AIRWAY EPITHELIAL RESPONSES TO
LPS-PARTICULATE EXPOSURE

Angela Fonceca B.Vis.Sci. (Hons)

This thesis is presented for the Degree of Doctor of Philosophy at the University of Western Australia, School of Paediatrics and Child Health, Faculty of Medicine and Dentistry.
For my friends and family
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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celcius</td>
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<tr>
<td>μm</td>
<td>Micrometre</td>
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<tr>
<td>μM</td>
<td>Micromolar</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>16HBE</td>
<td>Transformed human bronchial epithelial cells</td>
</tr>
<tr>
<td>AA</td>
<td>Atopic asthma</td>
</tr>
<tr>
<td>AEC</td>
<td>Airway epithelial cell</td>
</tr>
<tr>
<td>AM</td>
<td>Alveolar macrophage</td>
</tr>
<tr>
<td>ANA</td>
<td>Asthmatic non-atopic</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARC C3H/HeJ</td>
<td>TLR4 intact mice (TLR4&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BEBM</td>
<td>Bronchial epithelial basal medium</td>
</tr>
<tr>
<td>BEGM</td>
<td>Bronchial epithelial growth medium</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>TLR4 deficient mice (TLR4&lt;sup&gt;-&lt;/sup&gt;)</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric bead array</td>
</tr>
<tr>
<td>CD14</td>
<td>Cluster differentiation factor 14</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster differentiation factor 4</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>DEP</td>
<td>Diesel Exhaust Particles</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>EC150</td>
<td>Methacholine dose to induce 150% of Raw</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle minimum essential medium</td>
</tr>
<tr>
<td>ET</td>
<td>Endotracheal tube</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volumes over one second</td>
</tr>
<tr>
<td>FOT</td>
<td>Force oscillation technique</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HA</td>
<td>Healthy atopic</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HNA</td>
<td>Healthy non-atopic</td>
</tr>
<tr>
<td>hrs</td>
<td>hours</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IFNα</td>
<td>Interferon alpha</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>IL-1, 2, 3 ..</td>
<td>Interleukin 1, 2, 3 ..</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus amebocyte lysate</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipopolysaccharide Binding Protein</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LFOT</td>
<td>Low frequency forced oscillation technique</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine rich repeat</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic Acid</td>
</tr>
<tr>
<td>MAL</td>
<td>MyD88 adaptor-like protein</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MD-2</td>
<td>Myeloid differentiating factor 2</td>
</tr>
<tr>
<td>MCh</td>
<td>Methacholine</td>
</tr>
<tr>
<td>mg</td>
<td>Milligrams</td>
</tr>
<tr>
<td>MIF</td>
<td>Migration Inhibitory Factor</td>
</tr>
<tr>
<td>mins</td>
<td>Mins</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mPM&lt;sub&gt;0.5&lt;/sub&gt;</td>
<td>Model inert particulate matter</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NFκβ</td>
<td>Nuclear factor kappa beta.</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide oligmerization domain proteins (NOD) like receptor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligmerization domain proteins</td>
</tr>
<tr>
<td>pAEC</td>
<td>Paediatric derived airway epithelial cells</td>
</tr>
<tr>
<td>pAEC&lt;sub&gt;AA&lt;/sub&gt;</td>
<td>Paediatric derived airway epithelial cells from atopic asthmatics</td>
</tr>
<tr>
<td>pAEC&lt;sub&gt;ANA&lt;/sub&gt;</td>
<td>Paediatric derived airway epithelial cells from non-atopic asthmatics</td>
</tr>
<tr>
<td>pAEC&lt;sub&gt;CF&lt;/sub&gt;</td>
<td>Paediatric derived airway epithelial cells from patients with cystic fibrosis</td>
</tr>
<tr>
<td>pAEC&lt;sub&gt;HA&lt;/sub&gt;</td>
<td>Paediatric derived airway epithelial cells from healthy asthmatics</td>
</tr>
<tr>
<td>pAEC&lt;sub&gt;HNA&lt;/sub&gt;</td>
<td>Paediatric derived airway epithelial cells from healthy non-atopics</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive end expiratory pressure</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram</td>
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</table>
PM  Fine particulate matter
PRR  Pattern recognition receptors
Raw  Airway resistance
RNA  Ribonucleic acid
ROFA  Residual Oily Fly Ash
ROS  Reactive oxygen species
RPMI  Roswell Park Memorial Institute
RT  Reverse transcription
RT-PCR  Reverse transcription-polymerase chain reaction
sCD14  soluble cluster differentiation factor 14
secs  Seconds
T cells  Thymus derived lymphocytes
TBS  Tris buffered saline
TCC  Total cell count
Th  T helper
Th1  T helper 1
Th2  T helper 2
TIRAP  Toll Interleukin 1 receptor associated protein
TLR  Toll-like receptors
TLR2  Toll-like receptor 2
TLR3  Toll-like receptor 3
TLR4  Toll-like receptor 4
TLR5  Toll-like receptor 5
TLR9  Toll-like receptor 9
TLR4<sup>d</sup>  TLR4 deficient mice
TLR4<sup>i</sup>  TLR4 intact mice
TNFα  Tumor necrosis factor alpha
TRAF3  Tumor necrosis factor receptor associated factor 3
TRAM  Toll-receptor Associated Molecule
TRIF  Toll-receptor Associated Activator of Interferon
U937  Human monocytic transformed cell line
v/v  Volume per volume
Zrs  Impedence
Abstract

The overall incidence and prevalence of asthma have increased over the last three to four decades. Risk factors for the onset of asthma include environmental exposure to air pollution and viral infection while genetic factors include the hygiene hypothesis and raised serum IgE. Inhaled air containing lipopolysaccharide (LPS) and particulate matter (PM) has been shown to exacerbate asthma. Paradoxically, LPS exposure in early life may protect against the development of asthma and atopy.

The mucosa of the lower respiratory tract, which mainly consists of epithelial cells, provides a physical and functional barrier against inhaled pathogens, allergens and particulates. LPS is signalled when its lipid A moiety is recognised by Toll-like receptor 4 (TLR4). Despite LPS being attached to PM in ambient air, the immunological effect of LPS, PM or of an LPS-PM agglomerate have not been investigated in the lung. It was hypothesised that differential inflammatory effects to LPS, PM and LPS-PM exposure might account for some of the protective properties of LPS exposure observed. This thesis aims to examine the role of TLR4 in the bronchial epithelium to responses generated to LPS, PM and LPS attached to PM.

To adequately assess these responses in airway epithelium, TLR4 location and expression were investigated in freshly isolated airway epithelial cells from children. Individuals were grouped according to respiratory and atopic status into healthy non-atopic, healthy atopic, non-atopic asthmatic, atopic asthmatic and children with cystic fibrosis. TLR4 mRNA expression and intracellular
staining, assessed using real-time polymerase chain reaction and immuno-
histochemistry respectively were significantly greater in AECs from asthmatic
atopic children than from any other group. All freshly isolated AECs displayed
cell surface location of TLR4 while location in their cultured counterparts was
largely intracellular. On stimulating cultured cells with IL-13, TLR4 was found to
translocate to the cell surface, suggesting that signals present within the cellular
environment may determine receptor location.

Having established that TLR4 resides on the cell surface in vivo, the effect of
LPS and PM on TLR4 position was investigated in IL-13-stimulated and
unstimulated immortalised bronchial epithelial cells (16HBE). Commercially
available fluorescent polystyrene beads of 0.5μm (mPM_{0.5}) were used as a
model of inert PM. Upon exposure to mPM_{0.5}, LPS and an agglomerate of
mPM_{0.5} to LPS, IL-13 stimulated cells secreted less IL-8 than unstimulated cells
for the same exposures. These findings suggested TLR4 may be more
responsive when located intracellularly.

To investigate these effects further, animal studies were conducted.
Immunological responses to mPM_{0.5} and LPS were investigated in TLR4
deficient (C3H/HeJ) and normal mouse strains (ARC C3H/HeJ). Changes in
airway responsiveness, airway inflammation and secreted inflammatory
cytokines measured in collected BAL were significantly different in mice
exposed to LPS or mPM_{0.5}-LPS. Exposure to mPM_{0.5}-LPS induced significantly
more IFNγ and IL-12p70 secretion in TLR4 deficient mice compared to LPS
exposure of the same mice. Suggesting that LPS attached to mPM_{0.5}, is
signalled by a pathway other than TLR4 and this pathway is capable of inducing
a polarised Th1 response.

Overall, the results presented in this thesis reveal that airway epithelium from asthmatic children express relatively large amounts of TLR4 and that much of this is located intracellularly. Furthermore, in vitro experiments have shown that AEC responsiveness to cytokines (and by implication cytokines within the external cellular milieu) varies depending on the location of TLR4. Finally, animal studies have shown that LPS attached to mPM$_{0.5}$ can induce secretion of a different profile of inflammatory cytokines that is TLR4 independent and differs compared to LPS exposure alone. All these findings relate to the role of LPS exposure in the development and exacerbation of asthma and atopy, and implicate the bronchial epithelium as an integral cell type within this process.
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Chapter One

1 Introduction

1.1 The human airway epithelium

The airway epithelium interacts directly with inhaled air. It is a continuous pseudo-stratified sheet of cells that lines the upper and lower airways (Figure 1.1) forming an efficient barrier that impedes the penetration of microorganisms, macromolecules and toxins into the host tissue (Knight and Holgate 2003). The combined beating movement of cilia and secretion of airway epithelial lining fluid and mucus from these cells act to trap and sweep irritants into the oesphagus. These actions keep the humid moist environment of the airways sterile and prevent invasion of the circulatory system.

As an interface for the innate and adaptive immune systems the airway epithelium is an orchestrator of immune responses to contaminants in inhaled air (Zeldin et al. 2006). In addition, cytokine secretion allows the airway epithelial cells (AEC’s) to mediate the surrounding microenvironment and activity of other cells. By secreting particular cytokines in response to inhaled antigens, microbes or insults these cells can mount a primary immune response. Interleukin (IL)-8, interferon gamma (IFNγ) and granulocyte macrophage colony stimulating factor (GM-CSF) are examples of cytokines that recruit neutrophils, lymphocytes and dendritic cells to initiate necessary secondary responses such as T-cell maturation crucial for immune memory (Korn et al. 2001).
Secreted cellular signals can be part of signalling cascades that induce structural changes to the airways (Vignola 2003). For example, growth factors can cause an increase in cellular proliferation and apoptosis. Additionally, rates of secretory products can be affected. In turn these influence protein deposition in the epithelial basement membrane and mucus production (Jeffrey 2001). These activities can influence intrinsic airway function. Heavy mucus secretion can cause airway obstruction (Corry and Kheradmand 2007), whilst changes to airway wall thickness (due to increased smooth muscle or basement membrane layers) directly affect elasticity and compliance properties that shape breathing patterns (Jeffrey 2001) (Figure 1.2). The types of secretory mediators present in the airways are a reflection of airway epithelial homeostasis an element that is crucial to healthy breathing conditions.

### 1.2 Inhaled air triggers allergic and non-allergic disease

Inhaled air is purified, warmed and humidified by the airway epithelium for efficient gaseous exchange exposing AEC’s to all constituents carried by inhaled air such as viruses, bacteria, pollens and pollutants. The presence of these is subject to many factors including being either indoor or outdoor, time of year (seasonal), or in a rural versus an urban environment.

Based on size and type responses to inhaled air can be generalised as being either inflammatory or non-inflammatory; with subsets of these producing outcomes that characterise particular airway diseases.
Figure 1.1 Location of the airway epithelium

The airway epithelium lines the airways of the lower respiratory tract and is a ciliated pseudostratified epithelium. Goblet cells interspace the epithelium secreting mucus into the airways.
Abnormalities in the airway epithelium such as mucus cell hypertrophy (Aikawa et al. 1992), subepithelial cell fibrosis and epithelial cell metaplasia or hyperplasia (Kamm and Drazen 1992) are symptomatic of inflammatory airway diseases such as asthma, chronic obstructive pulmonary disease (COPD), chronic bronchitis and lung cancer. Inflammatory responses characterising airway disease can be further classified as either allergic or non-allergic. ‘Allergy’ describes the adverse health effects arising from exaggerated inflammatory responses to particular chemicals (allergens). These chemical specific immune responses can be genetic or a result of repeated allergen exposure over time known as ‘sensitisation’ of the immune response.

‘Atopy’ describes the potential allergic status of an individual often indicated by increased serum immunoglobulin E (IgE) titres and is predisposed by family history indicating a genetic influence. Birth cohort studies suggest atopy is a risk factor in developing asthma (Lowe et al. 2002; Martinez and Holberg 1995; Platts-Mills 1997; Wahn et al. 1998).

There has been an increase in prevalence and severity of asthma and allergy in global populations over the last few decades with some decline in asthma prevalence in the last decade (Smyth 2002). In the US alone the estimated total cost related to asthma was $12.7 billion in 2000 (US Department of Health and Human Services 2000).

The predisposition of atopy to asthma has furthered understanding of the relationships between inflammatory responses to allergic factors that lead to increases in IgE and asthma exacerbations (Lau et al. 2002; von 2000). House dust mite, cat dander and grass pollen are a few of the allergens linked to asthma exacerbation (Platts-Mills 2001).
1.2.1 Asthma

The key features of asthma include recurrent episodes of airway obstruction and wheezing with airway inflammation (Cohn et al. 2004b). In comparison to healthy airways, asthmatic airways show exaggerated narrowing after inhalation of various stimuli (hyper-responsive), have an increased cellular infiltrate and evidence of airway remodelling (Figure 1.2). These differences have been attributed to the presence of persistent inflammatory stimuli and aberrant inflammatory responses (Jeffery 2001; Vignola et al. 2003).

Asthma is a clinical diagnosis made by physicians on the basis of a patient’s medical history, physical examination and assessment of reversibility of airway obstruction. While many asthmatics have raised IgE levels and atopy, asthmatic symptoms can occur in their absence. The subjective nature of these symptoms makes a clear asthma diagnosis difficult. For this reason the hyper-responsive nature of asthmatic airways is used as an objective measure of asthma in research studies. Subjects undergo an airway challenge by inhaling increasing doses of a known inflammatory agent such as acetyl-β-methacholine chloride (MCh) to produce a dose response curve for airway responsiveness. Recordings of forced expired volume (FEV) are made using a spirometer after each dose. These measurements are used to produce a curve indicating hyper-responsiveness and airway obstruction when compared to control subjects. Lung function testing can be used to support a diagnosis of asthma (Cockcroft 2003).
Figure 1.2 Asthmatic airways and airway epithelium.

a. Asthmatic airways have a smaller lumen than normal due to a thicker airway wall. This is caused by a thickening of the basement membrane and proliferation of smooth muscle and mesenchymal cells.

b. Airway epithelium in asthmatics is easily sloughed and appears damaged in comparison to epithelium in a healthy individual.
While these methods are useful for diagnosing asthma in adults and older children, in younger children asthma and its diagnosis are often less clear. In a seminal study Martinez et al. studied a cohort of over 1000 newborn babies and found children who developed wheeze could be characterised into 3 different groups. Transient wheeze, non-atopic wheeze and persistent wheeze. Children with persistent wheeze were likely to be affected by atopy and wheezed on viral infection. Transient wheezers were characterised by respiratory syncytial virus (RSV) infection in early life causing bronchiolitis and a wheeze that lasted until school age. Whereas non-atopic wheeze made up 50-60% of all preschool wheeze but decreased in prevalence after school age (Martinez and Holberg 1995). The similar onset time for all groups coincided with interactions between environmental exposure and immune system development proposed by the hygiene hypothesis (Figure 1.3).

Based on epidemiological observations the hygiene hypothesis proposes the increased prevalence of allergy attributable to a lack of early childhood exposure to infectious agents, symbiotic micro-organisms (eg. gut flora) and parasites (Strachan 1989). Exposure to these was predicted to modify immune development. Support for this hypothesis has stemmed from the associations between increased rates of allergy/atopy and the increased use of antibiotics and reduced rates of allergy present in farming communities (Marra et al. 2006). Advances in molecular biology lead to discovery of distinct cytokine profiles secreted by T-helper (Th) cells in mice provided further support for the hygiene hypothesis. These classes of lymphocytes well established roles in amplifying immune responses.
Figure 1.3. Schematic chart of childhood wheezing phenotypes against age presented as a stacked area chart.

The prevalence of each phenotype at any age is the height of that band and the total prevalence of wheeze at any age is the sum of the three wheeze phenotypes (Taken from Morgan, Revue française d’allergologie et d’immunologie clinique 45 (2005) 542–546)(Morgan 2005).
Based on the type of profile induced these responses were classified as either T helper 1 (Th1) or T helper 2 (Th2) (Mosmann et al. 1986).

The Th1 pathway is proposed as being pivotal in cellular immunity, fighting infection from viruses and other intracellular pathogens as well as eliminating cancerous cells and stimulating delayed type hypersensitivity reactions. Th2 reactions are proposed to provide humoral immunity by upregulating antibody production to fight extracellular organisms. In humans Th-cells with a Th1 response inducing IFNγ and IL-2 on stimulation with a bacterial protein while T-helper 2 cells secrete IL-4 and IL-5 on exposure to helminth excretory/secretory antigen (Del Prete et al. 1991). The discovery of these T-cell subsets provided a mechanism that could be used to explain the development of allergy as defined by the hygiene hypothesis.

1.3 Skewed inflammatory responses causing inflammation: T-helper cells

Activated thymus derived lymphocytes (T cells) play a leading role in determining if asthma is allergic or non-allergic (Holt et al. 1999). Asthmatic airways are characterised by high numbers of a subset of these cells expressing cluster differentiation factor 4 (CD4+) (Holt et al. 1999).

Naive CD4+ T lymphocytes (T cells) differentiate into effector T cells based on cytokines released by innate immune cells in response to pathogens. These cells have an enhanced functional potential to orchestrate pathogen clearance providing a mechanism linking the innate and adaptive immune systems.
Further investigation revealed that Th responses were driven by particular cytokine signals to induce production and secretion of specific cytokine profiles (Luster et al. 2005). These profiles act to maintain particular Th response while inhibiting the opposing response. Maturation of Th1 cells is driven by secreted interferon (IFN)γ in response to pathogens (Luster et al. 2005). IFNγ induces interleukin (IL)-12, which activate T cell receptor (TCR) and the transcription factor Stat-1 to cause upregulation of Th1 cytokines. Naive CD4 cells mature to Th2 cells by interaction of IL-4 with TCR inducing Stat-6. By selectively producing IL-4, IL-5 and IL-13, Th2 cells promote an aggressive adaptive immune response – a response shown to lead to exaggerated and inappropriate responses to allergens (Luster et al. 2005).

The Th2 phenotype is thought to occur in the fetus and switches to a Th1 phenotype post-natally on exposure to environmental stimuli (Prescott et al. 1998) It is hypothesised that individuals who develop atopy have failed to make the usual post-natal switch from a Th2-skewed (allergic) T-cell phenotype toward a Th1 (non-allergic) T-cell phenotype (Holgate 2001). Raised numbers of CD4+ Th2 cells in asthmatic airways implicates these cells in the pathogenesis of asthma (Jeffrey 2001).

While each Th response can act to downregulate the other, a balance is needed to maintain immune homeostasis. Skewed Th1 responses have been found in autoimmune diseases such as inflammatory bowel disease, multiple sclerosis and type 1 diabetes (Guarner 2007).

As research into the Th cells has evolved a third Th subset Th17 has emerged (Weaver et al. 2007). These secrete a range of IL-17 proteins including IL-17A a cytokine that induces IL-8 secretion and recruits neutrophils, hallmarks of
airway inflammation. The homology of the IL-17 proteins makes these a distinct cytokine family. Mouse knockouts of IL-17A receptor (IL-17ra) have shown inadequate host defence responses (Ye et al. 2001) and overexpression in inflamed joints suggesting a role in autoimmune inflammation (Koenders et al. 2005; Lubberts et al. 2001).

As the Th2 cytokines are only found in mammals these cytokines may have evolved to protect multicellular organisms against more complex pathogens such as helminths. Th1 and Th17 are more primitive providing immunity to viruses, bacteria and fungi.

Long term exposure to distinct signal profiles such as those defining Th1, Th2 and Th17 responses are capable of shaping cell gene expression. Elucidating the consequences and involvement of the epithelium in initiating these signal profiles will allow for a better understanding for allergic airway disease aetiology.

While the ‘hygiene hypothesis’ described above provides a possible explanation for the onset of allergy and atopic asthma, mechanisms underlying non-allergic hyperresponsiveness remain unclear (Eisenbarth et al. 2002; Schwartz 2001). Non-allergic factors shown to exacerbate and contribute to asthma pathogenesis include environmental dust, ozone and fine particulate matter (PM) (Douwes et al. 2002). However, similar inflammatory changes are observed in asthmatic airways regardless of atopic status, suggesting a common response of the respiratory tract to injurious stimuli (Cohn et al. 2004a).
1.4 Injury causing inflammation

Airway epithelial cells are adept in repairing in response to injury or insults. Inadequate repair processes make the epithelium vulnerable to insults such as pollutants, allergens and viruses causing further injury. The combination of injury and insult has been shown to induce epithelial secretion of proinflammatory and profibrogenic mediators capable of causing chronic inflammation and long term tissue changes. This is supported by structural changes observed in asthmatic airways. Epithelial denudation, a thick basement reticulum and larger, more numerous smooth muscle cells together describe damaged, fragile tissue, features of aberrant repair and inflammation (Payne 2003). The onset of non-atopic asthma can be explained these abnormal repair responses. While the mechanisms influencing these processes are poorly understood the airway epithelium has a central role in the pathogenesis of this airway disease (Knight and Holgate 2003; Lane et al. 2005a) (Figure 1.2).

Asthma severity and augmented airway inflammation have also been linked to raised concentrations of ambient lipopolysaccharide (LPS) (Douwes et al. 2002; Michel et al. 1996; Rizzo et al. 1997; Schwartz et al. 1994; Thorne et al. 2005). LPS is a bacterial glycolipid found ubiquitously in the environment. Increased ambient LPS levels are present in farming communities because of animal faeces and organic waste (Rylander 2002). In contrast to the association between chronic environmental LPS exposure and augmented airway inflammation, atopy is not as prevalent in farming communities (Chen et al. 2007). These high LPS environments are shown to have a protective effect against atopy a regarded precursor for asthma (Chen et al. 2007). The
protective effect has been reported in farming environments across the globe attributing elevated LPS concentrations in preventing the onset of Th2 immune responses (Chen et al. 2007; Eduard et al. 2004; Filipiak et al. 2001; Kimbell-Dunn et al. 1999; Monso et al. 2000; Portengen et al. 2002).

Interestingly lower rates of reported atopy, have not correlated with reduced rates of asthma. The rate of asthma remains unchanged or greater in farming studies conducted in Australia, Europe, New Zealand and the United States (Chrischilles et al. 2004; Downs et al. 2001; Leynaert et al. 2001; Merchant et al. 2005; Wickens et al. 2002). Delineating how inhalation of LPS can induce protection against a precursor for asthma and the disease itself is widely regarded as the key to understanding asthma.

1.5 Lipopolysaccharide exposure: A model for inducing atopic and non-atopic asthma.

1.5.1 LPS structure and sources
Lipopolysaccharide (LPS), also known as endotoxin, (Figure 1.4) is a component of Gram-negative bacteria cell walls. LPS is found ubiquitously in the environment owing to the continual breakdown of bacteria at the end of their lifecycle. LPS has a polarised molecular structure characterised by lipid A, a core polysaccharide and an O-polysaccharide tail of variable length. Bacterial strains with long O-polysaccharide chains form smooth colonies and those without rough colonies, designating LPS as either smooth or rough (Chrischilles et al. 2004; Jiang et al. 2005) LPS is an extremely resilient product able to
persist in the environment for long periods of time. Prolonged baking of LPS at temperatures above 160°C for at least 4hrs is required to quell the immunostimulatory properties of its highly conserved pro-inflammatory agent lipid A (Liu 2002).

