Identification and Characterisation of Alveolar Epithelial Biomarkers

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i. Abstract

The alveolar epithelial surface has been shown to be important in many forms of lung injury (e.g. ARDS, pneumonia and fibrosis). Markers of cell specific injury to the lung that can be measured in bronchioalveolar lavage would be advantageous as they might allow study of damage to the alveoli in a non-invasive manner. In this thesis a strategy is described, using a bacterial model of pneumonia, for identifying markers specific for individual cell types within the alveoli. Also, two putative cell specific antibodies (MMC4 and MMC6) are further characterised to establish their potential as markers of alveoli injury.

A rat model of pneumonia, caused by instillation of *Staphylococcus aureus*, has shown a decrease in alveolar epithelial type I cells (AETI cells) and an increase in alveolar epithelial type II cells (AETII cells) seventy two hours post-infection.

Injured regions from seventy two hour post *Staphylococcus aureus* infected lungs were analysed via global gene analysis using Affymetrix® U34 gene chips. Overall 67 genes were found to increase greater than 2 fold ($F>0.05$, $P\leq0.001$); of which 49 encode from known proteins. 197 genes decreased 2 fold of greater ($F>0.05$, $P\leq0.001$) of which 125 translate to known proteins.

Searching the genes that decreased identified some proteins associated with AETI cells (e.g. caveolin: 7 fold decease, $P=0.017$ and aquaporin 5: 2 fold decrease, $P=0.01$). This suggests that this group of down regulated genes could potentially contain novel markers that could identify reduced numbers of AETI cells. Within this group CD9 was found to decrease. This was unexpected due to the influx of macrophages (known to be CD9 positive) into the site of injury. However, a literature search showed that CD9 is known to be expressed in the alveoli, but is not thought to be cell specific. Immunohistochemistry of frozen lung sections showed that anti-CD9 antibodies co-

localised with AETI markers (caveolin, MMC6) but not AETII markers ($RTII_{70}$, MMC4).

MMC4 monoclonal antibody (IgG2a) has been proposed as an AETII and Clara cell marker that recognises a protein novel to these two cell types within the lung, but the antigen has not been characterised.

MMC4 antigen was immunoprecipitated from detergent-solubilized fractions of organ homogenates using antibody-bound magnetic beads. The resulting purified protein was trypsin digested and analysed via MALDI-TOF and tandem mass spectrometry. The top 'hit' in SWISSPROT for the antigen was aminopeptidase N (APN). The MMC4 monoclonal antibody was found to recognise a commercially purified APN. Further analysis showed that the MMC4 monoclonal antibody was not a functional inhibitor of APN and that the antibody was not binding to a glycans attached to APN.

MMC6 monoclonal antibody (IgG2b) was created at the same time as the MMC4 antibody. Initial studies showed that MMC6 recognises AETI cells within the lung. This study shows that the antibody is recognising an antigen that is specific for the lung and is only located on AETI cells within the lung. The antigen has been found to be an integral membrane protein. Attempts at solubilizing the MMC6 antigen from the cell membranes were not successful; however mechanical disruption did partially remove the antigen from the cell membranes. Magnetic bead immuno-precipitation eluted a protein of 60kDa. Unfortunately, due to low yields of the protein, identification was not possible by MALDI-TOF or Edman degradation.

This thesis proposes a new way of interpreting global gene data to find proteins specific to different cell types. We cast doubts on the specificity of some previously proposed "specific" markers. It also highlights the pitfalls of claiming an antibody is a useful marker of injury until the candidate biomarker has been fully investigated. The continued emergence of CD proteins on alveolar epithelial cells within this study

suggest that the alveolar epithelium may have more influence over the immune response within the lung than previously realised.

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iii. Abbreviations

ACN	Acetonitrile
AETI	Alveolar epithelial type I cells
AETII	Alveolar epithelial type II cells
APN	Aminopeptidase N
ARDS	Acute respiratory distress syndrome
BSA	Bovine serum albumin
$C_{12}E_8$	Octaethleneglycol mono-n-dodecyl ether
Ca	Calcium
Cfu	Colony forming units
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Electrochemical luminescence
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and drug administration
IPF	Idiopathic pulmonary fibrosis
kDa	Kilo Daltons
MALDI-TOF	Matrix-assisted laser desorption/ ionization-time of flight
Mg	Magnesium
mRNA	Messenger ribonucleic acid
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
SD	Sprague Dawley
SDS	Sodium dodecyl sulphate
SPF	Specific pathogen free
TBS	Tris buffered saline
Tween	Polyethtlene sorbitan monolaurate

iv. Declaration

This thesis has been composed by myself from the results of my own work, except where stated otherwise, and has not been submitted in any other application for a degree.

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1. Contents

2. Introduction

Traditional drug development is expensive and time consuming; in 1992 the United States Food and Drug Administration (FDA) added previsions for accelerated approval of drugs based on data from biomarkers to be used in diseases with high mortality or morbidity (Food and Drug Administration, 1992). Since this ruling, the majority of cancer drugs that have been approved by the FDA have relied upon biomarkers to validate them (Pien, 2005). Biomarkers, however, do present a new set of problems in research as they can cause both false positive and false negative results due to poor understanding of what the biomarker is measuring, or poor understanding of the pathways that the disease follows. Better understanding of biomarkers during their development and more rigid testing of them is therefore needed prior to their use. My thesis looks at one method of identifying biomarkers (via genomics), highlighting areas where problems arise with this method. I also carried out further analysis on two potential biomarkers to asses their usefulness in classifying alveolar epithelial damage.

