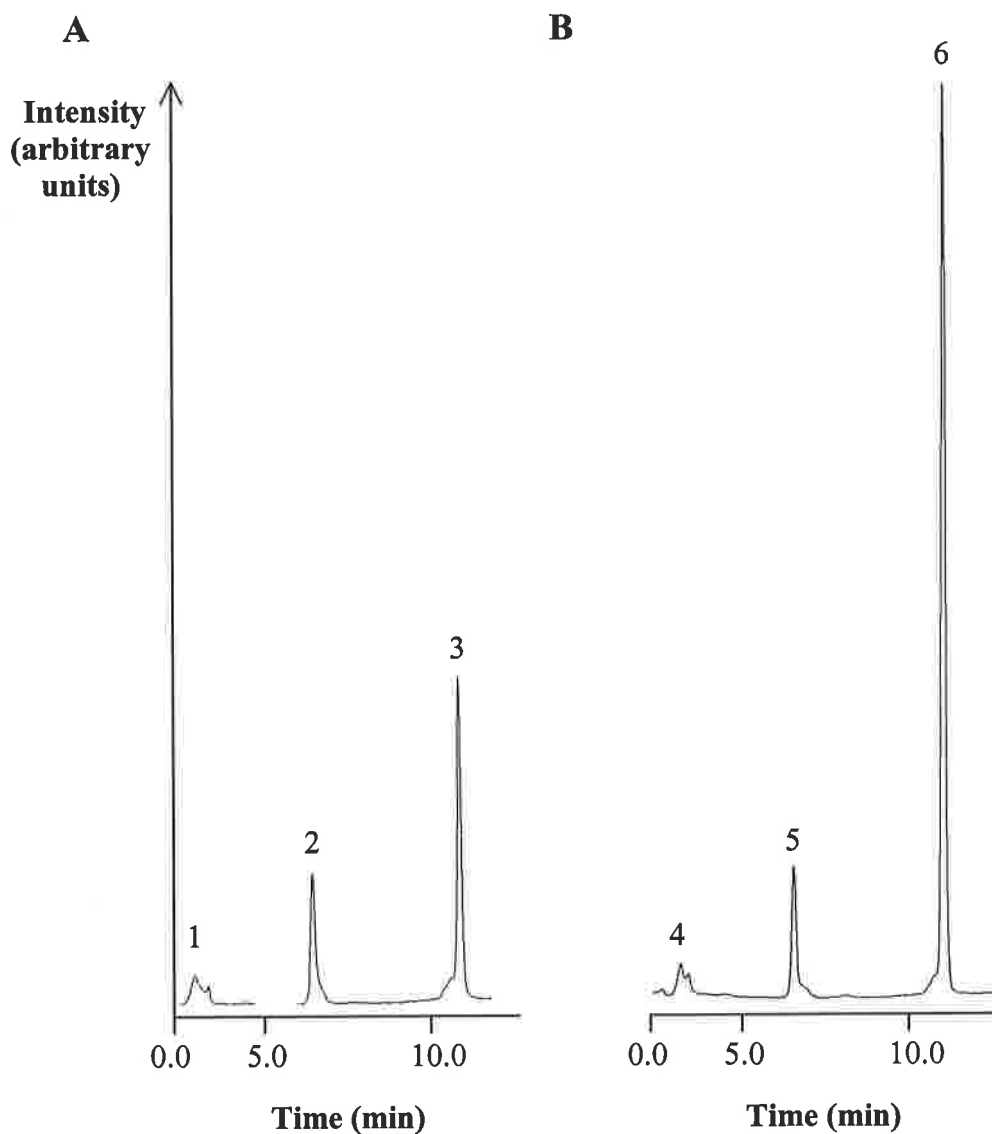
Figure 3.40: Uptake of DPPC into MM6 cell membranes as determined by HPLC.
Typical HPLC traces showing the separation of membrane PG, PE and PC in **(A)** control (untreated MM6 cells) and **(B)** MM6 cells pre incubated with 250 µg / ml DPPC for 2 hours. The increase in the amount of PC reflects uptake of DPPC into that phospholipid class. Data is representative of 3 experiments.

3.8   MEASUREMENT OF NADPH OXIDASE ACTIVITY IN A CELL-FREE SYSTEM

To investigate if DPPC could directly inhibit the NADPH oxidase, a cell free system was used. Following fractionation of activated cells, the NADPH oxidase is found in the plasma membrane fraction (Bolscher *et al*, 1989). This cell-free system was used to investigate whether DPPC impairs the assembly of the active enzyme. Unstimulated MM6 cells (100×10^6 cells) were fractionated by sonication and sucrose density centrifugation. Membranous and cytoplasmic components equivalent to 2×10^6 cells, when combined together in an appropriate buffer containing the anionic detergent SDS and NADPH, will consume oxygen and produce ROIs. NADPH oxidase activity was measured by consumption of oxygen as detected by EPR oximetry and confirmed with chemiluminescence studies for the production of ROIs.

3.8.1   EFFECT OF DPPC ON OXYGEN CONSUMPTION IN A CELL FREE SYSTEM

Electron paramagnetic resonance (EPR) coupled with the oxygen sensitive stable paramagnetic nitroxide probe ^{15}N PDT (4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-oxyl) is a quick and sensitive method for detecting dissolved oxygen concentrations [O_2]. The bimolecular collisions of oxygen with free radicals (spin probes) result in altered resonance characteristics of the radical and hence gives rise to oxygen-dependent EPR spectral line features (Glover *et al*, 1996). The calibration of the spin probe between 100% nitrogen and air saturated water was found be linear which enabled the EPR spectral linewidth to be equated to [O_2] (Figure 3.41).

$$y = 618.92x + 150$$
$$R^2 = 1$$
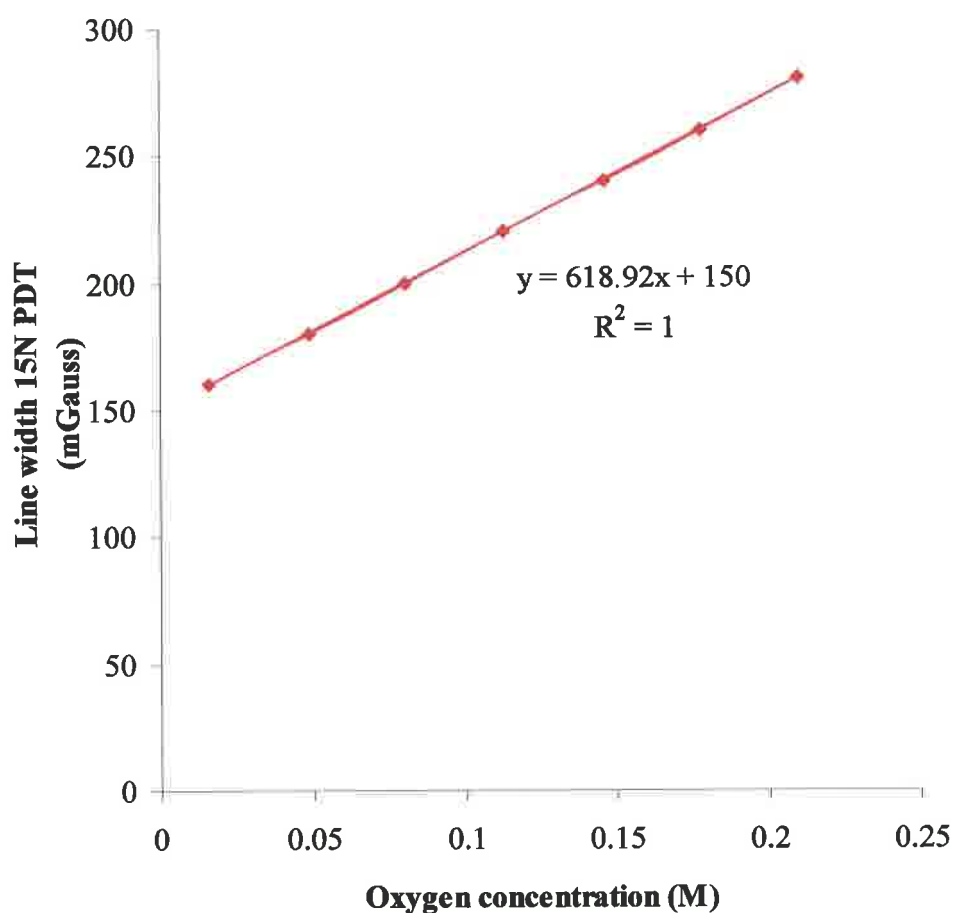
Figure 3.41: **EPR oximetry of ^{15}N PDT probe calibration.** The EPR linewidth of the ^{15}N PDT probe was scanned repeatedly at 30-s intervals. The probe was calibrated at various oxygen concentrations between 100% nitrogen and air (210 μM oxygen) so that linewidth measurements could be equated with oxygen concentration.

Figure 3.42 shows typical EPR spectra obtained by incubating the spin probe ^{15}N PDT with cell-free NADPH fractions (equivalent to 2×10^6 cells) in which the NADPH oxidase was assembled and activated by addition of SDS and the substrate NADPH. Only the lower-field line of the spectrum was monitored and the change in linewidth (a – b; Figure 3.42) reflects the consumption of oxygen. Addition of SDS to sonicates derived from resting MM6 cells stimulated the consumption of oxygen by the NADPH oxidase. Metabolic (mitochondrial) respiration was inhibited by addition of sodium azide to the reaction buffer. Further oxygen consumption was dependent on the presence of NADPH. Reaction buffers not containing SDS and NADPH were included in the assay as negative controls.

The effect of DPPC on oxygen consumption in this cell free system was studied by measuring the linewidth of the EPR spectra of the oxygen sensitive ^{15}N PDT probe with time. Oxygen consumption rates were obtained by measuring the oxygen concentration in a closed chamber over time and finding the slope of the linear plot. Membranous and cytoplasmic components were incubated with DPPC (100 or 500 µg / ml) prior to assembly of the components into a functional enzyme complex by SDS. The change in oxygen concentration for the cell-free NADPH oxidase samples incubated with DPPC is shown in Figure 3.43. The rate of oxygen consumption for control cell fractions incubated without DPPC consumed oxygen at a rate of 2.5 ± 0.61 µM per minute (slope measured over the first 6 minutes of consumption). The consumption of cell fractions incubated with DPPC (2.6 ± 0.31 µM per minute) was not significantly different from that of the control when compared by Mann-Whitney.
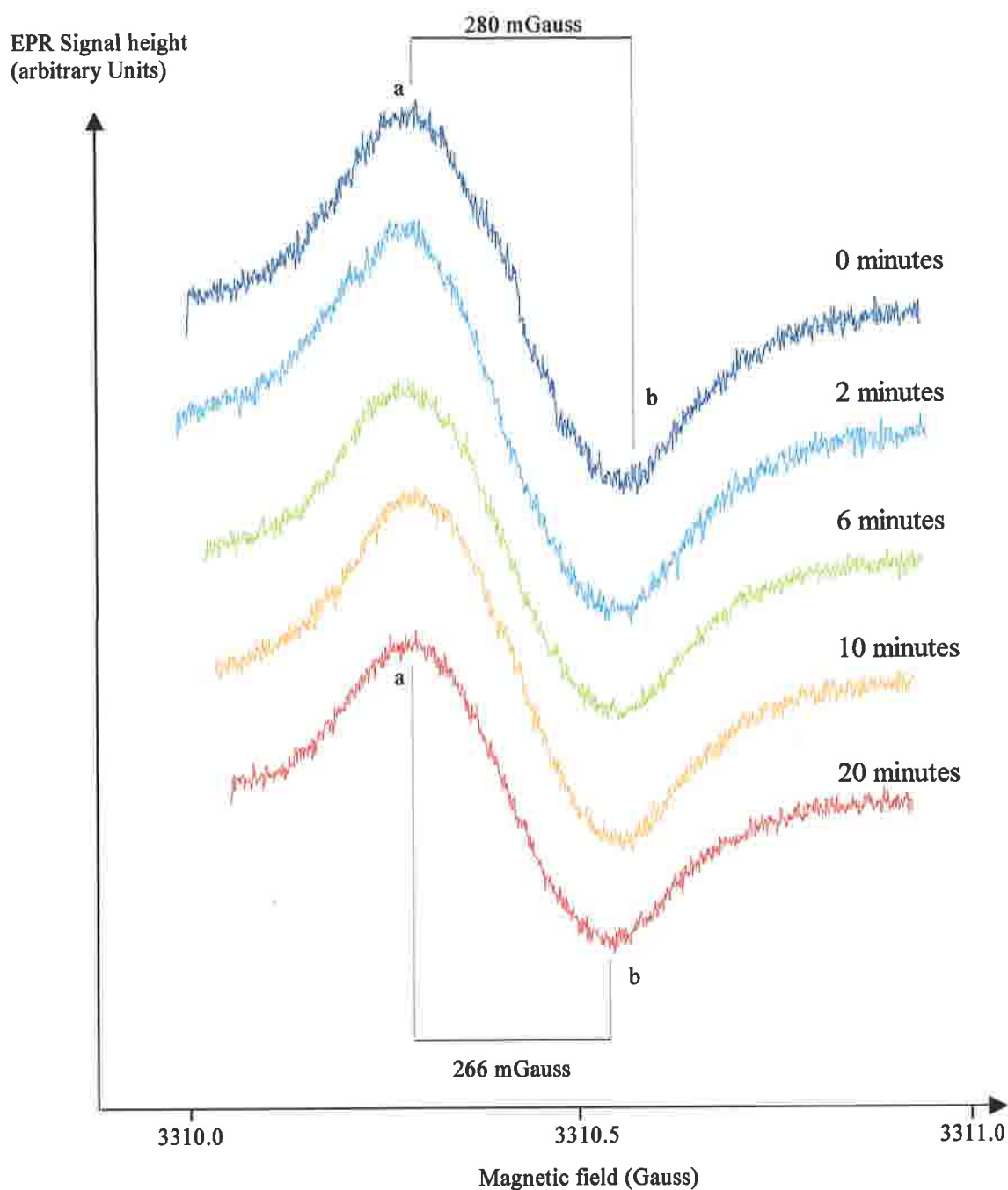
Figure 3.42: EPR oximetry of a cell free system. EPR spectra showing the change in peak to peak (a - b) linewidth of the lower field EPR line of ^{15}N PDT with time in a typical spin label oximetry experiment. 2×10^6 MM6 cell equivalents of membranous and cytoplasmic components were mixed with the spin probe and activated with SDS and NADPH. Spectra were measured every 2 minutes, for 20 minutes.
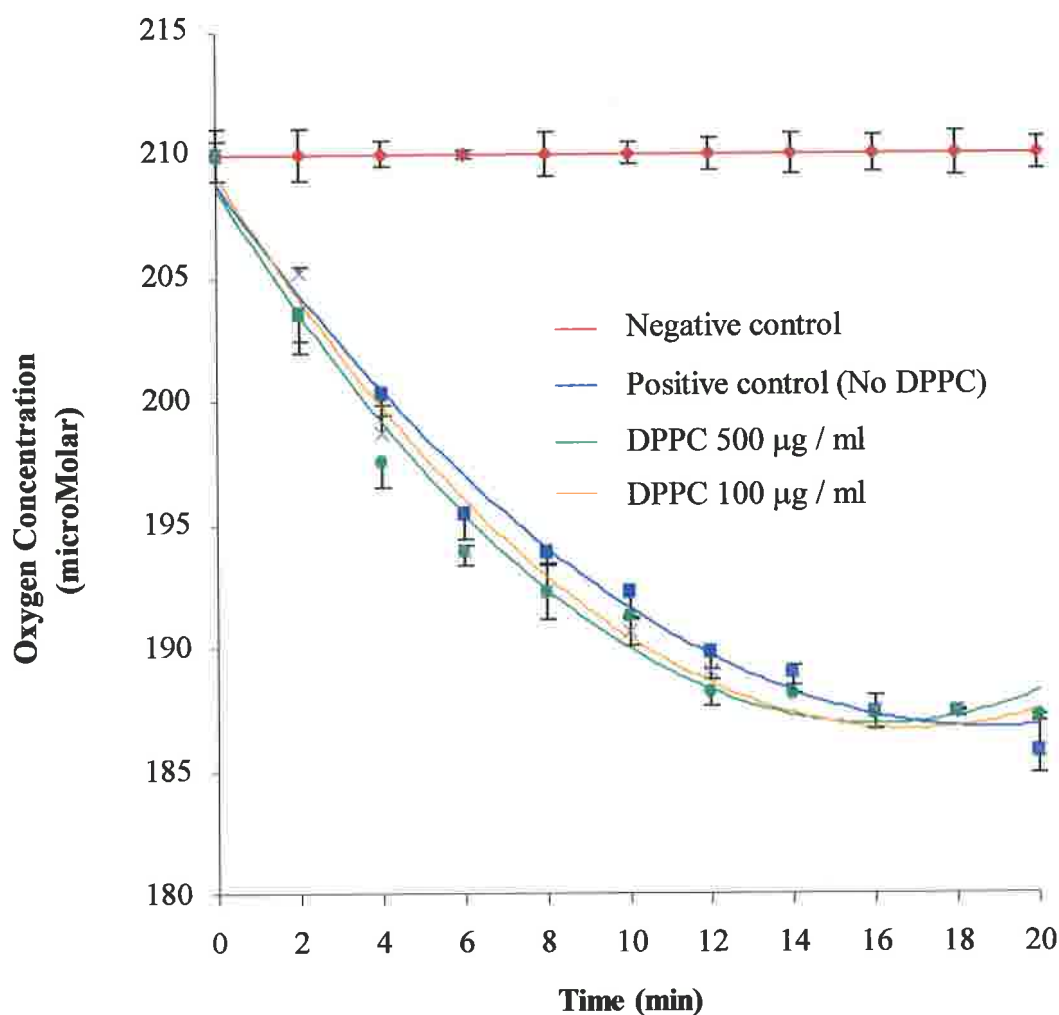
Figure 3.43: Oxygen consumption measured in cell-free NADPH oxidase samples incubated with DPPC. EPR oximetry was performed with a spin label (^{15}N PDT) in 2×10^6 MM6 cell equivalents pre-incubated with 100 or 500 µg / ml DPPC. Activation of the NADPH oxidase occurred only in the presence of SDS and NADPH. Negative control represents cell equivalents incubated without SDS / NADPH / DPPC. Data represents mean ± 1SD of 3 separate experiments.

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3.8.2   **EFFECT OF DPPC ON ROI PRODUCTION (CL) IN A CELL FREE SYSTEM**

The Diogenes™ cellular luminescence enhancement system (National Diagnostics, U.K.) enhances the sensitivity of the chemiluminescent assay to detect more accurately lower concentrations of the superoxide anion. DPPC did not significantly change the production of superoxide in the cell-free with the chemiluminescent model using Diogenes™ as a cellular probe for superoxide (Figure 3.44). Superoxide production was stimulated by SDS without a significant lag phase. Superoxide production started immediately and progressed at a linear rate for up to 5 - 6 minutes at which point peak superoxide production was seen and luminescence began to return to basal levels. Cell equivalents treated with DPPC did not differ in the kinetic response.

Taken together these results confirm that DPPC does not directly affect the activity of the NADPH oxidase in a MM6 cell free system. The results suggest DPPC does not affect the assembly of the individual components of this enzyme into a functional unit.
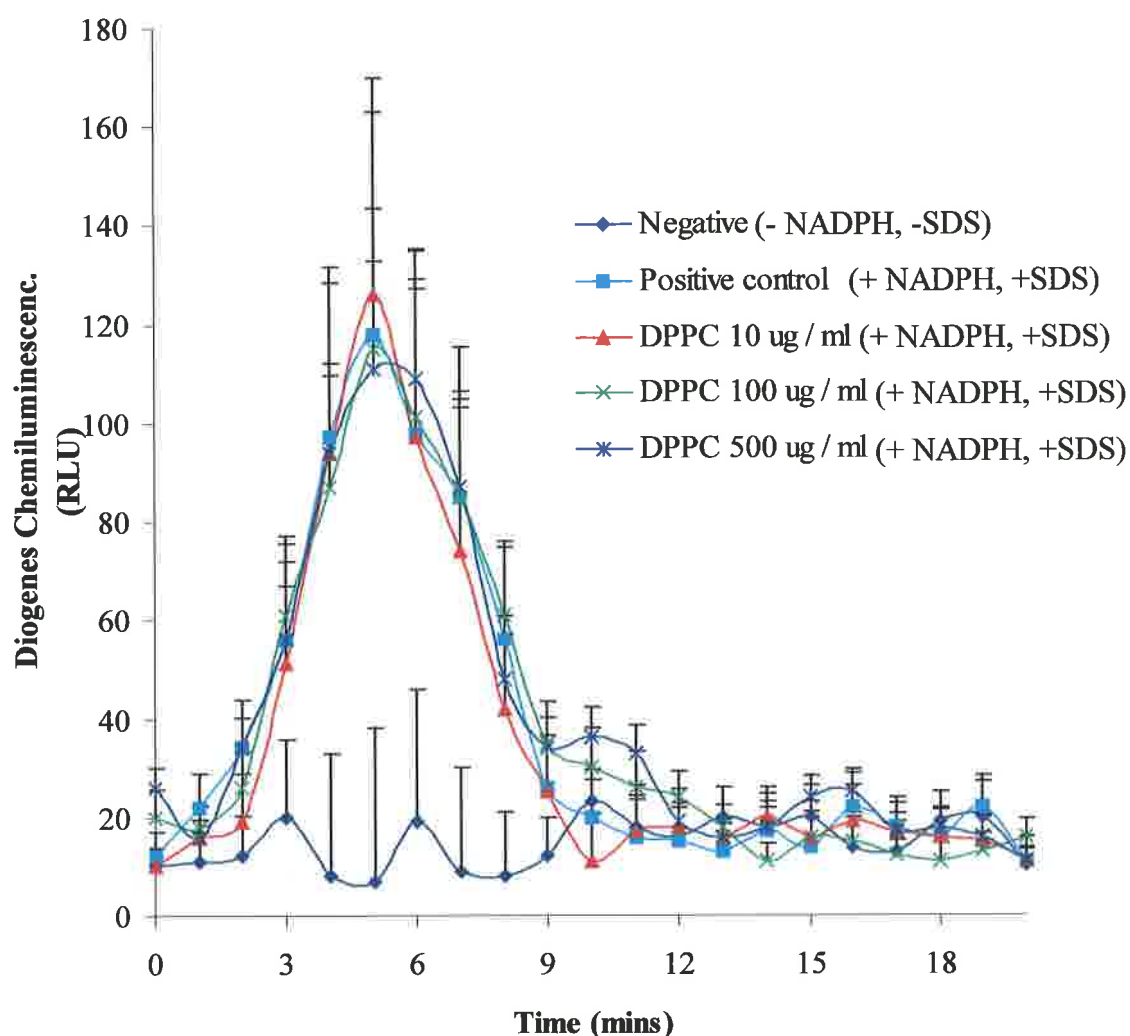
Figure 3.44: Superoxide production in a cell free system activated by SDS and detected by Diogenes™ enhanced cheiluminescence. Membrane and cytosol of MM6 cell equivalents (2×10^6) were preincubated with DPPC (100 or 500 μg / ml) prior to SDS activation and NADPH addition. Data represents mean $1 \pm$ SD of 3 independent experiments.

3.9 DETECTION OF MAPK ACTIVATION BY IMMUNOBLOTTING

The response of cells to extracellular stimuli is in part mediated by a number of intracellular kinase and phosphatase enzymes (Hunter, 1995). The MAPK superfamily of enzymes co-ordinates extracellular and intracellular signals, that are critical to a cells response to stimuli (Ono and Han, 2000). Complexities of signalling pathways and the probability that redundant pathways exist has led to difficulties in completely defining the pathways that lead to NADPH oxidase activation. Extracellular stimuli have been shown to transiently activate MAPK with different response profiles (Sommer *et al*, 1999; Coffer *et al*, 1998; McLeish *et al*, 1998; Chow *et al*, 1995; El Benna *et al*, 1994).

The yeast cell wall extract zymosan, is a particulate and activates the respiratory burst via receptor-mediated interactions. It has been reported that in rat alveolar macrophages, opsonised zymosan stimulates tyrosine phosphorylation and activation of extracellular regulated kinases (ERKs) (Torres and Forman, 1999). As shown in chemiluminescent experiments in this study, MM6 cells can respond to an activating stimulus with a greater capacity for ROI production if they are first 'primed' with LPS, by a mechanism that is not fully elucidated. Further, It has been suggested that phosphorylation of some oxidase components, particularly p47^{phox}, may be the signal necessary to initiate assembly of NADPH oxidase. MAPKs p44 / p42 and p38 can phosphorylate p47^{phox} *in vitro* (Yaffe *et al*, 1999; El Benna *et al*, 1996). Since activation of the NADPH oxidase is in part mediated through MAPK signalling, the effect of DPPC on these enzymes was determined. The active forms of MAPK were

initially optimised in this study so that peak stimulation of MAPK could be studied. Since DPPC modulates ROI production following LPS 'priming' and the binding of LPS was not impaired by DPPC, immunoblots for MAPK were performed following 18 hours LPS 'priming' and stimulation with OpZ or PMA.