When disassociated from its bacterial source LPS occurs in micelles or attached to other substances. LPS concentrations are high in sewerage treatment plants and swine confinement units (Liu 2002; Rylander 2002). It has also been found in house dust (Thome et al. 2005), grain dust (George et al. 2001; Schwartz et al. 1994) cotton dust (Simpson et al. 1999) and bakers dust (Marraccini et al. 2008). Due to an association with adverse respiratory health effects LPS is a well recognised occupational hazard in swine, poultry and dairy farms, grain handling facilities, vegetable and cotton processing sawmills, metal machining, composting and waste handling (Douwes et al. 2002; Mueller-Anneling et al. 2006).

The negative charge on the LPS molecule allows for easy attachment to other substances, including air pollution, organic dust and cigarette smoke (Vernooy et al. 2002). These airborne particulates act as a carrier for LPS into the distal lung. The large prevalence of these products in the environment means the general population is under constant low level environmental LPS exposure (Park et al. 2001). Under these conditions, the lung has efficient defence mechanisms, including mucociliary clearance, antimicrobial compounds in the epithelial lining fluid as well as alveolar macrophages.
1.6 Inflammatory responses: The dual nature of LPS induced responses

LPS is able to induce a potent inflammatory response capable of resulting in septic shock and death. Low level LPS exposure is shown to cultivate a Th1 response inhibiting the onset of atopy. This is illustrated by reduced rates of atopy in children that received *bacillus Calmette-Guerin* (BCG) vaccine for tuberculosis (O'Brien *et al.* 1995; Rosenstreich *et al.* 2003).

LPS in the vaccine caused a skewed Th1 response thereby inhibiting production of Th2 cytokines (Shirakawa *et al.* 1997). In addition repeated LPS exposure induces reduced inflammation known as LPS tolerance, another important facet of LPS biology. By preventing exaggerated systemic inflammation as a result of excess production by innate effector cells LPS tolerance serves to protect the host from septic shock and death. These immunomodulatory effects illustrate the diverse inflammatory responses this bacterial by-product is capable of.

An explanation for the dual (protective/exacerbatory) nature of LPS exposure remains at large. Since the discovery of the LPS receptor Toll-like receptor 4 there has been a rapid increase in understanding cellular pathways involved in LPS recognition and generated responses. As they continue to be elucidated the complexity of these signalling pathways is becoming more apparent (Arbibe and Sansonetti 2007). Recently published epigenetic mechanisms underlying LPS tolerance are an example of how molecular mechanisms may influence induced protective and inflammatory responses (Foster *et al.* 2008).
Figure 1.4 Lipopolysaccharide.

LPS is found on the outer wall of Gram negative bacteria cell walls.

LPS is characterised by a polysaccharide tail and lipid A head. The polarity of the molecule allows for easy adherence to other substances.
In primed immune systems dose and timing of LPS administration have proved a crux in the crossroad determining a proinflammatory or Th2 inhibitory immune pathway (Alexis et al. 2008; Eisenbarth et al. 2002; Gerhold et al. 2006; Tulic et al. 2002). Mouse models have proved useful in deciphering the affects of LPS inhalation in the onset of airway hyperresponsiveness. In a non allergic immune system LPS inhalation causes lung injury, reduced FEV and airway hyperresponsiveness (Baron et al. 2004; Savov et al. 2002). To investigate the effects of LPS exposure in immune systems with allergy systemic sensitisation is required. Intraperitoneal injection with ovalbumin is regarded as the best inducer of an allergy phenotype in mice. To induce an allergy asthma model systemic sensitisation is followed by airway sensitisation using a respiratory tract antigen challenge such as LPS (Epstein 2004). LPS exposures studied at various points of this process have clearly demonstrated the effect of LPS dose and timing on atopy and asthma phenotypes.

In unchallenged sensitised mice inhaled LPS was crucial in promoting lung inflammation implicating TLR4 signals in developing asthma-like airway inflammation (Eisenbarth et al. 2002). A dose-differential TLR4 inflammatory response was also shown. Th2 responses were induced on low level LPS exposure of OVA sensitised mice. Th1 responses were induced with high doses of LPS to similarly sensitised mice (Eisenbarth et al. 2002). A corresponding LPS dose-dependent dampening of airway responsiveness and reduced inflammatory cellular infiltrate was observed in sensitised and challenged rats (Tulic et al. 2002). These findings indicate that inhaled high LPS doses can induce a skew towards Th1 responses in an allergic phenotype and a low dose can exacerbate allergen induced inflammation in the lungs.
Timing of LPS exposure can prevent or exacerbate airway inflammation (Gerhold et al. 2002; Tulic et al. 2000). Administration of LPS before OVA sensitisation or challenge caused reduced IgE levels, prevented lung inflammation and hyper-responsiveness (Tulic et al. 2000). Similarly, LPS/OVA or OVA inhaled before sensitisation can completely suppress allergen sensitisation (Gerhold et al. 2002). In contrast LPS exposure after OVA challenge can cause a significant increase in airway inflammation while LPS inhaled before sensitisation does not prevent Th2 responses (Gerhold et al. 2002; Tulic et al. 2000).

Fetal exposure to LPS also effects immune response development. A skew away from Th2 responses has been observed in mice exposed to LPS in utero. On birth these mice were exposed to aerosolised LPS, then sensitised and challenged with OVA. They showed suppressed IgE, reduced airway inflammation and reduced airway responsiveness to methacholine (Gerhold et al. 2006). From these responses the critical influence of LPS exposure during gestation is implicated as a potential therapeutic strategy to prevent the onset of atopy and asthma.

In human subjects LPS inhalation causes acute lung inflammation and has a reported dose-response relationship (Michel et al. 1996; Michel et al. 2001). Subjects were observed to have increased lymphocytes, monocytes, C-reactive protein and TNFα compared to controls (Michel et al. 2001). These findings correlate with respiratory inflammation observed in workers exposed to elevated LPS measured in grain dust, swine dust and bakers dust (Jagielo et al. 1996; Marraccini et al. 2008). In asthmatic atopic subjects acute LPS inhalation causes exacerbation (Michel et al. 1992). Association studies have shown
endotoxin exposure to induce wheeze in the first year of life, raise the risk of respiratory infection in infants and raise exacerbatory asthma events in children (Douwes et al. 2002; Park et al. 2001; Rizzo et al. 1997). Exposure is also reported to reduce the risk of developing atopy (Gehring et al. 2002).

Whilst adsorption of LPS to particulates is recognised as a route for LPS to enter the lung, research studies have focused on responses to LPS and particulate exposure independently. Epidemiological studies have demonstrated associations between increased ambient particulate levels, impaired lung function and exacerbations of asthma. Furthermore the rise and dependence on motor vehicle use worldwide and resultant increase in motor vehicle PM correlates the increased prevalence of asthma (Salvi 2001). In urban areas, diesel exhaust particulate (DEP) contributes up to 90% of fine PM in ambient air (Airborne Particles Expert Group 1999). Together these studies build a strong case for the involvement of particulates as a non-allergic stimulant of airway inflammation. If LPS is adsorbed to another substance, binding sites to cellular recognition systems may change, inducing a different pattern of responses expected from TLR4. In addition, exposure to particulates has been shown to exacerbate pre-existing asthma and induce asthmatic symptoms in non-asthmatics illustrating a need to delineate the role of adsorbed LPS in this process (Pope, III and Dockery 1992a).
1.7 Particular Matter (PM): A vehicle for LPS exposure

1.7.1 PM composition and categorisation

Particulate matter (PM) describes all solid phase ambient particulates comprising a complex mixture of organic and inorganic compounds. While LPS is recognised and signalled by specific receptors located on the cell surface, PM has been shown to initiate inflammatory cellular responses by being phagocytosed and inducing reactive oxygen species (ROS) on presentation (Bayram et al. 1998; Fujii et al. 2002a; Li and Nel 2006).

PM is categorised base on size. Crudely, PM can be described as being either coarse (10-2.5μm), fine (2.5-0.1μm) or ultrafine (<0.1μm) (Bayram et al. 1998; Tao et al. 2003). The recognised health risks associated with exposure to small particulates and the importance of particulate size in determining deposition site, potential cellular interaction (based on surface area-to-volume mass ratio) and clearance rates within the respiratory tract led environmental authorities define these distinctions further (US Environmental Protection Agency 2001). Therefore, PM with an aerodynamic diameter of 10 microns or less is referred to as PM$_{10}$. The subfraction of PM$_{10}$ less than 2.5 microns is referred to as PM$_{2.5}$ (Figure 1.5).

PM$_{10}$ composition is associated with crustal material and organic debris (Committee on Environmental Health 2004). The specific composition of PM$_{10}$ depends on location as it is influenced by geography, climate, season and land use, factors that also affect the proportion of LPS. For example in areas surrounding sewerage plants ambient LPS levels are raised as they are in
areas where processing of organic matter or animals are present (Rylander 2002; Thorne et al. 2005; Wilson et al. 2000). Generally, the major component of PM$_{10}$ is black carbon derived from the combustion of fossil fuels (Artinano et al. 2004). Other components include hydrocarbons and metals, geological dust and organic matter, nitrogen salts, sulphur and chlorine (Harrison et al. 1995). In rural communities grain dust and windblown soil also contribute to PM$_{10}$ and are a source of LPS (Jagielo et al. 1996; Mueller-Anneling et al. 2006). Indoors, exposure to PM$_{10}$ results from activities such as cooking and cigarette smoking.

Particles greater than 5µm are deposited in the upper and larger lower airways (Zielinski et al. 1998). These are effectively cleared by mucociliary clearance and macrophage phagocytosis (Mukae et al. 2000; Zielinski et al. 1998). Despite this, all types of PM$_{10}$ listed have been linked to allergic responses, asthma exacerbations and wheezing (Churg and Brauer 2000; Churg and Wright 2003; Kim 2004; Mazzarella et al. 2007). As this larger PM fraction is more likely to consist of organic matter it also contains a greater proportion of LPS, a likely trigger of allergic inflammation and exacerbation.

A large proportion of PM$_{2.5}$ is composed of particles generated from combustion. Sources of these include diesel powered engines, power generation and wood burning (Committee on Environmental Health 2004). Due to the smaller size fraction these particles deposit in the lower airways and alveoli (Churg and Brauer 1997; Zielinski et al. 1998). As mucociliary clearance in these areas of the lung is slow particles can remain lodged for long periods of time. Combined with a larger bioactive surface area these particles have been linked to inducing a more severe inflammatory response (Wilson and Suh 1997).
Figure 1.5 Particulate matter

A. Electronmicrographs of ultrafine, fine and coarse particulate matter. These fractions are extracts taken from Residual oily fly ash (ROFA).

B. Schematic illustrating Diesel exhaust particulate (DEP). DEP is composed of many different chemicals combusted together to form a carbonaceous mass. Size variation is generally up to 10\(\mu\)m.
Long term exposure to PM$_{10}$ has been associated with decreased lung function (Churg and Brauer 2000; Downs et al. 2001). A study completed in Utah to assess the effects of PM$_{10}$ exposure found hospital admissions increased when PM$_{10}$ levels were raised due to operation of an intermittent steel mill. This effect was more pronounced in children and in people with asthma and bronchitis (Pope, III and Dockery 1992b). Similarly increased respiratory symptoms have been reported from workers exposed to dust in America, China and Spain (Korn et al. 1987; Sunyer et al. 1998; Xu et al. 1992). While there are many epidemiological studies correlating the role of PM$_{10}$ in respiratory disease a defined inflammatory mechanism induced by the interaction of particle and cell remains unknown.

1.7.2 PM inflammatory responses

Following exposure to PM, increases in pro-inflammatory cytokines associated with asthma symptoms have been demonstrated in vitro. For example, exposure of alveolar macrophages (Becker et al. 2003; Fujii et al. 2002b; van Eeden et al. 2001) and bronchial epithelia (Fujii et al. 2002b) to varying models of PM$_{10}$ and PM$_{2.5}$ have demonstrated increases in interleukin 6 (IL-6), IL-8, granulocyte macrophage colony stimulating factor (GM-CSF) and TNF$\alpha$ (Becker et al. 2003; Brown et al. 2001; van Eeden et al. 2001). Notably a synergistic inflammatory effect has been observed on co-culture of bronchial epithelial cells with macrophages exposed to carbon black PM$_{10}$ (Fujii et al. 2002b).

Consistent with both epidemiological and in vitro findings, PM$_{10}$ exposure has been found to induce airway responsiveness, airway inflammation and the
release of pro-inflammatory cytokines in several animal models (Archer et al. 2004; Gavett et al. 2003a; Gavett et al. 2003b; Mueller-Anneling et al. 2006; Wang et al. 2008). An enhanced inflammatory effect has been noted when PM$_{10}$ is delivered with LPS and in mice sensitised with ovalbumin (Archer et al. 2004; Wang et al. 2008). Inflammation caused by PM$_{10}$ inhalation is reduced in mice with LPS tolerance (Mueller-Anneling et al. 2006). Interestingly airway epithelial cells sensitised with LPS before being stimulated with PM$_{10}$ produced a greater inflammatory response than non-sensitised cells (Mukae et al. 2000). Together these findings illustrate that primed immune systems will react differently to PM$_{10}$ exposure.

Models of PM$_{10}$ vary between studies. Tested samples include collected ambient samples from particular cities and subtypes of PM$_{10}$ hypothesised to induce inflammation. Examples of these include Baltimore PM, residual oily fly ash (ROFA) and DEP. Carbon black makes up a large proportion of PM$_{10}$ and is commonly used as a model of PM$_{10}$ (Kulkarni et al. 2006; Mukae et al. 2000; van Eeden et al. 2001). Polystyrene beads are used to in studies aiming to elucidate the interaction of inert PM (Brown et al. 2001). These are commercially available in different sizes and with fluorescence allow for easy deposition observation.

Composition of PM$_{10}$ varies depending on collection sites and this influences biological activity. For example Baltimore PM$_{10}$ is collected from a sixth floor window in urban Baltimore. This ambient PM$_{10}$ is a heterogeneous mixture of transition metals, DEP and carbon (Walters et al. 2001). Similarly ROFA contains a large amount of transition metal traces and DEP is made up of over 18000 chemicals combusted together (Diesel Working Group 1995; Dye et al. 2001).
In addition samples of ambient PM can induce a greater pro-inflammatory response if adsorbed LPS are left untreated (Becker et al. 2002). Exposure to transition metals such as those present in ROFA generate reactive oxygen species (ROS) (Carter et al. 1997; Dye et al. 1997; Dye et al. 1999) induce raised levels of Th2 cytokines in BAL of exposed mice (Gavett et al. 1999). Therefore differences between models such as treatment to remove LPS, LPS contamination or presence of metals can give different results even though they may refer to the same product – PMₐ. This is an important consideration when comparing PM₁₀ studies and PM₁₀ study design. To date the presence of so many variables in PM₁₀ models have clouded attempts to provide a specific mechanism for the pathophysiological consequences of ambient PM₁₀ exposure (Graff et al. 2007; Peden 2002; Wilson et al. 2000; Wilson and Suh 1997).

ROS generate oxidative stress. The high toxicity of this environment makes the release of ROS a potent cellular defence mechanism used to combat infectious agents (Fujikawa et al. 1994; Sies et al. 1979). This toxicity extends to the host tissue implicating ROS as a stimulant for inflammation in the absence of a harmful pathogen (Folkerts et al. 2001).

The presence of ROS is measured by ratio of glutathione to its oxidised form glutathione disulfide (Shaik and Mehvar 2006). Generated ROS are postulated as the source of inflammation in PM₁₀ exposure studies due to the reduced measures of glutathione observed. Oxidative stress has also been attributed to the rapid influxes of calcium observed on PM₁₀ exposure (Brown et al. 2001). Therefore calcium signalling for oxidative stress caused by PM is proposed as a source of induction for pro-inflammatory cytokine secretion. The role of this
pathway in differential inflammatory effects induced on PM$_{10}$ exposure with sensitisation or tolerance to LPS remains unexplained. In addition inflammatory effects induced by substances able to adhere to particular types of PM$_{10}$ such as DEP to enter the host are also unaddressed.

DEP is characterised by a carbonic nucleus in which some 18000 high molecular weight organic compounds can be absorbed (Yanagisawa et al. 2003) (Figure 1.5B). Due to its uneven surfaces and easily accessible attachment sites, DEP is implicated as a carrier of aeroallergens, viruses and possible adjuvant (Marano et al. 2002). The range of chemicals combusted together to form DEP and the coating of this particulate with different chemical substances make it difficult to identify what is responsible for its observed effects (Fernvik et al. 2002b). Studies have suggested that DEP acts through polyaromatic hydrocarbons (PAH). These deposit on the mucosa and can cross the cell membrane due to their hydrophobic membranes allowing them to bind to a range of cytosolic receptors (Mazzarella et al. 2007). This binding could be responsible for the DEP-PAH effect on cell growth and differentiation observed (az-Sanchez 1997; Tsien et al. 1997). In mice exposed to aggregates of birch pollen and diesel carbon, increased IgE titres, bronchial hyperresponsiveness, IL-5 and eosinophilia when compared to mice exposed to these substances individually (Fernvik et al. 2002b; Fernvik et al. 2002a).

Like almost all forms of PM$_{10}$, DEP is a likely carrier for LPS. Differing responses to DEP aggregates compared to DEP alone verify the possibility that the protective/exacerabative effect of LPS exposure may be explained by its attachment to a PM source. This is substantiated by different inflammatory responses observed in PM administered with untreated LPS. As yet a study
investigating this possibility has not been published. A player critical in elucidating the inflammatory role of LPS attached to PM\textsubscript{10} is the LPS receptor TLR4.

### 1.8 Toll-like receptor 4: Understanding the ‘switch’ mechanism for allergy in airway epithelial cells.

#### 1.8.1 Toll-like receptor 4: Function and signalling

Toll-like receptor 4 (TLR4), was first discovered in \textit{Drosophila melanogaster} and is highly conserved through to humans (Lemaitre \textit{et al.} 1996). The discovery of TLR4 has been a key to understanding inflammation in response to bacterial exposure (Mayer \textit{et al.} 2007).

TLR4 is a protein within the Toll-like receptor (TLR) family. The TLRs are known as pattern recognition receptors (PRRs) recognising characteristic molecular signatures of particular microbial agents, termed pathogen-associated molecular patterns (PAMPs). One of the most studied PAMPs recognised by TLR4 is the lipid A motif of LPS. Lipid A anchors LPS to the outer membrane of Gram negative bacteria crucial for bacterial survival and is highly conserved across Gram negative bacteria. Different PRRs recognise different PAMPs thus TLR3 signals a response to viruses by recognising double stranded RNA. Whereas flagellin and unmethylated CpG motifs present on the DNA of particular bacteria are recognised and signalled by TLR5 and TLR9 respectively (Mayer \textit{et al.} 2007). Together these receptors act as an important surveillance system against invading micro-organisms (Figure 1.6).
TLR4 expression can be influenced by growth factors and cytokines, genetic polymorphisms and environmental exposure to LPS. A role for TLR4 in inflammatory airway disease has been suggested given its increased expression of the receptor in several chronic inflammatory diseases including artherosclerosis (Vink et al. 2002), periodontitis (Mori et al. 2003) and inflammatory bowel disease (Cario et al. 2002).

TLR4 expression in epithelial cells is less than that in dendritic cells and macrophages. Whereas TLR4 is membrane bound in these latter cells, in epithelial cells it is located intracellularly (Backhed et al. 2002a; Backhed and Hornef 2003). This is hypothesised to provide an additional regulatory barrier for mucosal surfaces, preventing unintended stimulation but preserving the ability to detect bacterial pathogens (Backhed and Hornef 2003). Consequently epithelial cells are largely hyporesponsive to LPS (Monick et al. 2003).

Lipopolysaccharide binding protein (LBP), CD14 (Cluster differentiation factor 14) and myeloid differentiating factor 2 (MD-2) are accessory proteins that present LPS to TLR4 efficiently (Figure 1.7). CD14 occurs in a soluble form and is membrane bound in myeloid cells. MD-2 is also found in a soluble and membrane bound form while LBP is soluble (Gradisar et al. 2007; Viriyakosol et al. 2006). All of these proteins bind circulating LPS. MD-2 is the only protein critical to TLR4 signalling for LPS (Gradisar et al. 2007). CD14 independent pathways have been shown for signalling rough LPS illustrating how accessory molecules are used to specify signalling by the innate immune system (Jiang et al. 2000).
The capacity for TLR4 recognition of LPS to generate different patterns of gene expression is possible by the number of intracellular pathways activated by TLR4 (Palsson-McDermott and O'Neill 2004).
Figure 1 6 TLR signalling pathways

The TLRs are characterised by the extra domain leucine rich repeat motifs and intracellular TIR domain. 11 members have been identified to date with signalling pathways documented for the first 9 of these. Similar in size and shape each is able to recognise and signal for a specific microbial signature. Signalling pathways are also similar, resulting in transcription of proinflammatory cytokines.
Figure 1.7 Accessory molecules and proteins in TLR4 signalling.

TLR4 signalling efficiency for the presence of LPS is raised but not dependant on interaction with LBP and CD14.

Differential responses in the TLR4 pathway can be generated by downstream signalling. These signals can be dependant or independent of signalling proteins such as MyD88.
The TLR extracellular domains generally comprise a leucine rich repeat (LRR) that confers ligand specificity. TLRs also share a highly conserved intracellular TIR (Toll/IL-1R) domain that is responsible for initiating an intracellular signalling cascade via the adapter protein MyD88 (myeloid differentiation factor 88) and its co-protein Mal (MyD88 adaptor like, also known as TIRAP -TIR containing adaptor protein) (Fitzgerald et al. 2001). The TLR4 intracellular signalling cascade can also involve a number of other proteins including TRIF (TIR containing adaptor protein inducing IFN-β), and TRAM (TRIF related adaptor molecule) before culminating in the activation of the transcription factor nuclear factor kappa-beta (NF-κβ) (Akira and Takeda 2004; Slack et al. 2000). NF-κβ induces transcription of a number of pro-inflammatory cytokines including interleukin-1 (IL-1), IL-8 and TNFα thereby inducing a proinflammatory response (Akira and Takeda 2004; Medzhitov et al. 1997; O’Neill 2006) (Figure 1.6).

TLR4 signalling pathways that are both independent and dependant on MyD88-Mal, TIRAP and TRAM have been observed (Fitzgerald et al. 2001; Horng et al. 2001; Yamamoto et al. 2003) (Figure 1.7). However the molecular mechanisms that modulate the involvement of these proteins remain unclear (Watts 2008). Understanding these mechanisms is integral to explaining how the innate immune system can prime select adaptive immune responses. In asthma, it has been suggested that dysregulated TLR4 signalling are the cause of exaggerated inflammatory responses that characterise this disease. For this reason research on the innate immune response (and particularly TLRs) has received renewed
attention for its potential to develop new treatments for chronic inflammatory conditions.

1.8.2 Factors affecting TLR4 function and signalling in the airway epithelium.
Despite the recent advances in innate immunity and continued unravelling of TLR function, the role of TLRs in airway epithelial cells remains largely unexplored. To date cystic fibrosis and COPD are the only inflammatory airway diseases in which airway epithelial cell TLR4 has been characterised (Greene et al. 2004; MacRedmond et al. 2007). Conflicting cellular localities for TLR4 have arisen. Epithelial cell culture models depict TLR4 to reside within the cell (Greene et al. 2004; Guillot et al. 2004) and biopsy tissue a cell surface expression (Hauber et al. 2005; MacRedmond et al. 2007).

TLR4 recognition of LPS is reported to occur despite an intracellular location. This is evidenced in intestinal epithelial cells by trafficking of the receptor between the cell surface and Golgi apparatus (Hornef et al. 2003; Latz et al. 2002). Soluble accessory proteins might play a role ferrying LPS to TLR4 and may provide additional extracellular signalling mechanisms affecting TLR4 signalling downstream (Latz et al. 2002). In an effort to explain activation of the MyD88-Mal independent (TRAM dependent) signalling pathway by TLR4 it was proposed that TLR4 endocytoses on LPS recognition. Therefore activation of TRAM dependant pathways would begin from TLR4 molecules in the endosome after being signalled by MyD88. Meaning that these proteins would signal LPS recognised by TLR4 sequentially but down different signalling routes and not exclusively as originally hypothesised (Kagan et al. 2008).
Increased surface expression of TLR4 in intestinal epithelial cells occurs in response to IFNγ stimulation (Bosisio et al. 2002; Suzuki et al. 2003; Tamai et al. 2002). Similar results have been shown for macrophages and fibroblasts, whilst reduced TLR4 mRNA expression in response to IFNγ has been shown in monocytes (Mita et al. 2002). Macrophage migration inhibitory factor (MIF) (Roger et al. 2001) and RSV infection (Monick et al. 2003) have been shown to increase TLR4 mRNA expression in airway epithelial cells. Together these results suggest that TLR4 expression varies depending on cell type, possibly reflecting specific roles within the immune system. Discrepancies between TLR4 protein and gene expression have been documented in airway and gut epithelial studies illustrating that protein expression cannot be used as a surrogate for gene expression or vice versa. For both cell types, changes in mRNA expression were greater than the measured protein expression (Abreu et al. 2002; Guillot et al. 2004).