2.1 Architecture of the lung

The lungs are a highly specialised organ, which is adapted to facilitate gas exchange between the blood and air. There are approximately forty different cell types within the lung which are organised in a highly structured formation (Williams, 2003). The lungs consist of a highly branching network of airways, which are made of rings of cartilage. Inside the cartilage there is an inner membrane, which is covered with various epithelial cell types. As the airways branch further they are no longer surrounded by cartilage and eventually they split into the alveoli, where gas exchange with the blood occurs. Alveoli are surrounded by a network of fine blood capillaries which covers approximately $126m^2$ (Schneeberger, 1991). Initially, it was thought that the air came into direct contact with the capillary network of endothelial cells, or that there was thin mucus forming the alveoli (Macklin, 1954). The presence of pulmonary epithelial cells forming a continuous lining to the alveoli was first described in 1952 by F.N. Low as

'Parent epithelial cells, located in the thicker, cellular portions of the alveolar wall attenuate to an average thickness of 0.1-0.2μ, forming a complete lining, and covering the alveolar walls, facing on the air spaces.' (Low, 1952); although the findings of this paper were not completely accepted at the time. The 'parent epithelial cells' he described are now called alveolar epithelial type II cells (AETII) and the 'attenuated cells' are the alveolar epithelial type I cells (AETI), both of which sit on the basement membrane of the capillaries' endothelial cells (Figure 1). Different cell populations are found in different areas of the lung and these perform specific functions. Below, I have described some of the cell types of the lung with emphasis on where in the lungs they are found and the cells' specialised roles within the lung.

2.2. Cell types found within the lung

2.2.1. Airway smooth muscle cells, alveolar myofibroblasts and pericytes

Airway smooth muscle cells, alveolar myofibroblasts and pericytes are all forms of contractile cells that are found associated with the blood capillaries surrounding the trachea, the bronchial passages and alveoli. Airway smooth muscle cells are found surrounding the trachea and the bronchia passages (Janssen, 1997) all the way down to the terminal bronchioles where they form an alveoli ring muscle (Kapanci, 1997). Smooth muscle cells are also found around the division of blood vessels into capillaries, where they are thought to be involved with regulating blood flow to the alveoli (Kapanci, 1997). Pericytes are mainly found around the post capillary venules while alveolar myofibroblasts are mainly found at the junction of three alveolar septa between the endothelial and epithelial cell layers. As with smooth muscle cells these cells are thought to be associated with regulation of blood flow through the capillaries (Kapanci, 1997).

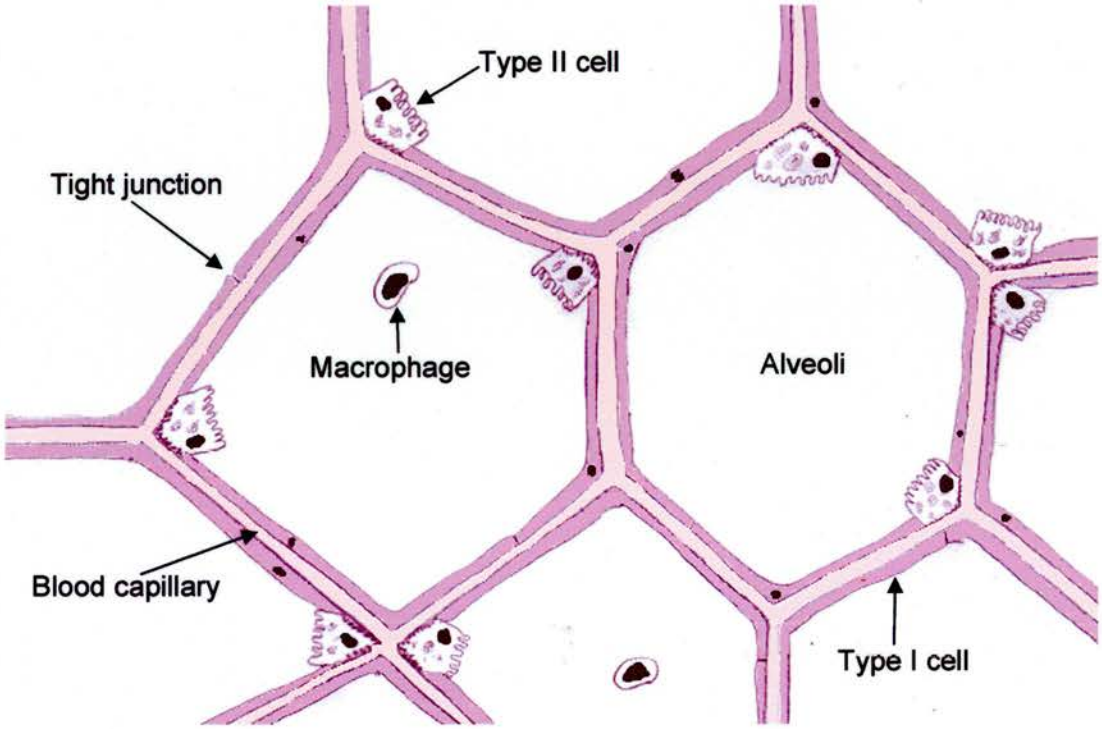


Figure 1: Cartoon representation of the alveoli of the lung showing AETI cells overlying the blood capillaries with AETII cells at the 'corners' of the alveoli. Endothelial cells have been omitted for clarity of picture. Cells are not drawn to scale.