3.9.1 MAPKs p44 / p42 ARE TRANSIENTLY ACTIVATED BY OpZ OR PMA

Stimulation of 'primed' MM6 cells with OpZ or PMA resulted in a modest increase in the activity of p44 / p42 MAPKs (Figures 3.45 and 3.46 respectively). The increase in p44 / p42 activity in response to OpZ was observed after 30 – 40 minutes and was maximal at 60 minutes. In PMA stimulated cells, a rapid activation of these kinases was seen (i.e. maximal expression within 10 minutes of stimulation) and these levels remained maximal for up to 60 minutes. Time points beyond 60 minutes were not investigated because ROI production detected by luminol enhanced chemiluminescence would have peaked and levels would be returning to basal rates. Negatively and positively phosphorylated control p42 proteins were included, demonstrating the specificity of the antibody used. In subsequent experiments, the stimulation of p44 / p42 MAPKs in MM6 cells was achieved with OpZ and PMA for 60 and 10 minutes respectively.
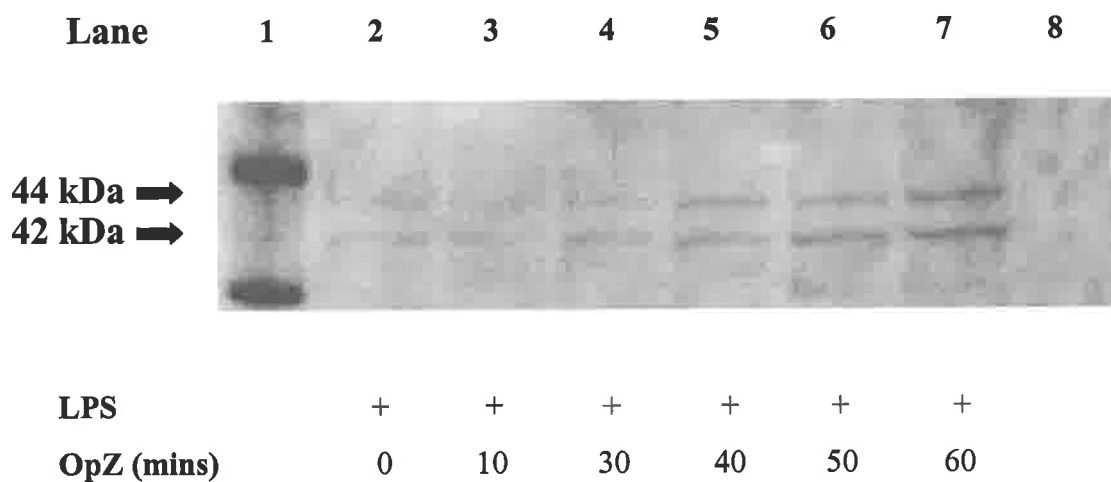
Figure 3.45: Immunoblot showing transient activation of p44 / p42 MAPK by OpZ in LPS primed MM6 cells. MM6 cells (5×10^6) were primed with LPS and stimulated with OpZ (125 μg / ml) for up to 60 minutes. Data shown are representative of three independent experiments. Lane 1, molecular weight markers; 8, non-phosphorylated p42 control protein.
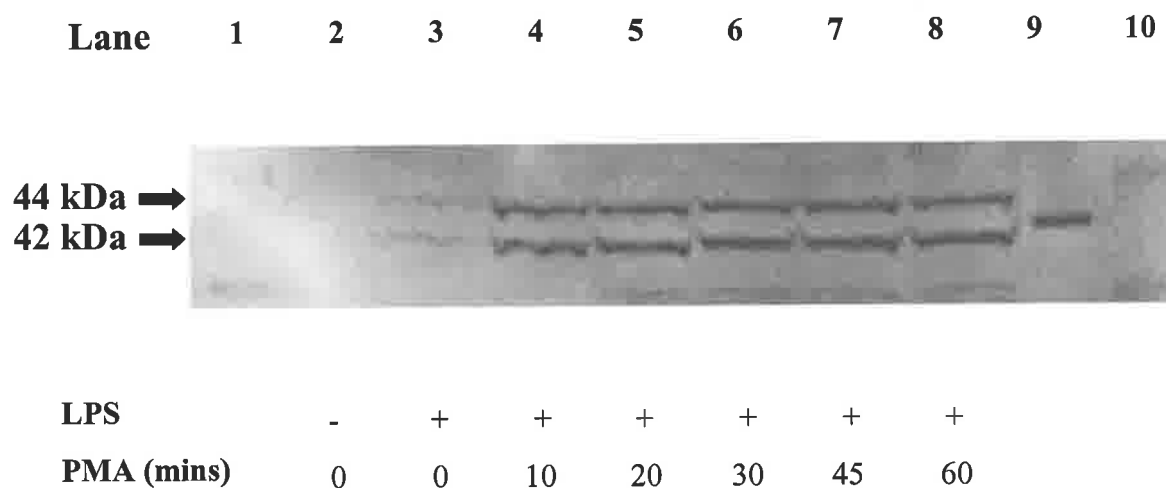
Figure 3.46: Immunoblot showing transient activation of p44 / p42 MAPK by PMA in LPS primed MM6 cells. MM6 cells (5×10^6) were primed with LPS and stimulated with PMA (100 ng / ml) for up to 60 minutes. Data shown are representative of three independent experiments. Lanes 1 and 10, molecular weight markers; 9, phosphorylated p42 control protein.

3.9.2   MAPKs p44 / p42 are activated by OpZ or PMA in a dose dependent manner

MM6 cells were 'primed' with LPS and activated with varying doses of OpZ or PMA for 60 and 10 minutes respectively. These times were determined to be optimal for MAPK expression and hence western blotting was performed at these time intervals following stimulation with PMA or OpZ. These experiments confirmed that p44 / p42 MAPK are phosphorylated and activated in a dose dependent manner when stimulated with these agonists (Figures 3.47 and 3.48). OpZ (125 µg / ml) and PMA (100 ng / ml) have been used throughout the study and stimulation of MM6 cells with these agonists at these levels resulted in significant phosphorylation of p44 / p42 MAPK. Therefore, subsequent studies on MAPK activity utilised these concentrations of agonist. Taken together these time and dose dependent experiments demonstrate the ability of OpZ or PMA to activate the p44 / p42 MAPKs.

3.9.3   Determination of DPPC effects on p44 / p42 MAPK activation

Using the optimal conditions for PMA and OpZ induced activation of p44 / 42 MAPK, the effect of DPPC on p44 / p42 activity was investigated. Preincubation of MM6 cells with DPPC at 10 – 500 µg / ml did not affect the activation of p44 / p42 when stimulated by either OpZ or PMA in LPS 'primed' cells (Figure 3.49 and 3.50 respectively). Control extracts showed the low (basal) levels of phosphorylated p44 / p42 MAPKs in cells not incubated with LPS or stimulated with an agonist.
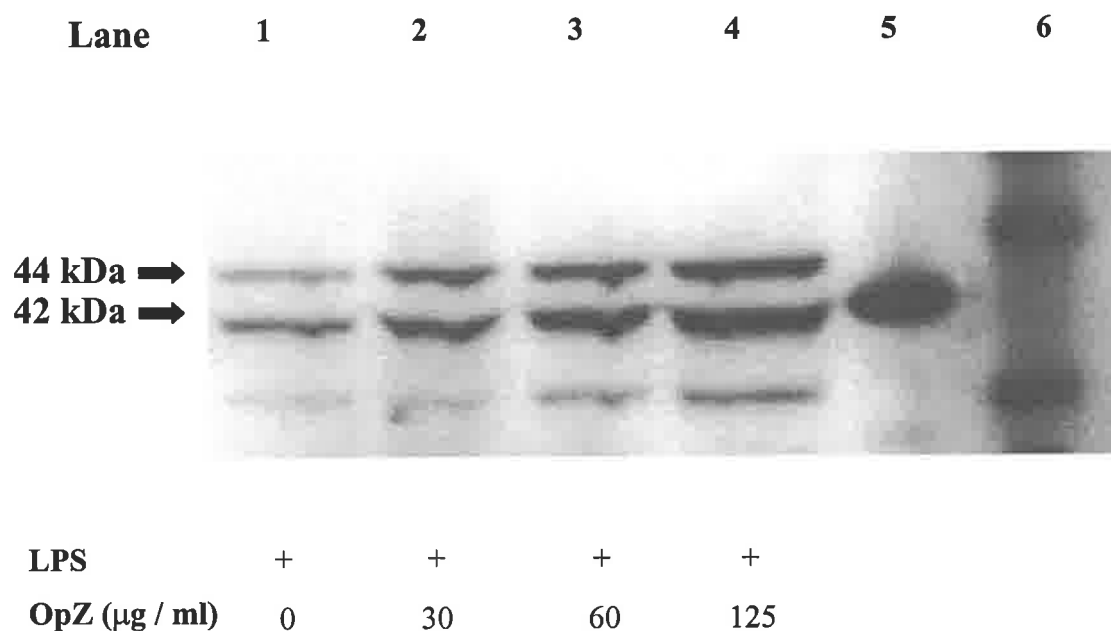
Figure 3.47: Immunoblot showing dose dependent activation of p44 / p42 MAPK by OpZ in LPS primed MM6 cells. MM6 cells (5×10^6) were primed with LPS and stimulated with OpZ for 60 minutes. Data shown are representative of three independent experiments. Lane 6, molecular weight markers; 5, phosphorylated p42 control protein.

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |

44 kDa ➡
42 kDa ➡

| LPS | | | - | - | + | + | + | + | + |
| PMA (ng / ml) | | | 0 | 100 | 0 | 1 | 10 | 100 | 1000 |
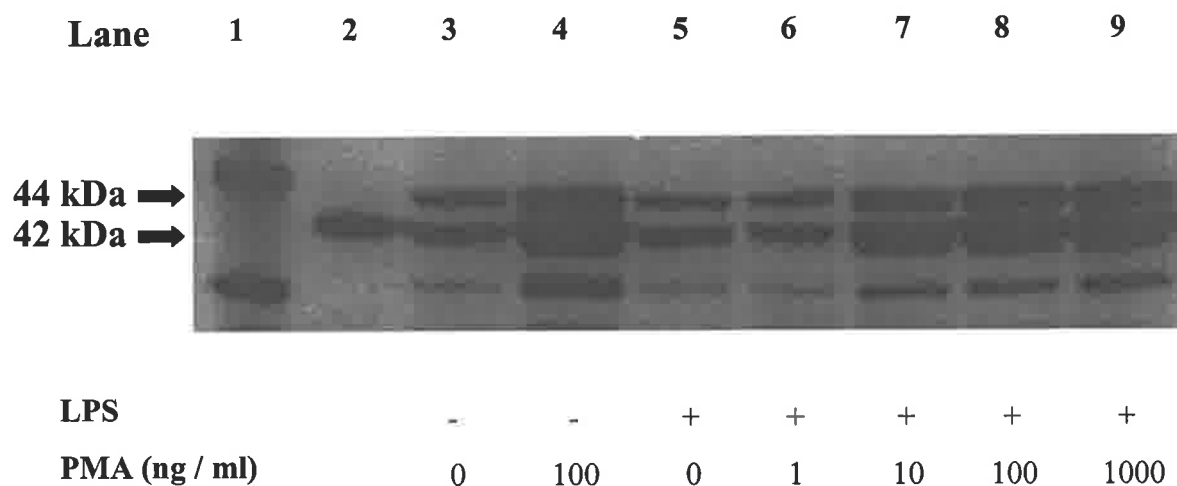
Figure 3.48: Immunoblot showing dose dependent activation of p44 / p42 MAPK by PMA in LPS primed MM6 cells. MM6 cells (5×10^6) were primed with LPS and stimulated with PMA for 10 minutes. Data shown are representative of three independent experiments. Lane 1, molecular weight markers; 2, phosphorylated p42 control protein.
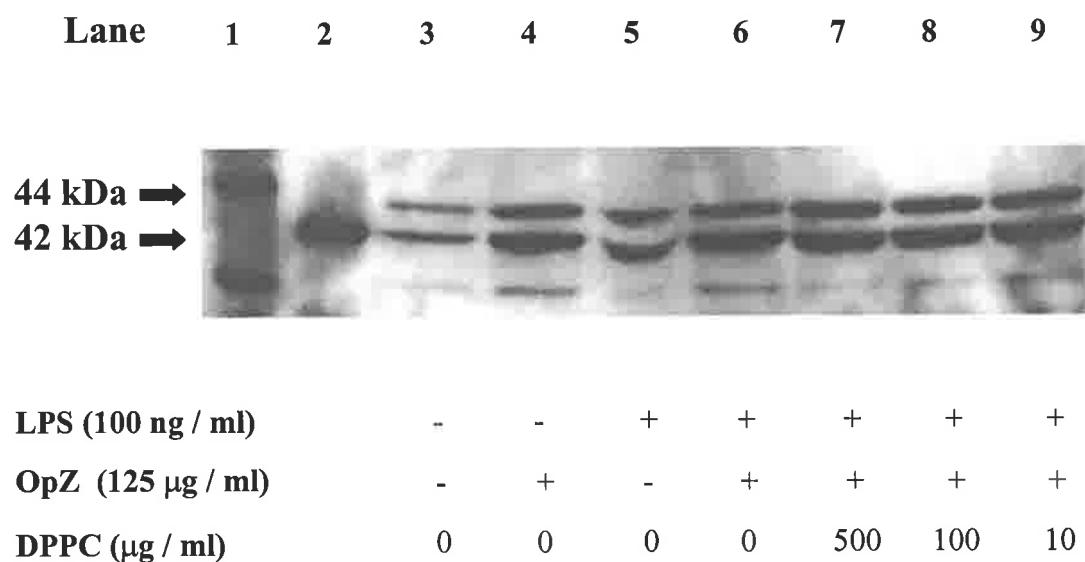
Figure 3.49: Immunoblot of OpZ stimulated p44 / p42 MAPK in DPPC treated MM6 cells. MM6 cells (5×10^6) were incubated with DPPC, washed (×3) in PBS and primed with LPS for 18 hours. Cells were stimulated with OpZ for 60 minutes. Data shown are representative of three independent experiments. Lane 1, molecular weight markers; 2, phosphorylated p42 control protein.

Lane	1	2	3	4	5	6	7	8	9

44 kDa ➡
42 kDa ➡

LPS (100 ng / ml)		--	-	+	+	+	+	+
PMA (100 ng / ml)		-	+	-	+	+	+	+
DPPC (µg / ml)		0	0	0	0	500	100	10
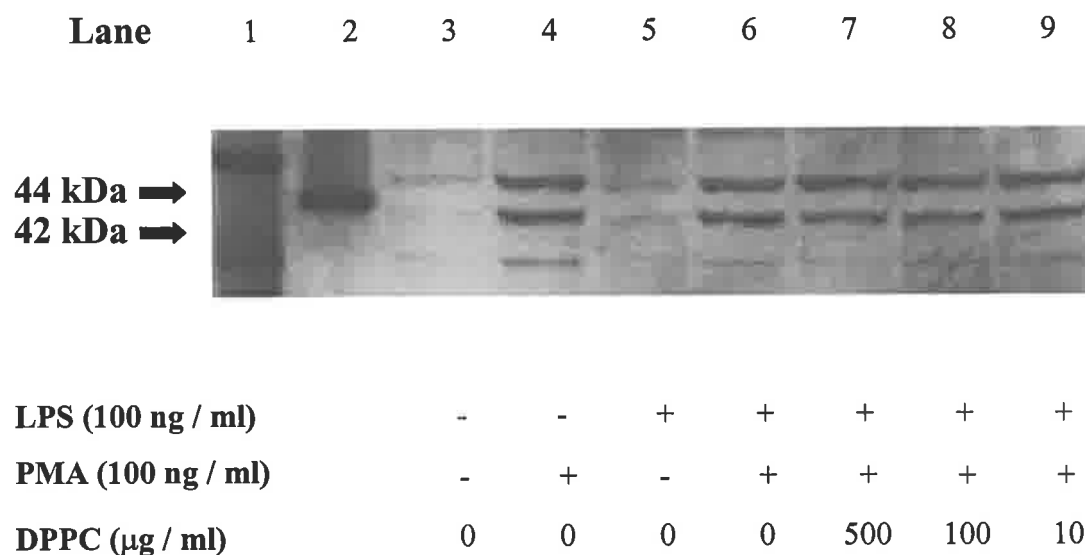
Figure 3.50: **Immunoblot of PMA stimulated p44 / p42 MAPK in DPPC treated MM6 cells.** MM6 cells (5×10^6) were incubated with DPPC, washed (×3) in PBS and primed with LPS for 18 hours. Cells were stimulated with PMA for 10 minutes. Data shown are representative of three independent experiments. Lane 1, molecular weight markers; 2, phosphorylated p42 control protein.

Further, the incubation with LPS for 18 hours results in basal levels of this phosphorylated MAPK. However, dephosphorylation of these proteins may occur over 18 hours. LPS 'priming' of MM6 cells for 18 hours does not enhance the activation of p44 / p42 MAPKs upon incubation with a stimulant. These results suggest the modulation of ROI production by DPPC is not mediated by affecting the activation of p44 / p42 by OpZ or PMA.

3.9.4 DETERMINATION OF DPPC EFFECTS ON P38 MAPK ACTIVATION

Optimal conditions for stimulation of p38 MAPKs were performed. However, the p38 MAPK protein in MM6 cells exhibited high constitutive phosphorylation. A number of experiments were performed in order to attempt to lower the phosphorylation of p38 MAPK in this cell line (Figure 3.51). This figure demonstrates the constitutive levels of p38 MAPK in MM6 cells cultured using various conditions. This representative experiment demonstrates that incubation of MM6 cells with 0.5% (*v/v*) serum for 1 day lowers the expression of this phosphorylated MAPK. A further incubation of these cells in serum depleted media did not lower basal phosphorylation of p38 MAPK. The basal levels of phosphorylated p38 under these conditions are moderate making comparisons between treated groups with controls difficult. Therefore the activity of p38 MAPK was tested using the conditions that were established for p44 / p42 MAPK activity.

Lane	1	2	3	4	5	6	7	8	9

38 kDa ➡

Days in culture		1	1	2	2	2	2	1	1
Serum in media (% v/v)		2	0.5	2	0.5	2	2	0.5	2
+ 2 hours in serum free media		-	-	-	-	-	-	+	+
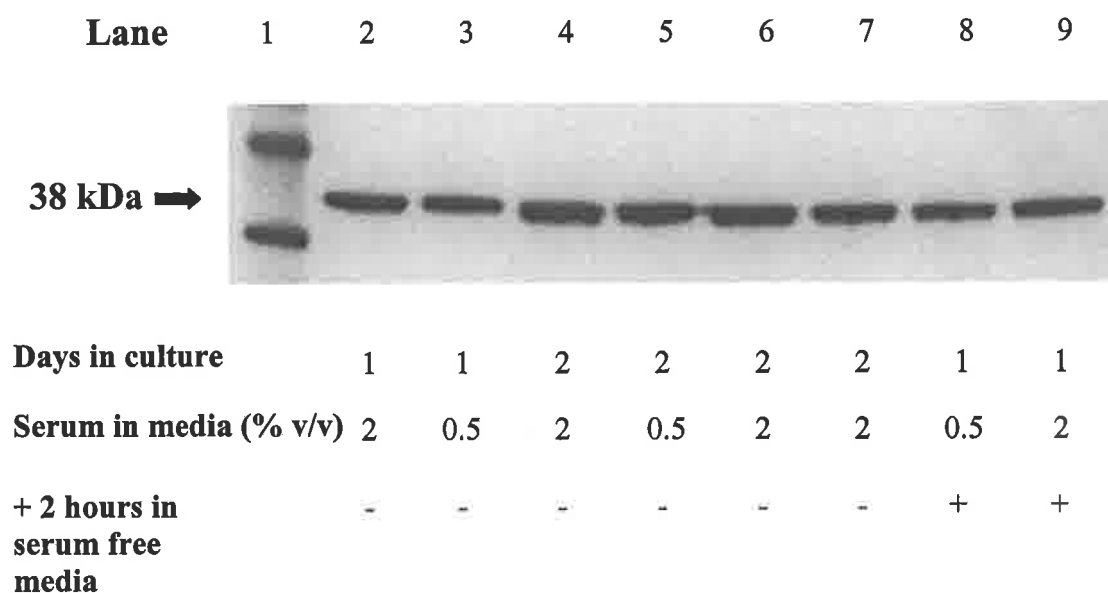
Figure 3.51: Immunoblot of basal levels of p38 MAPK. MM6 cells (5×10^6) were incubated in media containing either 0.5 or 2% (*v/v*) serum for 1 or 2 days and incubated with or without serum supplemented media for a further 2 hours. Data shown are representative of three independent experiments. Lane 1, molecular weight markers.

MM6 cells were incubated with DPPC (10 – 500 µg / ml) followed by washing in sterile PBS (×3) and 'priming' with LPS for 18 hours. Finally, cells were stimulated with PMA or OpZ as above. Figures 3.52 and 3.53 respectively show OpZ and PMA stimulation of these cells. In both instances, DPPC treated cells did not alter the expression of phosphorylated p38 MAPK. It is noteworthy, the expression between unstimulated cells and cells stimulated with PMA or OpZ made this comparison difficult. The responses were repeatable and the immunoblots are representative of at least three independent experiments.

Lane	1	2	3	4	5	6	7	8	9

38 kDa ➡

LPS (100 ng / ml)		-	-	+	+	+	+	+
OpZ (125 µg / ml)		-	+	+	-	+	+	+
DPPC (µg / ml)		-	-	-	-	500	100	10
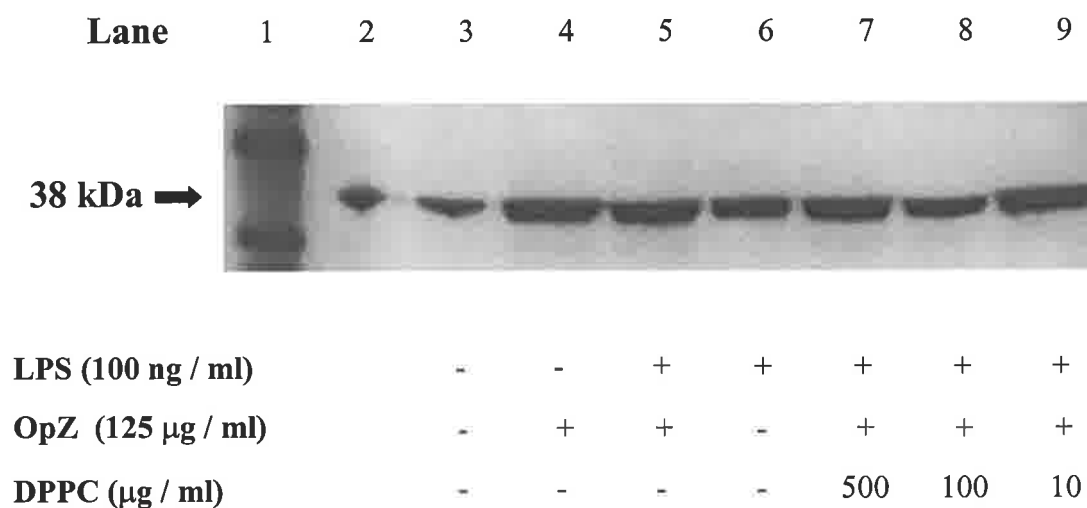
Figure 3.52: Immunoblot of OpZ stimulated p38 MAPK in DPPC treated MM6 cells. MM6 cells (5×10^6) were incubated with DPPC, washed (×3) in PBS and primed with LPS for 18 hours. Cells were stimulated with OpZ for 60 minutes. Data shown are representative of three independent experiments. Lane 1, molecular weight markers; 2, phosphorylated p38 control protein.

Lane	1	2	3	4	5	6	7	8	9

38 kDa ➡

LPS (100 ng / ml)		-	-	+	+	+	+	+
PMA (100 ng / ml)		-	+	-	+	+	+	+
DPPC (µg / ml)		-	-	-	-	500	100	10
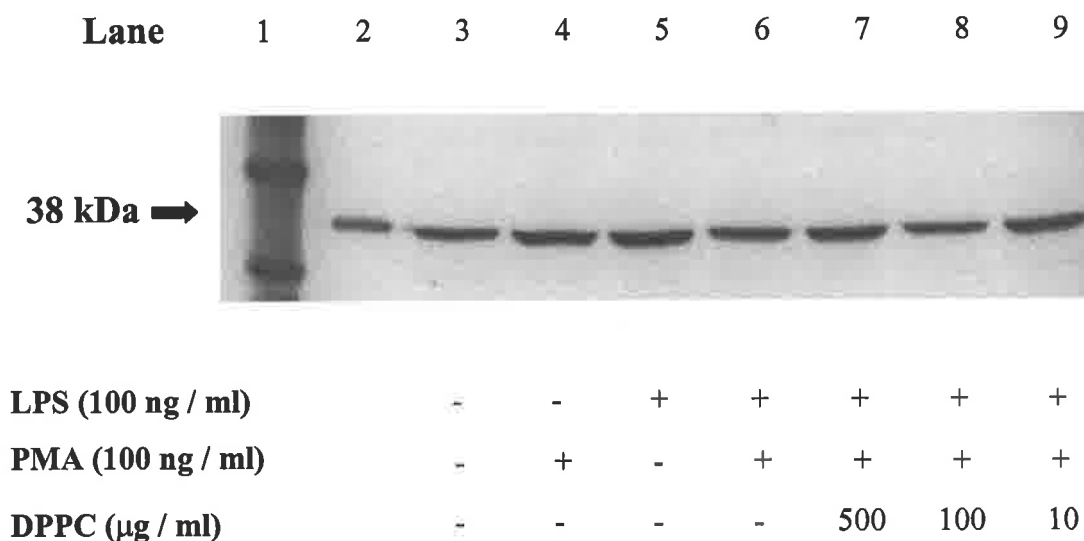
Figure 3.53: Immunoblot of PMA stimulated p38 MAPK in DPPC treated MM6 cells. MM6 cells (5×10^6) were incubated with DPPC, washed ($\times 3$) in PBS and primed with LPS for 18 hours. Cells were stimulated with PMA for 10 minutes. Data shown are representative of three independent experiments. Lane 1, molecular weight markers; 2, phosphorylated p38 control protein.

3.10   QUANTITATION OF PKC ACTIVITY BY RADIOASSAY

PMA, a direct activator of PKC is recognised as a potent stimulus for NAPDH oxidase activation (Castagna *et al*, 1982).   Oxidase activation induced by most neutrophil agonists is inhibited by pharmacological agents with specificity for PKC (Kessels *et al*, 1993).   Studies using PMA to stimulate phagocytes strongly support a link between phosphorylation of p47^{phox} and activation of NADPH oxidase (Park *et al*, 1998; Park *et al*, 1997; Chanock *et al*, 1994).   Since PMA primarily acts through activation of PKC, inhibition of PMA-stimulated production of ROI by MM6 cells incubated with DPPC (Figure 3.14) indicated that activation of PKC was impaired in these cells.   To investigate the effects of DPPC on activation of PKC, activity of this enzyme was determined in PMA and OpZ stimulated cells pre-treated with DPPC.   Calcium- and phospholipid-dependent protein kinase C (PKC) was quantified using the SignaTECT™ Protein Kinase C (PKC) Assay System.

3.10.1   OPTIMISATION OF PMA AND OPZ STIMULATION OF PKC ACTIVITY

In the first series of experiment, the PKC enzyme activity in response to PMA or OpZ was determined.   The study aimed to show the transient activation of this enzyme in response to these agonists.   To readily determine any difference between DPPC treated cells with controls it was important to assay PKC activity at its maximum.   Therefore, MM6 cells (5×10^6) were stimulated with PMA (100 ng / ml) or OpZ (125 µg / ml) over 0 – 60 minutes.   OpZ and PMA transiently activated PKC, respectively (Figures 3.54 and 3.55).