TLR4 may be involved in non-atopic asthma exacerbation. In idiopathic bowel inflammatory bowel disease there was an increase in TLR4 expression and IL-8 release following LPS exposure of pre-treated gut epithelial cells with IFNγ and IFNα (Abreu et al. 2002). This study illustrates inflammatory exacerbation with LPS stimulation of a polarised Th1 cytokine profile by increased expression of TLR4. If gut and airway epithelial cells were to show similar responses, the Th1 cytokine profile present in non-atopic asthma combined with LPS exposure could induce the same inflammatory outcome.

A predisposition to asthma and atopy based on polymorphisms of TLR4 provides another possible explanation for alteration of host responses to environmental LPS
exposure (Arbour et al. 2000). Two major polymorphisms in the TLR4 gene have been identified (Arbour et al. 2000; Cook et al. 2003). One is an A to G base transition at nucleotide 896 upstream from the start codon (Medzhitov et al. 1997). This single nucleotide polymorphism (SNP) results in an aspartic acid to glycine exchange at position 299 in the amino-acid sequence (referred to as Asp299Gly or A+896G). The second SNP is a co-segregating missense mutation that produces a C to T transition resulting in a threonine to isoleucine exchange at position 399 (referred to as Thr399Ile or C+11996T) (Arbour et al. 2000); with only the Asp299Gly mutation interrupting TLR4-mediated LPS signalling causing LPS hyporesponsiveness (Arbour et al. 2000; Michel et al. 2003; Schwartz 2001). The Asp299Gly polymorphism occurs at a frequency approaching 10% in Caucasian populations (Lorenz et al. 2000; Schwartz 2001) and has been linked to increased prevalence of asthma in school children (Bottcher et al. 2003) and increased atopy severity in adult asthmatics (Yang et al. 2004). CD14 polymorphisms and mutations to coding sequences downstream in the TLR4 signalling cascade have also been found to influence the signalling efficiency of TLR4 producing blunted responses to bacterial infections (Cook et al. 2003).

1.8.3 Other receptors for LPS
Although TLR4 is regarded as the primary PRR for LPS, other receptors are capable of recognising the inflammatory bacterial by-product. The involvement of these receptors may also play a role in the protective/exacerbative responses observed as a result of LPS exposure. Preliminary reports involving TLR2 suggested an interaction with LPS (Kirschning et al. 1998; Yang et al. 1999; Yang
et al. 1998) but subsequent studies have clearly demonstrated TLR4 is the primary receptor (Du et al. 1999; Kirschning et al. 1998; Takeuchi et al. 1999).

Scavenger receptors, primarily located in macrophages are also found in intestinal epithelial cells bind and signal LPS (Sankala et al. 2002). To date these have not been found on airway epithelial cells (Krieger and Stern 2001). Finally, the nucleotide-binding oligomerization domain (NOD) proteins and associated receptors (NOD-like receptors, NLRs) have been implicated as an intracellular PRR partner to the TLRs in signalling for microbes (Inohara and Nunez 2003b; Mitchell et al. 2007). NOD1 was originally reported to signal for cytosolic LPS (Inohara et al. 2001). However, recent studies have identified the key structure to be recognised by this receptor as diaminopimelate-containing N-acetylglucosamine-N-acetyl-muramic acid tripeptide. This structure is found in the peptidoglycans of gram-negative bacteria and the dipeptide y-D-glyutamyl-meso-deaminopimelic acid (Girardin et al. 2003).

1.9 Project Outline

The key points of the literature review are centred on the capacity of the bronchial epithelium to induce immune and physiological airway responses that sometimes characterise the onset and exacerbation of chronic airway inflammation. The influence of LPS exposure in the bronchial epithelium is of considered importance due to reported roles in the development, exacerbation and postulated protective effects in allergic and non-allergic asthma. Asthma accounts for some of the
highest morbidity rates in children in western countries. Therefore, understanding the role of LPS in the onset and exacerbation asthma is crucial in clarifying the mechanisms guiding development of this disease.

The ability of LPS exposure to induce both exacerbative and protective immune effects in the host has been a conundrum in studies investigating LPS responses. Most studies favour the use of relatively pure LPS preparations for response measures. However, due to its molecular structure LPS is likely to enter the lung attached to particulates carried in inhaled air. If LPS is attached to other substances when it enters the lung lipid A, crucial for TLR4 signalling is potentially hidden. Therefore activation of another inflammatory process is possible. This is likely and confounds extrapolations made between models using pure LPS aiming to replicate epidemiological data. While some investigations have successfully explained observed exacerbative/protective effect of LPS exposure in asthma and atopy by their findings, questions remain concerning the role of the adsorbed substances in induced airway inflammatory responses measured epidemiologically.

To date there has not been an in vivo study investigating the location of TLR4 of different airway disease states in airway epithelial cells. Single studies have been completed assessing TLR4 expression in cystic fibrosis and COPD bronchial epithelium. These studies have used bronchial biopsies and found the receptor to reside on the cell surface ((Hauber et al. 2005; MacRedmond et al. 2007). This conflicts with the intracellular location of the receptor in other epithelial cell models (Guillot et al. 2004). Determining TLR4 location is an important element to any
investigation aiming to draw conclusions concerning the role of TLR4 in inflammatory airway disease. Differences in TLR4 expression and/or location may explain differential immune responses observed in LPS exposure between disease states. It will also shape airway epithelial cell models used to elucidate TLR4 generated responses.

1.10 Aims and outcomes

The overall aim of this investigation is to understand the influence of LPS and LPS when attached to PM in inducing airway epithelial inflammatory responses regulated by TLR4. Elucidating the role of different inflammatory profiles on TLR4 expression will form part of the initial study that will assist in identifying if TLR4 is subject to influences in the surrounding microenvironment (Aim 1). An in vitro model will then be developed to replicate expression and location of TLR4. These cells can then be used for subsequent LPS and LPS-PM exposures. The in vitro model will portray in vivo breathing conditions as closely as possible (Aim 2). This is deemed pivotal to interpretations made from measured AEC responses that relate to airway disease.

In addition to in vitro responses the effects of LPS and PM-LPS on airway responsiveness will be assessed (Aim 3). Utilising an animal model this data will allow a comparison of isolated airway epithelial responses to these exposures with the entire airway system in terms of measured inflammatory cytokine profiles and induced airway sensitivity.
A number of smaller studies have been designed in an attempt to fulfil the aims and outcomes for this thesis. Specifically these studies are as follows:

Aim 1: Determine expression and location of TLR4 in airway epithelial cells from different inflammatory airway disease states, namely asthma, atopy and cystic fibrosis.

Immunohistochemical and flow cytometry techniques will be used to examine cellular location and reverse transcription real-time PCR to measure gene expression of the receptor in these cells.

Aim 2: Assess the role of Th1 and Th2 polarised cellular environments on TLR4 location.

Stimulation of immortalised airway epithelial cells with IFNγ and IL-13 will be used to assess the influence of Th1 and Th2 skewed stimuli on TLR4 location and expression in bronchial epithelial cells. Comparing the results of these findings to those in Aim 1 will confirm a role for polarised cytokine microenvironments in any differences in TLR4 expression that may be observed in vivo. Alternatively, correlating the results of these studies may point to other regulatory mechanisms controlling expression of the receptor in vivo.
Aim 3: Develop a suitable *in vitro* model to measure bronchial epithelial responses to LPS and PM exposure via TLR4.

Using the results of investigations described in Aims 1 and 2 a suitable airway epithelial model to represent TLR4 expression and location will be developed. This will involve using an immortalised human bronchial epithelial cell line allowing influences of environmental exposure on TLR4 to be measured, dispensing the need for precious primary cultures. An emphasis on recreating *in vivo* breathing conditions (using aerosolised exposures) and cellular environment by using cultures grown at an air-liquid interface will be maintained in order to obtain responses as close to those in the airways as possible.

Aim 4: Measure responses of single LPS and mPM\textsubscript{0.5} exposures measured in airway epithelial cells compared to the combined effects of LPS and mPM\textsubscript{0.5} exposure.

Commercially available 0.5\textmu m latex beads will be used as a model of inert PM (mPM\textsubscript{0.5}). The airway epithelial exposure model developed in Aim 3 will be used to measure responses to LPS, mPM\textsubscript{0.5} and mPM\textsubscript{0.5} attached to LPS. Changes in TLR4 gene expression and induced inflammatory responses (measured by IL-8) will be compared across the exposure types. This data will be used to assess if changes associated with LPS exposure are indicative of inflammation.
Aim 5: Determine TLR4 dependence of Induced airway responsiveness to exposure to PM or LPS individually and in combination.

Finally, in order to extend the *in vitro* findings to responses *in vivo*, mice with and without a functional TLR4 will be exposed to \( \text{mPM}_{0.5} \), LPS and \( \text{mPM}_{0.5} \)-LPS to determine whether changes in airway mechanics accompany cellular inflammatory responses measured in the lung. These results will also allow for a comparison to *in vitro* exposures, thus elucidating the role of the airway epithelium in inflammatory responses generated *in vivo*.
Chapter Two

2 Materials and Methods

To avoid repetition a description of methods applicable to all chapters are described below. Methods particular to individual chapters are described therein. General protocols regarding equipment and reagents, are located in Appendices 1 and 2 respectively.

2.1 Cell culture

The transformed human bronchial epithelial cell line, 16HBE were used throughout chapters 3 and 4 as a model for AEC’s. Cells were grown in EMEM media supplemented with FCS (10% v/v) and gentamicin (100U/mL) in submerged culture to confluence on cell culture inserts. The apical media was then removed and the cells differentiated into a mucoid epithelium with retinoic acid supplementation. Once differentiated the cells were used for experimentation purposes.

Based on previous investigations involving TLR4 the U937 monocytic cell line and embryonic human kidney epithelial cell line, HEK293 cells, were used as the respective positive and negative controls for TLR4 expression for work completed in chapters 3 and 4. Details pertaining to their origin are given below. All cells were grown in a NUAIRE incubator at 37°C in an atmosphere of 5% CO₂/95% air under strict aseptic conditions.
2.2.1 HEK293

The HEK293 cell line was kindly supplied by Associate Professor Lai (Centre of Vision Science, University of Western Australia, Perth, Australia). This cell line was originally derived from a human embryonic kidney that was transformed using adenovirus 5 DNA (Graham et al. 1977; Shaw et al. 2002). For purposes relevant to this investigation, the HEK293 cell line was utilized as a negative control for TLR4 expression.

2.2.2 U937

The cell line designated U937 was obtained from Dr Meri Tulic (Telethon Institute for Child Health Research, Perth, Australia). Originally, this cell line was derived from a 37 year old Caucasian male patient with histiocytic lymphoma and is capable of being induced to terminal monocytic differentiation (Sundstrom and Nilsson 1976). For purposes relevant to this investigation, the U937 cell line was utilized as a positive control for TLR4 expression.

2.1.3 16HBE140

The immortalized bronchial epithelial cell line, designated 16HBE140, was kindly donated by Dr Dieter Gruenert (University of Vermont, USA). This cell line was originally established from human bronchial epithelium that was subsequently transformed using a Simian Virus 40 (SV40) large T-antigen. Assessment of the resulting transformed cell line showed that cells retained typical epithelial cell line characteristics including; the presence of tight junctions and cilia, the ability to
generate transepithelial resistance as well as retaining β-adrenergic stimulation of cAMP-dependent chloride ion transport (Cozens et al. 1994). For purposes relevant to this investigation, the 16HBE cell line was utilized in the establishment and assessment of the in vitro bronchial epithelial cell model. Details regarding the in vitro model development and assessment are presented in Chapters 3 and 4. For brevity this cell line is referred to as 16HBE.

### 2.2 Cell culture maintenance Introduction

#### 2.2.1 DMEM
Dulbecco's modified Eagle's medium with L-glutamine (DMEM) was purchased from Gibco (Australia). The HEK293 cell line was maintained in DMEM containing FCS (10% v/v), penicillin (100U/mL), streptomycin (100µg/mL) and amphotericin B (2.8µg/mL). The media was stored between 2-4°C under sterile conditions until required.

#### 2.2.2 EMEM
Earle's minimal essential media without L-glutamine was purchased from Gibco (Australia). The 16HBE cell line was maintained in EMEM containing FCS (10% v/v), penicillin (100U/mL), streptomycin (100µg/mL) and amphotericin B (2.8µg/mL). The media was stored between 2-4°C under sterile conditions until required.
2.2.3 RPMI-1640

RPMI-1640 Medium containing NaHCO$_3$, phenol red and L-glutamine, was purchased from Gibco, (Australia). The U937 cell line was maintained in RPMI-1640 containing FCS (10% v/v), penicillin (100U/mL), streptomycin (100μg/mL) and amphotericin B (2.8μg/mL). The media was stored between 2-4°C under sterile conditions until required.

2.3 Cell line subculture

If adherent in nature, cells were released from flasks by incubating with 0.05% trypsin and 0.53mmol/L EDTA in PBS for between 5-8mins at 37°C depending on the cell type. Otherwise, the medium containing the suspension cultures was collected. Cell suspensions were centrifuged (1500rpm for 5mins at 4°C) and the pellet resuspended in the appropriate culture medium. Cell counts were then performed using a haemocytometer and the cells then subcultured into new flasks.

2.4 Storage of cultured cells

Stocks of cell lines of varying passage were frozen down in their appropriate culture medium containing DMSO (10% v/v). Cells were removed from their flasks using (0.05% trypsin and 0.53mM EDTA). The cell suspension was then washed, centrifuged in ice cold medium and finally resuspended in media containing DMSO. Cells were then frozen down overnight at -80°C in sterile cyrovials at a density of between 1-2 x 10$^6$ cells/vial. On the following day, vials were placed in liquid nitrogen for long term storage.
2.5 Freeze thawing of stored cells

Stock of cell lines were taken from liquid nitrogen storage and quickly thawed at 37°C and diluted 1:10 in culture medium. The cell suspension was centrifuged for 5mins and the supernatant including the residual DMSO removed. Cells were then seeded into new flasks with fresh medium and assessed under the microscope for viability and light refractivity. All adherent cell lines were observed to adhere to the tissue culture flasks within 3hrs.

2.6 Analysis

2.6.1 Immunohistochemistry

Staining for TLR4 was the primary method used to assess TLR4 location and to semi-quantitate protein expression. AEC’s were prepared on glass slides by cytopsin and fixed using 2% paraformaldehyde these were stored at -20°C until required. Following rehydration with TBS, fixed AEC’s were quenched for autofluorescence using 0.2% Sudan Black. Enzyme antigen retrieval was performed using Proteinase K (Progen) 35 µg/mL for 35mins at 37°C before the specimens were blocked for non-specific binding with 1% goat serum in TBS. For AEC preparations assessed for intracellular staining TBS with 1% saponin was used for the initial rehydration and as the washing buffer and diluent for blocking agents and antibodies. Rabbit anti-human/mouse/rat TLR4 (Santa Cruz) used as the primary antibody and diluted 1/100 in TBS and applied to sections. These were
left to incubate overnight at 4°C. Following multiple washes in TBS, a 1/50 dilution of Alexa 546-conjugated secondary antibody was and sections were incubated for 1hr at 4°C. A cytoplasmic marker, wheat germ-agglutinin, was diluted 1:1000 in 1% saponin in TBS. This stain was applied for 15mins for use in localising the TLR4 stain in permeabilised sections. After the final stain was applied, sections were washed in TBS and mounted in a fluorescent preserving mounting media before being viewed. Fluorescent staining was detected using appropriate filters of a Leica Microscope attached to a UV lamp. A Leica DC300F digital camera mounted to the microscope was used to produce the images by the Leica IM50 Image Manager software presented in these studies.

2.6.2 Reverse transcription real time PCR

Samples of interest were stored at –80°C in Qiagen RLT buffer until required. The Qiagen Rneasy kit was used for RNA extraction. Briefly, samples were thawed and placed on a Qiagen QIAshredder. Following this step RNA was precipitated out using 70% ethanol and then collected using a series of washing steps to elute the extracted RNA on the Qiagen mini-column.

Gene expression was analysed by two-step RT-PCR reactions. cDNA was synthesised using hexanucleotide primers and Multiscribe™ Reverse Transcriptase (Applied Biosystems, CA, USA) in a final reaction volume of 25μL containing 1 X RT buffer, 5.5mM MgCl₂, 0.5mM of each of the dNTPs, 2.5μM random hexamers, 0.4U/μL RNase inhibitor, 0.5U/μL Multiscribe reverse transcriptase and 200ng RNA. The reactions are performed under the following conditions: initial primer incubation step at 25°C for 10mins followed by RT
incubation at 48°C for 1hr and ended by reverse transcriptase inactivation at 95°C for 5 mins. The cDNA was then used in a final PCR reaction volume of 25μL containing 1 X Sybr green PCR master mix (Applied Biosystems), 0.7mM of human TLR4 primer and 0.5mM of human β-actin housekeeping primers each of forward and reverse primers and 5μL of cDNA. The conditions for the PCR include initial incubation at 50°C for 2mins, AmpliTaq Gold DNA polymerase activation at 95°C for 10 mins followed by 40 cycles of 15secs at 95°C and 1mins at 60°C. Primer sequences used for gene expression analysis are listed in Table 2.1. Amplification data generated was analyzed using the Comparative delta cycle threshold (Ct) method.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>CAACCAAGAACCTGGACCTG</td>
<td>GAGAGGTGGCTTAGGCTCTCTG</td>
</tr>
<tr>
<td>β-actin</td>
<td>TCTTTCCAGCCTTCTTCCTCG</td>
<td>AGCAGTGTGGGCGGTACAG</td>
</tr>
</tbody>
</table>

Table 2.1 Primer sequences used for gene expression analysis

2.6.3 IL-8 ELISA
Measurement of IL-8 in supernatants collected from cultures completed using the Pharmingen OPTeia IL-8 ELISA kit following the manufacturer’s instructions. Plates were coated with capture antibody overnight and then blocked while supernatants were thawed so that they could be added immediately to the plate after washing. After incubating at room temperature, the samples were removed and plates washed before addition of the detection antibody. Following this
incubation at room temperature plates were washed again and colour detection reagent added these were kept in the dark for 30mins to allow for colour development before the addition of the stop solution. Sample absorbance readings were then made at 405nm using a plate reader.

2.7 Statistical methods

The One Kolmogorov-Smirnov test was used to assess patient data for a normal distribution. This statistical method is used to test the null hypothesis that a sample is from a particular distribution. A significant Kolmogorov-Smirnov value indicates that a particular variable is not from a normal distribution. Using this test the data presented in this thesis was confirmed as being normally distributed.

Data was compared and presented as a comparison to controls using one-way ANOVA. These are reported as means ± SE, with means considered significantly different at the p<0.05 level. For analysis of differences between groups, the Tukey post-hoc statistical test was used with significance taken at the p<0.05 level.
Chapter Three

3 Characterising TLR4 location and expression in the human airway epithelium.

3.1 Introduction

Genes and proteins expressed by airway epithelial cells (AECs) are influenced by the external environment and their immediate cellular milieu. This chapter investigates the influence of internal environmental signals on TLR4 location and expression in AECs from healthy patients and those with atopic asthma and cystic fibrosis.

In recent years the hygiene hypothesis has formed a foundation for explaining the polarised cytokine profiles secreted by lymphocytes in response to specific microbial substrates (Del Prete et al. 1991). Microbial infections are thought to stimulate a Th1 profile by inducing secretion of IL-12 which causes IFN-γ release from a variety of cells including epithelial cells, dendritic cells, neutrophils and lymphocytes (Luster et al. 2005). This results in a cascade of microbicidal activity including macrophage and monocyte recruitment (Moreno et al 2006). Raised interleukin (IL)-4, IL-5, IL-10 and IL-13 have been shown to characterise a Th2 profile (Mosmann et al. 1986). In addition to lymphocytes these cytokines are released by epithelial cells, dendritic cells, eosinophils and macrophages (Luster et al. 2005). This profile has been shown to enhance antibody production and
eosinophil proliferation and is thereby associated with allergy. While raised IgE levels (used as a marker of atopy) are strongly correlated with a Th2 profile the mechanisms linking these two remain to be elucidated (1998; Coker et al. 2003; Luster et al. 2005).

Asthma severity increases with atopic sensitisation (Carroll et al. 2007; Larche et al. 2003). Of the Th2 cytokines IL-13 is significantly raised bronchoalveolar lavage (BAL) from asthmatics with atopy (Huang et al. 1995) and is considered a central mediator of allergic asthma (Wills-Karp et al. 1998). IL-13 has been shown to induce airway hyper-responsiveness and eosinophilia in mice, hallmarks of an asthma-like phenotype (Grunig et al. 1998; Zhu et al. 1999).

IFNγ recruits monocytes and macrophages to increase their secretion of proinflammatory cytokines (Trinchieri 1997) and is thought to drive the Th1 profile by this positive feedback loop. In cystic fibrosis (CF) patients suffer recurrent bacterial infections. Subsequently bronchoalveolar lavage from CF patients is characterised by a high neutrophil infiltrate and raised amounts of proinflammatory cytokines. Secreted cytokines measured in BAL from CF patients is associated with neutrophil recruitment and microbial infection such as IFNγ and IL-8 (Bonfield et al. 1995; Muller et al. 1999).

The Th1/Th2 paradigm serves as a tool for understanding potential interactions between the innate and adaptive immune systems in various disease states. For example, in atopic asthma, an airway disease thought to be caused by aberrant adaptive immune responses there is particular interest in understanding the role of
TLR4 (innate immunity). By adding IL-13 and IFN-γ to AEC cultures the effects of polarised cytokine environments on TLR4 expression can be investigated. This may increase our understanding of how AEC’s respond differently to dust borne LPS.

In this chapter, TLR4 expression and its location was examined in freshly isolated AECs from healthy children and from children with asthma and cystic fibrosis. The influence of extracellular signals on TLR4 location was investigated using IL-13 and IFNγ stimulated AEC cultures. Reverse transcription real-time (RT) PCR was used to examine TLR4 gene expression. Protein expression and location was examined using flow cytometry and immunohistochemistry analysis.
3.2 Methods

3.2.1 Patient samples

Paediatric derived airway epithelial cells bronchial epithelial cells (pAEC) were harvested via trans-laryngeal, non-bronchoscopic brushing of the tracheal mucosa through an endotracheal tube (Lane et al. 2005b). The method of obtaining epithelial cells was approved by the Princess Margaret Hospital for Children's Human Ethics Committee and written consent to all procedures described were obtained from each participant's legal guardian after being fully informed about the nature and purpose of this study. Patient consent and bronchial brushings for this study were completed by the Princess Margaret Hospital for Children Respiratory Medicine Research Fellows 2002-2005; Dr. Scott Burgess, Dr. Tonia Douglas, Dr. Amanda Griffiths, Dr. Julian Legg, Dr. Andrew Martin and Dr. Andre Schultz. The approved Ethics Application can be found in Appendices 3 and 4.

Children with a pre-existing bacterial or viral chest infection were excluded from this study. All subjects had spirometry and skin prick reactivity to common allergens measured prior to surgery or at the first outpatient follow-up.

3.2.1 Cell culture

A portion of the freshly isolated pAEC cells collected were cultured for experimentation purposes. The 16HBE cell line served as a model of the pAEC cultures for some experiments. The U937 monocytic cell line was used as the positive control for TLR4 expression in mRNA and protein analysis. HEK292 cells were used as the negative control for TLR4 staining in flow cytometry analysis.
Culturing and cell maintenance conditions for all cell lines listed here can be found in Chapter 2 section 2.1.