2.2.2. Airway epithelial cells

Epithelial cells, be they in the airway (trachea and bronchi) or alveoli, share one common role with all other epithelial cells of the body: they act as a barrier between the body and the outside environment. 'Airway epithelial cells' is a generalised term that covers a wide group of different cell types that are specialised to carry out other roles as well as acting as a continuous barrier in the epithelium. These include ciliated cells, taller cells, basal cells, goblet cells and Clara cells (Robbins, 1997). Ciliated cells make up approximately half of the airway epithelial population, where their function is to remove mucus secreted from the lung, taking with it any particles (dust, bacteria etc) from the inhaled breath (Robbins, 1997). Clara cells will be discussed in greater detail later.

2.2.3. Neuroendocrine cells

Neuroendocrine cells can be divided into two sub-populations: neuroendocrine epithelial cells (located on the luminal surface of the trachea and bronchial tract) and neuroendocrine cells (found in the pulmonary interstitial connective tissue). Neuroendocrine epithelial cells are found scattered throughout the epithelial lining of the airways and also in clusters with Clara cells in the intrapulmonary airways. The main function of these cells appears to be to decarboxylate amine precursor molecules. Neuroendocrine cells in the pulmonary interstitium are not located within the epithelial monolayer. Instead they are usually found in clusters with paraganglionic cells. These clusters are often found near the blood capillaries and bundles of smooth muscle fibres. Pulmonary interstitial neuroendocrine cells are also thought to be involved with the decarboxylation of amide precursors (Scheuermann, 1997).

2.2.4. Endothelial cells

Endothelial cells form the walls of the blood capillaries. In the lung they form part of the blood-air barrier along with the alveolar epithelial cells. Endothelial cells form a polarised monolayer of cells with cells joined by tight junctions to maintain cell polarity and prevent molecular diffusion. Endothelial cells are large attenuated cells which, in addition to their structural role, also are involved in the maintenance of the healthy lung (Simionescu, 1997). Many inflammatory mediators (e.g. interleukins 1-16, tumor necrosis factor and lymphotoxin) have an effect of endothelial cells. Tumor necrosis factor-α, for example causes activation of phospholipases (*in vivo*), alteration in thrombotic properties (*in vitro*), prostaglandin production (*in vitro*), increased cell permeability, leukocyte adhesion molecule expression and chemokine production (Silverman, 1997). The last three of these changes aid neutrophil and other leukocytes to pass from the blood capillaries into the lung air spaces in situations of infection within the lung.

2.2.5. Clara cells

Clara cells are non-cilliated cells found in the epithelial lining of the bronchioles interspaced between the other airway epithelial cell types. Most *in vitro* work has been carried out on Clara cells from the distal airways as these cells are found in greater number in this area; however, the similarity of these cells to the Clara cells found throughout the rest of the airways has been brought into question. (Plopper, 1997). The main function of Clara cells is thought to be to the removal of particles from the lungs. Clara cells share many of their functions with AETII cells (discussed later), such as producing the major components of pulmonary surfactant (i.e. lipids and surfactant proteins) and acting as progenitor cells (for ciliated cells). Clara cells also produce many proteins involved xenobiotic metabolism (i.e. P450s, Clara cell secretary protein, epoxide hydrolase and glutathione S-transferase) (Plopper, 1997).

2.2.6. Alveolar epithelial type I cells (AETI)

AETI cells are large, attenuated, flat cells which form the main gas exchange surface of the lung (Figure 1). In the rat, an average cell is 0.2μm thick, but has a surface area of 4500μm^2 (Schneeberger, 1991), which aids gas exchange as the gases only have a small depth of lung to diffuse through. The large surface area of AETI cells means that one cell may line more than one alveolus (Williams, 2003). AETI cells are polarised, with different proteins being expressed on the apical and basal membranes, aiding cells functions. There are few organelles in type I cells, which originally led scientists to believe that the type I cell was not involved in influencing the lung environment (Schneeberger, 1991). However work as early as 1980 was showing that AETI cells can influence the alveoli lining due to the presence of pinocytotic vesicles within the cells which export products from the cells (DeFouw, 1980). It has also been shown that type I cells have fluid transport proteins on the apical membrane e.g. AQP5 (see 2.2.6.1).

In the adult lung it is assumed that AETI cells do not undergo mitosis (Williams, 2003), however this is hard to prove as type I cells have an average turn over time of 40-120 days, making analysis difficult. Foetal type I cells have been shown to be able to differentiate back to type II cells (which is not thought to occur in the adult lung) and these cells are then able to undergo mitosis to form either type I or type II alveolar epithelial cells (Qiao, 2003). In the adult the main progenitor of type I cells are the type II cells, however bone marrow stem cells (Kotton, 2001) have also been shown to be able to differentiate into type I cells *in vivo*.