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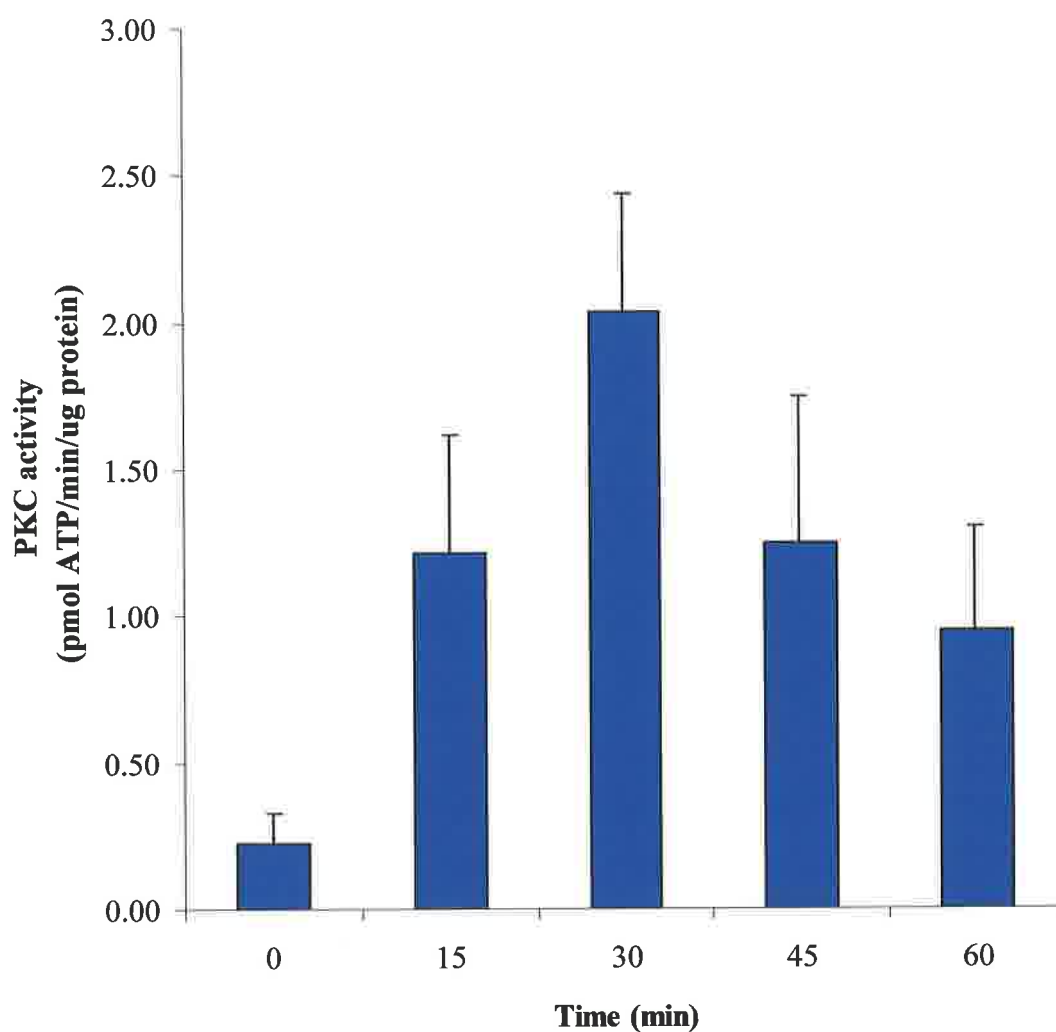
Figure 3.54: Time course of PKC activity in OpZ treated MM6 cells. MM6 cells (5×10^6) were incubated with OpZ (125 µg / ml) for 0 – 60 minutes followed by washing in PBS (×3). PKC activity was assayed as described in methods. Data represents mean ± 1 SD of 3 independent experiments.
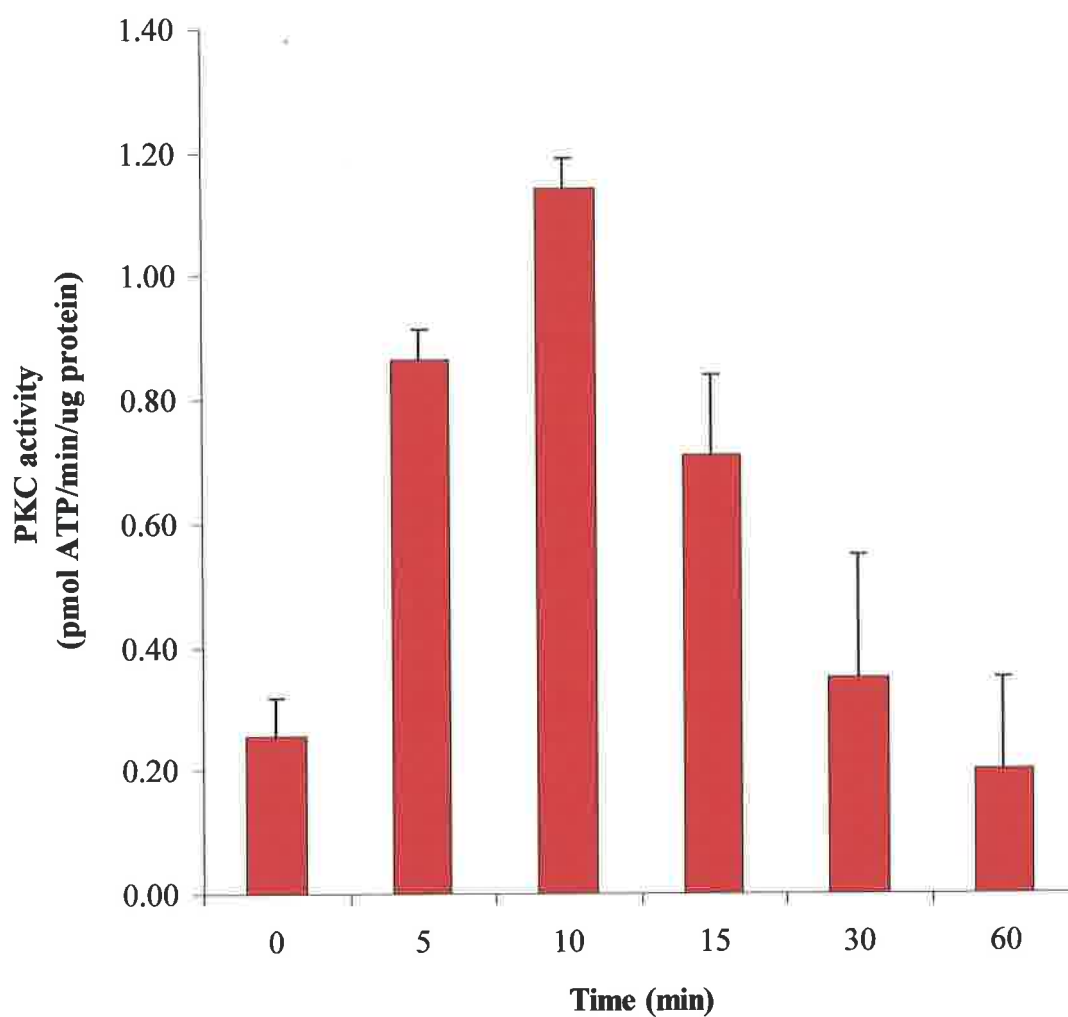
Figure 3.55: **Time course of PKC activity in PMA treated MM6 cells**. MM6 cells

(5×10^6) were incubated with PMA $(100 \text{ ng} / \text{ml})$ for $0 - 60$ minutes

followed by washing in PBS $(\times 3)$. PKC activity was assayed as

described in methods. Data represents mean $\pm$ 1 SD of 3 independent

experiments.

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OpZ activated a calcium-phospholipid dependent kinase within 15 minutes of the initial stimulus. The peak activity of PKC was seen to be at 30 minutes. At this time point there was a ten-fold increase in PKC activity when compared to MM6 cells stimulated without OpZ (time zero). The activity of this enzyme began to decline and at 60 minutes post initial stimulus a five-fold increase over control values was still seen. PMA activation of PKC was maximal at 10 minutes and returned to basal levels by 30 – 60 minutes post initial stimulus. Maximal activity was approximately four-fold greater than control levels.

These results demonstrate the ability of PMA and OpZ to stimulate the activation of a calcium and phospholipid PKC. PMA (100 ng / ml) activity was maximal at 10 minutes while stimulation of PKC activity with OpZ with 125 µg / ml was maximal at 30 minutes. It is noteworthy that OpZ, stimulated PKC activity by more than two-fold of that of PMA stimulation. These stimulating conditions were used in subsequent experiments.

3.10.2   PC INHIBITS PKC ACTIVITY IN OpZ STIMULATED LPS 'PRIMED' MM6 CELLS

The optimal conditions previously described for PKC activity in OpZ stimulated cells were used to assess whether phosphatidylcholine inhibits ROI production by modulating PKC activation. For OpZ stimulation, MM6 cells required 'priming' with LPS in order to observe a significant effect with respect to NADPH oxidase activity as measured by LCL. Therefore, similar conditions were used to that of the chemiluminescent studies in order to maintain comparable results. MM6 cells (5×10^6) were preincubated with 100 µg / ml of tPC or DPPC or 125 µg / ml PAPC. The cells were washed, 'primed' with LPS and stimulated with OpZ (125 µg / ml) for 30 minutes. Controls were incubated without lipid. In addition, controls were also performed with or without LPS and OpZ stimulation. The activity of PKC is significantly reduced in tPC, DPPC or PAPC treated cells ($P<0.01$ by ANOVA) (Figure 3.56). Using Tukey's multiple pairwise comparisons, PKC activity is significantly reduced by approximately 63% when compared to cells incubated without tPC ($P<0.05$). However the most significant reduction is seen in DPPC treated cells where approximately a 70% decrease is seen ($P<0.01$). Tukey's pairwise comparisons of this data provide evidence that incubation of MM6 cells with PAPC did not affect the activity of PKC when stimulated with OpZ.
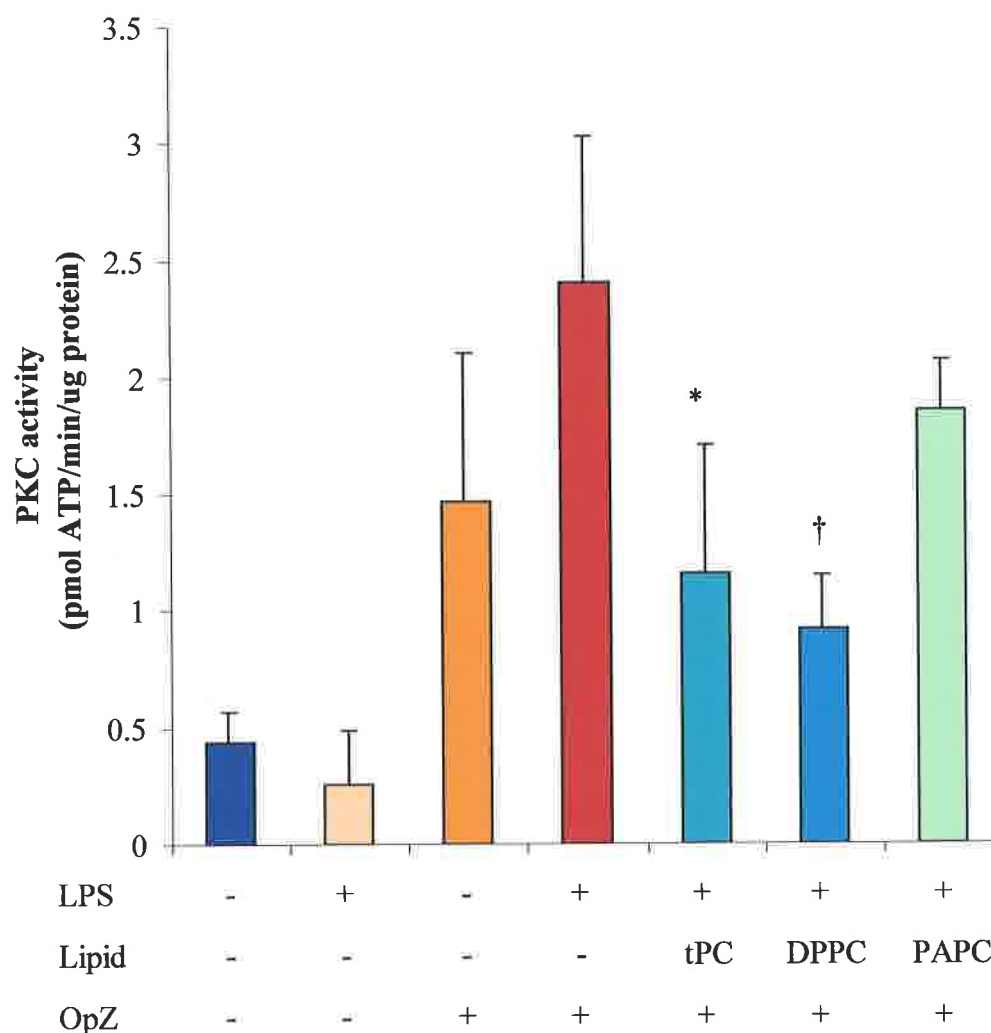
Figure 3.56: Effect of PC on OpZ stimulated PKC activity in LPS primed MM6 cells. MM6 cells (5×10^6) were pre-incubated with tPC or DPPC (100 µg / ml) or 125 µg / ml PAPC for 2 hours. Cells were washed in PBS ($\times 3$) and primed for 18 hours with LPS. OpZ (125 µg / ml) stimulation occurred for 30 minutes prior to assaying PKC activity by ^{32}P radioassay. Data represents mean ± 1 SD of 3 independent experiments. *P<0.05 and †P<0.01 by ANOVA and Tukey's pairwise comparisons.

Incubation of MM6 cells with LPS (100 ng / ml) for 18 hours did not stimulate the activity of PKC. Cells stimulated with OpZ (125 µg / ml) alone stimulated PKC activity by 66% compared to the basal levels (cells cultured in media alone). However, when cells were 'primed' with LPS and then stimulated with OpZ a synergistic effect was seen with respect to PKC activity. Under these conditions, there was approximately six-fold increase in PKC activity when compared to basal levels. These results reflect those seen in the OpZ stimulated LCL studies in that 'priming' has no or little effect on target activity but 'priming' with LPS greatly enhances the function. These results suggest PKC is likely to be involved in the 'priming' and / or activation of NADPH oxidase. Further, incubation with the phospholipid PC in particular the sub-species DPPC has a very significant effect on the activity of this enzyme when stimulated with the yeast extract OpZ.

3.10.3 PC INHIBITS PKC ACTIVITY IN PMA STIMULATED MM6 CELLS

Chemiluminescent studies provided evidence that DPPC inhibited ROI production in PMA stimulated cells. This production was significant in MM6 cells treated with or without LPS when compared to the control. In order to simplify the next series of experiments and therefore control the experiments better the effects of PC on PKC activity was determined in MM6 cells treated without LPS ('unprimed'). MM6 cells were incubated with 100 µg / ml or tPC or DPPC or 125 µg / ml of PAPC for 2 hours at 37°C. The cells were washed in PBS (×3) and stimulated with PMA for 10 minutes. As with OpZ stimulated cells, tPC and DPPC significantly reduced the activity of PKC ($P < 0.01$ analysed by ANOVA) (Figure 3.57).
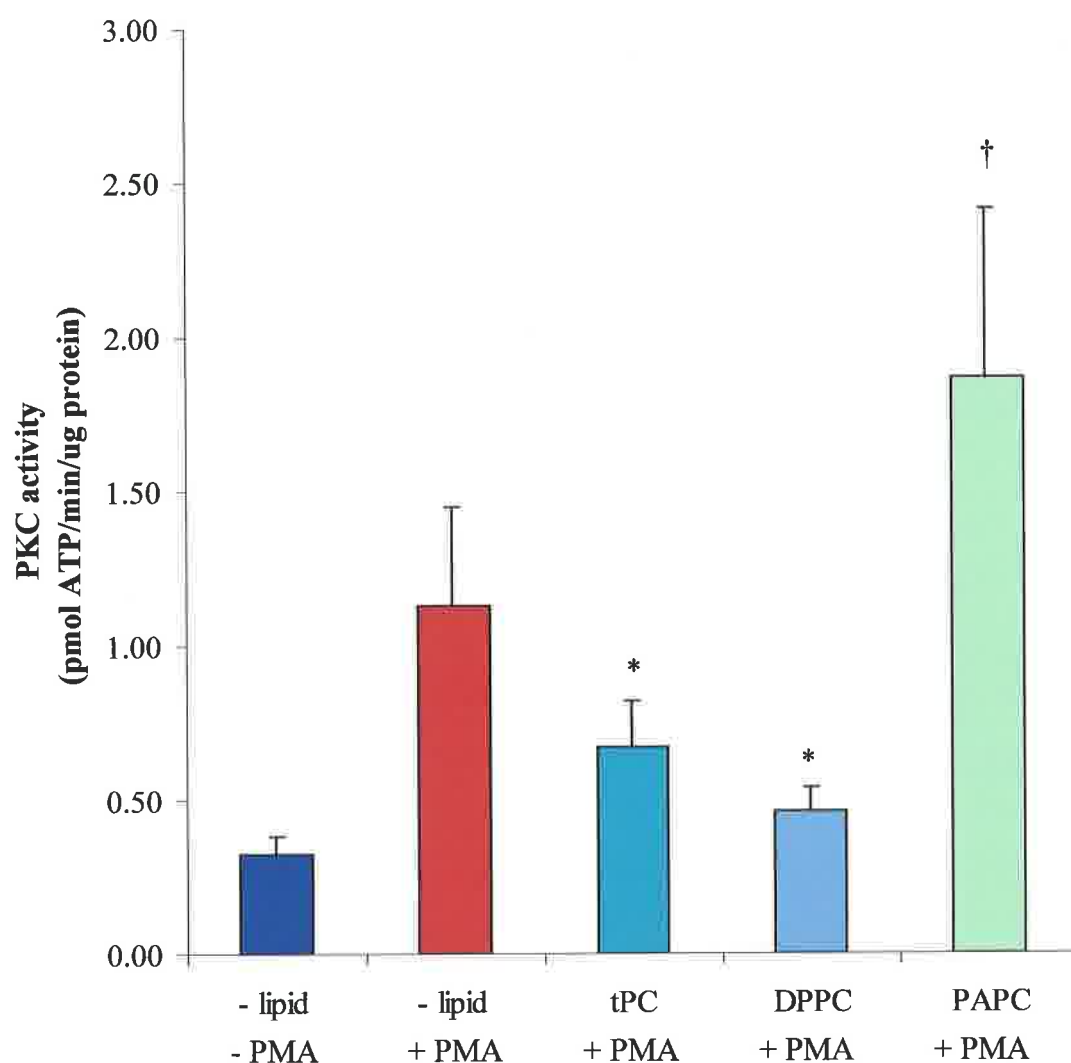
Figure 3.57: **Effect of PC on PKC activity in PMA stimulated MM6 cells.** MM6
cells (5×10^6) were pre-incubated with tPC or DPPC (100 µg / ml) or
PAPC (125 µg / ml) for 2 hours. Cells were washed in PBS (×3) and
stimulated with PMA (100 ng / ml) for 10 minutes prior to assaying PKC
by radioassay. Data represents mean ± 1 SD of 3 independent
experiments. *$P<0.05$ and †$P<0.01$ analysed by ANOVA and Tukey's
pairwise comparisons.

Tukey's analysis provides evidence that DPPC and tPC have significant inhibitory effects on PKC activity ($P<0.05$) when compared to MM6 cells incubated without lipid and stimulated with PMA. However, DPPC appeared to reduce the activity of PKC to complete or near complete inhibition. Contrary to this, PAPC was seen to significantly enhance the activity of PKC by approximately 50% over the control cells incubated without this phospholipid.

Taken together these results demonstrate the role of PC in PKC modulation. The results suggest PC may modulate ROI production through PKC mediated effects. PAPC had no effect on OpZ stimulated cells while PMA stimulated cells had significantly enhanced PKC activity. This suggests a specific role of certain sub-species of phospholipid in activation of PKC.

3.11 DETECTION OF NITRIC OXIDE BY THE GRIESS ASSAY

Nitric oxide (NO) release from mouse and rat macrophages is implicated in tumour cell cytotoxicity and the killing of intracellular organisms (Moncada *et al*, 1991). Evidence, however, suggests that human monocyte-derived macrophages or myeloid leukaemic cells differentiated along the monocytic lineage do not consistently release NO· (Zembala *et al*, 1994). LPS and / or interferon gamma (IFNγ) are potent stimulators of macrophage NO· production. NO· causes massive oxidative damage via reactions of NO· with oxygen (forming strong oxidants including nitrogen dioxide and peroxynitrites) (Anggard, 1994). To determine whether DPPC modulates the

inflammatory actions of macrophages by production of NO; this phospholipid was incubated with the murine macrophages RAW 264.7.

NO· is metabolically broken down into equal parts nitrite and nitrate. Assuming this occurs in RAW 264.7 cells, the production of nitrite measured by the Griess assay may be assumed to be halving of total NO· produced by stimulated macrophages. In this study, the stimulation of RAW cells with LPS for NO· production was not significantly different from untreated cells (Figure 3.58). Incubation with IFNγ alone produced a marked increase in NO· compared to cells incubated without this agonist. However, upon incubation with LPS and IFNγ there was a substantial increase (approximately eight-fold) in NO· produced compared to negative controls and RAW cells incubated with LPS alone. These results suggest the incubation of IFNγ and LPS together have a synergistic effect on nitrate production and hence NO· production. Therefore, to test the hypothesis that DPPC may modulate NO· production in macrophages, the LPS and IFNγ stimulated model was used on RAW cells. Preincubation of RAW cells with DPPC (10 – 500 μg / ml) did not significantly alter the production of NO· in LPS and IFNγ stimulated cells when compared to those cells incubated without DPPC (P>0.05 by ANOVA) (Figure 3.58). These results suggest that DPPC does not modulate the production of NO· in RAW cells.
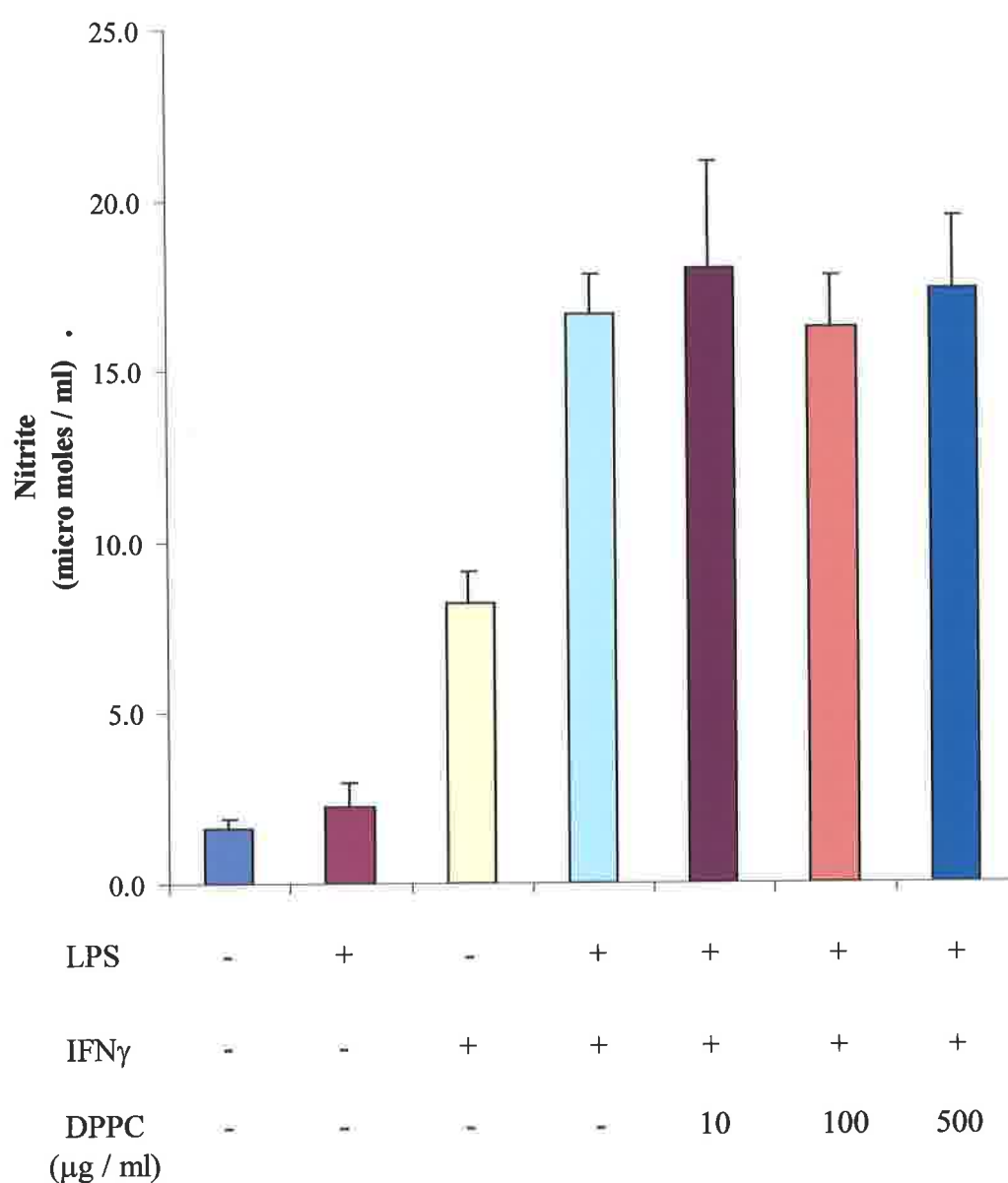
Figure 3.58: DPPC does not modulate nitrite production in RAW 264.7 murine macrophages stimulated with LPS and IFNγ. RAW cells (2×10^6) were incubated with DPPC for 2 hours. Cells were washed in PBS ($\times 3$) and stimulate with LPS (100 ng / ml) and IFNγ (units / ml). Controls were incubated without DPPC. Nitrite was detected by the Griess assay. Data represents mean ± 1 SD of 3 independent experiments.

3.12 DETERMINATION OF CYTOKINE PRODUCTION BY ELISA

After exposure to LPS and other inflammatory stimuli, monocyte / macrophages produce a number of regulatory mediators. These include cytokines, a group of naturally occurring proteins produced mainly by leukocytes, that have a variety of functions mostly involved in directing other immune cells to divide and differentiate. Selected cytokines with differing responses were assayed by enzyme linked immunosorbent assay (ELISA) in order to test the hypothesis that DPPC may affect the production of anti- or pro-inflammatory cytokines associated with phagocyte activation.

3.12.1 TUMOUR NECROSIS FACTOR ALPHA (TNF-α) AND INTERLEUKINS (IL) 1, 6 AND 10 ARE STIMULATED BY LPS IN A TIME DEPEDNDENT MANNER.

Prior to hypothesis testing, the production of TNF-α and IL-1, 6 and 10 in response to LPS in MM6 cells were optimised with respect to peak production over time. MM6 cells (1×10^6 cells) were stimulated with 100 ng / ml LPS for 0 – 36 hours at 37°C to stimulate the production of IL-1, 6 and 10. TNF-α was stimulated with 200 ng / ml LPS and 100 ng / ml PMA between 0 – 18 hours; these concentrations of LPS and PMA have previously been established within the laboratory to be optimal for TNF-α release. Controls (cells incubated without LPS / PMA) were performed at each time point. Following stimulation, cells were centrifuged and supernatants were collected, aliquoted and frozen at -80°C. ELISAs for each cytokine were performed on the assay supernatants.

The time dependent production of TNF-α, IL-1, 6 and 10 in response to LPS stimulation can be seen in figures 3.59 – 3.62 respectively. Each figure shows that very low (basal) levels of each cytokine are produced in the absence of LPS. Typically, these cells produced levels of cytokine below the lower limit of detection for the appropriate ELISA. Therefore the results of these basal levels were reported as 22, 6 or 12 pg / ml, the lowest standard concentration. Each interleukin assayed was produced in significant amounts by MM6 cells when stimulated with LPS. The maximum production of these interleukins was produced after 12 hours incubation with LPS. Generally, the levels of interleukin 1, 6 and 10 began to return towards basal levels following 18 – 24 hours incubation with LPS. Therefore, in order to test the hypothesis that DPPC preincubation of MM6 cells modulates IL-1, 6 or 10 production in LPS stimulated cells, MM6 cells were stimulated with LPS for 18 hours, an optima and convienient time point. The production and release of TNF-α was shown to be optimal at 4 hours following LPS / PMA stimulation. This time point was used in subsequent experiments.