3.2.1.1 Paediatric derived airway epithelial cells (pAEC)

Following consent and once each child was anaesthetized and intubated a disposable single sheath bronchial cytology brush (BC 25105, Olympus, Vic, Australia) was advanced through the endotracheal tube. After brushing and withdrawal from the endotracheal tube, the brush tip was inserted into cold sterile media (RPMI-1640) containing 10% (v/v) heat inactivated fetal calf serum. Cells were washed once in RPMI-1640 media and the cell pellet resuspended in bronchial epithelial basal medium (BEBM) supplemented with; bovine pituitary extract (50mM), 0.5μg/mL hydrocortisone, 20ng/mL human epidermal growth factor, 0.5μg/mL epinephrine, 6.5ng/mL triiodothyronine, 5μg/mL insulin, 10μg/mL transferrin, retinoic acid (0.3μM), 0.001% gentamycin (v/v) and amphotericin B (0.0005%). With added supplements, the media is referred to bronchial epithelial growth medium (BEGM). Both BEBM and BEGM were stored between 2-4°C under sterile conditions until required.

Macrophages were removed by incubating the cell suspension in a culture dish pre-coated with the CD-68 antibody (DAKO, New South Wales, Australia) for 20mins in a humidified incubator (37°C, 5% CO₂ / 95% air). A portion of the collected suspension of purified pAEC were stored for mRNA analysis, cytospun for immunohistochemical analysis or seeded for culturing purposes. Non-cultured pAEC cells are here on referred to as freshly isolated pAEC cells.
3.2.1.2 Cultured primary pAEC
Freshly isolated pAEC cells to be cultured were seeded into a culture vessel (25cm$^2$ growth surface area) that were pre-coated with a mixture of fibronectin (10mM), collagen S (30mM) and bovine serum albumin (100mM) and maintained at 37°C in a humidified atmosphere of 5% CO$_2$ / 95% air. Twenty four hours post-isolation, unattached cells residing in the supernatant were collected, pelleted by centrifugation and reseeded back into the same culture vessel. At this point, Ultroser G, a serum substitute, was also added (2% v/v) to the culture media. Subsequent cultures were then fed every second day and passaged at 70% confluence (approximately every 13-16 days) using trypsin dissociation.

3.2.2 Stimulation of 16HBE cells
16HBE cells were grown to 80% confluence at air liquid interface on 6 well Transwell inserts (Costar). At 72hrs prior to stimulation 16HBE cells were serum starved and a final media change performed 24hrs prior to stimulation. Cells were stimulated with doses representative of physiologically normal levels of either IFN$_\gamma$ (200U/mL) or IL-13 (10pg/mL) (Silva-Teixeira et al. 2004) and harvested at 1, 6 and 24hrs post stimulation. Cell pellets were collected for analysis by flow cytometry and gene expression by RT-PCR.

3.2.3 Analysis of collected samples
3.2.3.1 Immunohistochemistry
Freshly isolated pAECs, cultured pAECs and 16HBE cells were stained for TLR4 using the immunofluorescence method described in Chapter 2 (Section 2.6.1) to characterise receptor location. Non-specific binding was assessed by staining the
positive control with only the secondary antibody. This slide also served as the negative control. Semi-quantitative analysis of TLR4 protein expression was made by scoring staining intensities relative to the intensity of the positive control as outlined in Table 3.1. This scoring method was based on the HerceptTest®; a Federal Drug Agency (USA) approved in vitro diagnostic assay marketed by DAKO for semi-quantitative immunohistochemical analysis. This test permits breast cancer diagnosis by quantifying HER2 protein (human epidermal growth factor receptor) overexpression in breast cancer tissue. Due to a lack of quantitative methodology available for immunohistochemical staining this scoring system has been used to quantify staining intensities in studies of other proteins (Brustmann 2004; Kowalski et al. 2003).

In this study TLR4 staining intensities were scored relative to baseline scores assigned to the positive (+1) and negative (0) controls as outlined in Table 3.1. This was completed by at least 4 independent observers to ensure minimisation of any scoring bias. To restrict staining variability all groups were stained and photographed within a staining run. The normality of each run was assessed by comparing positive and negative controls between runs which were found to be highly reproducible. Receptor location was assessed by comparing TLR4 staining patterns present in permeable cells (saponin treated samples) with staining for a cytoplasmic marker (Wheat germ agglutinin). Co-localisation of these patterns was used to indicate an intracellular receptor domain.
<table>
<thead>
<tr>
<th>Score</th>
<th>Assessment of protein overexpression</th>
<th>Staining pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Negative</td>
<td>No staining is observed</td>
</tr>
<tr>
<td>1+</td>
<td>Negative</td>
<td>A faint barely perceptive staining is detected</td>
</tr>
<tr>
<td>2+</td>
<td>Positive</td>
<td>A weak to moderate complete staining is observed</td>
</tr>
<tr>
<td>3+</td>
<td>Positive</td>
<td>A strong complete staining is observed</td>
</tr>
</tbody>
</table>

Table 3.1 Scoring system used to semi-quantitate immunohistochemical staining intensity for TLR4 expression.

3.2.3.3 TLR4 mRNA expression

TLR4 mRNA expression was measured across all freshly isolated and cultured samples using RT-PCR. The specific protocol for gene expression analysis and primer sequences can be found in Chapter 2 (Section 2.6.2).

3.2.3.4 Flow cytometry analysis

Following stimulation with IL-13 and IFNγ, 16HBE cells were collected for analysis of TLR4 location by flow cytometry. This protocol required 3 staining steps, an initial incubation with the primary antibody (anti-human TLR4, 1:100) followed by an incubation for biotinylation (goat anti-human biotin, 1:200) to allow for
adherence of the strepavidin on phycoerythrin label (strepavidin PE, 1:50). All incubation steps were completed on ice. Between incubations cells were washed by adding 1mL of media wash (DMEM with 10% v/v FCS) and centrifuged for 5 mins at 5000 rpm at room temperature. After the final wash step the cell pellet was fixed in 0.2mL of 4% paraformaldehyde on ice for 15mins. These were then washed in PBS, centrifuged and the pellet resuspended in 0.3mL PBS ready for flow cytometry.

The U937 and HEK293 cell lines were used as the respective positive and negative controls for TLR4 staining. An isotype control (stem cell marker, anti-human p63, BD Biosciences) was included in every run to test for non-specific binding of the secondary antibody.

3.2.4 Statistical analysis

Experiments using cell lines were assessed for statistical significance using a normalised student t-test. The sample population was considered to have a normal distribution by the One Sample Kolmogorov-Smirnov test. Therefore differences between disease states were compared using ANOVA with post-hoc Tukey analysis. Means were considered significantly different at the p<0.05 level.
3.3 Results

3.3.1 Clinical characteristics

Bronchial brushings were obtained from children up to 16 years of age. Analysis of spirometry and skin prick tests from these patients revealed four distinct phenotypes namely, healthy non-atopic (pAEC_{HNA}), healthy atopic subjects (pAEC_{HA}), asthmatic non-atopic (pAEC_{ANA}) and mild atopic asthmatics (pAEC_{AA}).

Bronchial epithelial cells obtained from children with CF (pAEC_{CF}) (6-weeks to 6 years) were recruited from a cohort of 45 - 60 children who attend Princess Margaret Hospital as part of an annual bronchoalveolar lavage program. This included between 10 and 12 children who were newly diagnosed, with most being diagnosed through the newborn screening program and being identified within the first 4-6 weeks of life. The clinical characteristics for all the patient samples used in this study are presented in Table 3.2. Cultured pAEcs derived from this sample group and used for analysis in this study are also specified. A single sample collected from a patient with CF was positive for *Pseudomonas aeruginosa* colonisation following bacterial screening of all CF samples. This sample was included in all subsequent analysis completed for this study.
<table>
<thead>
<tr>
<th>pAEC phenotype</th>
<th>Sex</th>
<th>Average age (Yrs)</th>
<th>Age range (Yrs)</th>
<th>No.</th>
<th>Total No. for each phenotype (n)</th>
<th>No. used for culture analysis</th>
<th>No. of asthmatics on medication</th>
<th>No. of children with CF bacterial colonisation of the airways</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAEC_{HNA}</td>
<td>F</td>
<td>7.5</td>
<td>3.7-10.4</td>
<td>7</td>
<td>15</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>6</td>
<td>1.7-10.0</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAEC_{HA}</td>
<td>F</td>
<td>13.5</td>
<td>11.5-15.5</td>
<td>2</td>
<td>9</td>
<td>3</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>M</td>
<td>5</td>
<td>2-8.5</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAEC_{ANA}</td>
<td>F</td>
<td>11.8</td>
<td>10.4-13.1</td>
<td>2</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>4</td>
<td>2.0-5.9</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAEC_{AA}</td>
<td>F</td>
<td>8</td>
<td>3.6-12</td>
<td>7</td>
<td>10</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>7.8</td>
<td>2.4-16</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAEC_{CF}</td>
<td>F</td>
<td>3</td>
<td>2.2-4.0</td>
<td>3</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>3.6</td>
<td>2.9-4.3</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Clinical characteristics

This table shows the clinical information and characteristics of patients from whom pAECs were collected. The number of pAEC cultures derived from these groups used for analysis in this study is also specified (F= Female, M = Male).
3.3.2 TLR4 gene expression

TLR4 gene expression in freshly isolated and cultured pAEC from each airway disease state was normalised to the freshly isolated pAEC_{HNA} that served as the control. TLR4 mRNA expression was greatest in pAEC_{AA} and pAEC_{CF} freshly isolated pAEC cells compared to pAEC_{HNA}, pAEC_{HA} and pAEC_{ANA} cells. There were no measurable differences in mRNA expression between pAEC_{HNA}, pAEC_{HA} and pAEC_{ANA} freshly isolated cells (Figure 3.1A). Additionally, there were no differences observed in TLR4 mRNA between cultured pAEC cells from different airway disease states. Cultured pAEC cells had a similar TLR4 mRNA expression to the 16HBE cell line which was included for an airway epithelial cell line comparison (Figure 3.1B).

Freshly isolated pAEC_{AA} had a significantly increased TLR4 mRNA than the cultured pAEC_{AA}, illustrating a marked decrease of TLR4 mRNA expression \textit{in vitro} (Figures 3.1A and B). This trend did not hold for cultured pAEC cells from other disease states. Rather, mRNA expression was not significantly different from pAEC_{HNA}, pAEC_{HA} and pAEC_{ANA} cultured cells and their freshly isolated counterparts (Figures 3.1A and B). Cultured pAEC_{CF} patients were not available for comparison.
Figure 3.1A TLR4 gene expression in freshly isolated pAEC’s from different phenotypes.

RNA was extracted from freshly isolated pAEC collected from bronchial brushings. TLR4 mRNA expression was analyzed by real-time PCR using SYBR Green and normalised to the freshly isolated pAEC_HNA control group (n=5). Significantly more TLR4 mRNA was measured in cells from pAEC_CF and pAEC_AA’s (†) with pAEC_AA’s having the greatest TLR4 mRNA overall (#). Significance detected (p< 0.05)

Figure 3.1B TLR4 gene expression in cultured pAEC’s from different phenotypes.

pAEC’s cultured from freshly isolated cells collected by bronchial brushing were harvested at the second serial passage (p2) and RNA extracted (n=3). TLR4 mRNA expression was measured and normalised as for the freshly isolated pAEC. 16HBE transformed cell line TLR4 mRNA expression was included for comparison.
### 3.3.3 TLR4 Protein Expression

TLR4 protein (red) was present on the cell surface and intracellularly in freshly isolated cells as determined by comparison with a cytoplasmic marker (green) (Figure 3.2A, B). Staining was predominantly intracellular in cultured pAEC cells and the 16HBE cell line (Figure 3.2B). Freshly isolated pAEC_{HA} and pAEC_{CF} cells had the greatest cell surface staining intensity for TLR4. pAEC_{AA} cells had the greatest respective intracellular staining intensity. The least intense staining for cell surface and intracellular TLR4 was recorded for cultured pAEC_{HNA} and freshly isolated pAEC_{HA} respectively (Table 3.3). Cell surface TLR4 protein was less intense in cultured pAEC_{HNA} and cultured pAEC_{HA} compared to their freshly isolated counterparts while intracellular expression was unchanged (Table 3.3). Limited sample preparations in the cultured pAEC_{ANA} and pAEC_{AA} groups and unsuccessful culturing in the cultured pAEC_{CF} group prevented TLR4 protein analysis by immunohistochemistry in these groups.

Adding the relative staining intensity scores for a total protein score gives a semi-quantitative indication of overall protein expression in these cells. Cultured pAEC_{HNA} had the least TLR4 protein and freshly isolated pAEC_{AA} the greatest (Table 3.3).
Figure 3.2A Immunohistochemical staining of freshly isolated permeable and non-permeable pAEC cells for TLR4.

Freshly isolated pAEC_{HNA} pAEC_{HA} and pAEC_{ANA} cells were treated with 1% saponin to permeabilise the membrane to assess intracellular TLR4 (red) staining (Santa Cruz 1:100) relative to a cytoplasmic (green) marker (Wheatgerm agglutinin 1:1000). Cell surface staining was also completed on non-permeable cells (n=5) (200x magnification). Semi-quantitative analysis was completed by scoring TLR4 staining intensity relative to that observed in the U937 monocytic cell line (positive control) using the scoring panel in Table 3.2.
Figure 3.2B. Immunohistochemical staining for TLR4 in freshly isolated and cultured pAEC cells from different airway disease states.

Similarly to Figure 3.2A freshly isolated pAEC_{AA} and pAEC_{CF} patients were stained for TLR4 (red). Intracellular expression was assessed by comparison to a cytoplasmic marker (green) in cells treated with 1% saponin for permeabilisation. TLR4 surface staining was assessed in non-permeable cells (n=5). Cultured pAEC cells (p2) from healthy non-atopics (cultured pAEC_{HNA}) and atopics (cultured pAEC_{HA}) were similarly stained and scored as outlined in Table 3.2 for comparison (n=3)(200x magnification).
<table>
<thead>
<tr>
<th>Sample name</th>
<th>Surface TLR4 staining score</th>
<th>Intracellular TLR4 staining score</th>
<th>Overall protein score</th>
</tr>
</thead>
<tbody>
<tr>
<td>16HBE</td>
<td>0</td>
<td>2+</td>
<td>2</td>
</tr>
<tr>
<td>U937</td>
<td>3+</td>
<td>2+</td>
<td>5</td>
</tr>
<tr>
<td>pAEC_{HNA}</td>
<td>2+</td>
<td>2+</td>
<td>4</td>
</tr>
<tr>
<td>pAEC_{HA}</td>
<td>3+</td>
<td>1+</td>
<td>4</td>
</tr>
<tr>
<td>pAEC_{ANA}</td>
<td>1+</td>
<td>3+</td>
<td>4</td>
</tr>
<tr>
<td>pAEC_{AA}</td>
<td>3+</td>
<td>3++</td>
<td>6+</td>
</tr>
<tr>
<td>pAEC_{CF}</td>
<td>3+</td>
<td>3+</td>
<td>6</td>
</tr>
<tr>
<td>Cultured pAEC_{HNA}</td>
<td>1+</td>
<td>2+</td>
<td>3</td>
</tr>
<tr>
<td>Cultured pAEC_{HA}</td>
<td>2+</td>
<td>2+</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3.3. Summary of staining patterns for TLR4 in freshly isolated and cultured pAEC.

A summary of the staining patterns exhibited in Figures 3.2A and B are presented here. Staining intensity was semi-quantitated according to the guidelines in Table 3.2 by 4 observers. A more intense staining exceeding the scoring guidelines (as based on the Herceptest®) was noted for intracellular pAEC_{AA} and extracellular pAEC_{HA} stains as indicated by a 3++ score. An overall protein score was produced by adding surface and intracellular staining scores.
3.3.4 Stimulation of 16HBE cells

16HBE cells stimulated with IFNγ did not show a significant change in cell surface expression of TLR4 compared to control cells at any of the incubation times measured (Figure 3.3A-C and Figure 3.4). Following IL-13 stimulation there was a significant increase in cell surface staining for TLR4 at 6hrs stimulation (Figure 3.3E, Figure 3.4). This change was not present after stimulation for 24hrs and TLR4 staining appeared less intense than staining observed in control cells (Figure 3.3F and Figure 3.4). These trends were observed for all replicates of IFNγ and IL-13 stimulated 16HBE cells measured (n=3).
Figure 3.3 TLR4 location varies with cytokine stimulation.

16HBE cells were stimulated with IFNγ (A-C) or IL-13 (D-E). Cell surface staining for TLR4 expression was measured using flow cytometry at 0.5hrs (A, D), 6hrs (B, E) and 24hrs (C, F) post-stimulation. Non-stimulated 16HBE cells served as controls and are presented as shaded regions for each plot. Each experiment consisted of 10000 live cells. Together these plots form one representation of the data set completed (n=3).
Figure 3.4 Mean fluorescent density values for TLR4 staining in stimulated 16HBE cells analysed by flow cytometry.

16HBE cells were stimulated with IFNγ or IL-13 and cell surface staining for TLR4 expression was measured using flow cytometry at 0.5hrs, 6hrs and 24hrs post-stimulation. A significant change in TLR4 expression was observed for IL-13 stimulated 16HBE cells at 6hrs (p<0.05). These trends were observed for all replicates for each stimulation (n=3). Each bar represents TLR4 staining intensity for 10000 live cells.
3.4 Discussion

The results of this chapter show for the first time that the magnitude of TLR4 gene expression in the bronchial epithelium differs between different phenotypes (Figure 3.1), with a rank order of pAEC\textsubscript{AA}>>pAEC\textsubscript{CF}>>pAEC\textsubscript{ANA}, pAEC\textsubscript{HA}, pAEC\textsubscript{HNA}. Airway disease state also appears to influence TLR4 location in AECs. For example, intense TLR4 expression in pAEC\textsubscript{CF} cells was noted on the cell surface whereas staining for TLR4 in pAEC\textsubscript{AA} cells was predominantly intracellular.

Similarly, \textit{in vitro} conditions were shown to produce changes in TLR4 gene expression and receptor location. For example, freshly isolated pAEC\textsubscript{AA} had greater TLR4 mRNA compared to cultured pAEC\textsubscript{AA}. The significantly lower expression of TLR4 observed in cultured pAEC\textsubscript{AA} compared to their freshly isolated counterparts suggests missing stimulant(s) in the culture conditions. Overall, cells in culture displayed a predominant intracellular staining pattern for TLR4. The immortalised bronchial epithelial cell line 16HBE did not stain for TLR4 expression on the cell surface. As all of the cultured cells stained were monolayers and all contained a higher proportion of intracellular staining for TLR4, this result may indicate that the receptor resides in the intracellular domain in cells that are less differentiated. This may also be attributable to a lack of milieu signals keeping the receptor on the surface \textit{in vivo} that are not found \textit{in vitro}.

Surprisingly little variance in TLR4 mRNA expression was observed between freshly isolated or cultured pAEC\textsubscript{HNA}, pAEC\textsubscript{HA} and pAEC\textsubscript{ANA}. The sum of staining intensity scores for these groups was the same (Table 3.3). This semi-quantitative measure of protein expression supports the lack of variation in TLR4 mRNA.
expression observed between these groups. Interestingly trends between mRNA expression and TLR4 protein scores match for the freshly isolated pAEC cells but appear inversely proportional for cultured pAEC cells. Cultured pAEC_{RNA} and pAEC_{HA} cells have significantly greater amounts of TLR4 mRNA expression compared to their freshly isolated counterparts despite similar protein scores. This finding correlates with a study investigating TLR4 expression in cultured airway epithelial cell lines. Protein and gene expression were shown not to correlate across the cell four cell lines measured (Guillot et al. 2004; Kowalski et al. 2003). Combined with the findings presented here this suggests a possible 'culture' effect on TLR4 transcription.

The variation in cellular TLR4 location observed between airway disease states lead to investigating the possible influence of Th1 vs. Th2 signals. IL-13 was chosen as a stimulant since it is a dominant Th2 cytokine with a demonstrated role in the onset of asthma-like symptoms (Chung et al. 1999; Humbert et al. 1997; Wong et al. 2001). In 16HBE cells, stimulation with IL-13 induced translocation of TLR4 to the cell surface after 6hrs. The dose of IL-13 used for stimulation was based on normal physiological sera levels (Silva-Teixeira et al. 2004), suggesting a role for IL-13 in maintaining TLR4 protein expression on the cell surface. This data suggests raised levels of IL-13 and other signals present in the asthmatic phenotype contribute to an intracellular TLR4 location in pAEC_{AA}. IFNγ, a potent Th1 cytokine, did not induce TLR4 expression on the cell surface. Like IL-13 the dose of IFNγ was based on normal sera levels. There are many cell types that secrete a mixture of Th1 and Th2 cytokines. Some of these are located close to the
airway epithelium to assist with generating immune responses to combat invasion quickly. Dendritic cells, neutrophils, eosinophils, lymphocytes and macrophages are all sources of secreted cytokines able to influence concentrations bathing the epithelium (Luster et al. 2005). Therefore cytokine concentrations adjacent to the airway epithelium are likely to differ from those reported in sera levels. An extended dose response range of IL-13 and IFNγ on these cells is warranted to confirm the physiological relevance of the concentrations used here. In addition, a range of cytokines have been observed in polarised Th1/Th2 profiles. The influence of these individually or in combination would provide further insight into the regulatory mechanisms behind TLR4 location.

In this study 5 biological replicates were used for TLR4 gene and protein analysis. Sample size was limited by the invasive nature of the bronchial brushing procedure. An endo-tracheal tube is required to insert the cytology brush down the trachea into the bronchioles to perform the brushing. Therefore collections are restricted to children in surgery under general anaesthetic. To account for inter-sample variation and minimise the noise in data, microarray investigations recommend a sample size of 20 for human gene analysis (Wei et al. 2004). Inbred animals of the same strain and cell lines were shown to need 4 times less due to the genetic homogeny present in these samples (Wei et al. 2004). In this study TLR4 mRNA and protein expression appeared to have a robust expression trend within the grouped phenotypes suggesting a larger sample size would consolidate the differences shown here.
Primary cell and cell line work was completed on 3 biological replicates. This is lower than the recommended value \((n=5)\) however the low variances observed in cell line responses mean a lower number of replicates is acceptable and commonly published \((\text{Lee et al. 2000; Tan et al. 2003; Wei et al. 2004})\). At the time of this study optimising of pAEC culturing conditions had just completed \((\text{Kicic et al. 2006; Lane et al. 2005b})\). Therefore the number of primary cell cultures available had been few due to various deleterious factors on cell viability including routine contamination \((\text{cystic fibrosis cultures})\) and poor cell proliferation. Completing TLR4 protein expression in cultured \(\text{pAEC}_{\text{ANA}}, \text{pAEC}_{\text{AA}}\) and \(\text{pAEC}_{\text{CF}}\) samples would enhance the conclusions drawn from this study.

In summary, the results from this study demonstrate a variation in TLR4 expression and location between different airway disease states. Of those assessed, \(\text{pAEC}_{\text{AA}}\) and \(\text{pAEC}_{\text{CF}}\) were shown to have the greatest TLR4 gene and protein expression. The change in TLR4 location in 16HBE cells stimulated with IL-13 demonstrates the influential role of particular extrinsic stimuli on TLR4 location. Therefore, we believe further investigation into differences in TLR4 gene and protein expression observed in freshly isolated pAEC cells from different airway disease states are a likely result of the surrounding interstitial cytokine milieu. This is illustrated further by the difference in TLR4 mRNA expression in cultured \(\text{pAEC}_{\text{AA}}\) compared to freshly isolated \(\text{pAEC}_{\text{AA}}\) cells and the change in location of TLR4 in freshly isolated \(\text{pAEC}_{\text{HA}}\) to cultured \(\text{pAEC}_{\text{HA}}\). In these cells TLR4 expression and location appears linked to the donor atopic status, implicating microenvironment Th1/Th2 polarity. The impact of these differences on receptor generated responses remains unclear.
To investigate the role of TLR4 location on responsiveness, an *in vitro* model designed to replicate *in vivo* airway epithelial conditions has been established. This model will be used to assess the responses of airway epithelial cells to LPS as it commonly exists in inhaled air, as LPS attached to particulate matter. The validation of this model and measured responses in relation to TLR4 location are presented in Chapter 4.
Chapter 4

4 Responses of the human bronchial epithelium to environmental LPS exposure.

4.1 Introduction

Numerous epidemiological studies have linked chronic LPS exposure to the onset of COPD, chronic bronchitis and asthma, all of which are characterised by chronic inflammatory processes (Michel et al. 1996; Reed and Milton 2001; Rizzo et al. 1997). In support of this, murine models of chronic LPS exposure have demonstrated pathologic changes such as airway wall thickening, mucus cell metaplasia and irreversible alveolar enlargement (Brass et al. 2007; Savov et al. 2002). The negative charge on the LPS molecule allows for easy attachment to other substances, most likely to be some sort of airborne particulate (commonly regarded as fine PM). Despite a well established association of LPS exposure and airway disease (Baur et al. 1998; George et al. 2001; Kim et al. 2007) and suggestions LPS enters the lung attached to PM (Becker et al. 2002) a study clarifying the responses of attached LPS responses to non-attached has not been completed to date. In addition, alternate signalling pathways to TLR4, activated by LPS attached to PM have not been investigated.