Investigations into type I cell functions and properties have been hindered by the inability to isolate viable type I cells. The technique for isolation of AETI cells was first described in1986 (Welle,r 1986), however, few research groups have been able to isolate these cells with consistent results (Gonzalez, 2005). The majority of knowledge of type I cells has therefore come from work on either cell lines that are known to have type I cell markers on their surface (e.g. SV40 cells, a cancer cell line), or on type II cells that

have been allowed to differentiate in culture to a cell with type I cell morphology (hereafter referred to as a type I cell-like cells), which also expresses type I cell associate proteins. Both the cell lines and type I cell-like cells produce similar sugar binding response to fresh type I cells as shown by their ability to bind lectins. Lectin binding was used as an early method of differentiating type I and type II cells. *Ricinus communis 1* lectin binds to AETI cells but not AETII cells while *Maclura pomifera* binds to AETII cells, but not AETI cells (Brandt, 1982). This was one of the earliest indicators that type I cells were functionally different to type II cells and not just stretched out type II cells. Type I cells have since been shown to express proteins specific to type I cells, as well as proteins that are not expressed by type II cells but are found elsewhere in the lung (see table 1 for an overview of AETI cell proteins). Recently Gonzales *et al* (2005) have also shown that type I cell-like cells also express different proteins to both type I and type II cells, which brings into question the use of type I cell-like cells and cell lines in the investigation of type I cell functions. I have therefore split my description of type I cell functions into two groups: those where the findings were found in primary culture (not many) or were confirmed with *in vivo* results, and those where the findings have only been found *in vitro* on cell lines/type I cell-like cells and are therefore possible roles of the AETI cell.

2.2.6.1. Water transport

Water transport is important within the lungs to remove fluid just after birth, in prevention of illnesses, such as asthma and pulmonary edema (Crandall, 2000) and in resolution of illnesses by removing excess fluid released by necrotic cells. The level of water within the lungs has to be maintained carefully as too little water causes the cell surface of the alveoli to become dry and therefore gas exchange is reduced. If there is too much water then the alveoli become full of liquid and therefore the air can not come into close enough contact with the alveolar epithelial cells for gas exchange to take place.

Protein	Location in AETI cell	Expression in other cell types	Ref
RTI_{40}/TIα protein	Apical plasma membrane	None	Dobbs,1988;Kato, 2003; Kasper,1996
HTI_{56}	Apical plasma membrane	None	Dobbs, 1999
Caveolin-1/2	Microvesicles	Endothelial cells, fibroblasts, smooth muscle cells, bronchial epithelial cells	Kasper,1998; Newman,1999
Na^+/K^+-ATPase α_2-isoform	Basolateral membrane		Ridge, 2003
Aquaporin 5	Apical plasma membrane	Bronchial epithelial cells	Nielson, 1997
Cytochrome P_{450} 2B1	Cytoplasm	Bronchial epithelial cells	Takahashi,1994
Carboxypeptidase M	Apical plasma membrane	Alveolar macrophages	Nagae, 1993
ICAM-1	Apical plasma membrane	Alveolar macrophages, bronchial epithelial cells, endothelial cells, ATII cells?	Kang,1993; Christensen, 1993; Cunningham, 1993
Connexin 43	Cell membrane, cytoplasm	ATII cells	Abraham, 2001
p-glycoprotien	Apical plasma membrane	Smooth muscle cells, bronchial epithelium, ATII cells?	Campbell, 2003
RAGE	Basal membrane	Some endothelial cells of larger blood vessels, ATII cells (mRNA)	Fehrenbach, 1998; Shirasawa, 2004; Katsuoka, 1997
PAI-1	Apical plasma membrane	ATII cells?	Qiao, 2003
P2X4 purinoceptor	Plasma membrane	ATII cells?	Qiao, 2003
CDKN2B	Perinuclear	ATII cells?	Qiao, 2003
ATB^{0+}	Apical membrane	Ciliated cells, ATII cells?	Sloan, 2003
Cytochrome P_{450} 1A1		Bronchiolar epithelium, ATII cells	Saarikoski, 1998
Eotaxin		Airway epithelium, macrophages, lymphocytes, endothelial cells	Ganzalo, 1996
GGT	Apical plasma membrane	Clara cells, ATII cells	Ingbar, 1995; Joyce-Brady, 1994
IGFR-2	Apical plasma membrane		Maitre, 1995
Esterase			Schneeberger, 1991
Cytokeratin-18			Schneeberger, 1991
γ-glutamyltransferase			Schneeberger, 1991
Osteonectin			Schneeberger, 1991
Aquaporin 4			Williams 2003
B2-adrenergic receptors			Williams 2003
VAMP-2			Williams 2003
TIMP-3			Griffiths, 2005
Phospholipase A2 group			Griffiths, 2005

Table 1: Proteins known to be associated with AETI cells in normal adult lung

Water movement is thought to be primarily through aquaporins (AQP) which are water permeable integral membrane proteins that are unevenly distributed on cell membranes throughout the cells of the lungs (Verkman, 2000). Alveolar epithelial type I cells are known to have AQP 4 and AQP 5 on the apical surface (via immunohistochemical studies) (Yasui, 1997, Verkman, 2000, Williams, 2003), but as yet no AQP has been found on the basolateral surface of type I cells (King, 2001). AQP 4 is thought to be the main aquaporins involved in removal of post natal fluid, due to experiments on knockout mice (West, 2000). Aquaporin 5 knockout mice show no change in fluid clearance, suggesting that AQP4 (or as yet un-identified aquaporins) can compensate for lack of AQP5 throughout all stages of life (Williams, 2003).