3.12.2 DPPC MODULATION OF CYTOKINE PRODUCTION

TPC and its subspecies DPPC or PAPC (10 - 500 μg / ml) were preincubated with MM6 cells (1×10^6) for 2 hours followed by washing in PBS (×3). Cells were suspended in RPMI and stimulated to release IL-1, 6 and 10 with LPS for 18 hours. TNF-α release was determined following 4 hours stimulation with LPS and PMA. Following stimulation, the supernatants were harvested, aliquoted and frozen. TNF-α, IL-1, 6 and 10 were assayed in these supernatants using an ELISA.
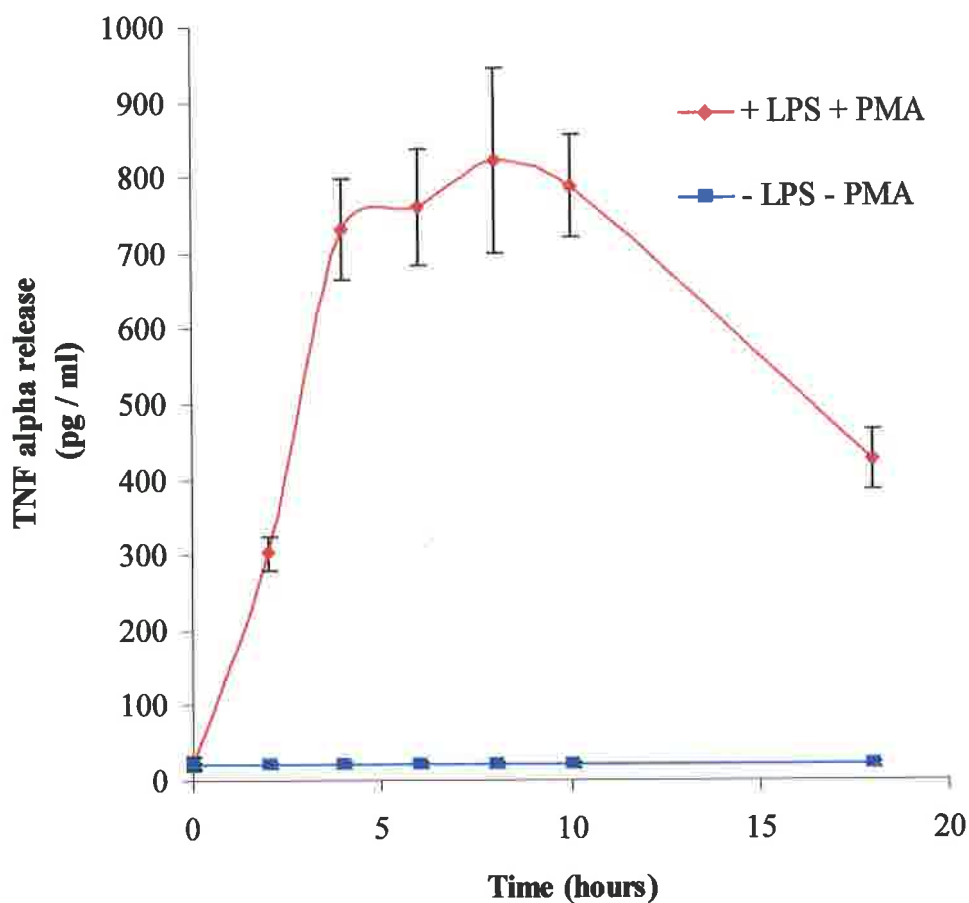
Figure 3.59: Effect of LPS incubation on TNF-α production in MM6 cells. MM6 cells (1×10^6) were incubated with LPS (200 ng / ml) and PMA (100 ng / ml) for various time periods. Controls were incubated without LPS / PMA. TNF-α production was assayed in the supernatant by an ELISA. Data represents mean ± 1SD of 3 independent experiments.
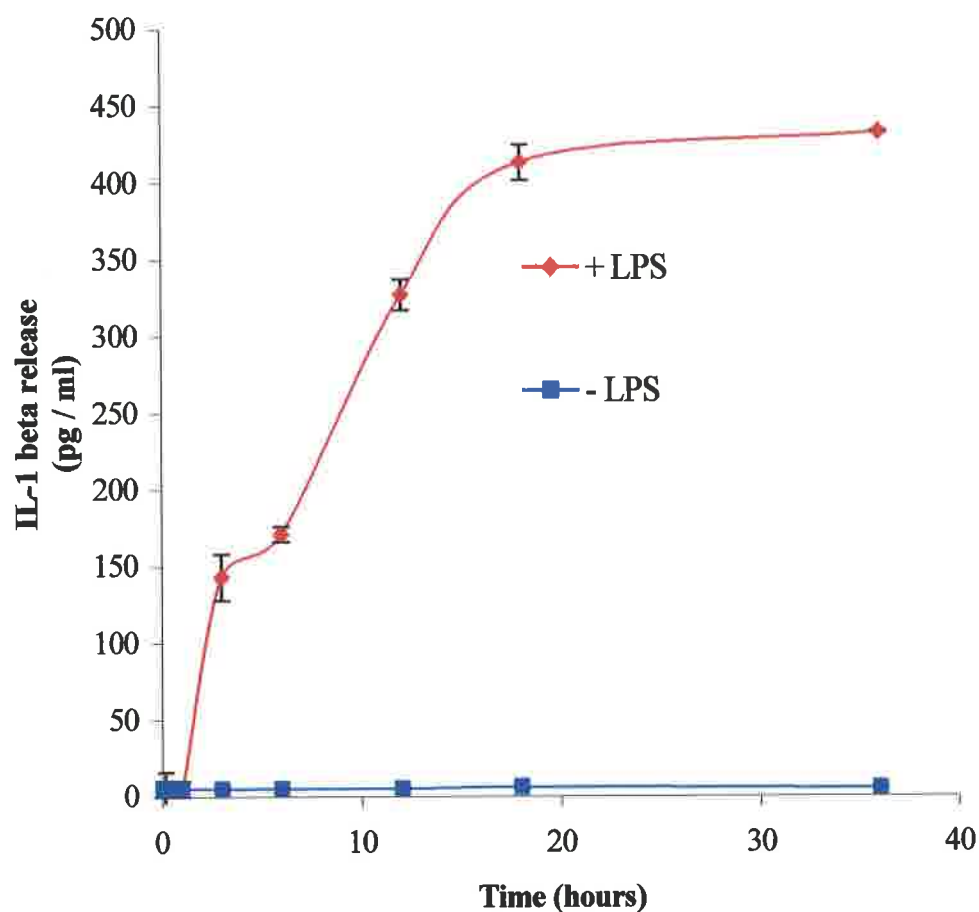
Figure 3.60: Effect of LPS incubation on IL-1β production in MM6 cells. MM6 cells (1 ×10⁶) were incubated with LPS (100 ng / ml) for various time periods. Controls were incubated without LPS. IL-1β production was assayed in the supernatant by an ELISA. Data represents mean ± 1SD of 3 independent experiments.
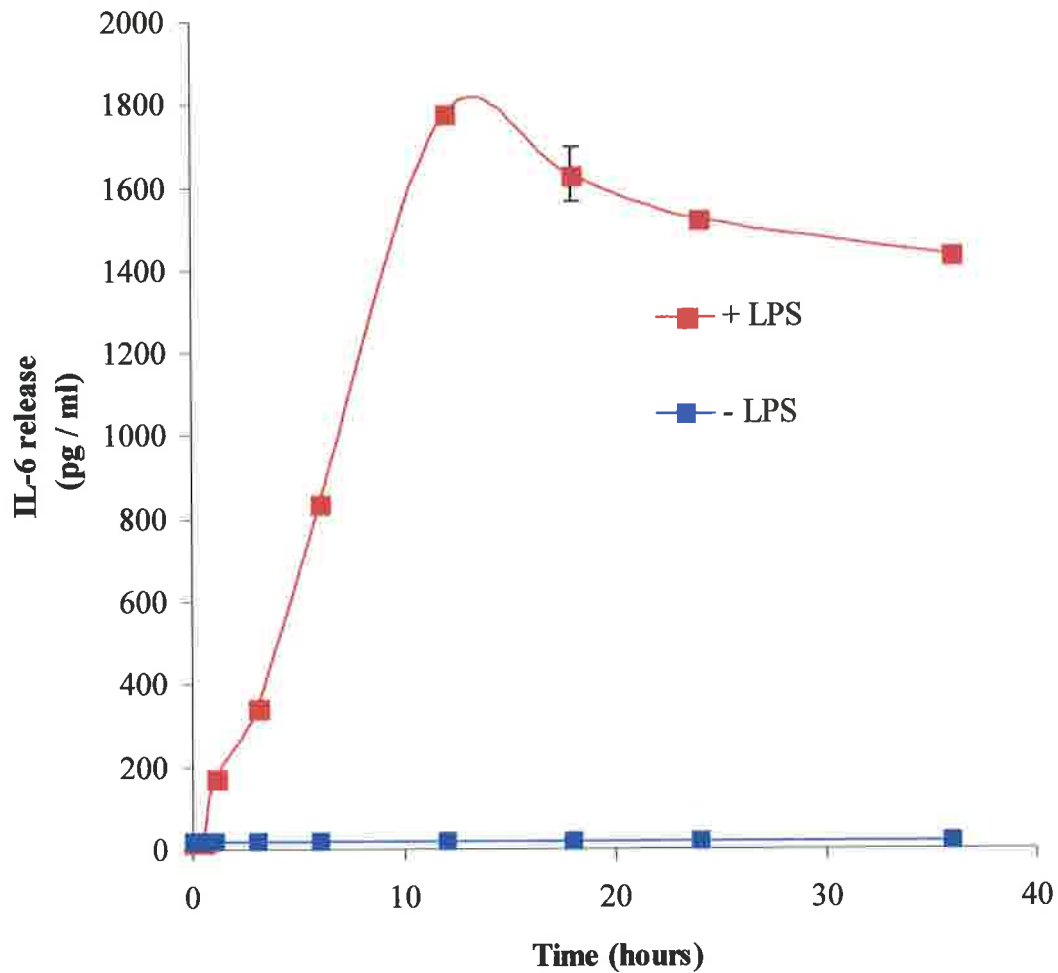
Figure 3.61: Effect of LPS incubation on IL-6 production in MM6 cells. MM6 cells (1×10^6) were incubated with LPS (100 ng / ml) for various time periods. Controls were incubated without LPS. IL-6 production was assayed in the supernatant by an ELISA. Data represents mean ± 1SD of 3 independent experiments.
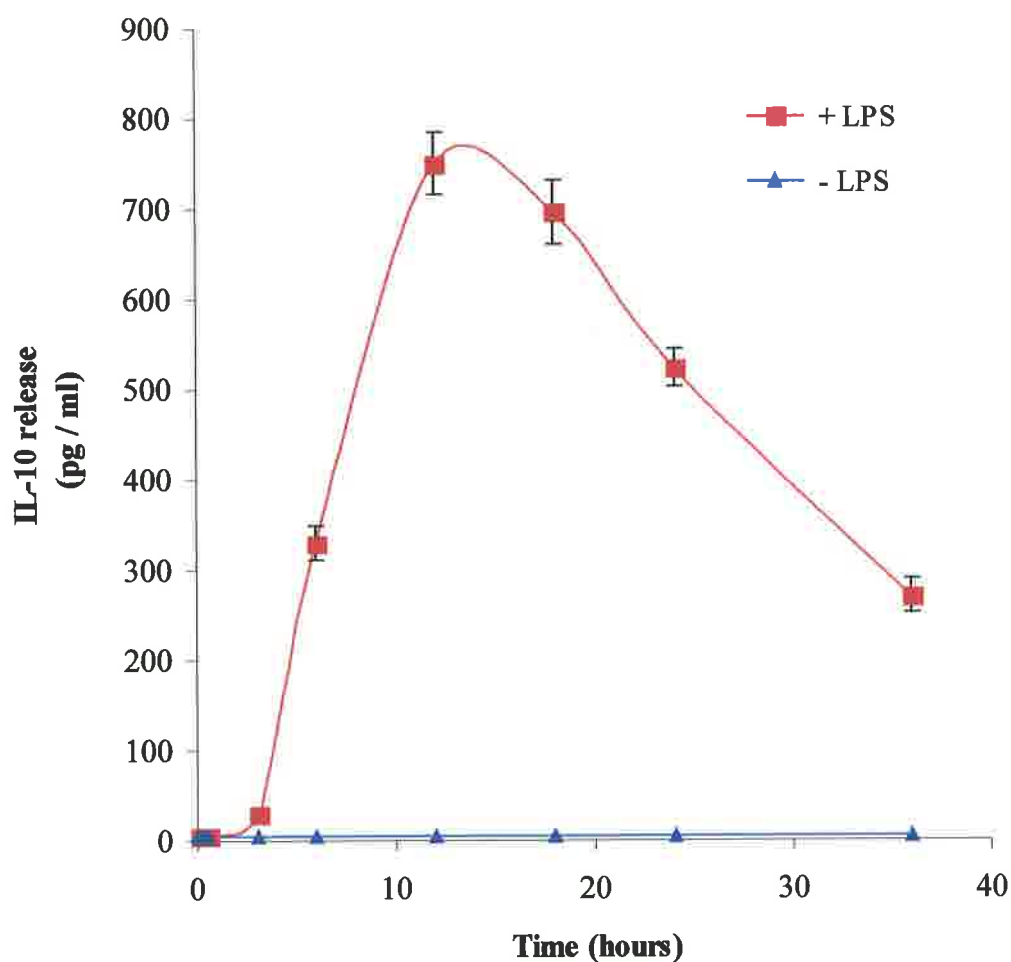
Figure 3.62: Effect of LPS incubation on IL-10 production in MM6 cells. MM6 cells (1 ×10^6) were incubated with LPS (100 ng / ml) for various time periods. Controls were incubated without LPS. IL-10 production was assayed in the supernatant by an ELISA. Data represents mean ± 1SD of 3 independent experiments.

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Figures 3.63 – 3.66 show the effect of the phospholipids tPC, DPPC or PAPC on the production of these cytokines. This study demonstrates that tPC and DPPC significantly reduce TNF-α release from MM6 cells after 2 hours incubation with these lipids. However, PAPC was not seen to affect TNF-α in the same way as DPPC. The maximum reduction in TNF-a release was seen after incubation with DPPC for 2 hours at a concentration of 125 – 500 μg / ml. In contrast, the production of the pro-inflammatory cytokine, IL-1 is significantly increased in MM6 cells pre-treated with phospholipids ($P<0.01$ analysed by ANOVA). Tukey's multiple pairwise comparisons indicates this significance is due to treatment with tPC or DPPC but only at a concentration of 500 μg / ml. The effect of PAPC is not significant on the production of this cytokine in LPS stimulated MM6 cells. Further levels below 500 μg / ml of the phospholipids tested did not affect the production of IL-1. As depicted in figures 3.64 and 3.65, incubation of the phospholipids tPC, DPPC or PAPC have no significant effect on the productions of IL-6 or IL-10. However, PAPC has a trend towards inhibiting the production of IL-10. This suggests PAPC may have a role in reducing the effects of the anti-inflammatory cytokine IL-10. This would support data that PAPC increases the pro-inflammatory role of superoxide production. However levels above 500 μg / ml of PAPC are unlikely to be seen *in vivo*. Taken together these results suggest that phospholipids that occur within pulmonary surfactant at physiological concentrations (i.e. approximately 125 μg / ml) have little or no effect on IL-1, 6 and 10 release but do however, modulate the production of the potent inflammatory cytokine TNF-α.
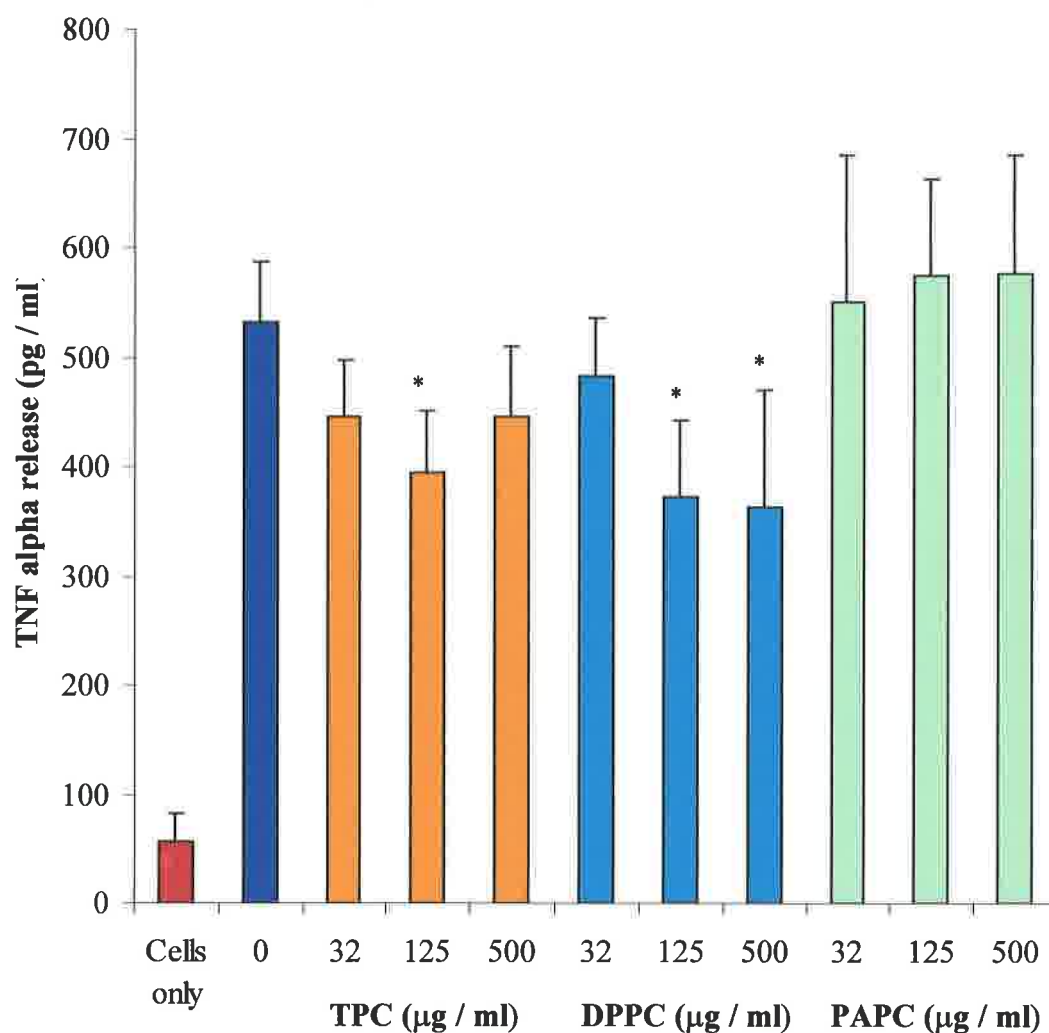
Figure 3.63: Effect of PC on TNF-α production in LPS and PMA stimulated MM6 cells. MM6 cells (1×10^6) were incubated with PC for 2 hours. Cells were washed in PBS and stimulated with 200 ng / ml LPS and 100 ng / ml PMA for 4 hours at 37°C. Controls were incubated without LPS / PMA and / or PC. TNF-α production was assayed in the supernatant by an ELISA. Data represents mean ± 1SD of 3 independent experiments. * $P<0.05$ by ANOVA with Tukey's multiple comparison method.
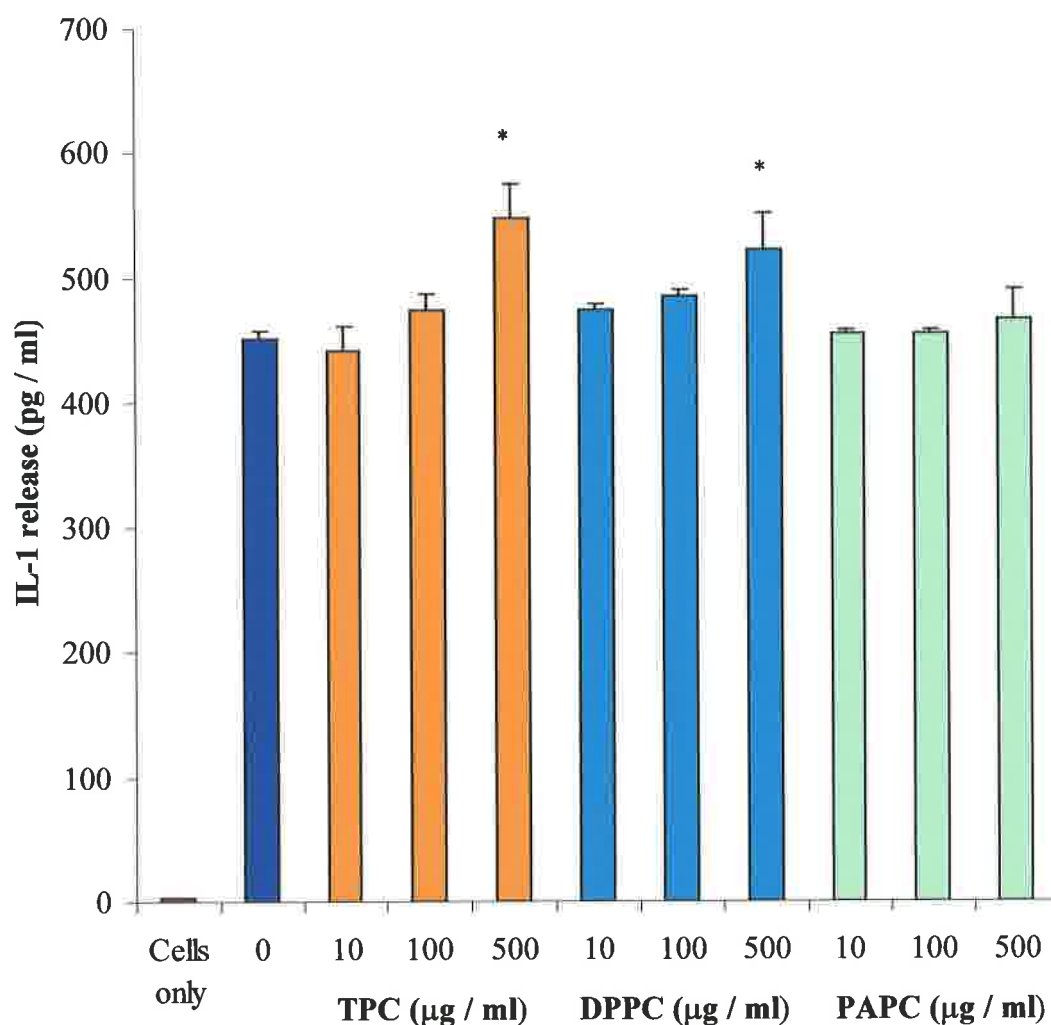
Figure 3.64: Effect of PC on IL-1β production in LPS stimulated MM6 cells.