As with LPS exposure, ambient levels of PM have been linked to increased respiratory symptoms (Brunekreef and Forsberg 2005; Jagielo et al. 1996; Pope, III and Dockery 1992b; Samet et al. 2007; Schwartz et al. 1994), impaired lung
function (Delfino 2002) and exacerbations of asthma (Moreland et al. 2001). The inflammatory effects of PM have also been shown in both in vitro (Becker et al. 2002; Fujii et al. 2002a; Mukae et al. 2000) and in vivo ((Dick et al. 2003) Tang et al. 2008) studies, suggesting the involvement of PM as a non-allergic stimulant of airway inflammation. However, the mechanism(s) that lead to airway inflammation following PM exposure remain tenuous. Research to date has identified the interaction of reactive oxygen species (ROS) with calcium channels inducing secretion of inflammatory cytokines in vitro (Brown et al. 2001). Interaction of PM with peripheral sensory nerves protruding the epithelial spaces have also been linked with inducing airway inflammation in vivo (Hsu et al. 1998; Lee and Pisarri 2001; Nurkiewicz et al. 2006). While cellular responses to LPS exposure are mediated through TLR4, mechanisms for cellular responses to PM remain to be elucidated (Wang et al. 2008).

Studies investigating the effects of PM$_{10}$ exposure have generally relied on residual oil fly ash (ROFA) (Gavett et al. 1999), diesel exhaust particulate (DEP) (Marano et al. 2002) or collected ambient PM$_{10}$ (Reibman et al. 2002). These were chosen by investigators for their ability to represent the environment being studied, such as responses to the presence of metals in industrial areas, traffic pollution, or ambient air. This rationale introduces multiple variables such as the types of metals or chemicals present and the presence of allergens. Therefore, while ROFA, DEP and collected ambient PM might represent PM; they may also contain TLR4 activating agents. Mechanistic differences in signalling of LPS when it is carried by PM are difficult to assess using any of these models. For this reason, we have
chosen to use polystyrene beads (0.5μm) to represent inert fine particulate matter (referred to as mPM$_{0.5}$).

In this chapter we establish a representative *in vitro* model of *in vivo* airway epithelial cell exposure to inhaled air. AEC’s in contact with inhaled air are ciliated and found at an air-liquid interface. To date, most studies of airway epithelial cells *in vitro* have used submerged cultures. This is a likely result of ease and time efficiency but overlooks the effect of differentiated cells on measured responses. The 16HBE cell line differentiates at an air liquid interface with retinoic acid supplemented medium (Wu *et al.* 1997). In this study differentiated 16HBE cells were subject to an aerosol exposure of either LPS and/or mPM$_{0.5}$.

Epithelial cell-derived IL-8 expression was used as a marker of TLR4 activation. TLR4 mRNA expression and TLR4 protein location were also measured in response to LPS, mPM$_{0.5}$ and an agglomerate of mPM$_{0.5}$ to LPS (referred to as mPM$_{0.5}$-LPS). To further examine the role of TLR4 location in responses generated to these exposures, IL-13 stimulated and non-stimulated 16HBE cells were also used. This follows on from findings in Chapter 3, whereby IL-13 was shown to induce a translocation of TLR4 to the cell surface, the location of the receptor *in vivo*. By aiming to re-create *in vivo* conditions as closely as possible, the results generated from this investigation allow the role of TLR4 in mPM$_{0.5}$-LPS exposure of AEC’s to be determined.
4.2 Methods

4.2.1 Aerosol preparations

Fifty micrograms per millilitre (50μg/mL) of *Salmonella typhimurium* LPS (Sigma-Aldrich) and 0.5μm Fluorobrite™ latex bead solutions (mPM0.5) were prepared for aerosol use. To prepare the mPM0.5-LPS solution, 10mg of LPS and 10mg of mPM0.5 were dry mixed together by vortexing for 15-20mins before being dissolved in distilled water to make a 50μg/mL solution to be used for exposure.

4.2.2 Exposure protocol

When cultures were ready for experimentation they were placed in a sealed Perspex chamber for a nebulised exposure to one of the pre-prepared aerosol solutions. The chamber contained an entry valve for the aerosol generator and was housed within a humidified incubator kept at 37°C. Cell cultures were exposed for 30 mins with the airflow over the cells maintained by a vacuum at 5L/minute. Cells were harvested for analysis at 0.5, 1, 6 and 24hrs.

4.2.3 Validation of aerosol preparations using the Limulus Amoebocyte Lysate assay

A commercially available Limulus Amoebocyte Lysate (LAL) assay (Sigma-Aldrich) was used to determine the amount of LPS delivered to the cell cultures by aerosol exposure and the proportion of LPS bound to mPM0.5 in the mPM0.5-LPS solution.

Limulus amoebocyte extract originates from crabs and coagulates in the presence of LPS. Using serial dilutions of a known LPS concentration and scoring these for
the presence or absence of a clot formed on addition of the Limulus extract permits an indication of the LPS concentration in solution. Gel clots were scored as instructed on the provided data sheet and the assay completed with the assistance of an experienced user (Professor Prue Manners) to ensure the results produced were valid.

To assess LPS binding to mPM$_{0.5}$ aerosol preparations of LPS and mPM$_{0.5}$-LPS were pulled by vacuum through a 0.2µm filter. This allowed for LPS flow through while preventing flow of the mPM$_{0.5}$ (diameter 0.5µm). Therefore unbound LPS from the mPM$_{0.5}$-LPS mixture could be measured in the filtrate using the LAL assay. These measurements were compared with a similarly filtered LPS preparation. The difference in LPS concentration between the LPS and mPM$_{0.5}$-LPS filtrates was then used to determine the proportion of bound LPS in the dissolved mPM$_{0.5}$-LPS mixture.

Serial dilutions of an aliquot of media collected from a culture dish exposed to a 50µg/mL LPS aerosol preparation for 30mins were assessed to determine the dose of LPS received by cells. Positive clot formation in these dilutions was compared relative to that in a LPS standard curve for a concentration value.

4.2.4 Cell Culture

16HBE cells were seeded onto Transwell inserts at a density of 48000 cells per well in EMEM with 10% FCS (v/v) and 0.1% gentamycin (v/v). These were grown as submerged cultures until 70% confluent. At this time, the apical media was removed and media changes made to the basal side of the culture. To encourage mucoid differentiation medium was supplemented with 0.5µg/mL retinoic acid (RA)
(Wu et al. 1997). Cells were grown to confluence for experimental use. Serum free supplemented media was used for a media change at 72hrs prior to stimulation and a final media change performed 24hrs prior to stimulation or aerosol exposure.

4.2.4.1 IL-13 Stimulation

To determine the influence of TLR4 location on AEC responses 16HBE cells were stimulated with IL-13 (10pg/mL) (Silva-Teixeira et al. 2004) for 6hrs before being nebulised to aerosols of LPS, mPM$_{0.5}$ and mPM$_{0.5}$ -LPS for 0.5hrs and then incubated for 1hrs. All incubations were completed at 37°C. Responses from these were compared to non-stimulated cultures that were similarly exposed.

4.2.5 Sample analysis

4.2.5.1 Lactate dehydrogenase (LDH) secretion

To assess cell health, cell culture supernatants were collected post-exposure for LDH analysis. These were measured using an InVitreos pathology instrument available for use through the Pathology Department at Princess Margaret Hospital for Children. Samples were processed under supervision according to the manufacturer instructions.

4.2.5.2 IL-8 secreted protein

Collected supernatants were also assayed for secreted IL-8 using a commercially available ELISA kit (OptEIA Human Set IL-8, BD Pharmingen). These are presented as a mean value of 3 wells in pg/mL unless stated otherwise.
4.2.5.3 Immunohistochemistry

Following exposure to the nebulised aerosols a portion of each cell culture sample was prepared as cytospins and stained for TLR4 protein as described in Chapter 2.

4.2.5.4 mRNA expression

A portion of each cell culture samples exposed to the nebulised aerosols were also collected for mRNA analysis. Cell pellets were dissolved in RLT buffer (Qiagen) and stored at -80°C until required as described in Chapter 2.

4.2.6 Statistical analysis

All of the data collected was completed in triplicate and compared for differences using ANOVA with Tukey post-hoc analysis for intra-nebulisations. Data are reported as means ± SE, with means considered significantly different at the p<0.05 level.
4.3 Results

4.3.1 Validation measurements

4.3.1.1 mPM$_{0.5}$-LPS adherence validation

The small difference in clot firmness measured between the LPS and mPM$_{0.5}$-LPS dilutions of the aerosol solution (Table 4.1) illustrates a portion of LPS was binding to the mPM$_{0.5}$ during dry mixing. The LPS remained attached when the mixture was dissolved as indicated by the less firm clot for the mPM$_{0.5}$-LPS mixture.
LPS Concentration (ng/mL) | Standard Curve gel clot result | Gel clot result for serial dilutions of 50µg/mL LPS aerosol preparations | Gel clot result for serial dilutions of 50µg/mL mPM₀.₅-LPS aerosol preparations
---|---|---|---
0.005 | - | - | -
0.05 | - | - | 1:10⁶ - 1:10⁶
0.5 | +++ | +++ | 1:10⁵ +/- 1:10⁵
5 | +++ | +++ | 1:10000 ++ 1:10000
50 | +++ | +++ | 1:1000 +++ 1:1000

Table 4.1 Validation of LPS binding to mPM₀.₅.

The mPM₀.₅-LPS mixture was assessed for LPS binding using the LAL gel clot assay. Four 10-fold serial dilutions were prepared from individual 50µg/mL preparations of LPS and mPM₀.₅-LPS that were filtered (0.2µm) to exclude the mPM₀.₅. A standard curve of known LPS concentrations was prepared with a positive clot indicating the presence of LPS from 0.5ng/mL LPS onwards. Corresponding dilutions of the LPS and mPM₀.₅-LPS filtered samples were then assessed for clotting to determine the presence of unbound LPS in the mPM₀.₅-LPS mixture. The amount of LPS detected in the filtrate of the mPM₀.₅-LPS mixture was less than that detected in the filtrate of the LPS sample. The lower amount of LPS present in the filtered mPM₀.₅-LPS solution indicates successful binding of a portion of LPS to mPM₀.₅ in the mPM₀.₅-LPS aerosol preparation.
4.3.1.2 LPS nebulised dose

The received dose of LPS was determined to be 1/10\textsuperscript{th} of the aerosol solution used. This result was demonstrated by the positive clot at the 1: 10000 dilution (Table 4.2) indicating that 0.5ng/mL of LPS is present at this dilution (as determined by the standard curve). For this reason, stated doses from this point onward correspond to the received concentration rather than the applied aerosol.
<table>
<thead>
<tr>
<th>LPS concentration (ng/mL)</th>
<th>LAL gel clot result</th>
<th>Serial dilutions of 50000ng/mL nebulised LPS used for sham media aerosol exposure (dilution factor)</th>
<th>Calculated LPS concentration in sham culture dish following LPS aerosol exposure (50000/10^5) (ng/mL)</th>
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Table 4.2 LPS dose of 30mins nebulised aerosol

The calculated dose received from a 50μg/mL LPS aerosol preparation applied for 30 minutes using this exposure model is 5μg/mL. This was done by matching the clot/liquid interfaces (+/-) of the LAL LPS standard curve and the sham serial dilutions. This match at the 1:10^5 dilution was then used to calculate the LPS dose the culture dishes in the chamber were being exposed to ie. Aerosol preparation concentration/1x 10^5.
4.3.1.3 Validation of ddH2O control

LPS, mPM0.5 and mPM0.5 -LPS were all prepared in ddH2O for use as a nebulised aerosol. Exposure of 16HBE cells to ddH2O did not induce IL-8 release that was significantly different from saline nebulised cells (Figure 4.1) (p<0.05). Therefore, ddH2O was used as the control for all subsequent in vitro studies.
Figure 4.1 Validation of ddH$_2$O control.

All aerosol solutions used for the *in vitro* exposures were prepared in ddH$_2$O. To ensure there were no adverse inflammatory effects for use of ddH$_2$O as the control nebulisation IL-8 secretion was measured in media collected from 16HBE cells nebulised with saline and ddH$_2$O (n=3). No differences were observed.
4.3.1.4 IL-8 dose response curve

IL-8 production increased with increasing doses of LPS (Figure 4.2, $R^2=0.9503$). Exposures of LPS doses that were 2.5µg/mL or more were significantly different compared to doses of 0.05µg/mL and 0.5µg/mL ($p<0.05$) (Figure 4.2). The 5µg/mL dose was the largest received dose. This data agrees with previously published dose response curves for epithelial cells exposed to LPS (Backhed et al. 2002b; Monick et al. 2003) justifying the 5µg/mL LPS dose for all subsequent studies.
Figure 4.2 IL-8 secretion increases with increasing LPS dose.

Significantly more IL-8 is secreted from 16HBE cells exposed to 25µg/mL and 50µg/mL preparations of LPS (p<0.05) compared to doses of 0.05µg/mL and 0.5µg/mL (n=3). A linear relationship exists between LPS dose and IL-8 secretion ($R^2=0.9503$).
4.3.2 16HBE Responses

4.3.2.1 Cytotoxicity

Cell viability was determined using LDH, a marker of cell stress ((Yusup et al. 2005) LDH readings of supernatants from cells exposed to LPS, mPM$_{0.5}$ and mPM$_{0.5}$-LPS were compared to those from unexposed cells at 0.5, 1, 6 and 24hrs post exposure (n=5) (Figure 4.3). Measured LDH from cells that were nebulised did not exceed control cell levels within any time point. Measured LDH appears elevated in control cells at 6 and 24hrs but these were not statistically significant to LDH measured in supernatants collected from control cells at 0.5 and 1hrs.
Figure 4.3 LDH is not significantly increased in 16HBE cells exposed to different aerosol preparations.

LDH was used to evaluate the cytotoxic effect of the exposure protocol and different aerosol preparations on cultures of 16HBE cells (n=5). For each incubation time there were no significant differences in LDH release between the control and aerosol exposure types including LPS exposure at 1hr (p>0.05).
4.3.2.2 IL-8 release

There were no detectable differences in IL-8 release between the mPM₀.₅ exposed cells and control cells for any of the time points (n=3) (Figure 4.4a). However, IL-8 secretion was significantly greater in cells nebulised with LPS at 6 and 24hrs compared to control cells (p<0.05). Cells nebulised with mPM₀.₅-LPS had a significantly raised level of IL-8 at all of the time points measured when compared to control cells (n=3) (p<0.05) (Figure 4.4b and c). Significantly greater amounts of IL-8 were released by mPM₀.₅-LPS exposed cells compared to cells nebulised with mPM₀.₅ and LPS respectively (p<0.05).
Figure 4.4 Secreted IL-8 from 16HBE cells nebulised with (A) mPM0.5 (B) LPS and (C) mPM0.5-LPS.

Supematants were collected from cells incubated for 0.5, 1, 6 and 24hrs post nebulisation and analysed for secreted IL-8 by ELISA (n=3). Significant increases were observed in cells nebulised with LPS at 6 and 24hrs and for all incubation times for cells nebulised with mPM0.5-LPS in comparison to controls (*p<0.05).
4.3.2.3 TLR4 protein expression

Permeable 16HBE cells were stained for TLR4 (red stain, bottom panel Figure 4.5) and a wheat germ agglutinin cytoplasmic marker (green stain, top panel Figure 4.5) to assess TLR4 protein location after nebulised exposure (n=3). Staining was observed in permeable 16HBE cells and remained intracellular in cells from all of the exposure groups at all time points. Staining in these cells appeared more evenly spread throughout the cytoplasm compared to the cytoplasmic stain (Figure 4.5 A-D, images at 0.5hrs). Staining for TLR4 was slightly more intense for cells exposed to LPS (C) and mPM$_{0.5}$-LPS (D). Staining for TLR4 in non-permeable cells was not observed (n=3) (data not shown).
Figure 4.5 TLR4 staining remains intracellular with aerosol exposure.

TLR4 (red stain, bottom panel) location remained intracellular in 16HBE cells nebulised to different aerosol preparations of mPM$_{0.5}$ (B), LPS (C) and mPM$_{0.5}$-LPS (D). Control cells show the greatest degree of co-localised staining with the cytoplasmic stain (green stain, top panel). Nebulisation mPM$_{0.5}$ (B) LPS (C) and mPM$_{0.5}$-LPS (D) producing a more evenly spread TLR4 stain (n=3). More intense staining was noted in cells nebulised with LPS (C) and mPM$_{0.5}$-LPS (D). These staining patterns were observed for all time points (0.5-24hrs post nebulised exposure). Images presented here are from 16HBE cells at 0.5hrs post nebulised exposure. TLR4 staining was not observed in similarly nebulised non-permeable cells (n=3, data not shown).
4.3.2.4 TLR4 gene expression

TLR4 mRNA expression was not significantly different between cells nebulised to LPS, mPM$_{0.5}$ or mPM$_{0.5}$-LPS for any of the incubation times measured after nebulisation (Figure 4.6). A significant increase in TLR4 mRNA expression was observed in cells nebulised to mPM$_{0.5}$-LPS at 6 and 24hrs incubation post nebulisation compared to the control cells. Similarly increased expression was observed at 24hrs post nebulisation for cells nebulised with LPS and mPM$_{0.5}$ compared to control cells (p<0.05).
Figure 4.6 TLR4 mRNA expression increases with incubation time following nebulised exposure.

16HBE cells nebulised to mPM$_{0.5}$-LPS had raised TLR4 mRNA expression compared to control cells at 6 and 24hrs post nebulisation (p<0.05). Cells nebulised to mPM$_{0.5}$ and LPS also had significantly raised TLR4 mRNA expression at 24hrs post incubation compared to control cells (p<0.05) (n=3 for all).
4.3.3 IL-13 stimulated 16HBE cell responses

4.3.3.1 IL-8 release

16HBE cells were stimulated with IL-13 (10pg/mL for 6hrs) to induce TLR4 translocation to the cell surface (Chapter 3). Following stimulation with IL-13, cells were exposed to mPM$_{0.5}$, LPS or mPM$_{0.5}$-LPS for 0.5hrs. Supernatants from these cells were collected 1hr after nebulisation and analysed for secreted IL-8 by ELISA (Figure 4.7). Secreted IL-8 was significantly greater in unstimulated in mPM$_{0.5}$, LPS and mPM$_{0.5}$-LPS nebulised cells compared to their unstimulated similarly nebulised counterparts (p<0.05). Interestingly, IL-13 stimulated cells nebulised mPM$_{0.5}$ and mPM$_{0.5}$-LPS had significantly reduced secreted IL-8 compared to stimulated control cells (p<0.05). Unstimulated cells nebulised with LPS and mPM$_{0.5}$-LPS had significantly raised secreted IL-8 compared to unstimulated control cells (p<0.05).
Figure 4.7 Lower level of IL-8 secretion in 16HBE cells stimulated with IL-13.

IL-8 secretion was significantly less in stimulated cells nebulised with mPM$_{0.5}$, LPS and mPM$_{0.5}$-LPS compared to their non-stimulated counterparts (p<0.05). IL-13 stimulated cells nebulised with mPM$_{0.5}$ and mPM$_{0.5}$-LPS had significantly less secreted IL-8 compared to IL-13 stimulated control cells (# p<0.05). Unstimulated cells nebulised with LPS and mPM$_{0.5}$-LPS had significantly more secreted IL-8 compared to unstimulated control cells (* p<0.05) (n=3).
4.3.3.2 TLR4 gene expression

After IL-13 stimulation (10pg/mL for 6hrs) and exposure to mPM_{0.5}, LPS or mPM_{0.5}-LPS for 30 mins followed by incubation for 1hr, cells were harvested for assessment of TLR4 mRNA expression (Figure 4.8). Gene expression of TLR4 was significantly increased in the control, LPS and mPM_{0.5} exposure groups when compared to the non-stimulated cells (p<0.05). IL-13 stimulation resulted in significantly increased TLR4 mRNA in mPM_{0.5} exposed cells compared to all other groups (p<0.05), while the mPM_{0.5}-LPS stimulated group measured TLR4 mRNA that was significantly lower compared to the stimulated control cells (p<0.05). There were no measurable differences in TLR4 mRNA between the control and LPS stimulated groups.
Figure 4.8 TLR4 mRNA in 16HBE cells increases with IL-13 stimulation.

A significant increase in TLR4 mRNA was observed with IL-13 stimulation of control cells and cells nebulised with mPM$_{0.5}$ and LPS compared with their non-stimulated counterparts (incubation time 1hr) ($p<0.05$). IL-13 stimulated cells nebulised with mPM$_{0.5}$-LPS had significantly less TLR4 mRNA expression compared to IL-13 stimulated control and IL-13 stimulated mPM$_{0.5}$ nebulised cells ($p<0.05$) (n=3).
4.4 Discussion

4.4.1 Major findings

The results of this chapter demonstrate that nebulised exposure of airway epithelial cells to LPS, mPM$_{0.5}$ or mPM$_{0.5}$–LPS does not affect TLR-4 location. Despite the receptor remaining intracellular, IL-8 was secreted in response to all nebulised exposures indicating receptor activity. Interestingly airway epithelial cells stimulated with IL-13 to induce translocation of TLR4 to the cell surface secreted less IL-8 than unstimulated cells suggesting TLR4 is less active when located on the cell surface.

4.4.2 Airway epithelial exposure model

This study has attempted to develop a model of ambient LPS exposure that involves binding LPS to a PM source. A published standardised protocol is not currently available. The ‘dry-mix’ method of binding LPS to mPM$_{0.5}$ was used based on the understanding that the polarised molecular structure of LPS would cause a degree of binding to almost any substance (Morrison et al. 1992). In addition random binding of LPS to ambient PM is an assumed process. Success of the ‘dry-mix’ method employed was confirmed by a lower amount of measured LPS in filtrate from a proportion of a mPM$_{0.5}$–LPS preparation compared to a LPS solution using the LAL gel clot method. This result justified use of the ‘dry mix’ method to bind LPS and mPM$_{0.5}$ together for use as the mPM$_{0.5}$–LPS aerosol.

The nebulised dose was calculated based on a 50μg/mL LPS nebulised exposure
of sham media. Serial dilutions of the nebulised sham media and LAL gel clot analysis was used to determine a corresponding positive clot relative to the LAL standard curve. By this method 5µg/mL of the initial 50µg/mL aerosol preparation was found to reach the sham media. Based on these calculations the one-tenth concentration loss was assumed across the aerosol preparations used for nebulisations.

All aerosol preparations used to nebulise the 16HBE cells were prepared in ddH₂O. Distilled water has been reported as an inducer of airway responsiveness however the mechanism for this remains unclear (Law et al. 2000). To ensure the ddH₂O nebulisation did not activate TLR4, IL-8 secretion was compared between ddH₂O and saline nebulisations. IL-8 secretion did not differ between 16HBE cells nebulised with ddH₂O and saline. Therefore ddH₂O was used as the control nebulisation for all experiments unless stated otherwise.