There are currently four theories as to why aquaporins are not found on the basolateral surface of type I cells:

1) They are there in small levels, but current methods for confirming their present (immunohistochemistry mainly) are not sensitive enough.

2) There is an aquaporin present, but it is one that is yet to be discovered.

3) Fluid is removed from the type I cells in an aquaporin independent manner.

4) Type I cells do not transport the water, but use it to maintain their shape (i.e. it stays within the cell).

Until recently, there had been no aquaporins found on AETII cells. However, Kreda *et al* recently found that AQP 3 is expressed on the basolateral membrane of AETII cells via immunohistochemistry (Kreda, 2001).

Aquaporins work by allowing water to move down an osmotic gradient (Kreda, 2001). Within the alveoli the osmotic gradient is made by ion pumps, ion channels and ion co-transporters. Just as AQP are unevenly distributed on cell surfaces, so are ion pumps, channels and co-transporters (Matthay, 1996). Most of the work on ion movement has been done on type II cells due to the difficulties in isolating type I cells.

Alveolar epithelial type II cells actively pump sodium from within the cell out of the basolateral membrane via Na, K-ATPase (an ion pump). However, sodium passively enters the cell via amiloride-sensitive channels on the apical surface. Sodium transport has also been shown to be important for the functioning of the type II cell as it is used in co-transporters to facilitate glucose, amino acid, protons and phosphate entry into the cell (Traebert, 1999).

In the last few years, papers from two different laboratories have been published that show that AETI cells also actively transport sodium, and therefore AETI cells must also affect water levels within the lung. Johnson *et al* (2002) showed that sodium uptake into AETI cells are sensitive to amiloride, suggesting a sodium channel is involved (*in vitro* data), and then later proved the presence of the sodium channel via immunohistochemistry. They also showed that Na, K-ATPase is present on AETI cells in the same way (activity blocked with ouabain) (Johnson, 2002). Borok *et al* (2002) have also shown that type I cells have Na, K-ATPase activity by showing that they produce mRNA for the protein via real time-polymerase chain reaction and immunohistochemistry showed that the protein is found on the surface of the cell.

2.2.7. Alveolar epithelial type II cells (AETII cells)

AETII cells are cuboidal cells found in the 'corners' of the alveoli (figure 1). The main distinguishing feature of type II cells are the characteristic lamellar bodies, which are the surfactant stores of the lung (Mason, 1991). AETII cell are, like AETI cells, polarised cells, however, they have a far smaller surface area of approximately $70\mu m^2$. Unlike AETI cells, AETII cells have microvilli on the apical surface and projections into the basement membrane which increase the surface area (Schneeberger, 1997). AETII cells were first isolated in the early 1970's (Kikkawa, 1974) so there is a large body of information on proteins known to be expressed by AETII cells. Fehrenbach *et al* (2001), has a comprehensive list of proteins found on type II cells, however table 2 is an abbreviated list of those proteins that are relevant to my thesis. AETII cells have long

Protein	Function	Location in AETII cell	Expression in other cell types
Surfactant protein A	Surfactant	Secreted/ Lamellar bodies	Clara cells
Surfactant protein B		Secreted/ Lamellar bodies	
Surfactant protein C		Secreted/ Lamellar bodies	
Surfactant protein D		Secreted/ Lamellar bodies	Clara cells
Cathepsin H	Surfactant maturation	Secreted/ Lamellar bodies	
B-adrenergic receptors	Surfactant secretion	Cell membrane	Clara cells
Annexin II	Exocytosis		
Receptor for SP-A	Surfactant recycling	Cell membrane	Clara cells
Alkaline phosphatase	Lysosomal enzyme	Lamellar body	
Aminopeptidase N	Differentiation?	Cell membrane	Macrophages
Retinoic acid receptor	Inhibits differentiation	Cell membrane	
CD95	Apoptosis	Cell membrane	Most
Bax		Intracellular	Most
Bcl-2		Intracellular	Most
AQP-1	Water transport	Cell membrane	Microvascular endothelial cells (Verkman, 2000)
Hg-insensitive channel (MIWC)		Cell membrane	
Hg-sensitive channel (CHIP28)		Cell membrane	
Plasmalogen	Antioxidant	Secreted	
Connexin 43	Gap junction protein	Cell membrane	AETI
CD44	Adhesion	Cell membrane	
E-cadherin		Cell membrane	
Ep-Cam		Cell membrane	
Entactin	Extracellular matrix	Basal membrane	
Laminin		Basal membrane	
Fibronectin			
C3	Innate immune system	Secreted	Macrophages
C4		Secreted	Macrophages
α_1-antitrypsin	Antiproteases	Secreted	Leukocytes
Elafin			
Matrix metalloproteinase		Secreted	
MMP-inhibitors (TIMP)		Secreted	
Mn superoxide dismutase (SOD)	Antioxidants	Secreted	

Table 2: Abridged list of AETII cell associated proteins from Fehrenbach (2001).

been known to be able to influence the environment around them. The most well known of these is surfactant production (discussed below) although AETII cells also influence the pH of the hypophase, fluid levels within the alveoli (discussed in relation to AETI cells), and the host defence of the lung.