MM6 cells (1 ×10⁶) were incubated with PC for 2 hours. Cells were washed in PBS and stimulated with 100 ng / ml LPS for 18 hours at 37°C. Controls were incubated without LPS and / or PC. IL-1β production was assayed in the supernatant by an ELISA. Data represents mean ± 1SD of 3 independent experiments. * P<0.05 by ANOVA with Tukey's multiple comparison method.
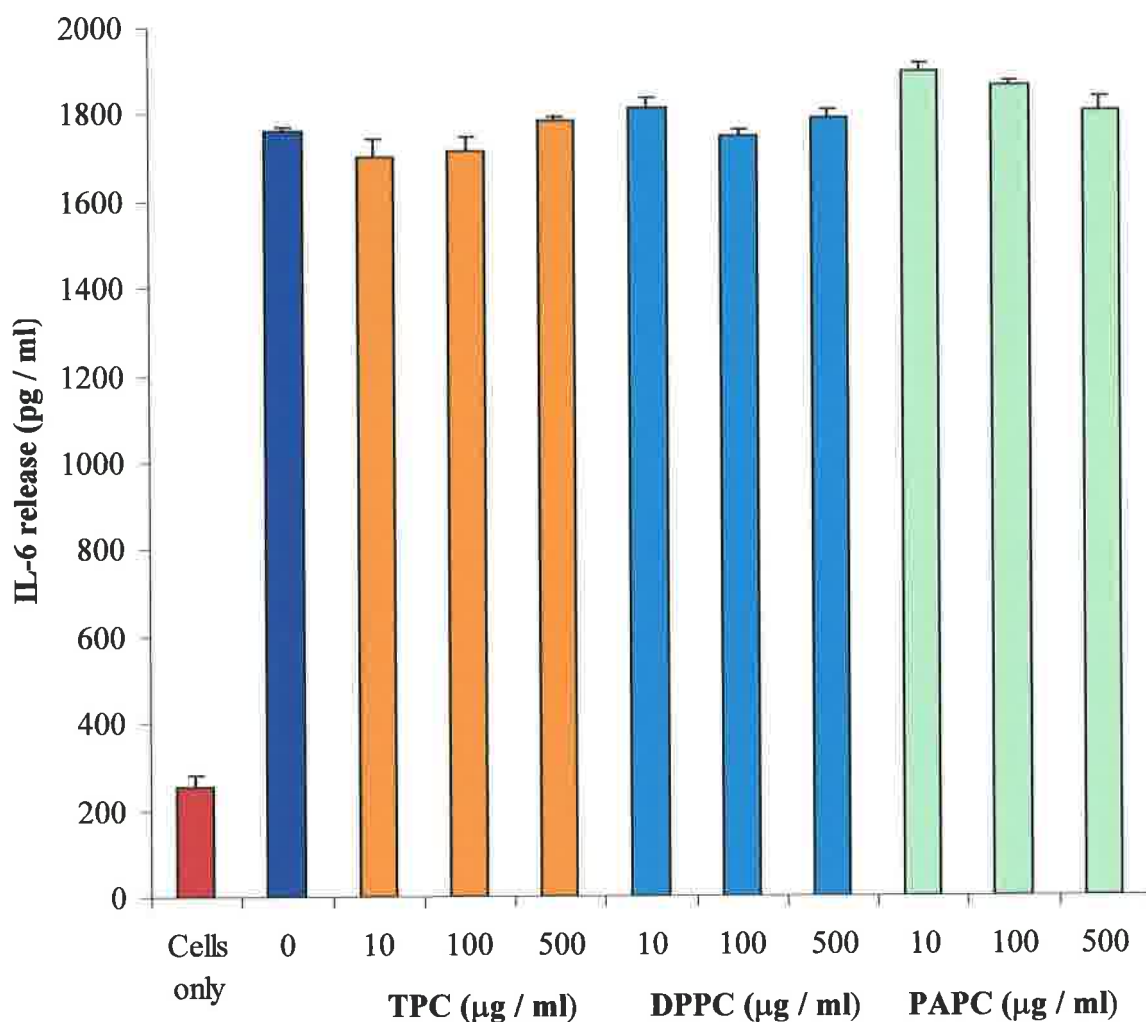
Figure 3.65: Effect of PC on IL-6 production in LPS stimulated MM6 cells. MM6 cells (1×10^6) were incubated with PC for 2 hours. Cells were washed in PBS and stimulated with 100 ng / ml LPS for 18 hours at 37°C. Controls were incubated without LPS and / or PC. IL-6 production was assayed in the supernatant by an ELISA. Data represents mean ± 1SD of 3 independent experiments.
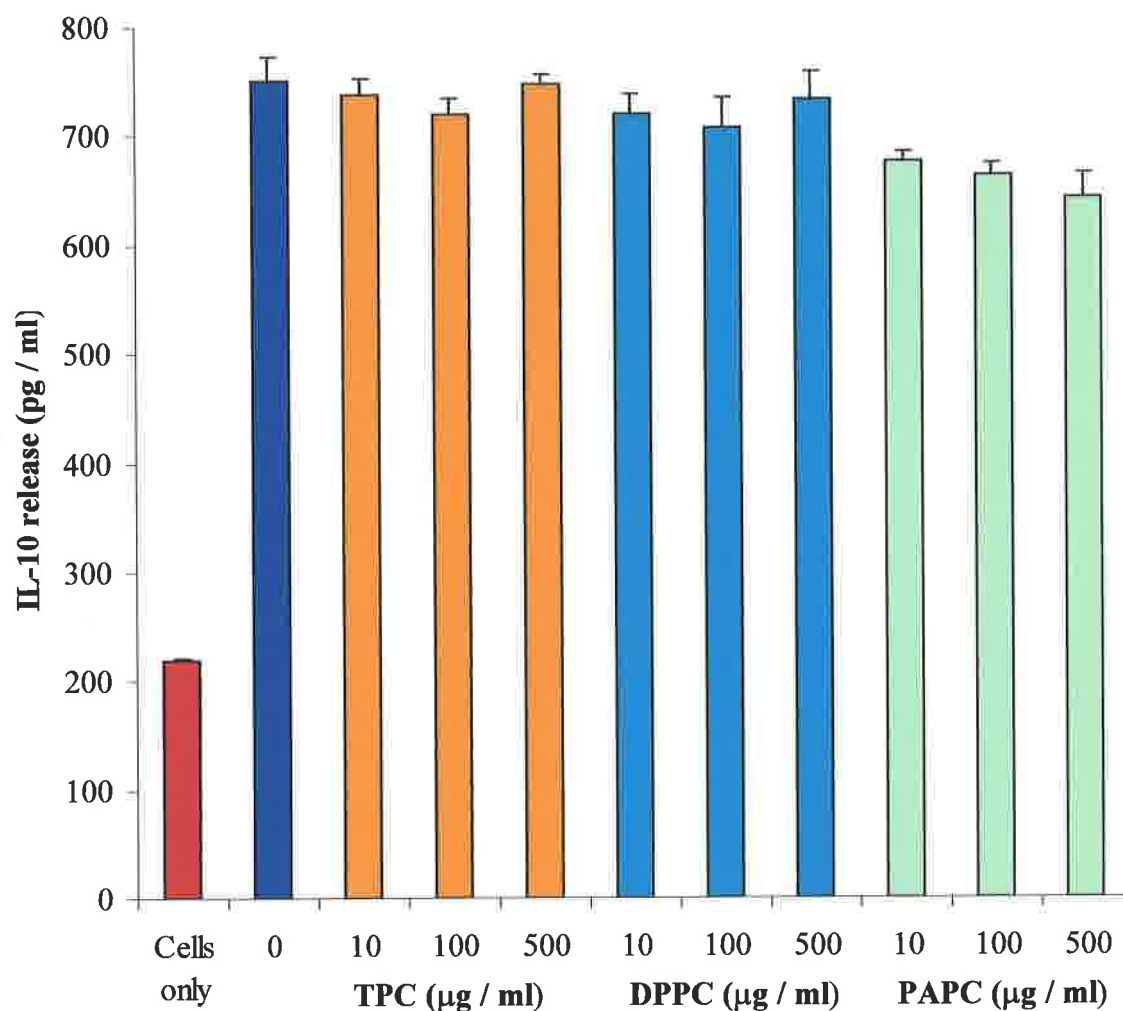
Figure 3.66: Effect of PC on IL-10 production in LPS stimulated MM6 cells. MM6 cells (1×10^6) were incubated with PC for 2 hours. Cells were washed in PBS and stimulated with 100 ng / ml LPS for 18 hours at 37°C. Controls were incubated without LPS and / or PC. IL-10 production was assayed in the supernatant by an ELISA. Data represents mean ± 1SD of 3 independent experiments.

CHAPTER 4: DISCUSSION

4.0   DISCUSSION

The increasing interest in the role of pulmonary surfactant in normal physiology and in its possible role in the pathogenesis of human lung disease has led to an increased need to develop our understanding of its mode of action in immunomodulation. Such knowledge may enable the development of better strategies in order to focus on those individuals most at risk. An example of such a strategy could include the improved characterisation of the inflammatory responses that occur within the pulmonary environment. The standardisation of analyses of lung surfactant composition and function will facilitate the recognition of the contribution of this complex material to the maintenance of normal lung function, as well as to disease and possibly the correlation of surfactant composition with risk of developing infections within the lung. It is being increasingly recognised that pulmonary surfactant may have an important role in the regulation of immune responsiveness within this delicate region of gaseous exchange. The results of this study have provided further evidence to enhance our understanding of immune regulation within the alveolar environment.

In summary, the data presented in this study demonstrates the ability of surfactant, phospholipids and the major surfactant phospholipid DPPC to modulate the release of reactive oxygen intermediates (ROIs) from inflammatory leukocytes in response to different stimuli. The ability of surfactant to modulate ROI production depends on an intricate interplay of many surfactant components including surfactant proteins and surfactant lipids. Lipids are the major component of pulmonary surfactant, and have been shown by this study to suppress the oxidative functions of monocytes and

macrophages in a dose- and time-dependent manner. The study also demonstrated that the major component of surfactant, 1,2-dipalmitoylphosphatidylcholine (DPPC), showed the greatest modulatory effect on ROI production in a human monocytic cell line (MonoMac-6; MM6). This occurred at concentrations of DPPC that reflect those levels normally seen in the lung *in vivo*. In addition, the data provides new insights on the mechanisms by which DPPC down regulates the inflammatory response of leukocytes. Finally, the effects of DPPC on cell activation appear to be specific since the production of tumour necrosis factor alpha (TNF-α) was significantly inhibited by this disaturated phospholipid whereas interleukin-1 beta (IL-1β) release was slightly increased. Other inflammatory functions such as the production of IL-6 and 10 or nitric oxide (NO) were not affected.

4.1 SURFACTANT LIPIDS MODULATE ROI PRODUCTION IN MONOCYTES AND MACROPHAGES

This study utilised the human monocytic cell line MM6. Established in the late 1980's, MM6 cells offer many advantages over currently available cell lines such as THP-1 or U937. For example MM6 cells proliferate rapidly, therefore generating virtually unlimited numbers of homogenous cell populations within a short period of time. This is in contrast to the rat alveolar cell line NR8383. These macrophages grow very slowly and require adherence to culture dishes in order to display their functional macrophage phenotype (Helmke *et al*, 1989). Rapidly proliferating cells such as MM6 are particularly advantageous in experiments that require large numbers of cells in order to generate a significant quantity of cellular material; for example Western blotting and

subsequent immunostaining of proteins. Further, MM6 cells respond to a variety of inflammatory stimuli including lipopolysaccharide (LPS), which interacts with the LPS receptor, CD14. The cell lines THP-1 and U937 cells are negative for CD14 as demonstrated by their lack of positive staining with the anti-CD14 clones My4, UCHM-1 and LeuM3 in immunofluorescence studies (Ziegler-Heitbrock *et al*, 1988). In addition, MM6 cells do not need to be induced to differentiate in order to display many of their characteristics of mature monocytes. Thus, MM6 cells provide an excellent tool for studying phenotypic and functional characteristics of human monocytes. Cultured human monocytes, which present functional similarities with alveolar macrophages, have been extensively used as a model system to study the biology of alveolar macrophages and in particular their response to pulmonary surfactant (Pinot *et al*, 2000; Walti *et al*, 1997; Geertsma *et al*, 1993).

Monocytes, the circulating precursors of macrophages, play an essential role in maintaining the sterility of tissues by the production of a range of mediators including cytokines and the release of ROIs and vasoactive lipids. The microenvironment in which monocytes mature into alveolar macrophages maybe important in determining their functional characteristics. A recent study has shown that alveolar macrophages have a greater capacity for leukotriene synthesis than peripheral blood monocytes. This elevated capacity only occurs after entry into the alveolar space and declines over time when cells are cultured outside this environment (Phare *et al*, 1998). Alveolar macrophages are constantly bathed in pulmonary surfactant, a complex mixture of phospholipids, neutral lipids and cholesterol and four genetically distinct surfactant specific proteins (Wright, 1997). Surfactant has been reported to either enhance (Webb

and Jeska, 1986), suppress (Hayakawa *et al*, 1992; Hayakawa *et al*, 1989) or not modify

the production of superoxide by alveolar macrophages or monocytes (Speer *et al*, 1991).

These studies have used a number of different animal models and methodologies for

estimating superoxide and ROI production, which may be responsible for the

conflicting reports. Using chemiluminescence (the study of light emitting reactions),

the present study indicates that the surfactants Survanta®, Curosurf® or Exosurf

Neonatal™, all used in the treatment of infant respiratory distress syndrome (IRDS), are

able to inhibit the release of ROIs from MM6 cells in a dose dependent manner.

Current data from this study indicates that surfactant composition (i.e. natural or

synthetic), the concentrations of surfactant used and the type of stimulus used to evoke

the production of ROIs by monocytes are important variables. Survanta®, Curosurf® or

Exosurf Neonatal™ pretreatment on MM6 cells inhibited ROI production to a greater

extent in opsonised zymosan evoked responses when compared to PMA stimulated

cells. Further, the suppression of PMA stimulated ROI production by surfactant, only

occurred when pretreatment of MM6 cells with surfactant was at a concentration of 500

µg / ml. The different responses of ROI production in surfactant treated cells stimulated

with the yeast particle extract zymosan compared to cells stimulated with the soluble

phorbol ester, PMA, suggests that surfactant maybe exerting its effects by interfering

with the plasma membrane (see section 4.4). This observation is supported by Walti *et*

al, 1997, who demonstrated that Curosurf® inhibited superoxide production induced by

the receptor mediated bacterial extract OM-85 but not by phorbol ester. However, the

present study has shown that at high surfactant concentrations (500 µg / ml), PMA

stimulated ROI production is also significantly inhibited. If the plasma membrane was

significantly altered by surfactant one might expect that certain signalling molecules,

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recruited at or near to the membrane may also be affected. An example of one important signalling molecule is protein kinase C. PMA acts directly on signal mechanisms within the cell by mimicking diacylglycerol (DAG) activity and hence directly activating protein kinase C (PKC). Possible alterations in membrane structure and signalling may explain why the effect of surfactant on ROI production in response to receptor-mediated stimuli is greater than that of soluble activators such as PMA.

Survanta® and Curosurf® are modified natural surfactants, which contain less than 1% of surfactant proteins B and C (by weight). In contrast, Exosurf Neonatal™ is a synthetic surfactant that lacks any of these proteins. Further, due to the hydrophilic nature of SP-A, the extraction procedure of natural surfactants excludes this protein in their final composition. Studies on the effects of the protein constituents of surfactant on inflammatory cell activity have largely focused on SP-A. This apoprotein binds to alveolar macrophages via a specific cell surface receptor that recognises the collagen domain of SP-A (Pison *et al*, 1992). SP-A has been shown to play a major immunomodulatory role in alveolar macrophages (Sasaki *et al*, 2001; Van Iwaarden *et al*, 2001). Exposure of alveolar macrophages to SP-A results in enhanced phagocytosis of a number of pathogenic bacteria such as *Pseudomonas aeruginosa* (Manz-Keinke *et al*, 1992). More recently, the pulmonary collectins SP-A and SP-D facilitate the resolution of inflammation by accelerating apoptotic PMN clearance (Schagat *et al*, 2001). SP-A has been suggested to act as an opsonin within pulmonary tissue, an environment that normally lacks serum opsonins. This observation is supported by the structural homology of SP-A with the complement component C1q (Tenner *et al*, 1989). Purified SP-A has also been shown to enhance alveolar macrophage lucigenin

chemiluminescence (van Iwaarden *et al*, 1990). In contrast Weber *et al*, 1990, reported that SP-A isolated from dog lavage inhibited the production of superoxide by alveolar macrophages. Subsequent studies have suggested butanol extraction of SP-A, results in a conformational change of this protein. Weissbach *et al*, 1994, reported that SP-A when attached to either the surface of a tissue culture dish or to zymosan, stimulated production of ROIs. This data demonstrates how important isolation techniques of surfactant components are and that a conformational change of SP-A when attached to a particle may be important in regulation of function. In Weissbach's study peripheral blood monocytes did not respond to SP-A with enhanced chemiluminescence. Further, the SP-A preparations used in some studies have not been examined for LPS content. SP-A preparations that contain significant quantities of LPS may be a reason why conflicting reports are seen on the effects of SP-A on macrophage functions. In addition to acting as an opsonising agent, SP-A also binds pollen and other aeroallergens such as the DerP1 from the house dust mite (Postle, 2000). Binding of DerP1 to SP-A prevents its subsequent binding to specific IgE suggesting a further anti-inflammatory role for this protein. It is possible that the proteins in natural surfactant preparations may modify uptake of surfactant containing phospholipids by alveolar macrophages. Wright *et al*, 1987, reported that a surfactant apoprotein (26 kDa - 36 kDa) enhanced uptake of DPPC by alveolar macrophages as well as alveolar type II cells obtained from the rat. Different uptake patterns may have resulted in the slightly different activity seen in natural and artificial surfactant. In the present study there appears to be little difference between natural surfactant effects on ROI production compared to the synthetic Exosurf Neonatal™ treatment. This suggests that in the

model used in this study, surfactant proteins B and C have a minimal effect on ROI production when in the presence of surfactant lipids.

Since surfactant is comprised mainly of phospholipids and other lipids, the effects of an artificial (or synthetic) surfactant, comprising of the main lipids found in surfactant, on ROI production was investigated. The artificial surfactant in this study decreased ROI production in LPS 'primed' and OpZ stimulated cells. This data supports the conclusion that lipid components of surfactant are responsible in whole or at least in part for the reduced oxidative functions in monocytes. To further characterise the nature of the inhibitory effect of surfactant lipids on ROI production, the effects of individual lipids on monocytic ROI production were analysed. The lipids used in this part of the study were previously reported to be the major constituents of human pulmonary surfactant (Harwood, 1987). This study reports that not all the lipids preincubated with MM6 cells were responsible for a suppressive effect on ROI production. In fact cholesterol, sphingomyelin and phosphatidylethanolamine (PE) were potent enhancers of ROI production in opsonised zymosan stimulated cells whereas phosphatidylglycerol (PG) suppressed ROI production by up to 70% when compared to control cells incubated without lipid. However, the concentrations of cholesterol, sphingomyelin, PE and PG required to cause these effects were in excess of their normal lung representation *in vivo*. Interestingly it was phosphatidylcholine (PC), the major surfactant phospholipid that accounted for the greatest decrease in ROI production (up to 40%) at concentrations that reflected those normally seen in human pulmonary surfactant (125 - 250 µg / ml). Many lung disorders are associated with surfactant deficiency, both qualitative and quantitative. For example in acute

respiratory distress syndrome (ARDS) the surfactant pool increased in early phase and decreased in the intermediate and late phase of the syndrome (Griese, 1999). The qualitative alterations of surfactant consist of reduced phospholipid content particularly that of PC and PG, followed by an increase in PE, PI and sphingomyelin. These factors undergoing alterations during the course of ARDS, could have a significant role in the pathogenesis and evolution of ARDS. Furthermore, chronic interstitial lung diseases such as idiopathic pulmonary fibrosis, share at least some inflammatory features with ARDS and have been associated with alterations in surfactant phospholipid composition (Gunther *et al*, 1999). In addition, the reduced lung function in asthma has also been associated with altered airway surfactant phospholipid composition (Heeley *et al*, 2000; Wright *et al*, 2000).

Although PC is the major component of surfactant, this phospholipid exists in different forms, depending on the structure of the fatty acyl chains esterified at the *sn*-1 and 2 positions on the glycerol backbone. It is known that dipalmitoyl PC accounts for approximately 90% of all disaturated species within lung surfactant. The present examined the effect of changing the fatty acid structure at the *sn*-2 position on the ability of PC to modulate ROI production. The production of ROIs was impaired in MM6 cells that were exposed to tPC (PC containing mixed species), DPPC and PAPC (1-palmitoyl-2-arachidonoyl phosphatidylcholine) between a concentration range of 0 – 500 μg / ml and stimulated to release ROIs with OpZ. These effects were seen within 30 minutes incubation in the presence of both DPPC and PAPC but only after 2 hours incubation in the presence of tPC. In addition PAPC demonstrates an ability to promote

ROI production only at a concentration of 125 µg / ml. This was not observed with tPC and DPPC treatment.

Using PMA as a stimulant of oxidative activity, it was observed that DPPC at 125 µg / ml is capable of partly inhibiting the respiratory burst. In *in vivo* conditions, alveolar macrophages constantly reside in pulmonary surfactant. Most investigators have only examined short-term incubation (<24 hours) of surfactant on inflammatory functions. This study reports, that DPPC continues to exert its suppressive effects on ROI production in cells cultured with DPPC for extended periods (>9 days of culture). Further the ability of DPPC to suppress ROI production is removed once the cells are incubated in non-DPPC containing media. However, this effect requires at least 36 hours in culture in the absence of lipid for it to be observed. This suggests that DPPC uptake is required for an effect on ROI production to take place and this effect is only slowly reversible.

This study has shown that the dose of phospholipid and time of incubation has a critical effect on oxidative functions. Webb and Jeska, 1986, found an increase in the monocyte oxidative response when incubated with alveolar lining material and provided evidence that unsaturated lipids were responsible. The results of the present study indicate that the different modulatory properties exerted by the phospholipids used in ROI production after LPS stimulation appears to be selective. The only difference between DPPC and PAPC is in the esterified fatty acid at the *sn*-2 position of PC, it would appear that these modulations might be associated with this substitution. Further studies comparing DPPC with other PC species (such as 1-palmitoyl, 2-oleoyl PC and

1,2 distearoyl PC) in the suppression of ROI production will elucidate more clearly the role of the fatty acyl moieties of PC in pulmonary surfactant in the down regulation of inflammatory leukocyte function. In addition, these further studies would clearly aid in defining a synthetic surfactant material with anti-inflammatory properties.

In this study, chemiluminescence was the technique used to study the production of ROIs. Native chemiluminescence is the relaxation of biologically excited molecules to a ground state by photon emission. However, chemiluminescence efficiency is very low in this system. The use of a chemiluminogenic probe enhances the chemiluminescent efficiency and yields a reliable, non-destructive, low cost yet sensitive method for studying ROI release. In our laboratory luminol (a cyclic hydrazide) has been the chemiluminogenic probe of choice as opposed to lucigenin (10,10'-dimethyl-9,9'biacridinium dinitrate). Both these probes appear to amplify chemiluminescence by different mechanisms and have different specificity for oxygen radicals. Luminol enhanced chemiluminescence (LCL) can be generated in a cell free system by the reaction of hydrogen peroxide with myeloperoxidase and a halide and can be abolished by hydrogen peroxide scavengers such as catalase. Although lucigenin and luminol are very cheap, luminol was the more reliable amplifier of chemiluminescence and is indicative of total oxidant production since this probe is small and lipophilic and thereby traverses the plasma membrane to be oxidised intracellularly. A disadvantage of LCL is that light emission cannot be correlated with a single type of oxygen metabolite.

In the current study the effects of surfactant or surfactant lipids on ROI production were not different whether surfactant lipid was present during the chemiluminescent assay itself, thus arguing against a direct scavenger-like effect of surfactant lipids. Further the decrease in ROI production was not attributed to a decrease in cell viability since viability assays of cells treated with surfactant (upto 1 mg / ml) was always greater than 90%. Finally, the effects of DPPC on ROI production were repeated in human peripheral blood monocytes, murine macrophages and rat alveolar macrophages. These studies confirmed and validated that phospholipid inhibition of ROI production is a consequence of incubation of inflammatory leukocytes with these lipids and not as a consequence specifically attributed to MM6 cells or assay interference. Although observed reductions in release of inflammatory products were not complete, a 20 – 40% reduction in the release of ROIs would offer a considerable protection against inflammatory damage in the delicate alveolar region of the lung. Indeed, complete inhibition of such responses would be deleterious to the individual as macrophage mediated responses are the central immune defence mechanism in this region of the respiratory tract. The data from this study suggests pulmonary surfactant lipids modulate ROI production in human monocytes and alveolar macrophages and highlights areas that may be responsible for the conflicting reports seen within the literature.

4.2 INFLUENCE OF DPPC ON OTHER INFLAMMATORY FUNCTIONS OF MONOCYTES

The present study provides new insights into the role of phospholipid on LPS induced cytokine expression. In this study, the effect of phospholipid incubation on LPS stimulated cytokine production was assessed in order to determine whether DPPC dampened many monocytic inflammatory responses or whether its effects were specific to ROI production. Conditions were initially optimised for maximal biosynthesis and release of tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6 and IL-10 in the MM6 cell model system. This study demonstrated that the optimal release of TNF-α in response to LPS was 4 hours following initial stimulation with LPS. The release of IL-1β, 6 and 10 in MM6 cells stimulated with LPS was optimal at approximately 18 hours following initial stimulation. These stimulation times were also considered to be the most convenient for assessing the effects of phospholipid on LPS stimulated cytokine production.

TNF-α is a potent pro-inflammatory cytokine generated by monocytes and macrophages. This cytokine is a crucial factor involved in the occurrence of intra-alveolar and pulmonary inflammation in preterm infants with respiratory distress syndrome. The release of TNF-α by MM6 cells has previously been shown to be dependent on the treatment of MM6 cells with PMA, as this stimulant specifically enhances the release of TNF-α from MM6 cells that have been co-stimulated with LPS (Pradines-Figueres and Raetz, 1992). The present study confirms previous findings that tPC suppresses the secretion of TNF-α in LPS-stimulated monocytes (Morris et al, 2000). Further, this study demonstrates that DPPC but not PAPC significantly inhibits

TNF-α production. Previously, Curosurf® and its phospholipid component were shown to inhibit the synthesis of TNF-α in monocytes (Baur *et al*, 1998; Speer *et al*, 1991) via a mechanism involving a decrease in TNF-α mRNA expression (Baur *et al.*, 1998). The present study also demonstrated that PC and its subspecies DPPC or PAPC did not inhibit the production of IL-1β, 6 and 10 at concentrations of PC that reflected those levels seen in vivo (i.e. approximately 100 μg / ml). However, Thomassen *et al*, 1992, observed that Exosurf Neonatal™ inhibited IL-1β and 6 release (as well as TNF-α) in alveolar macrophages in response to LPS. Subsequent investigations determined that inhibition of cytokine secretion and mRNA levels of LPS-stimulated alveolar macrophages were decreased by tyloxapol (nonionic dispersing agent) and not by DPPC, components of Exosurf Neonatal™ (Thomassen *et al*, 1995). In contrast, a recent study by Antal *et al*, 1998, reported that Survanta®, a natural bovine surfactant preparation that does not contain tyloxapol, diminished levels of IL-1β and IL-6 in an LPS stimulated human monocytic cell line. Such data suggests that natural surfactant preparations inhibit cytokine production by other mechanisms. Perhaps surfactant proteins found in natural surfactant play a role in suppression of cytokine production, particularly with respect to IL-1β and IL-6.

Nitric oxide (NO·) is a free radical essential for vasorelaxation, neurotransmission, inhibition of platelet and neutrophil adhesion and host defence. The regulation of NO· production by different mechanisms is therefore critical in maintaining homeostasis. Since, nitric oxide (NO·) is short lived and thus not amenable to direct detection, nitrite quantitation by the Griess assay was used to estimate NO· The determination of nitrite in cell supernatants is limited regarding sensitivity and its inability to detect nitrate the

other end product of NO· reaction in aqueous media. Consequently NO· is converted enzymatically or chemically to nitrite before total nitrite can be determined. It has been shown previously that the concentration of nitrite in biological samples such as cell culture supernatants was found to be at equal proportions with nitrate (Maru and Jackson, 1996). The nitrite assay is a simple and suitable method since it can be applied to a laboratory with little cost or need for expensive laboratory equipment.

The induction of NO· in murine macrophages has previously been shown to be triggered by LPS (Mullins *et al*, 1997). The use of IFN-gamma (IFNγ) and LPS has been shown to be synergistic described earlier by Lorsbach *et al*, 1993. Maru and Jackson, 1996, have shown previously that human peripheral blood monocytes and MM6 cells fail to produce NO· in response to LPS, IFNγ, TNF-α or PMA. These results have been supported by Gallay *et al*, 1993, and Schneeman *et al*, 1993. Zhang *et al*, 1993, have concluded that human monocytes are unable to produce NO· because they cannot initiate transcription of the inducible nitric oxide synthase (iNOS) gene in response to LPS and / or IFNγ. Therefore, to investigate the effect of DPPC on NO· production, the murine macrophage cell line RAW 267 was used. In this study, it was found that NO· production in DPPC treated cells in response to LPS and IFNγ was not significantly different from control cells incubated without DPPC. However, in another study by Miles *et al*, 1999, pulmonary surfactant was found to inhibit LPS-induced NO· production by rat alveolar macrophages. Interestingly, NO· formation was not affected by the major lipid components of surfactant or by two surfactant-associated proteins, SP-A or SP-C. However, Miles *et al*, demonstrated that SP-B was responsible for inhibiting NO· formation in a concentration-dependent manner, thus indicating this

hydrophobic protein is responsible for the observed reduction of NO· in pulmonary surfactant.

Taken together, the ability of phospholipids to differentially modulate aspects of inflammatory function suggests the actions of phospholipids (e.g. PC) are specific. Although such studies clearly demonstrate the regulatory effects of surfactant and surfactant lipids in controlling inflammatory responses within the lung, the mechanisms by which this is achieved remains elusive.

4.3   DPPC DOES NOT REDUCE LPS BINDING OR RECEPTOR EXPRESSION

The reduced production of ROIs by monocytes treated with surfactant may be due to aberrations caused by surfactant lipids at the agonist - receptor level. To address this point, studies were undertaken to assess the effect of LPS binding and receptor expression in MM6 cells treated with DPPC.