Data previously published for IL-8 secretion from epithelial cells in response to LPS stimulation show 5µg/mL LPS dose in the linear portion of the curve (Backhed et al. 2002b; Monick et al. 2003). In these studies 50µg/mL LPS was shown to produce a saturated IL-8 response (Backhed et al. 2002b; Monick et al. 2003). The IL-8 dose response graph generated here does not plateau indicating the doses of LPS used here did not induce a maximum IL-8 response from these cells (Figure 4.2). In combination with data from two other studies this suggests 5µg/mL of LPS induces IL-8 secretion in the linear portion of an IL-8 dose response curve. For this reason 5µg/mL was deemed a suitable dose for measuring LPS induced responses.
4.4.3 Airway epithelial responses

LDH was used in this investigation as a marker of cell stress to confirm cell viability during exposure. There was a trend for secreted LDH to increase with time in incubation. However there were no significant differences between control cells across the incubation times measured. While measured LDH appeared to be raised in LPS and mPM$_{0.5}$-LPS nebulised cells after incubation for 1hr these were not significantly different to secreted LDH measured in control cells at the same incubation time. It is possible that with an increased number of replicates these differences may be significant. As significant differences were not evident all of the nebulised exposure types (mPM$_{0.5}$, LPS and mPM$_{0.5}$-LPS) and the incubation times used following nebulisation (0.5, 1, 6 and 24hrs) were considered for analysis.

IL-8 is a potent chemokine, released following NFκB translocation into the nucleus and is an indirect measurement of TLR4 activation (Xu et al. 2000). A significant increase in IL-8 release was measured from cells nebulised with LPS after being incubated for 6hrs (Figure 4.4). In cells exposed to mPM$_{0.5}$-LPS an increase in IL-8 was measured from the initial incubation period (30 mins) onwards. In contrast, secreted IL-8 following mPM$_{0.5}$ challenge was not different compared to the control cells for any of the incubation times. This result suggests that LPS was the stimulus for IL-8 release in the mPM$_{0.5}$-LPS exposure group.

Secreted IL-8 was greater in cells nebulised with mPM$_{0.5}$-LPS compared to those nebulised with LPS across all of the incubation times. As the same concentration of LPS was present in both preparations additional cellular interactions possibly
explain the increased IL-8 observed in cells nebulised with mPM_{0.5}-LPS. IL-8 is known to be stored in Weibel-Palade bodies in endothelial cells (Utgaard et al. 1998). As part of a regulated secretory pathway this temporary storage site allows for immediate IL-8 secretion on stimulation eliminating lag time between stimulation and cytokine production (Utgaard et al. 1998). IL-8 secretion was increased in 16HBE cells incubated for 30mins post nebulisation with mPM_{0.5}-LPS. Weibel-Palade bodies are specific for endothelial cells and secretory vesicles of this nature have not been described in epithelial cells. Other investigations have also shown increased IL-8 in AEC’s stimulated with LPS and bacterial residues ≤3hrs (Wu et al. 2004). Together these findings suggest IL-8 cytoplasmic storage vesicles and in this study mPM_{0.5}-LPS may cause a greater release of IL-8 via this mechanism.

The sustained greater IL-8 response observed in cells nebulised with mPM_{0.5}-LPS compared to those nebulised with LPS alone may be attributed to the binding of LPS with mPM_{0.5}. Binding may facilitate more efficient signaling of LPS by TLR4. LPS activation of NOD1 also induces IL-8 release on meaning these results might represent a synergistic effect of TLR4 and NOD1 and has been alluded to previously (Opitz et al. 2006). A physiological explanation for this effect remains unknown.

While significantly more TLR4 mRNA expression was observed in cells nebulised to mPM_{0.5}, LPS and mPM_{0.5}-LPS at 24hrs compared to control cells there were no measurable differences between these nebulised exposures. This suggests measured TLR4 mRNA does not correlate with IL-8 secretion. Despite the same mRNA expression cells nebulised with LPS and mPM_{0.5}-LPS secreted greater
amounts of IL-8 compared to those nebulised with mPM$_{0.5}$ alone. Again this might reflect a contribution from another receptor/mechanism in response to these particular nebulisations such as the intracellular NOD receptors.

A large amount of variation is present in the TLR4 mRNA expression for the 30 minute and 1 hour incubation times following aerosolised exposure to LPS, mPM$_{0.5}$ and mPM$_{0.5}$-LPS. This variation is reduced at 6 hrs and 24 hrs. This variation can be attributed to differing rates of expression at these early time points between cells. This experiment warrants completion on further replicates or inclusion of a 3 hours time point to confirm the reduced variability of mRNA observed at these later time points.

4.4.4 Responses in IL-13 stimulated airway epithelial cells.

16HBE cells were stimulated with IL-13 to cause a translocation of the receptor to the cell surface to determine if TLR4 activity was affected by a cell surface compared to an intracellular location. TLR4 activity was gauged by the amount of IL-8 secreted by 16HBE cells nebulised to the different aerosol preparations.

Stimulation with IL-13 did not change IL-8 secretion in non-nebulised and ddH$_2$O control cells relative to their non-stimulated counterparts. However, in IL-13 stimulated cells nebulised with mPM$_{0.5}$, LPS and mPM$_{0.5}$-LPS a significantly lower amount of IL-8 was secreted compared to the non-stimulated nebulised cells. The amount of IL-8 secreted by the IL-13 stimulated LPS nebulised cells was similar to those observed in the control cells suggesting a return to baseline levels. However secreted IL-8 levels were lower in IL-13 stimulated mPM$_{0.5}$ and mPM$_{0.5}$-LPS
nebulised cells compared to the stimulated controls. This downregulation of IL-8 secretion appears to be specific to the IL-13 stimulated mPM\textsubscript{0.5} nebulisations and has not been reported before. Perhaps with greater numbers the significance of this effect would be reduced.

While IL-13 is a reported down regulator of IL-8 expression in peripheral blood mononucleocytes (PBMC) this affect is diminished in LPS activated PBMC’s where secreted IL-8 does not return to baseline levels (Marie et al. 1996; Ohta et al. 1998). Studies involving this affect in epithelial cells are not available. From these findings it is possible to conclude that intracellular TLR4 is more active than when located on the cell surface, however further investigation is warranted. As IL-8 expression is a result of NF-κB activation stimulated by TLR4 and the NOD receptors an expression profile for a range of TLR4 mediated transcription factors may provide a better indication of TLR4 activity and a more conclusive result.

In contrast to the lower levels of IL-8 secreted by IL-13 stimulated cells TLR4 gene expression was significantly greater in control and mPM\textsubscript{0.5} and LPS nebulised cells. This was not apparent in cells nebulised with mPM\textsubscript{0.5}-LPS and in these cells TLR4 gene expression was significantly lower in the stimulated cells relative to the control. Changes in protein expression are measurable only after a number of cellular processes. These are reported to take up to 4hrs before a measurable response can be observed (Hargrove 1993). In the case of gene expression signal transduction and RNA replication is shorter and depending on the gene and metabolic rate of the cell this process can take up to an hour for measurable responses to occur (Hargrove 1993). Cells were stimulated with IL-13 for 6hrs
before a 30 minute nebulisation followed by a 1hr incubation. Meaning changes in TLR4 mRNA expression can be attributed to the IL-13 incubation time.

There was no difference in TLR4 mRNA expression between IL-13 stimulated and non-stimulated cells nebulised with mPM0.5-LPS. The non-stimulated cells nebulised with mPM0.5-LPS secreted significantly more IL-8 than the stimulated cells despite having the same TLR4 mRNA expression. It might be that mPM0.5-LPS is able to interact more efficiently with TLR4 when it is located intracellularly to induce a response or it is interacting with a different receptor to induce IL-8 secretion ie NOD receptor family. This would explain the unchanged TLR4 mRNA expression between stimulated and non-stimulated cells. The lower IL-8 might be due to an affect of IL-13 stimulation on this other receptor/mechanism and requires further investigation.

TLR4 is not currently linked to signalling by PM; as such the increase in TLR4 mRNA following mPM0.5 exposure type is particularly interesting. Particulate exposure has strong links with lung hyperpermeability. By chromosomal locus analysis, susceptible genes for this condition are reported to be on Chromosome 4. Of these genes, TLRs are the most likely candidates, implicating these receptors in regulating responses to environmental pollutants, pointing to a possible explanation for this result (Kleeberger et al. 2000).

4.4.5 Conclusions

Cell culture models investigating responses to LPS exposure have traditionally relied on submerged monolayer cultures that are exposed to LPS dissolved in the
media bathing the cells. While useful, monolayer cultures are not truly representative of *in vivo* conditions. For this reason, we have developed and used an air-liquid interface culture system. This facilitates contact between aerosolized LPS and the epithelium, much like conditions *in vivo*. Using this system, IL-13 stimulated cells were hyporesponsive to LPS suggesting that TLR4 location may have an effect on the receptor activity. Lower IL-8 and TLR4 mRNA in IL-13 stimulated cells exposed to mPM$_{0.5}$-LPS compared to LPS exposed cells illustrates the possibility for alternate signalling pathways for LPS when it is attached to mPM$_{0.5}$. Elucidating these pathways may have important implications for understanding the reported protective and exacerbative role of LPS exposure in asthma and allergy.
Chapter Five

5 Physiological responses to LPS, PM and PM-LPS measured *in vivo.*

5.1 Introduction

In Chapter 3, it was demonstrated that TLR4 was primarily expressed on the surface of pAEC obtained from healthy individuals. This was in contrast to cultured AEC where the receptor was primarily localised intracellularly. Stimulation of cultured cells with the Th2 cytokine IL-13, induced translocation of TLR4 to the cell surface. This finding was used in chapter 4 to translocate TLR4 to the cell surface as a model to mimic TLR4 location in AEC *in vivo* in chapter 4. Responses to mPM$_{0.5}$, LPS and mPM$_{0.5}$-LPS exposure in these IL-13 stimulated and non-stimulated cells were subsequently measured. Following *in vitro* responses to exposure of LPS, mPM$_{0.5}$ and mPM$_{0.5}$-LPS, the aim of this chapter was to determine if these same exposures were responsible for generating inflammatory responses that may cause airway hyperresponsiveness.

There are numerous studies utilising animal models to examine the effects of LPS and PM exposures (Becker *et al.* 2002; Gavett *et al.* 1999; Gavett *et al.* 2003b; Hollingsworth *et al.* 2004; Tulic *et al.* 2001; Vernooy *et al.* 2002; Walters *et al.* 2002; Yanagisawa *et al.* 2003). However, this is the first study to measure responses to a combined exposure of PM-LPS. In addition, we have used TLR4 deficient mice (C3H/HeJ, referred to as the Tlr4$^d$ mice from here onwards) and
mice of the same strain with an intact TLR4 (ARC C3H/HeJ, referred to as the ‘Tlr4<sup>d</sup> mice) to assess the role of TLR4 in mediating these responses. The Tlr4<sup>d</sup> mouse is resistant to endotoxin due to a naturally occurring point mutation producing a proline to histidine switch in the TLR4 coding sequence, resulting in a dysfunctional receptor (Poltorak et al. 1998). A blunted response to LPS is still measured in these animals due to other receptors capable of signalling for LPS such as the NOD and scavenger receptors (Inohara et al. 2001; Krieger and Stern 2001; Sankala et al. 2002).

The exposure protocol used in this study is the same as that used by Tulic et al (Tulic et al. 2001). This protocol requires 6 consecutive 30mins exposures to aerosolised LPS every 24hrs. Airway mechanics, bronchoalveolar lavage (BAL) and tissue collection are taken 6 hrs after the final exposure. For the purposes of this study, mice were placed in individual Perspex chambers and exposed to either LPS, mPM<sub>0.5</sub> or mPM<sub>0.5</sub>-LPS. This type of exposure protocol differs to others that have used intratracheal instillation to deliver the stimuli. Respiratory mechanics were measured using forced oscillation technique (FOT) in response to methacholine challenge (MCh). The release of inflammatory cytokines and raised BAL cellular infiltrate were used as indices of lung inflammation.

By using Tlr4<sup>d</sup> and Tlr4<sup>i</sup> mice, this study will determine if mPM<sub>0.5</sub>-LPS exposure causes a larger inflammatory response than to LPS or mPM<sub>0.5</sub> exposure alone and if this response is TLR4 dependant. Particulates have been shown to enter cells via phagocytosis (Mukae et al. 2000), providing access to intracellular receptors as well as those on the cell surface. For these reasons exposure to mPM<sub>0.5</sub>-LPS is
expected to induce a greater inflammatory response in Tlr4<sup>d</sup> mice compared to LPS exposure alone.

5.2 Methods

5.2.1 Animals

Specific pathogen free female Tlr4<sup>d</sup> and Tlr4<sup>I</sup> mice aged between 7 and 9 weeks were purchased from the Animal Resource Centre, Murdoch, Western Australia. Mice were housed in a controlled environment with a 12hrs light to dark cycle. They were provided with food and acidified water ad libitum. All experiments presented were approved by the Telethon Institute for Child Research Animal Ethics and Experimentation Committee (AEEC #128).

5.2.2 Aerosol exposure protocol

Animals were exposed to 50μg/mL LPS (Salmonella typhimurium, Sigma), 50μg/mL 0.5μm fluorescent latex beads (mPM<sub>0.5</sub>) or 50μg/mL LPS-mPM<sub>0.5</sub> all dissolved in distilled water (section 4.2.1). As described in chapter 4, the 0.5μm fluorescent latex beads were chosen as a model for inert fine particulate matter exposure as has been used previously (Geiser et al 1994).

Aerosols were delivered to animals via an UltraNeb nebuliser (UltraNeb, DeVilbiss, Somerset, Pennsylvania) within a plexiglass chamber having separate compartments for each animal and thereby allowing for uniform exposure to each. Aerosols were pulled through the chamber by approximate 5L/minute vacuum (Figure 5.1). Aerosol exposures were conducted at room temperature.
Figure 5.1 Mouse exposure setup.

Mice are placed in individual compartments of a Plexiglas chamber. Pre-prepared aerosol solutions are placed in the nebuliser cups, this solution is nebulised via the Ultraneb nebuliser. The airflow is driven across the chamber by a 5L/minute vacuum.
5.2.3 Analysis

5.2.3.1 Immunohistochemistry

Paraffin embedded slides were deparaffinised with xylene and passed through graded alcohols (100%, 95%, 70%) for 3mins each. These were then rinsed with deionised water followed by a rinse in TBS. Endogenous peroxidases on the tissue sample blocked with 0.5% hydrogen peroxide in methanol for 30mins. All dilutions and washes were completed in TBS and at room temperature unless stated otherwise.

Enzyme antigen retrieval was performed using Proteinase K (Progen) 25µg/mL for 35mins at 37°C. Rabbit anti-human TLR4 (Santa Cruz) was used as the primary antibody and sections left to incubate overnight at 4°C. Washing was performed as mentioned previously and the secondary antibody (Alexor Flur Red goat anti-rabbit by Molecular Probes) applied. Sections were incubated for 1hrs and then washed before being mounted for viewing under fluorescent filter of a UV microscope (Leica).

5.2.3.2 Haematoxylin and eosin (H&E) staining

Paraffin embedded lung sections to be stained with H&E were dewaxed and rehydrated as for sections prepared for immunohistochemistry (section 5.2.3.1). Following hydration, sections were rinsed in distilled water and then placed in Mayer's haematoxylin for 4mins before being rinsed again in distilled water and
placed in Scott's tap water for 2mins. Sections were then rinsed in distilled water and dehydrated through a single change of 70% and 95% ethanol for 1mins at each change. Next, sections were placed in 1% alcoholic eosin for 1mins for counterstaining. Subsequently these were rinsed through 3 changes of 100% ethanol for 2mins at each change and put through 3 changes of xylene for 3 mins at each change before being mounted and viewed under a light microscope (Leica).

5.2.3.3 Inflammatory cell counts

Exposed animals that did not undergo FOT measurements were anaesthetised and tracheotomised for assessment of induced inflammation. Bronchoalveolar lavage (BAL) fluid was collected by slowly infusing and withdrawing a 1mL aliquot of 0.9% saline from the lung 3 times. The resulting fluid was then centrifuged at 2000 rpm for 4mins. The supernatant was removed and the pellet resuspended in saline. The cells were stained with trypan blue to determine viability and total cell count (TCC) obtained by counting cells with a haemocytometer.

5.2.3.4 Airway responsiveness

Airway responsiveness was assessed in mice anaesthetised with an intraperitoneal injection of a solution containing xylazine (2mg/mL, Troy Laboratories, NSW, Australia) and ketamine (40mg/mL, Troy Laboratories, NSW, Australia) at a dose of 0.01mg/mL. These mice were then tracheotomised with a 10mm section of polyethylene tubing (1.27mm OD, 0.86mm ID) and ventilated at 450 breaths/minute with a tidal volume of 8mL/kg and a positive end expiratory
pressure (PEEP) of 2cm water.

Impedance (Zrs) was measured to calculate airway resistance (Raw) using a modification of the low frequency forced oscillation technique (LFOT) as described previously (Pillow et al. 2001). Before measurements of lung mechanics, mouse lung history was standardised using two deep inflations and three pressure-volume curves. The respiratory system input impedance was measured during periods of apnoea using a 16s signal containing 19 mutually primed sinusoidal frequencies ranging from 0.25 to 19.625Hz. The constant phase model (Hantos et al. 1992) was then fit to the real and imaginary parts of the Zrs spectrum allowing calculation of Raw (van Noord et al. 2000) (Figure 5.2.2). At conclusion of the study, mice were sacrificed and lungs fixed with 10% buffered formalin at a pressure of 10cmH₂O for histology.

5.2.3.5 Methacholine challenge

Changes in airway mechanics to inhaled MCh were measured 6hrs after the last aerosol exposure, of either ddH₂O, mPM₀.₅, LPS or mPM₀.₅-LPS. Following measurement of baseline Zrs, mice were exposed to a 90 sec saline aerosol delivered with an ultrasonic nebuliser (Ultraneb, Devilbiss, Somerset, Pennsylvania). Zrs was then measured every minute for the next 5mins. This aerosol procedure was repeated with half log incremental doses of MCh from 0.1 to 30mg/mL with Zrs measured every minute for at least 5 mins after the aerosol until the parameters calculated from the constant phase model had peaked. Raw data are presented as changes in maximum responses and sensitivity.
Figure 5.2 Schematic setup for airway response measures.

After tracheostomy, the mouse is attached to the Flexivent that acts as the ventilator. The Flexivent is also responsible for generating the oscillatory pressures required for calculating the necessary tissue parameters. In this case, airway resistance is measured in response to MCh challenge as delivered by the nebuliser (Courtesy of Clinical Sciences, Telethon Institute for Child Health Research).
Airway sensitivity was determined by calculating the MCh dose required to produce a 150% increase in Raw from the range of MCh doses used for challenge (Schelegle et al. 2001).

5.2.3.6 Cytokine bead assay (CBA)

Analysis of IL-6, IL-12p70, IFNγ, MCP-1 and TNFα present in BALs was assessed using the BD Pharmingen Mouse Inflammation kit, as per manufacturers’ instructions. Briefly, this kit comprises a series of beads with discrete fluorescence intensities. Each bead in a CBA kit provides a capture surface for a specific protein, so that a mixture of these beads in a suspension allows for the detection of multiple analytes in a small volume sample. Once the incubation and washing steps were completed, measurement of the multiple fluorescent intensities by flow cytometry was used to generate a standard curve for each bead (cytokine) that were used as a reference to determine protein expression. Cytokine measures were presented as a percentage of the control or with the control set to zero.

5.2.4 Statistical analysis

All of the data collected were compared for differences using one-way ANOVA. Data were reported as means ± SE, with means considered significantly different at the p<0.05 level. For inter-strain analysis, the Tukey post-hoc statistical test was used with significance taken at the p<0.05 level.
5.3 Results

5.3.1 Validation of controls

In chapter 4, ddH$_2$O was used to prepare the LPS, mPM$_{0.5}$ and mPM$_{0.5}$-LPS solutions used for aerosol exposure of AECs. For continuity with the *in vitro* work the same aerosol preparations were used in these animal studies.

Distilled water is regarded as an inducer of airway responsiveness in humans (Law *et al.* 2000). While exposure to ddH$_2$O has been a control in a previous mouse study involving Tlr$^4^1$ mice, it is not common (Hollingsworth *et al.* 2004). Therefore, inflammatory responses to ddH$_2$O exposure were assessed here to ensure there were no changes due to ddH$_2$O exposure compared to the standard saline exposures used as controls in most published studies (Becker *et al.* 2002; Gavett *et al.* 1999; Gavett *et al.* 2003b; Tulic *et al.* 2000; Vernooy *et al.* 2002; Yanagisawa *et al.* 2003).

Measures of airway responsiveness and cellular infiltrate were compared between Tlr$^4^1$ and Tlr$^4^d$ mice exposed to ddH$_2$O and saline. Data for the saline exposed study were taken from a previous study conducted in the same laboratory (Burchell *et al.* 2002, unpublished data). A group of 5 mice were set aside for the ddH$_2$O experiment. Spirometry was successfully completed on 2 of these animals following nebulisation with ddH$_2$O. Lung function measurements were unsuccessful in the other mice. This was attributed to lung formation abnormalities observed
after dissection. In those animals that lung function test was completed changes from baseline were not observed.

There were no observable changes in inflammatory cell infiltrate or airway resistance in Tlr4\textsuperscript{d} mice exposed to ddH\textsubscript{2}O compared to Tlr4\textsuperscript{i} mice exposed to saline. In Tlr4\textsuperscript{d} mice, total cell counts were significantly increased in BAL taken from those mice exposed to ddH\textsubscript{2}O mice compared to the saline exposed Tlr4\textsuperscript{d} mice. Airway physiology measurements of Tlr4\textsuperscript{d} mice exposed to ddH\textsubscript{2}O were not significantly different to saline exposed Tlr4\textsuperscript{d} mice at 30mg/mL MCh.

Airway responsiveness was the measurement index in this study, as this remained the same between the ddH\textsubscript{2}O exposed and saline exposed Tlr4\textsuperscript{d} mice, ddH\textsubscript{2}O exposed mice were accepted as valid controls for this study. Therefore despite the low animal numbers ddH\textsubscript{2}O was deemed a suitable vehicle for nebulisation. Mice exposed to ddH\textsubscript{2}O are referred to as ‘controls’ throughout the remainder of this chapter.

5.3.2 TLR4 location in mouse airway epithelial cells

Immunohistochemical staining for TLR4 in TLR4\textsuperscript{i} mouse lung sections demonstrate surface location on airway epithelial cells (Figure 5.3.1). This was illustrated by the intense red staining located on the edge of the airway lumen, the location of airway epithelial cells.
Figure 5.3 Immunohistochemical staining of mouse airway for TLR4. Staining shows a clear surface location of TLR4 in the airway epithelium of TLR4\textsuperscript{−/−} control mouse lung (top, bottom picture is negative control) (200x magnification)
5.3.3 Cellular infiltration with exposure to mPM0.5, LPS and mPM0.5-LPS

In Tlr4\(^{+}\) mice total cell counts (TCC) were significantly greater in mice exposed to LPS and mPM0.5-LPS compared to control and mPM0.5 exposed mice (Figure 5.4 A). There were no significant differences in TCC’s between LPS and mPM0.5-LPS exposed mice. Similarly in Tlr4\(^{-}\) mice there were no significant differences in TCC’s for LPS and mPM0.5-LPS exposed mice compared to controls. For mPM0.5 exposed Tlr4\(^{+}\) mice, TCC’s were lower than the Tlr4\(^{-}\) control. Overall, TCC’s measured for Tlr4\(^{+}\) mice exposed to mPM0.5, LPS and mPM0.5-LPS were lower than their Tlr4\(^{+}\) counterparts.

Increased numbers of neutrophils and eosinophils in BAL of Tlr4\(^{+}\) mice exposed to LPS and mPM0.5-LPS are illustrated in Figure 5.4 B. These mice also had a lower number of macrophages compared to the control. There were no differences measured in Tlr4\(^{+}\) mice exposed to mPM0.5 compared to the control. In Tlr4\(^{-}\) mice there were no significant differences in differential cell counts in mPM0.5 or mPM0.5-LPS exposed mice compared to the control mice. Macrophages were the predominant cell type present in counts for these mice. A significantly increased number of macrophages were present in Tlr4\(^{-}\) mice exposed to LPS compared to the control (Figure 5.4B).

Lung sections from Tlr4\(^{+}\) mice exposed to LPS and mPM0.5-LPS and stained with haematoxylin and eosin, showed raised inflammatory cell infiltrate corresponding to raised differential cell counts observed in this mice (Figure 5.5).
Figure 5.4A. Total cell counts taken from BAL of Tlr4<sup>+</sup> and Tlr4<sup>+</sup> mice exposed to ddH<sub>2</sub>O, mPM<sub>0.5</sub>, LPS and mPM<sub>0.5</sub>-LPS.