2.2.7.1. Progenitor cell

As already described, unlike AETI cells, AETII cells can proliferate. AETII cells act as a progenitor cell for both new AETII cells and also AETI cells. It is unclear if it is all AETII cells that have the ability to proliferate, or if it is a specific sub-population. Reddy *et al* (2004) have found that, after injury with high oxygen levels, that they can isolate a sub-population of AETII cells that have a higher level of E-cadherin expression than other AETII cells. They predict that these cells are the sub-population of 'stem' cells within the lung. Critics of this argument say that the reason why only some of the AETII cells show this increase in proliferative proteins is due to the other AETII cells in the lung having been damaged.

2.2.7.2. Pulmonary surfactants

The inside of the alveoli are coated with a fluid bilayer with an aqueous subphase nearer the cells which is then covered with a film of pulmonary surfactant (Pison, 1996). The aqueous layer aids in gas exchange. This is where newly secreted surfactant material is stored while they undergo extra-cellular modification and is where components of the surfactant interact with host immune cells (Pison, 1996).

Pulmonary surfactant is a lipid rich layer, which prevents damage to the cells both within the alveoli (Sutherland, 2001) and in the small airways (Pison, 1996), regulates the surface tension within the lungs (Sutherland, 2001) and prevents the alveoli from collapsing (Pison, 1996). Surfactant is produced within the alveolar epithelial type II cells, where it is also stored in specialised organelles, the lamellar bodies. AETII can be

stimulated to secrete surfactant by many chemicals (such as β-adrenergic compounds (Saldías, 2001)) and by mechanical stretching of the cells, such as taking deep breaths or hyperventilating (Rose, 1999).

The major component of pulmonary surfactant is disaturated phosphatidychloride (DPPC) (Mason 1977, Saldías, 2001), which, along with other lipids, helps to maintain the low surface tension within the lung. DPPC is produced by a variety of different pathways and enzymes within AETII cells (Tonks, 2005) so it is always found in the lamellar bodies at high concentrations. As well lipids within the lamellar bodies, there is also a range of proteins, most notably surfactant proteins A, B and C. Type II alveolar epithelial cells also secretes surfactant protein D, complement components, lysozyme, plasminogen activator, basement membrane components, cytokines, growth factors, and hydrogen ions (See water transport) (Fehrenbach, 2001).

2.2.7.2.1. Surfactant protein A

Surfactant protein-A is the most abundant of the surfactant proteins (Tino, 1998, Pison, 1996). The main role of surfactant protein-A was thought to be to increase the surfactant uptake of AETII cells and decrease release of lamellar body contents into the alveolar air space (Pison, 1996). However surfactant protein-A double knockout mice do not show any differences with respect to the levels of surfactant within the lung when compared to the wild type (Tino, 1998).

Surfactant protein-A has also been shown to be involved in the innate immune system (Pison, 1996) due to its similar structure to mannose binding protein and C1q (both opsonins of the innate immune system) (Folkesson, 2002, Pison, 1996). Surfactant protein-A is thought to be able to bind to macrophages through the same receptor as C1q as macrophages co-incubated with C1q subsequently have reduced binding to surfactant protein-A (Tino, 1998). Surfactant protein-A can also directly affect macrophage function within the lung by promoting chemotaxis (the movement towards foreign

particles within the lung) and the oxygen burst within the macrophage (Pison, 1996). The surfactant protein-A knockout mouse has not been characterised with respect to the susceptibility to infections.

2.2.7.2.2. Surfactant protein B and C

Surfactant proteins B and C are hydrophobic proteins that appear to be involved in reducing the surface tension within the alveoli (Pison, 1996). Within the surfactant surfactant protein-B and surfactant protein-C make up approximately 8% and 4% of the protein content respectively. Surfactant protein-B is thought to be important for the correct formation of lamellar bodies within type II cells and is involved in the correct formation of tubular myelin with surfactant protein-A (Ikegami, 1998). Surfactant protein-B deficiency causes lethal respiratory failure at birth so it must be essential (Ikegami, 1998). The roles of surfactant protein-C have not been classified due to difficulty in purifying the protein and its tendency to denature and aggregate quickly (Ikegami, 1998, Saldías, 2001).

2.2.7.2.3. Surfactant protein D

Surfactant protein-D, unlike all the other surfactant proteins, is not stored within lamellar bodies in type II alveolar epithelial cells (Folkesson, 2002). It is, however, synthesized by AETII cells as well as Clara cells (Folkesson, 2002, Pison, 1996). Unlike the other surfactant proteins it is not specific to the lung and is also found in the gastric mucosa (Pison, 1996). It does not have a role in maintaining the surface tension of the lung, but like surfactant protein-A it has a role in the innate immune system. Like surfactant protein-A it is a collagenous glycoprotein and calcium dependent (Folkesson, 2002). It is also similar in structure to mannose binding protein and thought to act in a similar way to it.

2.2.7.3. Repair of the damaged epithelium

After injury to the epithelium via a variety of agents it has been shown that the alveoli looses AETI cells as shown by the 'denuded' basement membrane characteristically seen by electron microscopy. Over time, this 'denuded' membrane is covered with cuboidal epithelial cells, which have been shown to produce many of the proteins associated with AETII cells. Therefore, these cells have long been believed to be AETII cells. However, recent findings by Clegg *et al* suggest that not all of these cells are the same (Clegg, 2005). Clegg *et al* showed that some of these cells co-express RTI_{40} (an AETI cell marker) and MMC4 (a potential AETII cell marker) possibly indicating cells that are trans-differentiating into AETI cells. Other suspected AETII cells are thought to undergo apoptosis to 'make room' for the new elongated AETI cells enabling the lung to return to 'normal'.