In this study MM6 cells were first 'primed' with lipopolysaccharide (LPS) in order to attain maximal ROI production. 'Primed' phagocytes produce more ROIs than 'unprimed' cells and thus allow a more sensitive discrimination between treatment groups. The data demonstrates that 'priming' of MM6 cells with LPS requires 18 hours incubation with this agonist in order to increase ROI production by approximately 10 fold over 'unprimed' cells. In addition, the 'priming' agent (LPS) does not induce ROI production following the 18-hour LPS incubation. This data is consistent with previous investigators and indicates the suitability of LPS as a 'priming' agent for ROI production (Zughaier et al, 1999; Hughes et al, 1997). The mechanisms of DPPC's

inhibition may include the perturbation of LPS binding to its receptor CD14 thereby affecting the 'priming' process of the phagocyte. To assess the effects of DPPC on LPS binding, MM6 cells were incubated with DPPC followed by incubation with radiolabelled LPS (^{3}[H]LPS). The number of LPS molecules able to bind to MM6 cells following pretreatment with DPPC was not significantly different from control cells incubated without DPPC. Therefore DPPC is unlikely to prevent the binding of LPS to MM6 cells. This conclusion is supported by the present study's LCL data. DPPC suppressed ROI production in MM6 cells after addition of LPS i.e. the mechanisms of DPPC suppression on ROI production must be independent of LPS binding since DPPC was added after LPS. In contrast, it has been suggested that surfactant was responsible for inhibiting 'priming' of rabbit alveolar macrophages (Hayakawa et al, 1989). In Hayakawa's study, 'priming' was interpreted as the escape from surfactant suppression as opposed to an enhanced release of mediator over basal levels.

LPS 'priming' may be suppressed by down regulation of LPS receptor expression, thereby limiting the amount of interaction between LPS and the cell. The expression of the LPS receptor CD14 was estimated by fluorescent activated cell scanning (FACS) following preincubation with DPPC. The FACS data from this experiment shows there was no significant difference in the expression of CD14 in DPPC treated cells compared with control cells. Therefore, the clearly suppressive effect of DPPC on ROI production seems to be regulated by mechanisms other than interference in LPS binding and 'priming'.

Efficient interaction between monocytes and particles require the participation of numerous factors including opsonins and serum proteins such as immunoglobulin G, which become attached to the surface of micro-organisms or particles, and interacts with specific receptors on phagocytes. Perturbations of the membrane receptors responsiveness from exposure to pulmonary surfactant have been reported in lymphocytes. Roth *et al*, 1993, reported that modified natural bovine surfactant reduces the binding of lymphokine activated killer cells to tumour targets and induces a parallel reduction in the expression of adhesion molecules involved in inflammation such as ICAM-1. This may also explain the time-dependent gradual loss of the adherence capacity in human monocytes cultured with Curosurf$^{®}$ (Walti *et al*, 1997). In this present study, we examined by FACS, the expression of CD11b/Mac1 (iC3b receptor), CD16 (FcγRIII receptor) and CD64 (FcγRI receptor), receptors important in particle recognition and phagocytosis. Data presented within this study demonstrated that DPPC preincubation of MM6 cells did not change the expression of these receptors. This data suggests that suppression of ROI production is not due to an inefficient interaction between important phagocytic receptors with target stimuli.

4.4   DPPC UPTAKE DOES NOT DIRECTLY AFFECT THE ASSEMBLY OF THE

NADPH OXIDASE

The LCL time course observations of DPPC's effect on ROI production suggest that a time-dependent incorporation of DPPC into monocytes may represent a crucial event that causes impairment of ROI production in MM6 cells. Previous studies within the department regarding quantitation of DPPC by HPLC in MM6 cells indicated that there was an increase of DPPC uptake in MM6 cells following preincubation with this lipid (Tonks *et al*, 2001). This increase suggested cellular uptake of this particular phospholipid during incubation. Coupled with this, spin-labelled EPR spectroscopy showed an increase in order parameter suggesting a significant change in membrane composition associated with preincubation with DPPC. This interpretation is supported by morphological observations in this present study, showing that monocytes incubated with DPPC presented as round cells with an apparent increase in lipid staining of the membrane, compared to control monocytes. In addition as the concentration of DPPC increased, there was a relative increase in DPPC uptake. This was particularly noted in cells incubated with 500 µg / ml of DPPC, in which the presence of lamellar bodies, an intracellular storage vehicle for lipids was observed. These lipid storage structures are commonly seen in alveolar macrophages and type II epithelial cells in which surfactant undergoes re-uptake as part of its normal 'life' cycle (Wright, 1997). These membrane changes may be associated with incorporation of phospholipids into the membrane. A more detailed analysis of membrane lipids after exposure to surfactant lipids would provide a more complete picture of phospholipid turnover within the membrane. Such studies may include a time course study of PC incubation with MM6 cells followed by HPLC analysis and / or electron microscopy studies. Further, the use of confocal

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microscopy may aid in the intracellular localisation of fluorescently labelled lipids. These studies may provide evidence as to the role of membrane modifications induced by surfactant lipids.

Functional consequences of lipid heterogeneity have started to emerge such as the non-random mixing of lipids and phospholipids in the membrane bilayer leading to the formation of lipid microdomains (Horejsi *et al*, 1999). These microdomains are thought to be involved in numerous signalling events associated with the cell membrane (Brown and London, 1998). The response of a cell to its environment occurs via proteins that are embedded in the plasma membrane. This includes interactions between cells, ligand-mediated signalling, as well as attack by pathogens and their toxins. However, besides protein-mediated interactions, the last decade has highlighted the importance of lipids and in particular lipid rafts in these signalling processes (Simons and Ikonen, 1997). The various phases of lipid bilayers represent physical states that differ in the packing, the degree of order and the mobility of the constituting lipids. The two extreme phases are the quasi-solid gel phase and the fluid liquid-crystalline (l_c) or liquid disordered (l_d) phase. For homogenous bilayers formed by purified phospholipids or sphingolipids, a sharp transition between gel and liquid phases occurs at a characteristic melting temperature. In the l_d membrane phase, lipid acyl chains adopt non-extended conformations due to trans-gauche isomerisations at their C-C bonds (Goot and Harder, 2001). Lipid bilayers in the l_d phase are highly fluid and their lipids have a rotational and lateral mobility. In contrast, in the solid gel phase the hydrocarbon chains are in their all-trans extended conformation. The lipids are then highly ordered, densely packed and therefore have strongly reduced mobility in the plane of the bilayer. In the

presence of cholesterol, lipid bilayers can also adopt a third, intermediate phase, the liquid-ordered phase (l_o). This latter phase is thought to mimic ordered raft like lipid phases in the plasma membrane (Brown and London, 1998). Ordered domains within the membrane are normally associated with saturated lipids, increased amounts of saturated lipids may cause perturbations in these domains and consequently affect such signalling responses. A primary effect of changing the acyl chain saturation of a fatty acid is to change the phase transition of the lipid (Hamilton, 1989). The phase of a lipid bilayer depends not only on factors such as temperature or the presence of cholesterol, but also on the chemical nature of the hydrocarbon chains constituting phospholipid and / or sphingolipids. In particular, natural phospholipids generally contain at their *sn*-2 positions polyunsaturated fatty acids, the kinked structure of which impedes straightening and tight packing of the chains, an example of such a phospholipid is PAPC. Natural phospholipids therefore tend to promote fluid phases. Levels of PAPC required to cause membrane alterations are very unlikely to be encountered in pulmonary surfactant under normal conditions. However, increased concentrations of PC species containing linoleic acid (16:0/18:2 PC, 18:0/18:2 PC, and 18:1/18:2 PC) have been observed in asthmatic patients (Heeley *et al*, 2000). These phospholipid species or in combination with other lipids and / or proteins may have the potential to modulate ROI production in monocytes and macrophages and therefore provides an area of further research.

The analysis of single detergent-resistant membranes has proven to be an invaluable tool to identify raft components and to determine parameters that affect partitioning of proteins and lipids into raft membranes. These rafts appear to serve as platforms for

GPI-anchored proteins such as CD14, some transmembrane proteins (e.g. a Fcγ receptor -CD36), receptors (e.g. FcαR) and doubly acylated protein tyrosine kinases of the Src family (e.g. Lyn, Lck, Fyn, Hck). The first two groups are incorporated into rafts in the endoplasmic reticulum and are transported via the Golgi to the cell surface. Tyrosine kinases and receptor structures (e.g. FcεRI) translocate into rafts during the course of cell activation (Prieschl and Baumruker, 2000). Lipids associated with the receptor coalesce with lipid rafts, where the tyrosine kinase Lyn is located, enabling a stable interaction of both molecules in this microdomain. Thus, protein-lipid and lipid-lipid interactions are the first essential steps in initiating cellular signalling events, but the specificity is still unclear. However, the importance of these interactions is underlined by cholesterol deprivation, a procedure that destroys the raft organisation (Ilangumaran and Hoessli, 1998). Therefore altering the composition of monocyte / macrophage cell membranes with phospholipids such as DPPC leads to a change in its fluidic properties. Such alterations may include perturbation of lipid raft formation and hence the selective partitioning of proteins and receptors such as some Fcγ receptors that are important in binding to opsonised particles and subsequent signalling transduction.

Membrane changes may also affect enzyme systems associated with the membrane, in particular NADPH oxidase the enzyme responsible for the production of ROIs. Surfactant and / or lipid preparations might alter NADPH oxidase activity by either inhibiting signal transduction events, which trigger oxidase assembly, or blocking the assembly steps directly. The NADPH oxidase consists of cytoplasmic and membranous components. Following the addition of stimulus, these components assemble to form a functional enzyme within the plasma membrane. A cell free system

was used in this study to investigate whether DPPC impairs the assembly of the components of the NADPH oxidase. DPPC did not inhibit the oxygen consumption or the production of superoxide by the NADPH oxidase complex when this material was added to a mixture of the components of the NADPH oxidase before addition of sodium dodecyl sulphate (SDS), i.e. before assembly of components into a functional enzyme complex (Figure 1.6). Therefore, DPPC does not inhibit the production of ROIs by inhibiting the assembly of cytoplasmic and membranous components into a functional enzyme in a cell free system. In contrast, Geertsma *et al*, 1993, showed that sheep surfactant inhibited the assembly of the NADPH oxidase by an unknown mechanism. A response that may be related to the different components within sheep surfactant including surfactant proteins or the extraction procedure for surfactant which has previously been discussed and may be responsible for changes in surfactant protein structure and regulatory properties.

4.5   DPPC REDUCES PKC ACTIVITY, A MECHANISM FOR SURFACTANT SUPPRESSION OF ROI

The failure of DPPC to suppress ROI production in a cell free system containing NADPH oxidase, demonstrates that DPPC did not directly interfere with activated NADPH oxidase but may have instead inhibited an event preceding oxidase activation. Chao *et al*, 1995, demonstrated by immunoblotting that surfactant treatment of intact neutrophils induced a decrease in the association of two cytoplasmic NADPH oxidase components p47^{phox} and p67^{phox}, with the isolated plasma membrane. Thus, DPPC may exert its effect by inhibiting the translocation and binding of cytoplasmic components to the membrane components by attenuating the signal transduction mechanisms involved

in phosphorylating these cytoplasmic components. PMA, an analogue of diacyl glycerol and activator of protein kinase C, is believed to induce translocation of oxidase subunits and oxidase activation through both PKC dependent and independent pathways which ultimately lead to phosphorylation of multiple serine residues on p47^{phox} (Nishizuka, 1988). In many cells, activation of PKC leads to activation of mitogen activated protein kinases (MAPK) (Chow *et al*, 1995; Han *et al*, 1994). The yeast cell wall extract zymosan, is particulate and activates the respiratory burst via receptor-mediated interactions. It has been reported that in rat alveolar macrophages, opsonised zymosan stimulates tyrosine phosphorylation and activation of extracellular regulated kinases (ERK) and p38 MAPK pathways (Torres and Forman, 1999). The present study demonstrates that MM6 cells can respond to an activating stimulus with a greater capacity for ROI production if they are first 'primed' with LPS, by a mechanism that is not fully elucidated. It has been suggested that the 'priming' process may involve both p44 / p42 and p38 MAPKs (Yaffe *et al*, 1999). The MAPK have also been shown to be transiently activated by extracellular stimuli. Using Western blotting and immunostaining of proteins, our data demonstrates that opsonised zymosan and PMA activate a number of MAPK in MM6 cells, in a dose and time dependent manner. Using the optimal conditions established in this study for Western blotting and subsequent MAPK activity, the effects of DPPC on these important intracellular kinases were determined.

The levels of phosphorylated MAPK protein in MM6 cells were assessed by Western blotting coupled with immunostaining and chemiluminescent detection. Electrophoretic techniques have traditionally been used to separate individual protein components in

biological fluids, based on size and charge of the proteins. The electrophoretic methods used in this study utilised sodium dodecyl sulphate (SDS), which masks the native charges of a protein with its own negative charge, thus allowing separation based on size. In addition, samples in this study were also treated with a reducing agent in order to cleave disulphide bonds and allow protein unfolding. These proteins no longer possess their native shape and charge and are referred to as denatured. Polyacrylamide has been the most popular and superior matrix to perform protein separation. However, the sole separation of proteins within a gel matrix does not allow for sufficient characterisation of phosphorylated MAPK proteins. Immobilisation of proteins of interest on a suitable membrane is required for protein immunostaining. A technique termed Western blotting (or immunoblotting) is the most prevalent immunochemical method for the detection of proteins electrically transferred from a polyacrylamide gel onto a porous nitrocellulose membrane (Towbin *et al*, 1979). Nitrocellulose membranes provide a suitable support for immunochemical characterisation as opposed to nylon membranes, which can be somewhat harder to work with. Binding of proteins to nitrocellulose is predominantly mediated by hydrophobic interactions and electrostatic forces (Schneider, 1980).

Following electroblotting the phosphorylated MAPK proteins immobilised on nitrocellulose were detected by the reaction with a specific monoclonal antibody followed by a secondary antibody that was conjugated to horse-radish peroxidase. The antibody - antigen interactions were then visualised by enhanced chemiluminescence and detected photographically. Comparison of various Western blot detection systems have shown that chemiluminescent detection may be ten times more sensitive than

standard colorimetric assays (Cunningham, 1992). The disadvantages of Western blotting include the requirement of expensive reagents, materials and apparatus used for electrophoresis, blotting, and immunodetection.

An interesting finding of this study was that DPPC uptake and membrane changes did not appear to affect the major signalling pathways associated with the MAPKs, p44 (ERK 1) / p42 (ERK 2). The data in this study demonstrates that DPPC did not alter the expression of these doubly phosphorylated MAPKs in MM6 cells 'primed' with LPS and stimulated with PMA or OpZ. This effect was also seen with p38 MAPK. However, the detection of phosphorylated p38 MAPK in MM6 cells was hindered by the basal level of p38 phosphorylation. MM6 cells exhibited high levels of constitutive p38 MAPK phosphorylation. These levels were lowered slightly by modifying the incubation conditions of MM6 cells. For example, lowering the percentage of serum in the culture media improved the basal levels of phosphorylated p38 MAPK. This data suggests that DPPC inhibition of ROI generation is not related to such MAPK signalling events.

The intracellular killing of bacteria by phagocytes is regulated by both stimulatory and inhibitory intracellular signals. The pathway including calcium and phospholipid-dependent activation of PKC is generally considered inhibitory and the pathway including cAMP-dependent activation of PKA (PKA) inhibitory for the regulation of antibacterial phagocytes. Since PMA is a direct agonist of conventional and novel isoforms of PKC (Newton, 1995) and that DPPC suppresses PMA induced ROI responses, the PKC pathway may be a possible target for the action of DPPC. The

inhibitory effects of pulmonary surfactant on ROI production have previously been attributed to stimulation of protein kinase A (PKA) and inhibition of PKC (Geertsma *et al*, 1994). This study did not investigate the effects of surfactant lipids directly on PKC activity. Further conclusions were based on surfactant attenuation of PMA-mediated translocation of PKC. Our study investigated both opsonised zymosan and PMA stimulated responses.

The results described in this study in human monocytes suggest that the PKC pathway is involved in PMA and opsonised zymosan stimulation. This study demonstrated that PKC activity is transiently increased in a time dependent manner in response to these agonists. The activity of PKC is maximal at 30 minutes in OpZ stimulated cells as opposed to 10 minutes in PMA stimulated monocytes. The difference in peak times may be a reflection in the difference of receptor mediated and soluble responses. This data is supported by the LCL results presented in this study, in that the time to peak LCL production was approximately 40 minutes longer than the PMA response that peaked at 10-15 minutes following PMA addition to the system. Further, previous studies within the department have shown that in monocytes, oxidase activation involves PKC activity as PMA or zymosan induced chemiluminescence was inhibited by Bisindolylmaleimide I (GF-109203X), Bis-tyrphostin, Genistein, Gö 6976 and Rottlerin (data not shown).

The present study demonstrated that tPC and DPPC but not PAPC inhibited PKC activity in LPS 'primed' and opsonised zymosan stimulated cells. In addition tPC and DPPC inhibited PKC activity in PMA stimulated cells. However, PAPC increased the

activity of this kinase when stimulated with PMA. Results of this study further suggest an additional specific effect of different PC species on ROI production as DPPC inhibited PKC activity more strongly than tPC while PAPC was shown to increase PKC activity. These results demonstrate that the PKC pathway is a target of DPPC in human monocytes. The mechanism by which DPPC interacts with PKC is unclear. A major proportion of PKC activity is constitutively associated with the plasma membrane in rat alveolar macrophages (Peters-Golden *et al*, 1991). Membrane perturbation by surfactant phospholipid would therefore be an attractive mechanism for the suppression of inflammatory responses to LPS and opsonised zymosan. Such a notion is supported by a recent study, that demonstrated surfactant modulates cAMP accumulation in monocytes through a membrane-controlled mechanism (Pinot *et al*, 2000). The suppression of PKC activity may explain why nuclear factor kappa beta (NF-κB) was suppressed by Survanta® and Exosurf Neonatal™ in a study by (Antal *et al*, 1996). NF-κB plays a pivotal role in the transcription of genes involved in inflammation. However, incubation of freshly derived monocytes with LPS and Curosurf® resulted in nearly identical activation of NF-κB, when compared to monocytes exposed to LPS alone. Therefore the clearly suppressive effects of Curosurf® on transcription and protein synthesis seems to be regulated by other, yet undefined signalling pathways. However, the individual lipids may play a role in regulation of NF-κB activity.

4.6   GENERAL CONCLUSIONS

In conclusion, this study has shown that surfactant inhibits the production of ROIs in monocytes. Specifically, the results of this study suggest that particular phospholipids found in surfactant may modulate the production of ROIs by monocytes and macrophages. In particular, DPPC, the major surfactant phospholipid inhibits the production of ROIs and the release of TNF-α in monocytes and macrophages. However, DPPC failed to modulate nitric oxide production and release of IL-1β, 6 and 10 demonstrating the selective nature of the effect. The effects of DPPC where not due to decreased LPS binding or to reduced expression of receptors important in the recognition and phagocytosis of bacteria.

This study showed that DPPC was taken up by monocytes in a dose dependent manner and uptake results in the formation of lamellar bodies, the intracellular storage granule of surfactant. This is similar to normal surfactant homeostasis associated with the alveolar macrophage. Electron microscopy studies also indicated that DPPC affects the membrane ultrastructure of monocytes. Further investigations, studying the effect of lipids on membrane fluidity and the consequence of lipid incubations on phase transitions of lipids within the membrane are required. Such studies may lead us to equate particular changes in membrane characteristics on the ability of a cell to mount an inflammatory response.

This study demonstrated that DPPC did not interfere with the assembly of the NADPH oxidase, the membrane associated enzyme responsible for the production of ROIs. In addition, MAPK signalling pathways associated with NADPH oxidase activation are

not modulated by DPPC. However, this study has provided evidence that the activity of protein kinase C, an important intracellular signalling enzyme is inhibited by PC and DPPC whilst PKC activity was increased by preincubation with PAPC. How surfactant phospholipids modulate PKC activity warrants further investigation. Taken together these findings indicate that surfactant, and the surfactant lipid DPPC suppresses monocyte NADPH oxidase activation by down-regulation of PKC. Surfactant phospholipids may therefore act as an immunomodulator for leukocyte inflammatory responses in the lungs.

The present study suggests that many different facets of surfactant function in the airways and the alveolus need to be considered in the interpretation of pathological mechanisms of lung diseases. Thus, there are a number of considerations for the design of therapeutic surfactants in lung diseases. For example, in addition to their physical effects on lung function, surfactant phospholipids especially PC and PG are also potent regulators of inflammatory cell functions in the lungs. These phospholipids significantly inhibit the production of ROIs in response to zymosan stimulation. Quantitative and / or local deficiencies in PC and PG such as those seen in ARDS (Nakos *et al*, 1998) may facilitate lung destruction and loss of function since the attenuation of ROI production will have been removed. Inflammatory cells such as neutrophils migrate out of the circulation and accumulate in large numbers in the lung during episodes of ARDS. These neutrophils will exist and respond within a surfactant-rich environment in which the release ROIs, that have potential to damage lung tissue, may be modulated in such an environment. The observation that lung surfactant, particularly DPPC can markedly attenuate monocyte respiratory burst activity through

inhibition of PKC suggests additional rationale for the administration of surfactant to patients with these types of diseases. Further the results of the study suggest that new therapies aimed at attenuating PKC activity may also lead to reduced ROI production in monocytes and macrophages. Pneumonia involves similar pathophysiology and abnormalities as described in patients with ARDS. Although technically not bacterial, lung infection from *Mycoplasma pneumoniae* also results in abnormal composition of surfactant but is characterised by increases in phospholipids with increased palmitic acid fractions of PC (Somerson *et al*, 1971).

Exogenous surfactant replacement therapy with formulations based on different lipid compositions may be tailored to specific lung diseases depending on the preferred outcome. For example, individuals with lung disease with an underlying risk of infection may be treated with exogenous surfactant therapy based on a lipid formulation that promotes an enhanced bactericidal activity of inflammatory cells within the lung. Such formulations may include PAPC and perhaps SP-A, which have both been shown to enhance phagocytosis and ROI production in monocytes and macrophages. Obviously, there will be a need to carefully balance the pro- and anti-inflammatory functions of these cells such that treatment will not be detrimental to lung stricture and function. Further, any formulation of an active surfactant for treating lung diseases such as ARDS must take into account its overall effect on the inflammatory cell response. Consideration should be given to exploring in more detail the extent of any surfactant dysfunction in asthma and other lung diseases. Further, the influence of both normal and abnormal lung surfactant on the inflammatory reaction characteristic of lung pathology should be investigated further. Such information is essential before any

possible therapeutic intervention with exogenous surfactant is attempted. Understanding the mechanisms by which pulmonary surfactant lipids alter macrophage responses to infectious stimuli will advance our understanding of innate immune responses in the lung and might aid the development of lipid-based agents as therapies for inflammatory lung diseases.

It seems likely that in the near future, a new generation of artificial substitutes will be competing on the market with currently available modified natural surfactants. These new preparations may be based on copies of the surfactant associated hydrophobic proteins, produced by recombinant technology or protein synthesis, reconstituted with selected synthetic lipids such as DPPC. An example of a new surfactant substitute based on recombinant SP-C is the 'KL$_4$' peptide. This artificial peptide is physiologically active when recombined with DPPC, PG and palmitic acid and has been very effective in the treatment for neonatal RDS (Cochrane and Revak, 1999; Cochrane *et al*, 1996). In principle, any lung disorder interfering with surfactant function could become a potential target for surfactant therapy. Experimental data from this study suggests the potential role of different lipids and phospholipids in modulating immune responses within the lung. If surfactant could be safely and effectively administered preferably as an aerosol, worldwide cases of pneumonia, asthma, chronic bronchitis with or without emphysema would constitute huge numbers of potential recipients.

Finally, surfactant liposomes may also prove to be an important method for the dispersion of antibiotics and other drugs in the airways. The possible use of liposomes as a vehicle to facilitate gene therapy for cystic fibrosis or familiar SP-B deficiency

offers an attractive mechanism to treat the lung disorders. The full potential of surfactant therapy for various forms of lung diseases thus remains to be explored, and the development of synthetic substitutes is still an important challenge for future research.

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Dipalmitoylphosphatidylcholine modulates inflammatory functions of monocytic cells independently of mitogen activated protein kinases

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SUMMARY

Phosphatidylcholine (PC) is the major phospholipid of pulmonary surfactant and it is hypothesized that PC and its subspecies modulate the functions of alveolar macrophages. The most abundant of these subspecies is dipalmitoylphosphatidylcholine (DPPC). This study was undertaken to determine the effect of PC on monocyte function using a human monocytic cell line, MonoMac-6 (MM6). This study showed that preincubation of MM6 cells with DPPC at 125 μg/ml for 2 h inhibited the oxidative response to either zymosan or phorbol-12-myristate-13-acetate (PMA) by 30% ($P < 0.001$). This inhibition with DPPC was independent of LPS priming. When DPPC was replaced with 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC) there was no inhibition and in contrast a significant increase in oxidant production was observed. We also demonstrated that total PC (tPC; a heterogeneous species of PC from egg) and DPPC but not PAPC significantly inhibited the release of TNF-α from MM6 cells ($P < 0.05$). DPPC did not inhibit phosphorylation of the mitogen activated protein kinases (MAPKs) p44/p42 or p38 in stimulated cells. Measurements of membrane fluidity with spin label EPR spectroscopy indicate that DPPC incorporation significantly alters the membrane fluidity of MM6 cells. These results suggest that DPPC, the major component of pulmonary surfactant, may play a role in modulating leucocyte inflammatory responses in the lung. This may in part be related to membrane effects but does not include alterations in p44/p42 or p38 MAPK signalling.

Keywords phospholipids monocyte respiratory burst signal transduction mitogen activated protein kinases (MAPK)

INTRODUCTION

The thin epithelial barrier, which comprises the alveolar surface for gaseous exchange, is uniquely vulnerable to damage related to inflammatory changes. These may result from particulate insults, oxidant gases or damage secondary to infection. The immune response within this region of the lung must carefully balance pro- and anti-inflammatory responses without compromising host defences. It is well established that surfactant plays a major role in preventing lung collapse at the end of expiration, however, it has been suggested that the lipid component within surfactant may have a role in the regulation of lymphocyte function [1]. There is evidence indicating pulmonary surfactant lipids and surfactant proteins (SP-A, B, C and D) alter the bactericidal activity of the alveolar macrophage [2–4]. Recent reports suggest surfactant proteins (SP-A and SP-D) regulate a variety of immune cell functions *in vitro* including enhanced chemotaxis and phagocytosis

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and alterations in the production of free radicals and cytokines [5,6].

Phospholipids are the major component of surfactant, accounting for approximately 90% composition by weight [7]. The most abundant phospholipid in pulmonary surfactant is phosphatidylcholine (PC) comprising approximately 70% (by weight) of the total phospholipids which is mainly in the form of a diacyl species dipalmitoylphosphatidylcholine (DPPC) [8]. The immunomodulatory role of DPPC within the lung is not fully elucidated but a number of studies have indicated its importance with respect to inflammatory cell function as opposed to its primary role in reducing surface tension [9,10]. An early study by Juers, provided evidence that alveolar-lining material could enhance bactericidal killing and this was specifically attributed to its lipid content [11]. Subsequent studies have provided conflicting reports on the bactericidal modulating activity of surfactant and surfactant components on phagocytic cells, possibly due to differences in experimental designs [9,12]. Phagocyte oxidative responses as well as production of other inflammatory mediators are altered in ARDS and may be related

to changes in surfactant or surfactant phospholipid composition [13].