In Tlr4<sup>+</sup> mice, counts taken from mice exposed to LPS and mPM<sub>0.5</sub>-LPS were significantly increased compared to ddH<sub>2</sub>O controls (α, β: p<0.05). There were no significant differences in total cell counts from Tlr4<sup>-</sup> mice between ddH<sub>2</sub>O, LPS, or mPM<sub>0.5</sub>-LPS exposures. For Tlr4<sup>-</sup> mice, mPM<sub>0.5</sub> exposure induced a smaller total cell count than the ddH<sub>2</sub>O controls (χ: p<0.05).
Figure 5.4B. Differential cell counts taken from BAL of Tlr4\textsuperscript{i} and Tlr4\textsuperscript{d} mice exposed to ddH\textsubscript{2}O, mPM\textsubscript{0.5}, LPS and mPM\textsubscript{0.5}-LPS.

Significantly increased numbers of neutrophils and eosinophils were measured in the Tlr4\textsuperscript{i} mice exposed to LPS and mPM\textsubscript{0.5}-LPS compared to the ddH\textsubscript{2}O controls (δ, ε, η, i: p<0.05). These mice also had a significantly reduced number of macrophages compared to the controls (ϕ, γ: p<0.05). In the Tlr4\textsuperscript{d} strain, the only significant difference was an increased number of macrophages in the LPS exposed mice compared to the controls (ϕ: p<0.05).
Figure 5.5 H&E staining of lung sections taken from a control and LPS exposed Tlr4−/− mouse to illustrate inflammatory cellular infiltrate in the lungs.

Alveolar spaces are filled with cellular infiltrate in the lung section of a LPS exposed mouse (top) compared to the open spaces observed in the control mouse lung section (bottom) (200x magnification).
5.3.4 Airway responsiveness with exposure to mPM\textsubscript{0.5}, LPS and mPM\textsubscript{0.5} - LPS

5.3.4.1 Summary of inter-exposure differences in airway resistance.

To measure changes in airway mechanics both mice strains were challenged with MCh 6hrs post exposure as described in section 5.2.2. The results demonstrate that nebulisation of Tlr4\textsuperscript{i} mice with mPM\textsubscript{0.5}-LPS induced a significant difference in Raw at 30mg/mL compared to the controls (\( \alpha: p<0.05 \)) (Figure 5.6). There were no significant differences observed between mice of either strain exposed to LPS, mPM\textsubscript{0.5} or mPM\textsubscript{0.5}-LPS (Figure 5.6).
Figure 5.6 Summary graph of maximum airway responses measured at 30mg/mL MCh in response to various exposures.

In the Tlr4^i mouse, the magnitude of maximal response is significantly increased in those mice exposed to mPM_{0.5}-LPS (α: p<0.05). No significant differences were measured between exposure types and ddH_{2}O controls in the Tlr4^d strain.
5.3.4.2 Summary of inter-exposure differences in airway sensitivity.

It has been reported that the dose of MCh to induce airway responsiveness can be used as a measure of airway sensitivity (Mochizuki et al. 2005). In this case the MCh dose to induce 150% increase in airway resistance is presented (Figure 5.7). In the Tlr4<sup>d</sup> strain, mice responded at significantly lower doses of MCh compared to their Tlr4<sup>i</sup> counterparts indicating the airways of the Tlr4<sup>d</sup> mice are more sensitive overall.

Mice from the Tlr4<sup>d</sup> strain nebulised with mPM<sub>0.5</sub> (1.65 ± 0.22 mg/mL) and mPM<sub>0.5</sub>-LPS (1.25 ± 0.35 mg/mL) required a lower dose of MCh induce 150% of maximum airway resistance compared to Tlr4<sup>d</sup> control mice (ε, δ p<0.05). Similarly a lower dose of MCh was needed to induce 150% maximum airway responsiveness in Tlr4<sup>i</sup> mice nebulised with LPS (3.56 ± 0.62 mg/mL) and mPM<sub>0.5</sub>-LPS (4.32 ± 0.83 mg/mL) compared to control mice of the same strain (χ, β p<0.05).
Figure 5.7 Summary graph of MCh dose required to induce 150% of maximum response for all types of exposures.

The dose of MCh required to induce 150% of the maximum airway response was significantly reduced in Tlr4\(^i\) mice exposed to LPS and mPM\(_{0.5}\)-LPS compared to the control, indicating greater airway sensitivity in these mice (\(\beta, \chi\): p<0.05). In Tlr4\(^d\) mice, airway sensitivity was increased in mPM\(_{0.5}\) and mPM\(_{0.5}\)-LPS exposed mice compared to the controls (\(\delta, \varepsilon\): p<0.05). Overall, Tlr4\(^d\) mice were significantly more sensitive to MCh than their Tlr4\(^i\) mice counterparts.
5.3.5 Measured cytokines in BAL with exposure to mPM_{0.5}, LPS and mPM_{0.5}-LPS

Significantly raised IL-6, IL-12p70, IFN\textsubscript{\gamma} and MCP-1 measurements were obtained in BAL from both strains of mice exposed to LPS and mPM_{0.5}-LPS compared to control and mPM_{0.5} exposed animals. There were no differences observed in secreted cytokines measured in BAL of mPM_{0.5} exposed mice compared to control mice of either mouse strain.

Significant differences between the LPS and mPM_{0.5}-LPS Tlr4\textsuperscript{i} exposed animals were measured for IL-12p70 and IFN\textsubscript{\gamma} (ι, o, p<0.05) (Figure 5.8). Elevated levels of IL-12p70 and IFN\textsubscript{\gamma} were measured in the BAL of Tlr4\textsuperscript{d} mice exposed to mPM_{0.5}-LPS compared to those exposed to LPS (φ, π: p<0.05). Similarly raised IL-6 and MCP-1 levels were observed in both strains of mice exposed to LPS and mPM_{0.5}-LPS. In Tlr4\textsuperscript{i} mice, TNF\textsubscript{\alpha} was elevated in LPS exposed mice compared to the control mice and similarly exposed Tlr4\textsuperscript{d} mice (τ p<0.05).
Figure 5.8 Summary graphs of cytokine levels measured in the BAL of exposed mice using CBA.

In Tlr4<sup>i</sup> mice, significant increases were measured for IL-6, IL-12p70, IFN<sub>γ</sub> and MCP-1 following exposure to LPS and mPM<sub>0.5</sub>-LPS (α, χ, ε, γ, κ, ν, ω, ξ: p<0.05). Similarly, significant increases in IL-6, IL-12p70, IFN<sub>γ</sub> and MCP-1 were also measured in Tlr4<sup>d</sup> mice exposed to LPS and mPM<sub>0.5</sub>-LPS (β, δ, φ, η, λ, μ, ψ, Δ: p<0.05) compared to the controls. Significantly more IL-12p70, IFN<sub>γ</sub> and TNFα were measured in the Tlr4<sup>i</sup> LPS exposed mouse compared to the mPM<sub>0.5</sub>-LPS exposure (τ, o, τ: p<0.05). Significantly more IL-12p70 and IFN<sub>γ</sub> were measured in the Tlr4<sup>d</sup> mPM<sub>0.5</sub>-LPS exposed mouse compared to the LPS exposure in the same strain (φ, π: p<0.05).
5.4 Discussion

5.4.1 Morphological changes

In this chapter, we have shown in the airways of naïve mice, epithelial cells express TLR4 on the cell surface in a pattern similar to human airway epithelial cells (Chapter 3). In contrast to the human airway epithelial cells stained in chapter 3 intracellular staining of TLR4 in the mouse airway epithelial cells was not observed. This suggests recognition and signal transduction by TLR4 for LPS occurs at the cell surface.

5.4.2 Inflammatory cell infiltrate

In this study both Tlr4\textsuperscript{t} and Tlr4\textsuperscript{d} mice were exposed to mPM\textsubscript{0.5}, LPS and mPM\textsubscript{0.5}-LPS aerosols to determine the influence of these exposures on airway inflammatory responses. Mice exposed to mPM\textsubscript{0.5} did not show any change in inflammatory cell infiltrate or lung morphology when compared to control mice. In contrast, mice exposed to LPS and mPM\textsubscript{0.5}-LPS an increased number of neutrophils in their BAL. These results were in agreement with other reports that show an increased number of neutrophils in response to LPS exposure ((Brass et al. 2007; Savov et al. 2002; Vernooy et al. 2002) suggesting LPS is driving the inflammatory response in the mPM\textsubscript{0.5}-LPS exposed mice. This may be due to the large portion of unbound LPS in the mixture as validated in Chapter 4 and is further supported by the low TCC measures for Tlr4\textsuperscript{t} mice exposed to mPM\textsubscript{0.5}.
5.4.3 Airway responsiveness

Mice challenged with mPM$_{0.5}$-LPS were the only group to show a significant increase in Raw in response to MCh. Since mice challenged with mPM$_{0.5}$ did not show any changes in airway resistance, our data suggests that LPS present in the mPM$_{0.5}$-LPS combination is likely to be responsible for the increased magnitude of responsiveness measured for this (mPM$_{0.5}$-LPS) exposure type.

The required dose of MCh to produce a 150% increase in Raw was not significantly different between control and mPM$_{0.5}$ Tlr4$^l$ mice. In contrast, the airways of mPM$_{0.5}$ exposed Tlr4$^d$ mice responded to a significantly reduced dose of MCh compared to Tlr4$^d$ controls; demonstrating increased airway sensitivity in the absence of TLR4 to mPM$_{0.5}$ exposure. The lower cell counts and lack of observable morphological changes on H&E stains of lung sections in these mice suggest the increased airway sensitivity is unlikely to be attributable to inflammatory processes. However, it is plausible that background TLR4 responses may contribute to some stabilising effect on the sensory nerves. In the absence of these signals (Tlr4$^d$ mice) the nerves may be more responsive. Particulate exposure has been linked with increased sensitivity of peripheral nerves for some time now. However, despite numerous studies in mice and guinea pigs, the exact mechanisms are still unclear (Lee and Pisarri 2001; Li et al. 1997; Nurkiewicz et al. 2006).

Interestingly, exposure to mPM$_{0.5}$-LPS resulted in more sensitive airways in both Tlr4$^l$ and Tlr4$^d$ mice. The increased sensitivity of exposure to mPM$_{0.5}$-LPS in the Tlr4$^l$ mice was probably due to the presence of LPS whilst in the Tlr4$^d$
mouse due to mPM$_{0.5}$. In support of this, studies by Becker et al 1996 and Soukup and Becker 2001 have demonstrated enhanced PM induced inflammation due to the presence of endotoxin (Becker et al. 2002; Nurkiewicz et al. 2006; Soukup and Becker 2001).

5.4.4 Cytokines measured from BAL

To assess the role of TLR4 in inducing cytokine secretion in response to mPM$_{0.5}$, LPS and mPM$_{0.5}$-LPS exposure in Tlr4$^i$ and Tlr4$^d$ mice BAL was collected to compare levels of secreted inflammatory cytokines. The presence of inflammatory cytokines measured in BAL followed a similar trend to that observed in the TCC’s. In Tlr4$^d$ mice, for example, LPS and mPM$_{0.5}$-LPS exposure resulted in the greatest TCC’s corresponding to the greatest inflammatory cytokine levels.

None of the measured secreted cytokines were raised in mPM$_{0.5}$ exposed mice of either strain. This contrasts with other PM exposure studies in mice that show raised levels of IL-6 and TNF$_\alpha$ (Dick et al. 2003; Walters et al. 2001) Tang et al 2008). These studies use collected ambient PM that contains a heterogenous mixture of metals. Transition metals are known to stimulate ROS species that in turn cause secretion of pro-inflammatory agents (Carter et al. 1997; Dye et al. 1999; Gavett et al. 1997). The smaller changes observed using the model inert polystyrene beads in this study suggests an influence of PM composition on inflammatory responses. In addition there were no changes in baseline TCC and differential counts of BAL from mice nebulised with mPM$_{0.5}$. In contrast, studies using other models involving ambient PM collections have shown significantly raised cell counts (Dick et al. 2003; Gavett et al. 2003b; Wang et al. 2008).
and all PM size fractions have been found to induce lung inflammation in mice (Dick et al. 2003). Therefore it was expected that the ultrafine sized particles used in this study would induce an inflammatory response. The lack of response measured in this study suggests lung inflammation in response to PM exposure requires a particular chemistry.

Elevated IL-6, IL-12p70, IFNγ and TNFα were present in the BAL of Tlr4⁺ and Tlr4⁻ LPS exposed mice with greater amounts measured in the Tlr4⁺ strain. There was at a 2-fold greater level of secretion of these cytokines in the BAL of these two mouse strains demonstrating the potency of TLR4 induced cytokine responses. MCP-1 levels were not significantly different between Tlr4⁺ and Tlr4⁻ LPS exposed mice, illustrating a mechanism other than TLR4 for inducing secretion of this cytokine in response to LPS exposure. Scavenger and NOD receptors present in alveolar macrophages are likely candidates for the non-TLR4 dependant MCP-1 response observed in the LPS exposed Tlr4⁻ mice.

In both mouse strains mPM₀.₅-LPS exposure induced increased levels of; IL-6, IL-12p70, IFNγ and MCP-1. Of these cytokines, there were significantly greater levels of IL-6 and IFNγ in the Tlr4⁺ mPM₀.₅-LPS exposed mice compared to the Tlr4⁻ strain. There was not a significant difference in secreted IL-12p70, TNFα and MCP-1 between strains for mPM₀.₅-LPS exposure. Significantly different measures for IL-12p70 and IFNγ in the mPM₀.₅-LPS exposed mice compared to the LPS exposed mice lend support for an alternate pathway for LPS signalling when attached to mPM₀.₅. Raised levels of IFNγ and IL-12p70 measured in the Tlr4⁻ mPM₀.₅-LPS exposed mice in comparison to the LPS exposed mice.
suggests microbial recognition by a mechanism other than TLR4. Microbial invasion induces IFNγ secretion that in turn causes raised IL-12p70. This response sequence implies activation of the innate immune system. Although this is likely IFNγ and IL-12p70 are also potent inducers of Th1 responses demonstrating the possibility of a particle dependant LPS driven Th1 activation in the absence of TLR4. Further investigation into the activity of this non-TLR4 system(s) may explain how LPS exposure is able to generate a Th1 or Th2 response as has been reported in epidemiological studies (Chen et al. 2007; Schwartz 2001; Strachan 1989; von 2000). One explanation is a carrier effect of mPM0.5 when attached to LPS. It is possible that an endocytotic process allows LPS passage into the cell. In this case the opportunity to bypass some cell surface mechanisms against microbial invasion such as TLR4 may cause the activation of another intracellular microbial surveillance mechanism such as the NOD-like receptors (NLRs).

Recognition of LPS attached to mPM0.5 by TLR4 depends on conformational interaction of lipid A with the receptor. If the mPM0.5-LPS complex is endocytosed cytoplasmic PRRs such as NOD1 and NOD2 can recognise motifs in the lipid regions of LPS. In chapter 3 TLR4 was shown to reside intracellularly in addition to being on the cell surface in pAEC’s. Immunohistochemical staining of TLR4 in these mice showed clear TLR4 expression in AEC’s. Staining was completed on saponin treated specimens but intracellular TLR4 was not obvious. Further investigation using another technique may clarify this result. A recent report suggests TLR4 recedes to an intracellular endosomal location for signalling purposes (Kagan et al 2008). The activity of TLR4 within these endosomes was not discussed however from data presented in chapter 4 it is
likely that TLR4 is still active when cytosol bound. Therefore intracellular TLR4 recognition of LPS attached to mPM$_{0.5}$ is possible.

While TLRs and NLRs induce inflammation via translocation of NF-κβ into the cell nucleus different adaptor proteins activating particular signalling pathways are used (Inohara and Nunez 2003a; Strober et al. 2006). Together these provide signal induced response specificity. Therefore adherence of LPS to mPM$_{0.5}$ within the cytosol is a critical determinant for activation of PRRs. Occlusion of LPS PAMPs used for signalling by these receptors can alter induced inflammatory responses. This process may be evident in the raised IFNγ and IL-12p70 levels observed in the Tlr4$^d$ mPM$_{0.5}$-LPS exposed mouse. However, for a true molecular understanding of these processes the exact binding sites and bond strength between LPS and mPM$_{0.5}$ is needed.

5.4.5 Aerosol model used

Traditionally saline has been used as the control aerosol preparation for clinical assessment of hyperresponsiveness in humans. This has been successfully adapted for mouse studies aiming to replicate this clinical setting using FOT. However for this study, aerosol preparations in saline do not truly represent the conditions by which particulates are carried in inhaled air. Since water droplets are more common in inhaled air rather than physiologically controlled solutions such as saline, an initial investigation into the preparation of aerosols in ddH$_2$O was performed.

Whilst ddH$_2$O has been recognised to induce airway responsiveness clinically (Law et al. 2000), others have justified using ddH$_2$O as a control parameter. For example, Hollingsworth and colleagues (Hollingsworth et al. 2004) successfully
prepared ROFA particulates in ddH₂O in their mouse study to investigate aerosolised effects of particulate and ozone on mouse lung. Results from this study showed that aerosolised ddH₂O did not have any adverse effects on the mice studied.

Similarly, results from this present study did not find ddH₂O to have a detrimental effect on mouse airway physiology. In Tlr4ᵢ mice there was no hyperresponsiveness, no inflammatory response and more importantly no morbidity observed with ddH₂O exposure. In Tlr4ᵈ mice the only observable difference between ddH₂O and saline exposure was an elevated TCC in BAL of ddH₂O exposed mice, which did not translate to any alteration in airway responsiveness.

Historical data from the Clinical Sciences laboratory was used to show that there were no differences in TCC’s between Tlr4ᵢ mice exposed to ddH₂O and Tlr4ᵢ mice exposed to saline. Although some caution is needed in drawing conclusions from data collected at a different time point, these results suggests the observed increase in inflammatory cell infiltrate of theTlr4ᵈ mice exposed to ddH₂O is a likely result of the mutation. More importantly, airway responsiveness in the Tlr4ᵈ mice exposed to ddH₂O did not differ to Tlr4ᵈ mice exposed to saline. Since airway responsiveness was the measure of interest in this particular study, the use of aerosols prepared in ddH₂O was deemed an appropriate control parameter.

At present, the studies by Tulic et al are the only ones to have used FOT to evaluate changes in airway responses to LPS (Tulic et al. 2000; Tulic et al. 2001; Tulic et al. 2002). The literature currently available relies heavily on full
body plethysmography (Penh) for inferences into airway responses resulting from LPS and PM$_{0.5}$ exposures (Hollingsworth et al. 2004; Savov et al. 2002). Discrepancies in using this method have been well discussed in the literature, from a lack of correlation with lung weight or histological changes (Adler et al. 2004; DeLorme and Moss 2002; Flandre et al. 2003; Pauluhn 2004; Petak et al. 2001) to more validated measurements of airway mechanics. Previous studies have illustrated inflammatory effects of LPS in mice as measured by increased inflammatory cell infiltrate and increased airway resistance (Becker et al. 2002; Hollingsworth et al. 2004; Tulic et al. 2000). Other studies investigating PM exposure have relied on cytokine measures from the BAL without airway response measures (Brass et al. 2007; Dick et al. 2003; Gavett et al. 2003a). The results from this study are the first to fully address the effects on airway inflammation and responsiveness due to exposure of LPS, mPM$_{0.5}$ or mPM$_{0.5}$ - LPS.

Documented differences in baseline airway mechanics between inbred mouse strains attributed to genetic factors (Held and Uhlig 2000; Tankersley et al. 1999). Significant differences between sexes within mouse strain have also been shown (Card et al. 2007). The large differences in baseline responses between the two strains of mice observed in this study have not been described before. Conflicting reports exist regarding Tlr4$^d$ (C3H/HeJ) airway sensitivity (Bergner and Sanderson 2003; Gavett and Wills-Karp 1993). This difference suggests raised airway sensitivity in the Tlr4$^d$ mice as reported previously (Bergner and Sanderson 2003). As the baseline response is raised differences in lung function between mice strains observed here may not accurately reflect the role of TLR4 in airway responses induced on exposure to these aerosol
preparations. It is likely that in the absence of TLR4 airway responses would be significantly diminished in response to LPS compared to those mice with TLR4.

Exposure models vary between studies. Nasal and tracheal instillations of the exposure substance of interest are common (Dick et al. 2003; Yanagisawa et al. 2003). Tracheal instillation bypasses filtration of inhaled air by the upper respiratory system. In these cases the received dose might be greater and there is a potential to avoid systemic responses generated by exposure of the upper respiratory tract. The aerosol method used in this study is superior as it replicates Tlr4 breathing of inhaled substances carried in the air more closely. Ideally this protocol would have used a dry aerosol exposure but due to equipment constraints a wet aerosol was used. This method relied on water as a vehicle for the substance of interest, whereby pre-prepared solutions are nebulised over the animals. The presence of water droplets may act to mask or add to the responses generated in some way by interacting with substances in solution. As these droplets would not normally be found in inhaled air, future studies clarifying the effects of mPM0.5-LPS exposure should preferably use a dry aerosol generator.

5.4.6 Conclusions

In conclusion, this study has shown that LPS attached to mPM0.5 induces different inflammatory responses to mPM0.5 or LPS exposures alone. This finding suggests activation of a signalling pathway other than TLR4 in response to mPM0.5-LPS exposure. This is an important consideration as LPS is commonly found attached to particulates in inhaled air. The interaction of LPS with another signalling pathway may explain why LPS exposure can generate
polarised Th1 or Th2 inflammatory immune responses as has been previously reported. Future studies investigating the effect of PM size on PM$_{0.5}$-LPS responses and possible endocytotic pathways that might be involved in PM$_{0.5}$-LPS signalling may elucidate the mechanisms present here.
Final Discussion

6.1 Summary of main findings in relation to the specific aims of this investigation.

The main objectives of this thesis were to, (1) characterise the expression and location of TLR4 in AEC’s obtained by bronchial brushing from children with different airway conditions; asthma, allergic asthma and cystic fibrosis. Changes in TLR4 expression were also assessed when these AEC’s were placed in monolayer culture to evaluate the validity of studying this receptor in culture systems. (2) TLR4 signalling was investigated in AEC’s following exposure to LPS, mPM0.5 and mPM0.5-LPS and (3) the effects of these exposures on pulmonary inflammation and function were then determined in normal and TLR4 deficient mice.

6.1.1 To examine the level of TLR4 gene expression and pattern of TLR4 protein location on freshly isolated and primary cultured AEC’s.

TLR4 gene expression and pattern of TLR4 protein location was examined on freshly isolated and primary cultured pAEC’s from healthy (pAEC_{HNA}), healthy atopic (pAEC_{HA}), asthmatic (pAEC_{ANA}), atopic asthmatic () or cells from patients with cystic fibrosis (pAEC_{CF}). Using real time PCR, gene expression levels of TLR4 was greatest in freshly isolated pAEC_{AA} and pAEC_{CF} compared to pAEC’s from the other phenotypes assessed. Of the cultured cells, TLR4 gene expression was upregulated in pAEC_{HA}, pAEC_{HNA} and pAEC_{ANA} primary cell
cultures relative to their freshly isolated counterparts after being normalised to freshly isolated pAEC_{HNA} TLR4 gene expression. In contrast, cultured pAEC_{AA} cells had significantly lower TLR4 gene expression relative to freshly isolated pAEC_{AA}.

TLR4 protein was shown to reside predominantly on the cell surface by immunohistochemistry. However, there was variability in staining intensity and receptor location depending on phenotype of pAEC's (Figure 6.1a). The most intense surface staining was observed in pAEC_{HA} and pAEC_{CF} cells. In contrast, pAEC_{AA}, pAEC_{ANA} and pAEC_{CF} cells had the most intense intracellular staining pattern. In pAEC_{HNA} cells, staining appeared equally distributed between the cell surface and cytosol region. Using a previously published immunohistochemical scoring method, semi-quantitative analysis of freshly isolated pAEC_{AA} and pAEC_{CF} recorded the greatest overall TLR4 protein scores. Together these findings indicate raised TLR4 expression in pAEC_{CF} and a possible role of asthma (pAEC_{ANA}, pAEC_{AA}) in skewing toward a predominant intracellular TLR4 location.