2.2.7.4. Host defence

AETII cells produce many components of the innate immune system, such as C3, C4 (Fehrenbach 2001) which aid bacterial clearance form the lung by facilitating recognition of the bacteria by neutrophils and macrophages. As already mentioned, AETII cells also produce surfactant proteins A and D which also act as opsonins within the lung to aid partial and pathogen removal. AETII cells also produce proteins that aid

in the recruitment of neutrophils and macrophages into the lung. MHC class II has been shown to be produced by AETII cells under a variety of different conditions (Sherman, 1992) suggesting that AETII cells could have a role in antigen presenting and therefore in the recruitment of T-lymphocytes into the lung.

2.3. Regulation of cell numbers within the lung

The proportion of the lung covered by AETI and AETII cells seem to differ slightly depending on which species being studied and the technique used. An example of this is Crapo *et al* (1982) who used electron microscopy to count the number and type of cells over 50 fields to determine the number of type I and type II cells within different species (rat and human). In the human they found that AETI cells covered 92.9% of the lung surface and make up 8.3% of the total lung cells, and type II cells covered 7.1%, equalling 15.9% of the total lung cells. However, in the rat they found that AETI cells covered 96.2% (8.9% of lung cells) and AETII cells covered 3.8% (14.2% of lung cells) (Crapo, 1980). General consensus over type I /type II cell numbers seem to suggest that 95% of the alveolar surface is type I cells and 5% are type II cells and that these cells are found in equal numbers (Williams, 2003).

2.4. Lung epithelia in studies of lung injury

Changes in the alveolar epithelium are found as a result of a large range of lung injuries. Best known examples are acute respiratory distress syndrome (ARDS), thought to cause 36,000 deaths per year in the US (Matthay, 2003), idiopathic pulmonary fibrosis, which affects 7-10/100,000 of the population (Pardo, 2002) and pneumonia which causes approximately 1,000,000 hospital admissions per year in the UK (Davidson's, 2002).

2.4.1. Acute respiratory distress syndrome (ARDS)/ acute lung injury

ARDS is characterised by acute respiratory distress, decreased lung compliance, diffuse infiltrates evident on the chest radiograph and reduced response to oxygen therapy (Matthay, 2005) ARDS can be caused by a wide range of direct and indirect methods (table 3). The most common cause is sepsis from Gram negative bacteria (Canonico, 1997). Studies into ARDS have shown a high influx of fluid into the lung (Matthay, 2005) which suggests that the alveolar epithelium is damaged, as epithelial cells have a

Direct mechanism	Indirect mechanism
Aspiration	Sepsis
Diffuse pulmonary infection	Severe non-thoracic trauma
Near drowning	Hypertransfusion for emergency resuscitation
Toxic inhalation	Cardiopulmonary bypass (rare)
Lung contusion	

Table 3: Major direct and indirect causes of ARDS in humans (Canonico, 1997)

lower water permeability than the underlying endothelial cells. There is also a decreased clearance of water from the alveolar air space as shown by the decrease in the salt ion transport from the alveoli. Ion transport from the lung is via AETI cells, indicating that AETI cells are damaged in ARDS. Surfactant production is also reduced in ARDS, as surfactant is produced by AETII cells, this could also imply a decrease in Type II cells. Electron microscopy studies from patents that have died from ARDS also show a decrease in the levels in type I and type II cells, with areas of the lung showing denuded basement membrane. This is not found in electron microscopy's of the normal lung.

Current methods for predicting ARDS outcome include presence of chronic liver damage, non-pulmonary organ dysfunction and advanced age. Other factors currently examined, such as blood oxygen levels compared to oxygen levels being ventilated and the lung injury score have shown no clear correlation to outcome of injury. Degree of alveolar epithelial damage has been shown to be linked to patent outcome (Ware, 2000), however, these studies have not characterised which cells within the lung are being affected; nor do they give an indication of the likelihood of the patent progressing to pulmonary fibrosis. A better knowledge of which cells within the alveoli are damaged within ARDS could help clinicians plan suitable treatments more efficiently.

2.4.2. Idiopathic pulmonary fibrosis

Normal fibrosis in the lung is characterised by an increase in mesenchymal cells and connective tissue matrix in a localised area of the lung. There is also injury to the epithelial surface of the alveoli, with the alveoli being covered in cuboidal epithelial cells instead of AETI cells. In severe fibrosis there is also a loss of blood capillaries in the fibrotic area (Wolff, 1997). Idiopathic pulmonary fibrosis (IPF, also called cryptogenic fibrosing aveolitis) is an end stage disease that can be reached via many pathways depending on the type of initial injury to the lung. IPF is characterized by

epithelial injury, formation of fibroblasts, myofibroblasts and extra cellular matrix accumulation (Pardo, 2002). Patients with IPF normally survive for 4-5 years from onset of symptoms with the ability of the lungs to transfer oxygen getting progressively worse over time (Wolff, 1997).