The inhibitory effect of pulmonary surfactant on ROI generation in neutrophils and monocytes has been attributed to alterations in NADPH assembly [10,14,15]. It has also been shown that mitogen activated protein kinases (MAPK) may participate in the regulation of oxidant production by phosphorylation of p47^{phox}, a component of the NADPH oxidase [16,17]. The mechanisms of respiratory burst inhibition by surfactant lipids have not been investigated in terms of signalling pathways. Three MAPK signal pathways have been identified in mammals including extracellular signal-regulated kinases (ERK) p44/p42, c-Jun NH2-terminal kinase and p38 MAPK [18–20]. Activation of p44/p42 and p38 MAPKs have shown to be involved in respiratory burst activation by opsonized zymosan [21].

Therefore this study examines the possible regulatory effects of PC (including total tPC, a heterogeneous sample of PC subspecies in which DPPC accounts for approximately 33%) and its subspecies on the respiratory burst and tumour necrosis factor alpha (TNF-α) release in the human monocytic cell line MonoMac6 (MM6). In addition this study seeks to identify the regulatory properties that DPPC may have on ERK p44/p42 or p38 MAPK activity.

MATERIALS AND METHODS

Materials

L-α-phosphatidylcholine (TPC; type XVI-E from egg yolk), l-α-phosphatidylethanolamine, l-α-phosphatidylcholine dipalmitoyl (DPPC), l-α-phosphatidylcholine β-arachidonoyl-γ-palmitoyl (PAPC), sphingomeylin, lyso-phosphatidylcholine, lipopolysaccharide (*Escherichia coli* O111:B4), phenylmethylsulphonyl fluoride, sodium orthovanadate, deoxycholate and tergitol NP-40 were purchased from Sigma Chemical Co. (Dorset, UK). chloroform, methanol and ammonium hydroxide were purchased from BDH (Dorset, UK). silica gel column was obtained from Jones Chromatography (Hengoed, UK)

Cell culture

The human monocytic cell line MonoMac-6 (MM6) was obtained from the German collection of microorganisms and cell cultures (DSM; Braunschweig, Germany). MM6 cells were maintained in RPMI 1640 medium without L-glutamine (Sigma, UK). RPMI was supplemented with 1% bovine insulin, 10% heat inactivated foetal bovine serum (FBS), 1% 2 mM L-glutamine, 1% nonessential amino acids, 1% penicillin (50 IU/ml)/streptomycin (100 μg/ml) and 1% sodium pyruvate (purchased from Gibco, Paisley, UK) at 37°C in 5% CO_2 humidified atmosphere. Cells were subcultured every 3 days at a density of 0·4 × 10^6 cells/ml. Unless otherwise stated, prior to experimentation MM6 were 'weaned' onto a serum free medium, Ultraculture (supplemented as for RPMI 1640 media without FBS) as indicated by the manufacturer BioWhittaker UK Ltd, Wokingham, UK.

Isolation of human monocytes from peripheral blood was performed by density centrifugation over Ficoll Paque® and negative selection of monocytes using midiMACS (Miltenyi Biotec, Camberley, U.K.). Briefly, EDTA treated whole blood from healthy donors was diluted with 2–4 volumes of PBS. The diluted cell suspension was layered over Ficoll Paque® (1·077 density) and centrifuged at 400 × g for 30–40 min at 20°C. The interface cells (lymphocytes, monocytes and thrombocytes) were

carefully aspirated, washed and resuspended in PBS containing 2 mM EDTA. The cells were indirectly magnetically labelled with a cocktail of hapten-conjugated CD3, CD7, CD19, CD45RA, CD56 and anti-IgE antibodies and MACS MicroBeads coupled to an antihapten monoclonal antibody. The magnetically labelled T-cells, NK cells, B cells, dendritic cells and basophils were retained on a MACS column in the magnetic field of the midiMACS. The isolated monocytes were resuspended at 1 × 10^6 cells/ml in supplemented RPMI medium and used immediately.

Viability of cells was assessed by trypan blue dye exclusion and the CellTiter AQ$_{ueous}$-one solution proliferation assay (Promega, Southampton, UK). The viability of MM6 cells exposed to LPS and/or lipid was greater than 90% in all experiments.

Preparation of lipid media

The physiological concentrations of PC within the lung have been previously estimated to be between 100 and 350 μg/ml [2,22]. The concentrations of phospholipids utilized were approximately those encountered by alveolar macrophages within the lung. The desired amounts of lipids dissolved in chloroform ($CHCl_3$) were dried as a thin film under nitrogen on ice in acid washed bijoux tubes treated with RepelCote (BDH, Dorset, UK) and stored in the dark at $-70°C$ until required. Lipid preparations were hydrated in supplemented medium and sonicated on ice to minimize potential oxidation and breakdown of the lipid.

Preparation of stimulants

An opsonized zymosan (OpZ) suspension was prepared according to a method as previously reported by Allen, 1986 [23]. OpZ suspensions (2·5 mg/ml) were prepared in advance and stored frozen at $-70°C$. Phorbol 12-myristate 13-acetate (PMA; Sigma, UK) was dissolved in DMSO at a stock concentration of 1 mg/ml and diluted in RPMI 1640 to give a final working concentration of 100 ng/ml

Chemiluminescence

MM6 cells prepared at a density of 1 × 10^6/ml were preincubated with tPC, DPPC or PAPC at concentrations of 500, 250, 125, 63 and 2 μg/ml for 2 h at 37°C in 5% CO_2 atmosphere. Cells were washed in PBS (×3) and resuspended in ultraculture, primed for 18 h with LPS (100 ng/ml). Luminol enhanced chemiluminescence (LCL) according to the method of Allen, 1986 [23] with minor modifications was used to quantify the release of ROIs. LCL was detected at room temperature using a standard luminometer. Experiments were performed in quadruplicate and each experiment was repeated three times. Briefly, following treatment, the cells were washed twice with phosphate buffered saline (PBS) and resuspended in standard buffer (4·58 mM KH_2PO_4, 8·03 mM Na_2HPO_4, 0·76% NaCl, 0·033% KCl 0·033, 0·1% glucose, 0·1% endotoxin free albumin 0·1, 0·5 mM $MgCl_2$, and 0·45 mM $CaCl_2$, pH 7·3) at 2·0 × 10^6 cells/ml. To every ml of cell suspension 100 ml of a 3·5-mM solution of luminol (Sigma, UK) was added. Following gentle mixing and a 10 minute adaptation period in the dark, 150 μl cell/luminol suspension were transferred to a 96 well plate (FluoroNunc-PolySorb, Gibco, UK). LCL was initiated by the addition of OpZ (125 μg/ml) or PMA (100 ng/ml). Temporal traces of evolving chemiluminescent reactions were recorded every 5 min for 60 min or until it demonstrated a definite decline. There was no appreciable light emission in this system in the absence of cells or stimulants. LCL results are expressed as

relative light units (RLUs) or:

$$\% \text{ of control} = \frac{\text{Peak LCL response of experimental group}}{\text{Peak LCL response of control group}} \times 100$$

Time course study of PC effects on LCL

In order to determine the effects of a period of incubation on productions of ROIs, preincubation of cells with lipids was performed using the optimal dose of lipid as determined by the previous experiment. MM6 cells were preincubated with the PC species for 0–18 h at 37°C in a 5% CO_2 atmosphere.

Effect of DPPC on ROI production by human peripheral blood monocytes

To extend the effects seen with DPPC on a continuous cell line, ROI production was assessed in isolated human peripheral blood monocytes by the oxidation of $2'7'$-dichlorofluoroscein diacetate (DCFH-DA) to the highly fluorescent $2'7'$-dichlorofluoroscein (DCF) by ROIs. ROI production was measured using FACS analysis as basal levels of isolated monocytes were elevated using the luminol chemiluminescent technique. Briefly, isolated human peripheral blood monocytes (1×10^6/ml) were preincubated with DPPC (125 μg/ml) for 2 h at 37°C in 5% CO_2 atmosphere. Cells were washed in PBS ($\times 3$) and resuspended in supplemented RPMI media at (2×10^6/ml). DCFH-DA (100 μM) was preincubated with the cells for 15 min at 37°C followed by stimulation with PMA (100 ng/ml) for 15 min at 37°C. Fluorescence (FL1) from single cells were collected using a logarithmic amplifier after gating on the combination of forward light scatter and perpendicular light scatter. A total of 10 000 cells were analysed per tube and data was acquired and analysed using Cellquest (Becton Dickinson, U.K.). The fluorescence distribution was analysed and displayed as a single histogram. Controls were monocytes incubated without lipids and/or DCFH-DA or PMA.

Control experiments

Two control experiments were carried out. The first to determine whether any observed oxidative response was independent of priming with LPS (100 ng/ml). MM6 cells prepared at a density of 1×10^6/ml were primed as above followed by washing in PBS ($\times 3$) and incubation with DPPC (125 μg/ml) for 2 h at 37°C in 5% CO_2 atmosphere. Cells were washed and resuspended in standard buffer and chemiluminescence assays performed. The second experiment was to determine whether phospholipids interfered directly with the chemiluminescence assay. MM6 cells prepared at a density of 1×10^6/ml were primed as described above, followed by washing in PBS ($\times 3$). Cells were resuspended in standard buffer containing appropriate lipids and chemiluminescent assays were performed immediately.

Measurement of TNF-α release from MM6

MM6 cells prepared at a concentration of 1×10^6/ml, were preincubated with the same concentrations of PC subspecies and at the optimal preincubation time previously used. Following lipid preincubation, MM6 cells were stimulated with LPS (200 ng/ml) and PMA (100 ng/ml) for 4 h at 37°C in 5% CO_2 atmosphere. Previous experiments have shown that PMA alone at this concentration does not stimulate TNF-α production from MM6 cells [24]. TNF-α in MM6 culture supernatants were quantified by a commercially available ELISA kit (R & D, Minneapolis, USA) according to the manufacturer's instruction.

Quantification of uptake of phospholipids into MM6 membrane by HPLC

MM6 cells prepared at a density of 1×10^6/ml were pretreated with 250 μg/ml DPPC for 2 h at 37°C in a 5% CO_2 atmosphere. Cells were washed in PBS ($\times 3$) and membrane phospholipids were extracted using a modification to the method of Bligh and Dyer, 1959 [25]. The separation and quantification of membrane phospholipids was determined by HPLC on a silica gel column with a mobile phase consisting of chloroform, methanol and ammonium hydroxide. The uptake of DPPC into MM6 membranes was detected by a light scattering evaporating detector as described previously [26]. A standard phospholipid mixture containing DPPC, PC, sphingomyelin and phosphatidylethanolamine over a range of 5–200 μg/ml were used to obtain a standard curve. This was calculated by regressing the peak areas to obtain the best-fit quadratic line. The quantity of DPPC in MM6 cells after treatment was compared to the mean obtained from untreated cells and reported as a mean percentage increase by weight.

Measurement of membrane fluidity by EPR spectroscopy

MM6 cells at a density of 1×10^6/ml were preincubated with 125 or 250 μg/ml of DPPC for 2 h. After washing ($\times 3$) in PBS, the cells were resuspended in PBS at 5×10^6 cells/ml to which 10 μl of 20 mg/ml doxyl stearate in ethanol was added. The concentration of ethanol in the final cell suspension was less than 1%. The cells were incubated for 15 min at room temperature followed by washing in PBS ($\times 3$) to remove free spin label. Cells were resuspended in 100 ml of PBS and membrane fluidity was measured by electron paramagnetic resonance (EPR) spectroscopy as previously described by Darmani, 1993 [27]. The order parameter (S) was calculated from the relationship, $S = (A// - A\perp)/25G$ where $A//$ and $A\perp$ are the maximal and minimal coupling constants, respectively, obtained from the EPR spectra of the spin labelled cells [27].

Western blot analysis of p44/p42 or p38 MAP kinase

The phosphorylated forms of the P44 (ERK1)/P42 (ERK 2) or P38 MAPK proteins were analysed by a commercially available kit (phosphoplus P44/P42 MAPK or phospho P38 Antibody kit; New England Biolabs UK Ltd, Hertfordshire, UK). Analysis of these MAPKS was performed according to the manufacturer's instructions. MM6 (1×10^6 cells/ml) were preincubated with or without 500, 100 or 10 μg/ml of DPPC for 2 h. Cells were washed in PBS ($\times 3$) and primed with or without LPS (100 ng/ml) for 18 h. Cells were stimulated with 100 ng/ml of PMA for 10 min or OpZ for 60 min. These times were determined as a result of experiments to ascertain the optimal conditions for MAPK expression (data not shown). Following stimulation with PMA or OpZ the cells were washed twice in ice cold PBS and lysed in lysing buffer (containing 100 μl of 100 mM NaCl, 10 mM tris-HCL (pH 7·2), 2 mM EDTA, 0·5% (w/v) deoxycholate, 1% (v/v) NP40, 10 mM MgCl$_2$, 1 mM phenylsulphonyl fluoride and 100 mM sodium orthovanadate) for 15 min on ice, followed by sonication (Soniprep, Sanyo, Watford, UK) for 10s on ice to reduce sample viscosity. Cells were centrifuged at 13 500 × g. For 5 min at 4°C and the lysate supernatant fraction was stored at − 70°C. Protein concentration of the soluble cytosolic extract was estimated using a modified lowry method (Bio-rad, California, USA). Protein concentration

of sample lysate supernatants were adjusted to 5 mg/ml with LDS sample buffer and heated for 10 min at 70°C. Samples were then loaded onto precast 10% SDS PAGE gels (Novex, Groningen, Netherlands) and electrophoresed at 200 V for 50 min. Gels were electroblotted on nitrocellulose membranes (Amersham, UK) and washed in washing buffer (Tris-buffered saline with 0·1% Tween-20). To prevent non-specific protein binding, the membrane was incubated in blocking buffer (Tris-buffered saline, 5% w/v non-fat dry milk and 0·1% Tween-20) for 1 h at room temperature. The appropriate phosphorylated anti-MAPK antibody was incubated with the membrane overnight at 4°C. The membrane was then rinsed with wash buffer (3 × 5 min). Detection of MAPK was achieved by incubating the membrane with horseradish peroxidase (HRP)-conjugated anti-mouse antibody (1 : 2000 dilution) for one hour at room temperature. The membrane was rinsed (3 × 5 min) in wash buffer. Visualization of antibody complex was achieved by enhanced chemiluminescence (ECL) LumiGLO® (1 minute, room temperature) and subsequent exposure on hyperfilm (Amersham, UK). MAPKS were identified by comparison with pre-stained molecular weight markers and MAPK control proteins.

Statistics
For multiple group comparisons, the data were subjected to one way analysis of variance (ANOVA) to determine overall difference between the group means and Tukey's honestly significant difference (HSD) for pairwise differences for within group comparisons. Differences in medians were analysed by Mann–Whitney. Minitab software version 12·0 (Minitab Inc.) was used for all analyses.

RESULTS

Effect of phosphatidylcholine tPC, DPPC and PAPC on luminol enhanced chemiluminescence (LCL)
Control experiments showed that lipid preparations did not interfere with the chemiluminescent assay under these conditions (data not shown). LCL generation peaked at approximately 55 min after stimulating cells with OpZ. PC exerted no significant effect on the time to peak responses when compared to the primed MM6 control. However, the luminescence generated from MM6 pretreated in this manner with tPC, DPPC and PAPC exhibited significantly ($P < 0.0001$) different LCL than untreated MM6 primed with LPS. The modulation of peak light emitted was found to be dose dependent in MM6 preincubated with tPC, DPPC and PAPC (Fig. 1). Further analyses of this data using Tukey's pairwise comparison, demonstrated a significant difference
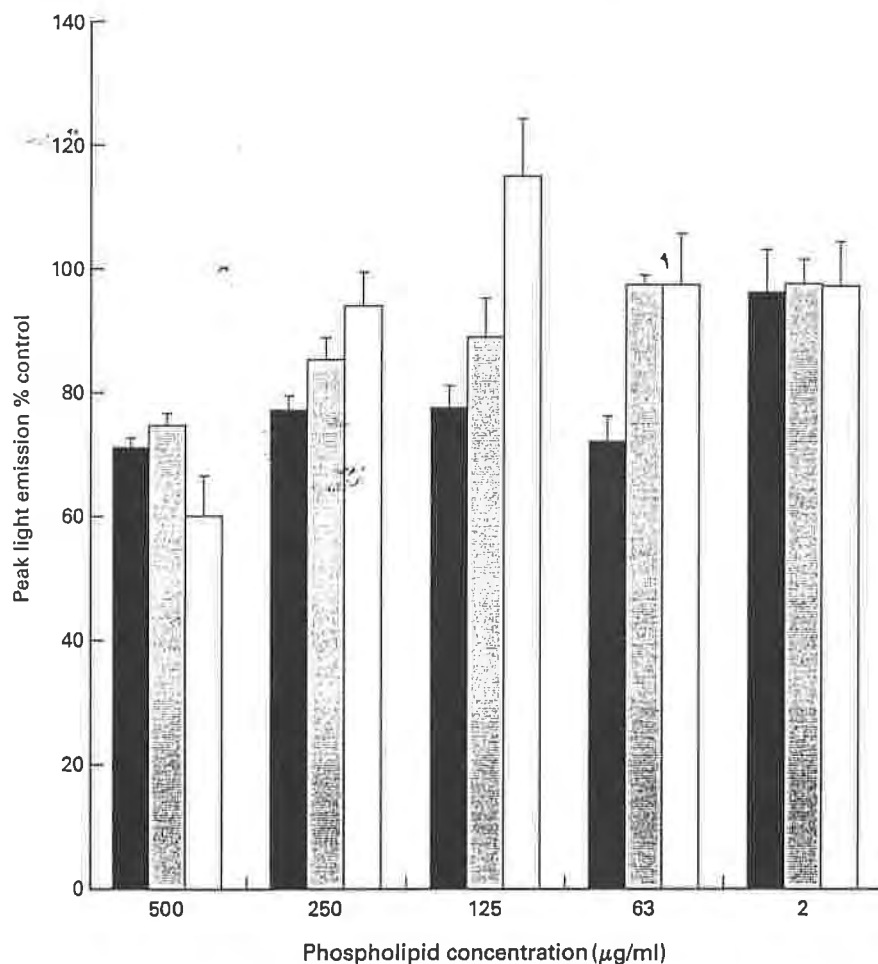

Fig. 1. Dose response of phospholipid treatment on respiratory burst activity of MM6 cells primed with LPS and stimulated with OpZ. Results are expressed as mean (± SD) of 3 separate experiments. Control values (100%) are MM6 cells incubated in the absence of lipid before priming with LPS. ■ DPPC; ▣ tPC; □ PAPC.
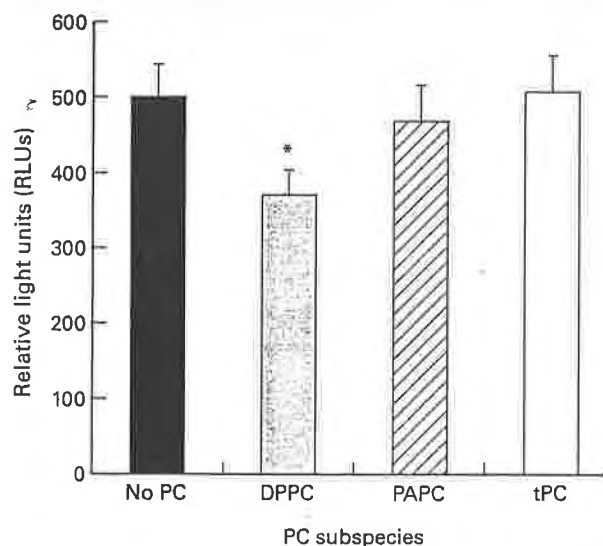
Fig. 2. Effect of phospholipid treatment on respiratory burst activity in MM6 cells primed with LPS and stimulated with PMA. Results are expressed as mean ($\pm$ SD) of 3 separate experiments. Control value represents MM6 cells incubated in the absence of lipid before priming with LPS. All phospholipids were used at a concentration of 125 μg/ml. *$P < 0.001$ analysed by ANOVA.

($P < 0.001$) amongst the means as the concentration of the phospholipid increased to 63, 125 and 250 μg/ml. There were significant differences between tPC and PAPC when compared to DPPC ($P < 0.01$) again when using Tukey's pairwise comparisons. Using PMA as a stimulant, preincubation of each PC subspecies at 125 μg/ml for 2 h gave a significant reduction in the release of ROIs as determined by LCL ($P < 0.0001$ using ANOVA) (Fig. 2). Tukey's indicated that DPPC was responsible for the significant decrease in LCL in this part of the study.

Time course of phosphatidylcholine effects on LCL
Using opsonized zymosan as stimulus (125 μg/ml), peak light emission of MM6 cells treated with tPC and DPPC for 2, 6 and 18 h was significantly inhibited ($P < 0.01$ and $P < 0.001$, respectively) from that of untreated MM6 (Fig. 3) when analysed by Mann–Whitney. However, 125 μg/ml PAPC significantly enhanced LCL ($P < 0.001$) at 30 min, 2, 6 and 18 h again when comparing the medians against control.

Control experiments
Experiments were performed to ascertain if the DPPC was affecting the LPS priming process or the stimulation of the cells with PMA or OpZ. Incubation of MM6 cells with DPPC (125 μg/ml) after they were primed with LPS gave significant inhibition of LCL ($P < 0.005$). This suggests that the DPPC was not affecting the priming process. In addition incorporation of phospholipids into the chemiluminescence buffer had no direct effect on the subsequent LCL from MM6 cells stimulated with PMA or OpZ. This showed that DPPC does not inhibit or quench the LCL once it has been produced.

Effect of DPPC on ROI production in isolated human peripheral blood monocytes
DCF-DA treated human peripheral blood monocytes were

stimulated with PMA to induce ROI production and analysed by FACS for the fluorescent oxidized DCF product. Treatment of these cells with DPPC (125 μg/ml) inhibited the ROI response by approximately 30% ($P < 0.05$ analysed by Mann–Whitney) (data not shown). This is a similar inhibition to that obtained from chemiluminescence assays in which DPPC inhibited the LCL response of the human monocytic cell line MM6.

Secretion of TNF-α in MM6 cells precultured with PC and its subspecies
Total PC and DPPC significantly reduced TNF-α release by MM6 when compared to controls ($P < 0.05$). A significant reduction of TNF-α release was seen after preincubation with DPPC and tPC at a concentration of 125 and 250 μg/ml (Table 1). No significant reduction was seen in PAPC treated cells at any of the concentrations tested.

Uptake of DPPC by MM6 cells and effects on membrane fluidity
HPLC analysis of MM6 cell extracts demonstrated that total membrane DPPC was increased by 30 $\pm$ 2.8% by weight ($n = 3$) after 2 h incubation with 250 μg/ml of DPPC (results not shown). No measurable increase in any other quantified phospholipids was seen. EPR spectroscopy showed that the order parameter (S) increased from 0.655 in untreated cells to 0.783 and 0.787 in cells preincubated for 2 h with 125 and 250 μg/ml DPPC, respectively ($n = 3$). This represents a significant decrease in membrane fluidity (results not shown).

The effect of DPPC on p44 (ERK 1)/p42 (ERK 2) or p38 MAPK activity
The preincubation times shown to affect LCL and TNF-α release were used to study MAPK signalling in DPPC treated cells. There was no observable effect of 10, 100 or 500 μg/ml of DPPC on p44 (ERK 1)/p42 (ERK 2) phosphorylation (Fig. 4) when primed with LPS and stimulated with PMA or OpZ. No observable effect of DPPC on p38 phosphorylation was seen using similar experimental conditions (Fig. 5). Similar results were obtained in cells that were not primed with LPS. Cells not stimulated with PMA or OpZ had little or no activated MAPK.

DISCUSSION

This study provides evidence of the ability of the phospholipid PC to modulate the release of ROIs in a monocytic cell line and in isolated human peripheral blood monocytes. In addition, we have demonstrated that tPC, DPPC, but not PAPC reduce TNF-α release from MM6 cells. The data presented also provides new insights on the mechanisms by which DPPC down regulates inflammatory responses that may be associated with the fatty acid component of PC. Additionally, we have shown that DPPC is incorporated into the cell membrane and causes significant alterations in membrane fluidity.

This study utilized the human monocytic cell line MM6. This is a well characterized cell line which displays many characteristics of mature peripheral blood monocytes and macrophages. Monocytes, the circulating precursors of macrophages play an essential role in maintaining the sterility of tissues by the production of a range of cytokines, including TNF-α, and the release of ROIs and vasoactive lipids. The microenvironment in which monocytes mature into macrophages may be important in determining their functional characteristics. A recent study has shown
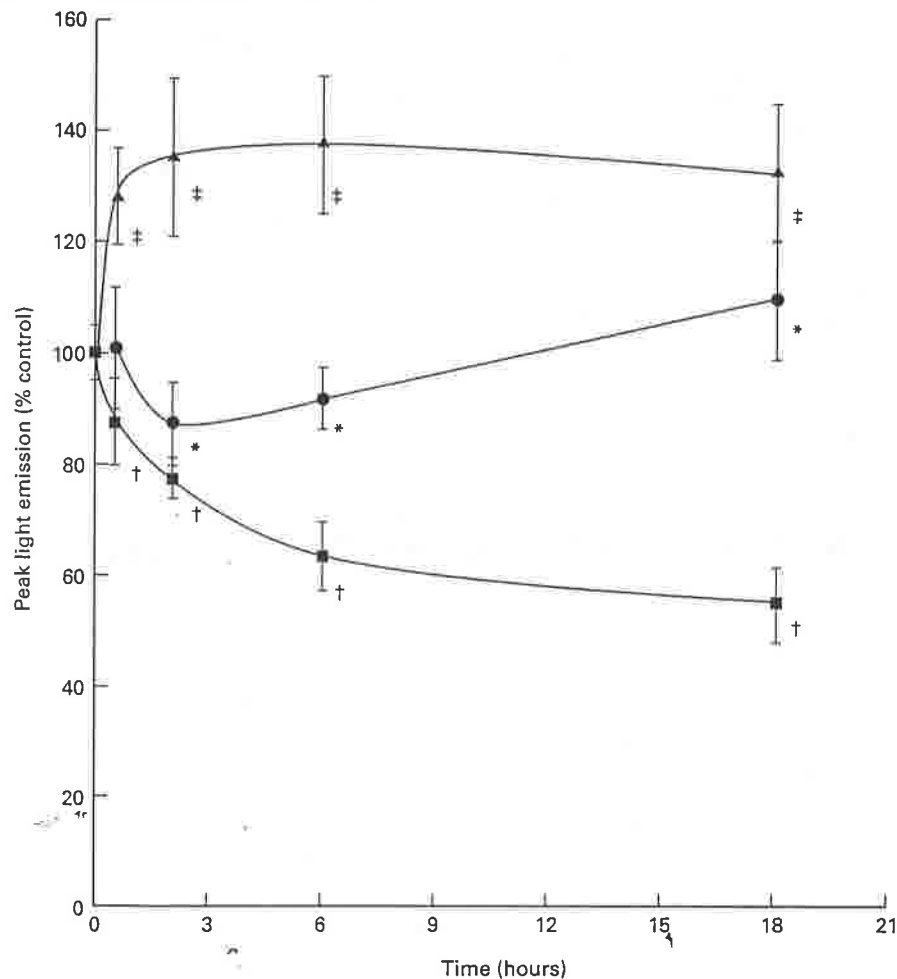
Fig. 3. Time course of phospholipid treatment on respiratory burst activity in MM6 cells primed with LPS and stimulated with OpZ for production of ROIs. Results are expressed as mean (± SD) of 3 separate experiments. Control values (100%) are MM6 cells incubated in the absence of lipid before priming with LPS. ● tPC; ■ DPPC; ▲ PAPC. $*P < 0.01$, $†P < 0.001$, $‡P < 0.001$ analysed by Mann–Whitney.

that alveolar macrophages have a greater capacity for leukotriene synthesis than peripheral blood monocytes. This elevated capacity only occurs after entry into the alveolar space and declines over time when cells are cultured outside this environment [28].