Recently TLR4 has been reported to recede into the cytoplasm to an endosomal vesicle for signalling purposes (Kagan et al. 2008). This has been proposed based upon cytosolic location of tumor necrosis factor receptor associated factor (TRAF)-3. TRAF3 is an essential adaptor involved in downstream signalling of TLR4 and does not readily access plasma membrane signalling complexes. Activation of TRAF3 leads to interferon production and is a reported inhibitor of NF-κB. This has not been in shown in airway epithelial cells however it may explain why TLR4 can be found intracellularly. The marked
upregulation of pAEC\textsubscript{AA} TLR4 gene expression may be a result from many different influences therefore further investigation elucidating relationships between TLR4 location, expression and AEC sensitivity to LPS is warranted. Outcomes from such studies may show induced TLR4 translocation to the cell surface reduces LPS sensitivity. This finding may lead to possible therapeutic benefits by reducing potential exacerbatory events observed in asthmatics in response to LPS.

As well as influencing gene expression, culturing conditions also appeared to affect the staining pattern of TLR4. Thus, in contrast to freshly isolated cells, TLR4 expression was predominantly intracellular when pAEC were placed in submerged culture. Cells isolated from the airways may behave differently in culture as compared with \textit{in vivo} responses when they are part of a tissue/organ, because the cell–cell interactions that exist in tissue are lost \textit{in vitro}. This gene and protein data demonstrate the influence of the cellular microenvironment on cellular characteristics (Figure 6.1B).

6.1.2 To assess the role of Th1 and Th2 polarised cellular environments on TLR4 location.

IFNy and IL-13 were chosen to compare the effects of a Th1 and Th2 environment on TLR4 location and expression. IFNy is secreted by Th1 lymphocytes and is known for its ability to exert an inhibitory effect on Th2 cells, inhibiting allergic inflammation and IgE synthesis (Kips et al. 1996; Lewkowich and HayGlass 2002). These properties contrast with those of IL-13, used to emulate a Th2 polarised environment (section 6.1.1). IL-13 was chosen to
stimulate cultured cells because it is the cytokine with the strongest correlation to wheeze and atopy of all of the cytokines investigated to date (Wills-Karp et al. 1998; Wong et al. 2001; Zhu et al. 1999). For this reason it was deemed the most appropriate stimulus to create a Th2 polarised environment. For both cytokines, concentrations were chosen to reflect normal sera levels to assess changes induced at baseline.

Based on studies observing an upregulation of TLR4 expression by IFNγ in gut epithelium (Abreu et al. 2002), a similar effect was expected in the airway epithelium. However, in this investigation, cultured epithelial cells showed a greater shift in TLR4 expression on the cell surface following IL-13 stimulation (Chapter 3). Surprisingly no changes were observed with IFNγ stimulation in 16HBE cells. This observation contrasts those in the gut epithelium, indicating this may be a cell type dependant response to stimulus.

Exposure to IL-13 induced a translocation of TLR4 to the cell surface illustrating the possible effect of an ongoing extrinsic stimulus could have on receptor location in a cell (Figure 6.1C). While TLR4 mRNA expression was increased in the IL-13 stimulated cells, LPS responsiveness, as measured by IL-8 release did not. Rather, IL-13 stimulated cells appeared hyporesponsive to LPS suggesting that location may have an effect on the receptor activity.

It is also possible that IL-13 may have an inhibitory effect on IL-8 secretion. IL-13 has been shown to inhibit IL-8 secretion along with other proinflammatory cytokines in studies involving airway smooth muscle cells and monocytes (de
The mechanism explaining this effect remains unknown. It is possibly via the same pathway as that involved in the inhibition of IL-8 by IL-4 (Aman et al. 1996). IL-4 and IL-13 share subunits of the same receptor indicating similar signalling pathways and suggesting similar induced responses (Debinski et al. 1995; Obiri et al. 1997). At least one study has attributed the inhibitory effect of IL-4 on IL-8 to the transcription factor Stat 6 in fibroblasts and keratinocytes (Hoeck and Woisetschlager 2001; Raingeaud and Pierre 2005). This effect is reportedly cell type specific but has not been conducted on airway epithelial cells, pointing to a need for further study in this area (Raingeaud and Pierre 2005).

From these results, IL-13 stimulation was found to induce translocation of TLR4 to the cell surface. Results from chapter 3 indicate that TLR4 resides on the airway epithelial surface in vivo. In chapter 4 it was shown that when located on the cell surface TLR4 is less responsive to LPS as measured by IL-8 secretion. These results suggest that pAEC AA should be more responsive to LPS exposure as they have the greatest amount of intracellular TLR4 (Chapter 3). Indeed, several studies have demonstrated increased airway hyperresponsiveness in asthmatics following LPS exposure and greater IL-8 levels in induced sputum of asthmatics (Birring et al. 2004; Nightingale et al. 1998)(Figure 6.2). Together these data link TLR4 location as a determining factor in LPS induced exacerbations in asthma. By understanding which signal(s) cause the receptor to localise intracellularly in pAEC AA a potential target site for therapeutic intervention may be possible.
6.1.3 To characterise an in vitro model designed to measure bronchial epithelial responses to LPS and PM$_{0.5}$ exposure via TLR4.

The *in vitro* model established in Chapter 4 was used to investigate the responses of airway epithelial cells to LPS and PM$_{0.5}$ exposures. This model utilised cells at an air liquid interface in an attempt to reflect *in vivo* conditions. The 16HBE cell line was used in these studies because the available primary cell culture numbers were not sufficient for the requirements of this investigation. The 16HBE transformed airway epithelial cell line is able to differentiate with retinoic acid stimulation, making it one of the cell lines best able to represent freshly isolated cells.

Preliminary experiments were conducted to determine an appropriate concentration of LPS for the aerosol preparation. From these experiments the effective dose was found to be $1/10^{th}$ of the prepared aerosol solution concentration. LPS aerosol solutions were prepared at 50µg/mL concentration. This yielded a 5µg/mL effective LPS dose that induced secretion of IL-8 within the linear portion of reported dose response curves for epithelial cells (Backhed *et al.* 2002a; Monick *et al.* 2003). For consistency, mPM$_{0.5}$ and mPM$_{0.5}$-LPS were also prepared at 50µg/mL. In this exposure model, polystyrene beads (0.5µm diameter) were used (referred to as mPM$_{0.5}$) to assess responses to inert PM. In the air-liquid interface culture system, LPS exposure induced a much larger IL-8 response then exposure of cells to mPM$_{0.5}$.

Ambient PM particle size is an important consideration as it alludes to composition. The PM$_{10}$ fraction is more likely to consist of organic matter and
thereby greater proportion of LPS. Whereas PM$_{2.5}$ consists largely of particles generated from combustion processes (Samet et al. 2007). Sources of these include diesel powered engines, power generation and wood burning (Committee on Environmental Health, Pediatrics 2007). Presence of metals, DEP and LPS can affect inflammatory responses. This has been noted in investigations concerned with measuring ambient PM size fraction dependent responses (Becker et al. 2002; Becker et al. 2005; Dick et al. 2003; Soukup and Becker 2001). Secreted IL-8 released by cells nebulised with mPM$_{0.5}$ was not significantly different from control cells. This contrasts with inflammatory cytokine data demonstrated in other studies but is a likely result of the chemically inert composition of the polystyrene bead model compared to the mixed composition of collected ambient PM fractions used elsewhere (Dick et al. 2003; Fujii et al. 2002b; Mukae et al. 2000; Soukup and Becker 2001).

6.1.4 To investigate if airway epithelial responses are different to LPS and PM$_{0.5}$ exposures compared to an exposure of LPS attached to PM$_{0.5}$.

These experiments were performed to assess the airway epithelial responses of exposure to LPS as it would be carried in inhaled air. These results demonstrate that dissimilar signalling pathways are activated as for mPM$_{0.5}$-LPS exposure compared to LPS and PM$_{0.5}$ exposures.

Adherence of mPM$_{0.5}$ to LPS was low but significant as shown in Chapter 4. Exposure of airway epithelial cells to mPM$_{0.5}$-LPS induced IL-8 secretion that was greater than exposure to mPM$_{0.5}$ or LPS. This result indicates that unbound
LPS present in the mPM$_{0.5}$-LPS mixture were probably driving the overall response (Figure 6.2A, B, C).

IL-13 stimulated 16HBE cells were used as a model of healthy airway epithelial cells in vivo, whereby TLR4 is found on the cell surface. Interestingly, in cells either stimulated with IL-13 or left unstimulated, exposure to mPM$_{0.5}$-LPS did not induce a significant change in TLR4 mRNA, whilst in the LPS and mPM$_{0.5}$ exposed cells a significant elevation of TLR4 mRNA was observed. This suggests an increase in protein cycling due to stimulation. That this did not occur in cells that were exposed to mPM$_{0.5}$-LPS suggests an interaction of a different signalling mechanism (responsible for mPM$_{0.5}$-LPS recognition) that is disruptive to IL-13 induced effects (Figure 6.3A, B). It is possible that phagocytosis of mPM$_{0.5}$-LPS allows LPS to bind to internal receptors such as the NOD1. Activation of the NOD1 signalling pathway may be responsible for lower TLR4 mRNA observed for this exposure type. As NOD1 recognises motifs on the polysaccharide chain of LPS occlusion of lipid A by mPM$_{0.5}$ would still allow LPS recognition. Similar to TLR4 signalling NOD1 results in NF-κβ activation inducing inflammatory cytokine production. In this case the presence of NOD1 and TLR4 signalling proteins would need to be targeted in order to more accurately determine the roles of these in mPM$_{0.5}$-LPS responses.
6.1.5 To determine if mPM$_{0.5}$-LPS exposure induces a greater airway response in vivo than exposure to mPM$_{0.5}$ or LPS individually and if these responses are TLR4 dependant.

Mice with (normal) and deficient functional TLR4 were used to assess the role of exposure and receptor in airway and inflammatory responses. Airway resistance was measured using the forced oscillation technique (FOT) and inflammatory changes were assessed from bronchoalveolar lavage (BAL) by differential cell counts and cytokine levels. Immunohistochemistry confirmed that TLR4 was located on the cell surface of airway epithelial cells in these mice.

Increases in airway hyperresponsiveness and inflammatory responses were only observed in normal mice exposed to LPS or mPM$_{0.5}$-LPS. The similarity in responses for these exposures and taking into consideration the presence of unbound LPS in the mPM$_{0.5}$-LPS mixture, strongly suggests that LPS is the major stimulus for airway inflammation. IL-12p70 and IFN$_\gamma$ were increased in the mPM$_{0.5}$-LPS exposed group of mutant mice. These were the only cytokine measures to refute LPS as the responsible agent in responses generated from mPM$_{0.5}$-LPS exposure.

IL-12p70 is a potent inducer of IFN$_\gamma$ and increased IFN$_\gamma$ is suggestive of a Th1 polarisation. The greater IFN$_\gamma$ levels in the mPM$_{0.5}$-LPS exposed mutant mouse suggests attachment to mPM$_{0.5}$ allows LPS to bypass signalling by TLR4, possibly activating another microbial surveillance mechanisms (Figure 6.3c). It is plausible that an interaction of attached LPS with scavenger (or other LPS) receptors such as NOD1 may be a more favourable interaction, permitting a
greater response then exposure to unbound LPS (Figure 6.3d). This finding may also provide a possible explanation for the dual mechanism reported in LPS exposure; ie the ability to generate Th1 and Th2 skewed responses as observed in epidemiological studies (Chen et al. 2007; Delfino 2002; Michel et al. 2001; Rizzo et al. 1997; Strachan 1989; von 2000; Zeldin et al. 2006).

6.2 Conclusions

In conclusion, the work described in this thesis suggests LPS induced TLR4 responses in human bronchial epithelial cells depends on receptor location. In addition, signalling by TLR4 may be bypassed when LPS is attached to PM$_{0.5}$. While tenuous, both of these findings are relevant to our understanding of asthma and atopy. The exacerbation of asthma following LPS exposure may be explained by TLR4 receptor location, while the development of polarised cytokine responses in epithelial cells (ability of LPS to bypass TLR4) might contribute to the observed protective effect of LPS exposure against the development of asthma and atopy. This was demonstrated by the elevated IL-12p70 and IFN$_\gamma$ in Tlr4$^d$ mice exposed to mPM$_{0.5}$-LPS compared to the Tlr4$^i$ mice.

6.3 Future studies

Having completed studies on immortalised cell lines such as 16HBE to establish the in vitro exposure model, future studies could incorporate the use of primary bronchial epithelial cells of different airway disease states. Exposure studies on these cells would allow confirmation of the influence TLR4 location and expression has on inflammatory responses. During the course of this investigation, preliminary studies assessing the effects of LPS and mPM$_{0.5}$
exposure of NHBE cells was conducted. However, not knowing the atopic status of the cells prevented accurate conclusions to be made and the study was abandoned. Our laboratory has developed a cryobank of paediatric bronchial epithelial cells where the atopic status is known (Kicic et al. 2006; Lane et al. 2005b). This resource allows for the use of primary cells of different disease states to be exposed and responses compared.

To validate the use of IL-8 as a measure of TLR4 responses in the in vitro model, a knockout or silencing effect on TLR4 should be investigated. Levels of IL-8 in control cells after 1hr were unexpectedly high, and as such, results from time points after 1hr were not considered. Having investigated the initial responses generated on exposure of 16HBE cells to mPM$_{0.5}$, LPS and mPM$_{0.5}$-LPS, the analysis of responses from longer time points may provide differences based on changes in gene expression that may have been missed in this study.

Similarly, responses to exposures of mPM$_{0.5}$-LPS have been completed in a non-sensitised murine model. These can now be used as a comparison to exposures completed on sensitised animals with and without TLR4, providing further insight into the influence of allergy on responses to LPS and PM. Determining whether TLR4 binding is bypassed when LPS is attached to PM$_{0.5}$ in sensitized animals would be of particular relevance in understanding how LPS interacts with intracellular to influence airway inflammatory responses. Further studies clarifying these responses should take possible binding to the non-functional TLR4 into account. Unable to induce signalling, this decoy may have an inhibitory effect.
Figure 6.1 Changes in TLR4 expression with disease status and in cultured human bronchial epithelial cells.

Immunohistochemical staining was used in Chapter 3 to characterise TLR4 location in bronchial epithelial cells from freshly isolated cells of different phenotypes (A) and in cultured cells (B). Staining for TLR4 was predominantly on the cell surface in the freshly isolated pAEC's of all the phenotypes (see above), however, intense intracellular staining was noted for pAEC_{AA} and pAEC_{CF}. Following culture, these same cells revealed a translocation of the receptor to a predominantly intracellular location, although some surface staining for TLR4 remained in cells from atopic donors. In context of IL-13 causing a translocation of the receptor to the cell surface at 6hrs (C) (as also found in Chapter 3), and taking into consideration the different microenvironment present for atopic and CF cells our data suggests that extrinsic cellular stimuli may play an important role in determining TLR4 location \textit{in vivo} in AEC's.
Figure 6.2. Summary of LPS interactions with TLR4 in AEC’s that are (A) freshly isolated, (B) in culture or (C) cultured and stimulated.

A. Interaction of LPS with bronchial epithelial cells. LPS is recognised by TLR4 on the cell surface of AEC’s. This causes leads to secretion of pro-inflammatory cytokines such as IL-8, causing an inflammatory response.

B. Interaction of LPS with AEC’s in culture. TLR4 is not present on the cell surface therefore LPS is presented to the receptor after being phagocytosed. IL-8 responses measured in these cells may also be due to another receptor such as NOD1.

C. IL-13 stimulation downregulates IL-8 secretion in response to LPS. IL-13 causes TLR4 to translocate to the cell surface. Cell responsiveness to LPS decreases as measured by IL-8 secretion.
Figure 6.3. Summary of responses to mPM$_{0.5}$-LPS in (A) AEC's and (B) stimulated AEC's in culture compared to responses measured in the mouse model (C) and a possible mechanism explaining these responses (D). (A) Unstimulated AEC's are less responsive to mPM$_{0.5}$-LPS than to LPS exposure. LPS exposure produces a greater amount of IL-8 secretion indication a stronger interaction with TLR4 than mPM$_{0.5}$-LPS. (B) Sensitivity to LPS as measured by IL-8 secretion in IL-13 stimulated AEC's decreases when exposed to mPM$_{0.5}$-LPS. IL-8 secretion is significantly decreased in stimulated cells in response to mPM$_{0.5}$-LPS compared to LPS exposure alone. This indicates that LPS attached to mPM$_{0.5}$ is presented and thereby processed differently. (C) mPM$_{0.5}$-LPS exposure in mice induces more IL-12p70 and IFN$_{\gamma}$. IL-12p70 is a potent inducer of IFN$_{\gamma}$. This response is replicated in mutant mice suggesting LPS is presented differently to cells when attached to mPM$_{0.5}$ and can induce different responses to when it is presented alone. (D) When LPS is attached to mPM$_{0.5}$ a more favourable conformational interaction is possible between LPS and another type of receptor. This explains the raised levels of IFN$_{\gamma}$ and IL-12p70 observed in exposures of mutant mice to mPM$_{0.5}$-LPS in comparison to nebulisation with LPS alone.
References


Ref Type: Report


223. Tamai, R., Sakuta, T., Matsushita, K., Torii, M., Takeuchi, O., Akira, S., Akashi, S., Espevik, T., Sugawara, S., and Takada, H. (2002). Human gingival CD14(+) fibroblasts primed with gamma interferon increase production of interleukin-8 in response to lipopolysaccharide through up-


Appendices

Appendix 1: Materials

General laboratory reagents purchased from Sigma (Sigma-Aldrich Chemical Co., USA) were of analytical grade, with specific chemicals/reagents and their sources listed below:

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<td>Goat anti-mouse biotinylated IgG</td>
<td>BioLegend, CA USA</td>
</tr>
<tr>
<td>Mouse anti-human TLR4 monoclonal</td>
<td>BioLegend, CA USA</td>
</tr>
<tr>
<td>PE Streptavidin</td>
<td>BD Biosciences, CA USA</td>
</tr>
<tr>
<td>Rabbit anti-human TLR4 polyclonal</td>
<td>Santa Cruz Biotechnology Inc., CA USA</td>
</tr>
<tr>
<td>IL-13 recombinant protein</td>
<td>BD Biosciences, CA USA</td>
</tr>
<tr>
<td>IFNy</td>
<td>BD Biosciences, CA USA</td>
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</tbody>
</table>

**RNA EXTRACTION KITS AND GENE expression analysis**

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAshredder</td>
<td>QIAGEN, Hilden Germany</td>
</tr>
<tr>
<td>Reverse Transcription reagents</td>
<td>Applied Biosystems, CA USA</td>
</tr>
<tr>
<td>RNase-free DNase kit</td>
<td>QIAGEN, Hilden Germany</td>
</tr>
<tr>
<td>RNeasy RNA extraction kit</td>
<td>QIAGEN, Hilden Germany</td>
</tr>
<tr>
<td>SYBR® Green PCR master mix</td>
<td>Applied Biosystems, CA USA</td>
</tr>
</tbody>
</table>

**KITS**

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytometric Bead Array Mouse Inflammation kit</td>
<td>BD Biosciences, CA USA</td>
</tr>
<tr>
<td>IL-8 OptEIA ELISA kit</td>
<td>BD Biosciences, CA USA</td>
</tr>
<tr>
<td>Limulus amebocyte lysate (LAL) assay</td>
<td>Sigma Aldrich, MO USA</td>
</tr>
</tbody>
</table>
### Appendix 2: Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C incubator</td>
<td>Contherm (Contherm Scientific Ltd., Wellington New Zealand)</td>
</tr>
<tr>
<td>Biophotometer</td>
<td>Eppendorf, Hamburg Germany</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Eppendorf, Hamburg Germany</td>
</tr>
<tr>
<td>CO₂ incubator</td>
<td>DH Autoflow™ (Nuaire, MN USA)</td>
</tr>
<tr>
<td>Compound microscope</td>
<td>Leica Microsystems Pty. Ltd., Bensheim Germany</td>
</tr>
<tr>
<td>Cytospinner</td>
<td>Rotofix 32 Hettich Zentrifugen (GMI Inc., MN USA)</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>FACS Calibur, Becton Dickinson, CA USA</td>
</tr>
<tr>
<td>Flow meter</td>
<td>Crown Scientific, Australia</td>
</tr>
<tr>
<td>Fluorescence microscope</td>
<td>Leica Microsystems Pty. Ltd., Bensheim Germany</td>
</tr>
<tr>
<td>Laminar Flow (Biological Safety Cabinet Class II)</td>
<td>AES Environmental Pty. Ltd., Australia</td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td>(Eppendorf, Hamburg Germany)</td>
</tr>
<tr>
<td>Nebuliser</td>
<td>Aerflo nebuliser (Maersk Medical Ltd., Worcestershire, England)</td>
</tr>
<tr>
<td>Perspex chambers</td>
<td>Crown Scientific, Australia</td>
</tr>
<tr>
<td>pH meter</td>
<td>Jenway 3310 (Jenway, Essex England)</td>
</tr>
<tr>
<td>Pipettes</td>
<td>Gilson Inc., WI USA</td>
</tr>
<tr>
<td>Plate reader</td>
<td>ELx800 (BioTek Instruments Pty.)</td>
</tr>
<tr>
<td>Instrument</td>
<td>Manufacturer/Location</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Plebox</td>
<td>Crown Scientific, Australia</td>
</tr>
<tr>
<td>Real Time Machine</td>
<td>ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, CA USA)</td>
</tr>
<tr>
<td>Flexivent</td>
<td>SCIREQ™, Montreal, Canada</td>
</tr>
<tr>
<td>Thermocycler</td>
<td>PTC100™ Peltier Thermal Cycler (Bio-Rad, MA USA)</td>
</tr>
<tr>
<td>Tissue culture wares</td>
<td>BD Falcon™, BD Biosciences, CA USA</td>
</tr>
<tr>
<td>MS1 Mini-shaker Vortex</td>
<td>IKA®, Staufen, Germany</td>
</tr>
</tbody>
</table>
Appendix 3: Ethics approval for asthma study

Dear Professor Stick,

REGISTRATION NUMBER: 937/EP

TITLE: Developmental and cellular mechanisms involved in the pathological changes to the epithelium in asthma

REFERENCE NUMBER: EC04-37.2

MEETING DATE: 17 June 2004

The Ethics Committee has recommended approval for an amendment and extension to the abovenamed study. This recommendation has been ratified by the Women’s and Children’s Health Service.

The amendment related to an additional blood sample being taken from subjects of this study while they are under anaesthetic. This would be used by another study (Reg. No: 891/EP, Immune Surveillance in CF – the role of macrophages and dendritic cells) for analysis. The investigators have combined the information sheets for these two studies so that parents have only one sheet to read.

It should be noted that all other aspects of the approval remain unchanged. This is so, in particular, in relation to the progress reports required and regarding any amendments to the protocol.

Please quote the above registration number on all correspondence.

Yours sincerely,

Dr Geoff Masters
Executive Director
Medical Services
21 June 2004

cc Dr Siobhain Brennan, ICHR

* The Ethics Committee is constituted, and operates in accordance with the NHMRC National Statement on Ethical Conduct in Research Involving Humans.
Appendix 4: Ethics approval for cystic fibrosis study.

Dear Professor Stick,

REGISTRATION NUMBER: 976/EP
TITLE: Detection of early lung disease in cystic fibrosis
REFERENCE NUMBER: EC04-37.5
MEETING DATE: 20 May 2004

The Ethics Committee has recommended approval for an amendment to the abovenamed study. This recommendation has been ratified by the Women’s and Children’s Health Service.

This study is being amended to reflect current practice for cystic fibrosis patients. As the current practice for cystic fibrosis patients includes tracheal brushing to collect and RNA and culture epithelial cells the investigators wished to include this in the documentation for this study.

It should be noted that all other aspects of the approval remain unchanged. This is so, in particular, in relation to the progress reports required and regarding any amendments to the protocol.

Please quote the above registration number on all correspondence.

Yours sincerely,

Dr Geoff Masters
Executive Director
Medical Services
27 May 2004

cc Professor Peter Sly

The Ethics Committee is constituted, and operates in accordance with the NHMRC National Statement on Ethical Conduct in Research Involving Humans.