Table 1 Dose dependent suppression of TNF-α release by LPS stimulated MM6 cells exposed to a range of concentrations of PC*

Concentration of lipid (μg / ml)	TNF-α release (pg / ml)		
	TPC	DPPC	PAPC
Control (no lipid)	532 ± 56	527 ± 32	545 ± 64
32	446 ± 51	483 ± 53	—
63	450 ± 103	469 ± 33	548 ± 134
125	395 ± 56†	373 ± 70†	542 ± 77
250	371 ± 58†	382 ± 22†	574 ± 89
500	446 ± 64	363 ± 106	576 ± 108

*Data shown represents mean (± SD) for 3 experiments performed in triplicate.

†$P < 0.05$ by ANOVA with Tukey's multiple comparison method ($n = 3$).

Pulmonary surfactant is a complex mixture of phospholipids, neutral lipids and cholesterol and four genetically distinct surfactant specific proteins [6]. The major surfactant phospholipid is PC of which 90% of the disaturated species within the lung is dipalmitoyl [29]. The concentrations of phospholipids used in this study approximate to the levels known to occur in pulmonary surfactant [2,22].

Activation of the respiratory burst can occur in response to both particulate and soluble agents. The mechanism of signal transduction however, varies according to the type of agent used. The phorbol ester PMA, an analogue of the intracellular messenger diacylglycerol (DAG) activates the respiratory burst through activation of protein kinase C (PKC) [30]. In many cells, activation of PKC leads to activation of MAPK [20,31]. The yeast cell wall extracts zymosan, is a particulate and activates the respiratory burst via receptor–mediated interactions. It has been reported that in rat alveolar macrophages, opsonized zymosan stimulates tyrosine phosphorylation and activation of ERK and p38 MAPK pathways [21]. MM6 cells can respond to an activating stimulus with a greater capacity for ROI production if they are first primed with an agonist (LPS), by a mechanism that is not fully elucidated. It has been suggested that the priming
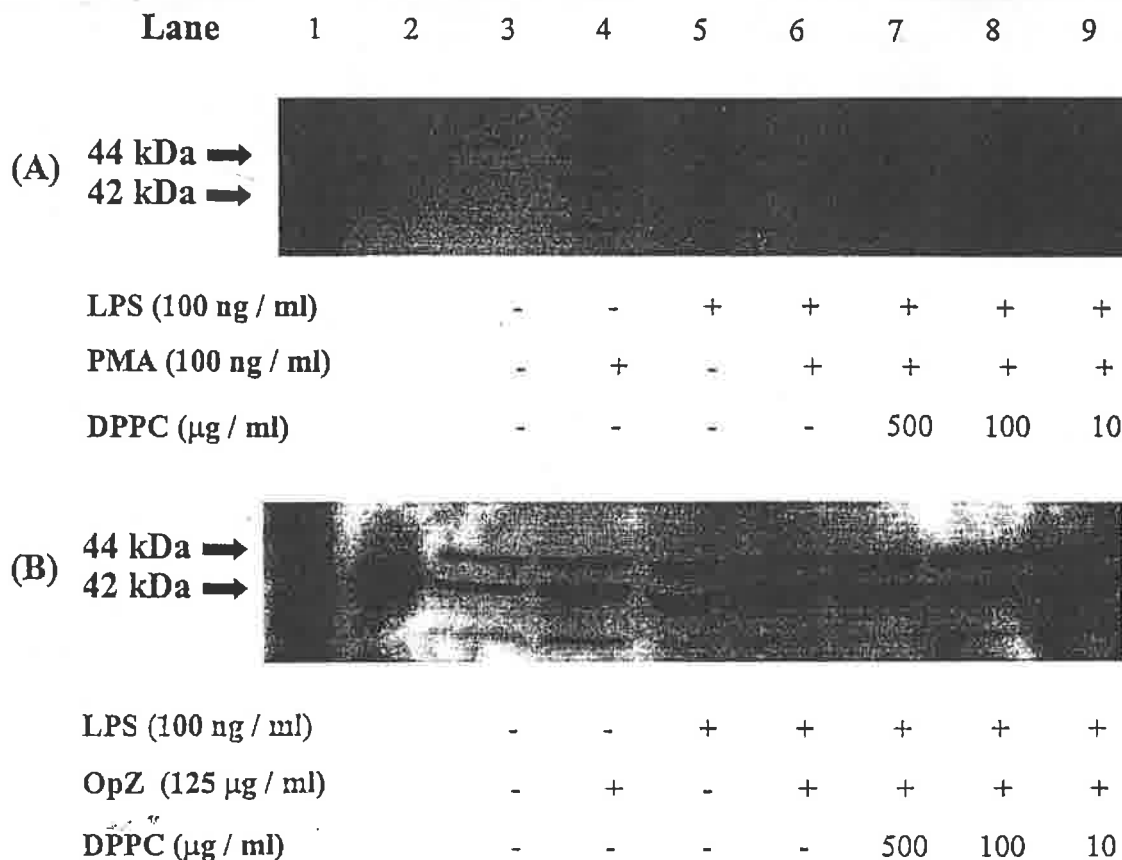
Fig. 4. Immunoblot of p44/p42 MAPkinase activity. A, PMA stimulated MM6 cells; B, OpZ stimulated MM6 cells. Data shown are representative of three independent experiments. Lane 1, molecular weight markers; 2, p42 control protein.

process may involve both p44/p42 and p38 MAPKs [32]. The production of ROIs was impaired in MM6 cells that were exposed to tPC, DPPC and PAPC between a concentration range of 0–500 μg/ml and stimulated to release ROIs with OpZ. These effects were seen within 30 min incubation in the presence of both DPPC and PAPC but only after 2 h incubation in the presence of tPC. In addition PAPC demonstrates an ability to promote ROI production only at a concentration of 125 μg/ml. This was not observed with tPC and DPPC treatment. Using PMA as a stimulant of oxidative activity, we observed that only DPPC is capable of inhibiting the respiratory burst. These experiments were extended to isolated human peripheral blood monocytes where a similar reduction in ROI production by DPPC treated cells was seen. Interestingly, this appears to be independent of cell priming with LPS as significant inhibition was seen in MM6 cells primed with LPS prior to incubation with DPPC. These effects are directly related to the phospholipid interaction with MM6 cells since control experiments performed with the same lipid preparations did not interfere with the chemiluminescence assay itself. Cytotoxicity studies confirmed that the viability of MM6 cells exposed to lipid was greater than 90% at all concentrations of lipids used.

We have demonstrated in this study that tPC and DPPC significantly reduce TNF-α release from MM6 cells after 2 h preincubation with these lipids. However, PAPC was not seen to affect TNF-α release in the same way as DPPC. The maximum reduction in oxidative metabolism and TNF-α release was seen after incubation with DPPC for 2 h in the concentration range of

125–250 μg/ml. Quantification of DPPC by HPLC in MM6 cells indicated that there was an increase of DPPC uptake by MM6 cells following preincubation with this lipid. This increase suggests cellular uptake of this particular phospholipid during incubation. Coupled with this, EPR spectroscopy from spin-labelled MM6 cells showed an increase in order parameter, suggesting a significant change in membrane composition associated with preincubation with DPPC. These membrane changes may be associated with incorporation of saturated phospholipids into the membrane. A primary effect of changing the acyl chain saturation is to change the phase transition of the lipid [33]. Functional consequences of lipid heterogeneity have started to emerge such as the nonrandom mixing of lipids and phospholipids in the membrane bilayer leading to the formation of lipid microdomains [34]. These microdomains are thought to be involved in numerous signalling events associated with the cell membrane [35]. Ordered domains within the membrane are normally associated with saturated lipids, increased amounts of saturated lipids may cause perturbations in these domains and consequently affect such responses. It has been reported that surfactant inhibits the assembly of cytoplasmic and membraneous components of the NADPH oxidase into a functional enzyme [14]. The mechanism of surfactant inhibition of NADPH oxidase assembly is unknown.

An interesting finding was that these membrane changes did not appear to affect the major signalling pathways associated with the MAPKs p44 (ERK 1)/p42 (ERK 2). In this study DPPC did not alter the expression of these doubly phosphorylated MAPKs in
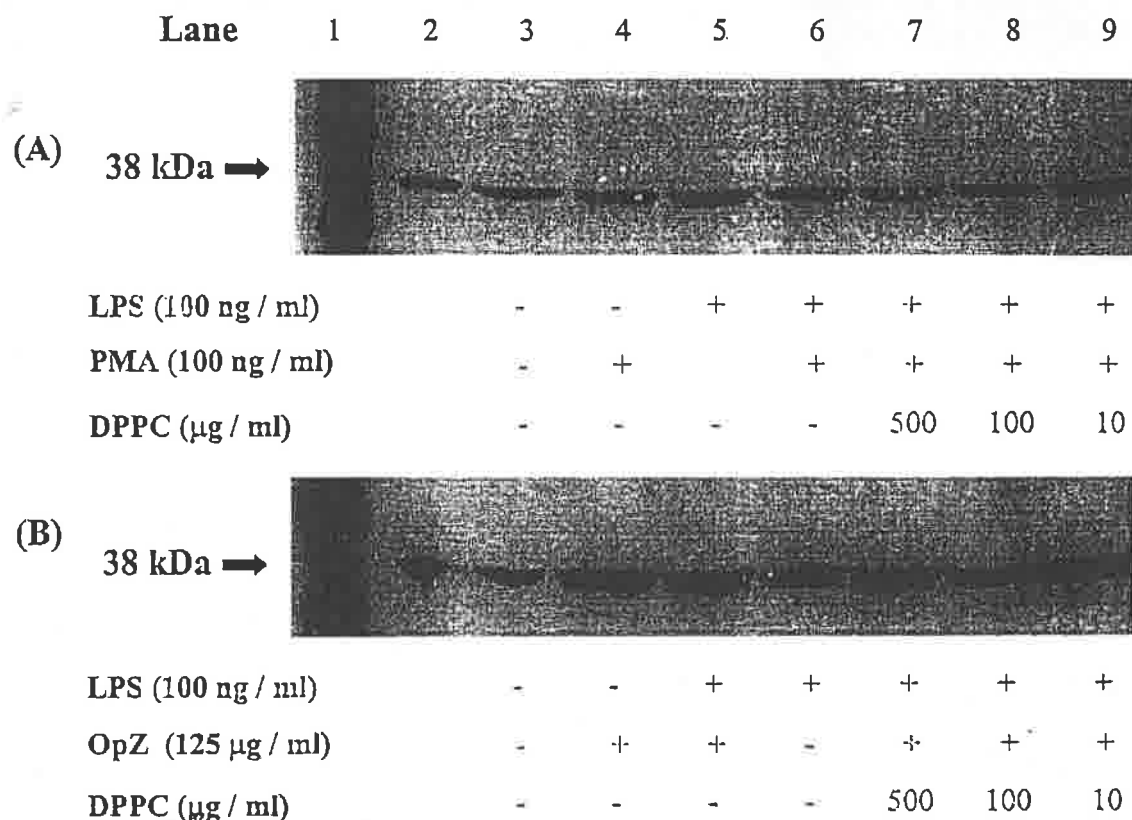
Fig. 5. Immunoblot of p38 MAPkinase activity. A, PMA stimulated MM6 cells; B, OpZ stimulated MM6 cells. Data shown are representative of three independent experiments. Lane 1, molecular weight markers; 2, p38 control protein.

MM6 cells primed with LPS and stimulated with PMA or OpZ. This effect was also seen with p38 MAPK. This suggests that DPPC inhibition of ROI generation and TNF-α release is not related to such signalling events.

Previous studies have used a number of different animal models and methodologies for estimating superoxide and ROI production, this may be responsible for conflicting reports [2,4,36]. We have shown that the dose of phospholipid and time of incubation has a critical effect on oxidative functions, e.g. PAPC has a stimulatory or inhibitory role on the respiratory burst that depends on lipid dose and time of incubation. Webb and Jeska [12] found an increase in the monocyte oxidative response when incubated with alveolar lining material and provided evidence that unsaturated lipids were responsible. The results of our study indicate that the different modulatory properties exerted by the phospholipids used in ROI production and TNF-α release after LPS stimulation appears to be selective. The only difference between DPPC and PAPC is in the esterified fatty acid at the *sn*-2 position of PC, it would appear that these modulations might be associated with this substitution.

Although DPPC reduced ROI production and TNF-α release, it did not inhibit phosphorylation of the MAPKs in MM6 cells stimulated with LPS and OpZ or PMA. However, preliminary membrane fluidity studies indicate that DPPC incorporation significantly altered the membrane of MM6 cells. These membrane alterations may affect the release of TNF-α from the interior of the cell, however, Baur *et al.* [37] have shown that DPPC pretreatment of monocytes results in a decrease in TNF-α mRNA production. Such changes may also affect enzyme systems associated with the membrane in particular NADPH oxidase, the enzyme responsible for the production of ROIs. Further studies are required to determine whether this is indeed the case.

In summary, this study has shown that DPPC, the major phospholipid species in pulmonary surfactant, can down-regulate oxidative functions in monocytes and may therefore act as an immunomodulator for leucocyte inflammatory responses in the lungs. Although observed reductions in release of inflammatory products were not complete, a 20–40% reduction in the release of these mediators would offer a considerable protection against inflammatory damage in the delicate alveolar region of the lung. Indeed, complete inhibition of such responses would be deleterious to the individual as macrophage mediated responses are the central immune defence mechanism in this region of the respiratory tract.

ACKNOWLEDGEMENT

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SHORT COMMUNICATION

PROSTAGLANDIN E₂ AND TUMOUR NECROSIS FACTOR-α RELEASE BY MONOCYTES ARE MODULATED BY PHOSPHOLIPIDS

R. H. K. Morris,[1] A. J. Price,[1] A. Tonks,[1] S. K. Jackson,[2] K. P. Jones[1]

The regulation of pro- and anti-mediator release from cells within the alveolar space would represent a desirable mechanism serving to protect this delicate gas-exchanging region of the lung. This study investigates the effect of alveolar surfactant lipids on the regulation of tumour necrosis factor alpha (TNF-α), a potent inflammatory cytokine, and prostaglandin E_2 (PGE_2), a lipid mediator with anti-inflammatory properties. The results of this investigation reveal a marked effect on the release of these two important mediators from a monocytic cell line, MonoMac 6 (MM6), by phosphatidylcholine (PC), phosphatidylethanolamine (PE), cholesterol (Chol) and sphingomyelin (SM). PC, PE and Chol demonstrated marked downregulation of TNF-α production at lipid concentrations of 125 and 250 μg/ml. Interestingly, SM significantly up regulated the release of TNF-α at these concentrations. However, the release of PGE_2 in MM6 cells incubated with the same lipids was significantly increased with PC and Chol, and significantly decreased in cells pre-treated with SM. This indicates a role for these lipids in alveolar immunoregulation.

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The major role of pulmonary surfactant is to prevent lung collapse at the end of expiration, however the phospholipid component within surfactant may have a role in the regulation of immune function.[1] Surfactant is approximately 90% phospholipid, with neutral lipids and surfactant proteins.[2] The majority of surfactant phospholipid (approximately 70% W/V) is in the form of phosphatidylcholine (PC), with smaller quantities of phosphatidylethanolamine (PE) and sphingomyelin (SM).

During the progression of acute respiratory distress syndrome (ARDS) there is a decrease in the PC content.[3] This has suggested a role for such changes in disease. It has been shown that phospholipids modulate the production of reactive oxygen intermediates in monocytes.[4,5] Synthetic surfactant preparations exert suppressive effects on oxidative function, TNF-α secretion and mRNA production.[6] Alveolar macrophages are less responsive in the production of TNF-α. The effects of surfactant phospholipids on anti-inflammatory mediators such as prostaglandin E_2 (PGE_2) have received little attention.

PGE_2, is a metabolite of arachidonic acid, seen in inflammation, particularly in fever, oedema, pain and the regulation of leukocyte function.[7]

Inflammatory stimuli, including lipopolysaccharide (LPS), are capable of stimulating monocytes/macrophages to synthesise PGE_2. In trauma or sepsis, monocytes and macrophages release PGE_2, which is considered to be a principal mediator of post-traumatic immunosuppression.[8] PGE_2 regulates the synthesis and secretion of inflammatory cytokines, such as TNF-α in monocytes/macrophages and other cell lines.[9]

We have investigated the effect of pre-incubating Monomac-6 cells (MM6), a human monocytic cell line, with synthetic phospholipids PC, PE, SM and the neutral lipid cholesterol on the release of PGE_2 and TNF-α in response to LPS.
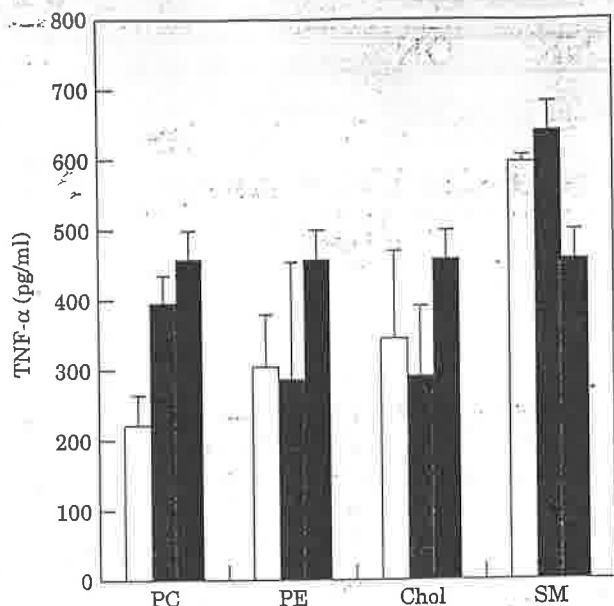
Figure 1. Effect of lipid pre-incubation on the release of TNF-α from MM6 cells.

(☐), 250 μg/ml; (▨), 125 μg/ml; (■), control.

Figure 2. Effect of lipid pre-incubation on the release of PGE2 from MM6 cells.

(☐), 250 μg/ml; (▨),125 μg/ml; (■), control.

RESULTS

Viability

MM6 cell viability was greater than 90% throughout the study and was not affected by lipid pre-incubation or by the addition of interferon gamma, LPS or PMA as determined by Trypan blue exclusion assay and the CellTiter AQ$_{ueous}$ one solution proliferation assay (Promega, UK).

TNF-α release

The release of TNF-α was significantly decreased following the incubation of MM6 cells with PC, PE and cholesterol at 125 and 250 μg/ml ($P<0.01$; $n=6$). Pre-incubation of MM6 cells with SM significantly increased the release of this cytokine ($P<0.001$; $n=6$) (Figure 1).

PGE2 release

The release of PGE2 was significantly increased following pre-incubation with PC and cholesterol at 250 μg/ml ($P<0.01$; $n=6$). Only cholesterol gave a significant increase at 125 μg/ml ($P<0.01$; $n=6$). No significant differences were seen in MM6 pre-treated with PE at either concentration. However, SM at 125 and 250 μg/ml gave a significant decrease in PGE2 release ($P<0.05$; $n=6$) (Figure 2).

DISCUSSION

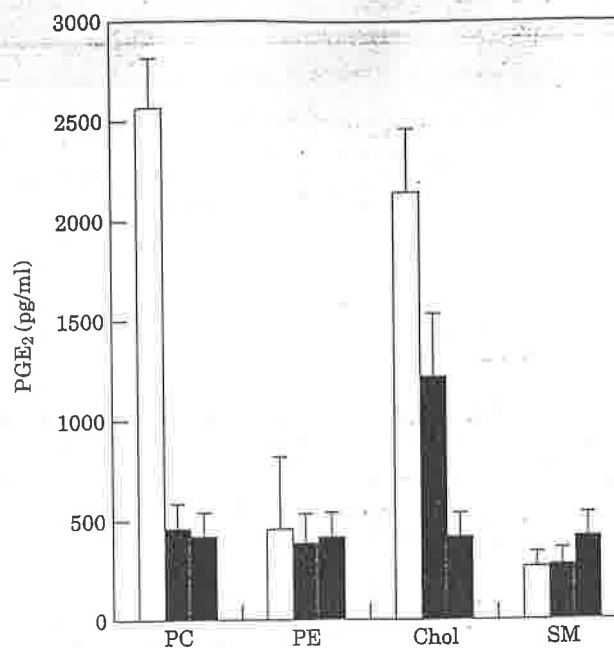The results demonstrate the ability of phospholipids to modulate the secretion of TNF-α and PGE2.

Release of TNF-α in MM6 cells was inhibited by pre-incubation with PC, PE and cholesterol. The phospholipid levels used in this study approximate to those seen in surfactant, where total phospholipids are 325 μg/ml.[10] PGE2 was increased in cells pre-cultured with PC and cholesterol. Conversely, SM both up regulated TNF-α and down regulated PGE2 release.

Within the alveolar space, resident macrophages are constantly bathed in the phospholipid-rich surfactant; regulatory effects are important in protecting these delicate gas exchange regions from inflammatory damage. Surfactant preparations have been shown to exert modulatory effects on lymphocytes.[11] Phagocytosis, as well as the production of inflammatory mediators, is altered in ARDS as is surfactant phospholipid composition.[12] Alveolar lining fluid modulates 5-lipoxygenase metabolism,[13] and natural porcine surfactant downregulates mRNA for TNF-α and TNF-α type II receptors.[14]

A recent report has shown that indomethacin induced inhibition of PGE2 increases TNF-α production in stimulated macrophages.[15] The effects on PGE2 and TNF-α seen in this study suggest a regulatory role for surfactant lipids on cells within the alveolar space.

MATERIALS AND METHODS

Cell Culture

MM6 cells (DSM; Braunschweig, Germany) were maintained in RPMI 1640 medium supplemented with 1% bovine insulin, 10% heat inactivated fetal bovine serum (FBS),

1% 2 mM L-glutamine, 1% non-essential amino acids, 1% penicillin (50 IU/ml)/streptomycin (100 µg/ml) and 1% sodium pyruvate (Gibco, Paisley, Scotland), at 37°C in 5% CO$_2$ humidified atmosphere. Cell viability was assessed by Trypan blue exclusion and CellTiter AQ$_{ueous}$ 96 proliferation assay (Promega, UK).

Preparation of lipid media

Phospholipids were dissolved in chloroform, dried under nitrogen and stored in the dark at −70°C. Phospholipid preparations were hydrated in supplemented medium as described above and sonicated on ice to minimize oxidation.

Stimulation of TNF-α and PGE$_2$ release

MM6 cells prepared at 1×10^6/ml were pre-incubated with PC, PE, SM or cholesterol at 250, 125, 31.5 and 16.25 µg/ml for 2 h at 37°C in 5% CO$_2$. Cells were washed in PBS (×3) and resuspended in RPMI containing interferon gamma (IFN-γ) 100 IU/ml for 12 h. TNF-α release was stimulated by addition of PMA (Phorbol-12-myristate 13-acetate) (50 ng/ml) and LPS (50 ng/ml). PGE$_2$ release was stimulated by the addition of LPS (50 ng/ml) and incubated for 4 h. Culture supernatants were stored at −70°C.

Determination of TNF-α release

TNF-α was quantified by a commercial ELISA (R&D Laboratories, Minneapolis, USA). Procedure was carried out in accordance with manufacturer instructions.

Determination of PGE$_2$ release

PGE$_2$ was measured by a commercial EIA (R&D Laboratories, Minneapolis, USA). Procedure was carried out in accordance with manufacturer instructions.

Statistical analysis

For multiple group comparisons, the data were subjected to one way analysis of variance (ANOVA) to determine the overall difference between the group means. Minitab software version 12.0 (Minitab Inc.) was used for all analyses. Statistical significance was defined as being $P < 0.05$.

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Phospholipid Modulation of Monocyte Oxidative Activity Measured by Luminol-Enhanced Chemiluminescence

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The lung is susceptible to inflammatory and oxidative injury leading to tissue damage and decreased function. Infiltrating neutrophils and monocytes and resident macrophages are a likely source of the damaging molecules. It is hypothesized that pulmonary surfactant rich in phospholipid may modulate the cytotoxic and inflammatory function of alveolar macrophages. Changes in membrane lipid composition may alter the immunological responses of monocytes to infection, including endotoxin (lipopolysaccharide, LPS). The present study was undertaken to determine the role of phospholipids in the modulation of monocyte respiratory burst activity.

Materials and methods. Human monocyte-derived cell line (MonoMac 6) was from the German Cell and Tissue Culture Collection, Braunsweig, Germany. Phospholipid species luminol and LPS were from Sigma Chemical Co. (Poole, United Kingdom). Phospholipids (10–500 μg/mL) were dissolved in RPMI 1640 medium and added to 1×10^6/mL MonoMac 6 cells with or without 100 ng/mL LPS for 12 h at 37°C. The cells were then washed three times, and the respiratory burst was triggered by the addition of zymosan in the presence of luminol. The production of reactive oxygen species was measured by chemiluminescence using an automated luminometer. Cells not preincubated with LPS to prime them produced little superoxide and acted as controls.

Results. The phospholipid species investigated all had effects on the priming of monocytes for superoxide production in the respiratory burst. At the concentrations used (10–500 mg/mL), they had no direct effect on the triggering of the respiratory burst with zymosan or phorbol 12,myristate 13,acetate. Therefore the phospholipids altered the monocyte responses to LPS, resulting in a primed state for enhanced superoxide generation.

Mixed lipid species of phosphatidylcholine inhibited the LPS-induced priming of the monocyte respiratory burst in a dose-dependent manner from 100–500 μg/mL. However, dipalmitoylphosphatidylcholine, the major phospholipid species in pulmonary surfactant, was stimulatory at 100–500 μg/mL but was inhibitory at concentrations below this. Mixed species of phosphatidylethanolamine had a suppressive effect on the respiratory burst in a dose-dependent manner up to 100 μg/mL, whereas at high concentrations (250–500 μg/mL) sphingomyelin had a stimulatory effect. The effects of other molecular species of the phospholipids are currently being investigated.

Conclusions. This study shows that phospholipids can modulate the respiratory burst in monocytes and that this is at the level of monocyte priming. The effect of the phospholipids was dependent both on the lipid acyl composition and on the polar headgroup composition. These results suggest that phospholipid species may regulate monocyte and macrophage oxidative responses *in vivo*, and alterations in cellular phospholipid content and composition may affect the host responses to infection. Manipulation of the extracellular lipid content may be a novel strategy in the treatment of inflammatory lung diseases.