The most common hypothesis of the pathgogenesis of fibrotic lung disease is that there is a lung injury, followed by inflammation, then fibroproliferation and eventually fibrosis (Selman, 2003). This assumes that inflammation precedes the fibrosis and that it is necessary for fibrosis to occur. In most types of fibrosis this appears to be true, however IPF does not always require a sustained inflammation stage (Selman, 2003). Although inflammation can cause IPF, the alveolar epithelium is now also recognised as a factor in the progression from lung injury to IPF (Pardo, 2002). Alveolar cell proteins plasminogen activator inhibitor 1 (AETI cells) and 2 have been shown to be increased in IPF, which may aid in a procoagulant/ anti-fibrinolytic environment being established, which in turn, promotes fibrosis. Alveolar epithelial cells also produce many proteins involved in the migration, proliferation and phenotype change of fibroblasts as well as influencing the extracellular matrix. Examples of these proteins are: platelet-derived growth factor, transforming growth factor beta, tumor necrosis factor alpha, connective tissue growth factor and endothelin-1 (Pardo, 2002).

The alveolar epithelial cells themselves also appear different in the IPF lung. IPF lungs have been shown to have hyperplastic type II cells, reactive large and elongated epithelial cells (presumably AETII cells transdifferentiating into AETI cells) and flat AETI-like cells overlying the fibroblast foci (Selman, 2003). All three of these have links to AETI and AETII cells. Greater understanding of AETII cell migration, proliferation and transdifferentiation could give insight into how these three situations occur.

2.4.3 Pneumonia

Pneumonia causes approximately 1,000,000 hospital admissions per year in the UK (Davidson's, 2002) and many more people are treated within the community. Pneumonia can be caused by a wide grange of different bacterial and viral pathogens, the main ones are listed in table 4. More recently, the occurrence of colonisation by more than one pathogen has also increased (Gutiérrez, 2005).

Pneumonia is characterised by a new and persistent cough, often with sputum production, fever and altered breathing sound. The 'gold standard' for diagnosis of pneumonia is a chest radiograph showing shadowing within the lungs (British Thoracic Society, 2001). This shadowing is due to a build up of fluid within the lungs. In animal models it has been show that distal fluid removal is not affected by pneumonia, therefore this reduction of fluid clearance is likely to be at the alveoli level (Boyer, 2005). As AETI and AETII cells are the main site of fluid removal within the alveoli this suggests that the alveoli are affected in pneumonia. A *Staphylococcus aureus* model of pneumonia found increased levels of a AETI cell marker (RTI_{40}) within the bronchioalveolar lavage fluid 24 hours after instillation (McElroy, 1999) suggesting that it is the AETI cells that are affected in pneumonia.

2.4.3.1 Animal models of pneumonia

Pneumonia is induced within animals by a variety of methods. The main three methods are: instillation of the bacteria suspended in a saline solution via a tube inserted down the trachea from the mouth (e.g. McElroy, 1999), instillation of bacteria bound to agar beads via a tube surgically inserted into the trachea (e.g. Boyer, 2005), and insertion of a fine thread into the lungs (e.g. Zagorul'ko, 1990). Instillation via the mouth of bacteria suspended in saline carries the risk of accidentally instilling into the stomach in inexperienced hands; however it does avoid the use of surgery needed to instil directly into the trachea. Using agar beads also adds the injury caused by large particles being

Causes of pneumonia	Reference	Cause of pneumonia	Reference
Psuedomonas aeruginosa	Chastre, 2002	*Haemophilus inluenzae*	Gutiérrez, 2005
Acinetobacter spp	Chastre, 2002	*Moraxella catarralis*	Gutiérrez, 2005
Staphylococcus aureus	Chastre, 2002	Adenovirus	Gutiérrez, 2005
Streptococcus pneumoniae	Chastre, 2002	Varicella-zoster virus	Gutiérrez, 2005
Mycoplasma pneumoniae	Gutiérrez, 2005	*Chlamydophila pneumoniae*	Gutiérrez, 2005
Legionella pneumophilia	Gutiérrez, 2005	*Chlamydophila psittaci*	Gutiérrez, 2005

Table 4: Common causes of pneumonia in humans.

added into the lung. Use of a fine thread (such as fishing line) does appear to cause pneumonia, however as pneumonia is rarely caused by insertion of fine thread in humans, this method is not as favourable as the previous two for making comparisons between human and animal pneumonia.

2.4.3.2 *S. aureus* induced pneumonia

Within our research group there is a previously established model for inducing acute pneumonia within SD rats via instilling *S. aureus* bacteria into the lung via a tube inserted into the mouth and down the trachea. Previous studies of the injury induced by this technique has shown that levels of an AETI marker (RTI_{40}) within the BAL fluid increase 3 fold at 24 hours post instillation (innoculum of $4x10^9$) (McElroy, 1999). This suggests that either AETI cells are being lost from the basement membrane into the alveolar air space, or that the RTI_{40} antigen (T1α) is selectively cleaved from the AETI cells after *S. aureus* induced injury. Electron microscopy studies of the lungs 24 hours pos instillation show a basement membrane denuded of AETI cells suggesting that the increase in T1α protein in the BAL is due to AETI cells in the airspace.

Three days post instillation lung homogenates from the injured regions of the *S. aureus* infected lungs showed greater than 18 fold decrease in RTI_{40} reactivity via western blot analysis (Clegg, 2005), suggesting that the damage to AETI cells carries on past the first 24 hours despite the animals behaving normally.

Immunohistochemistry of lungs 3 days post instillation showed a possible increase in AETII cells shown by the increase in a potential AETII cell markers (MMC4) binding to the lung samples. Western blot analysis also showed an increase in MMC4 antigenicity (Clegg, 2005). This would fit in with the current thinking of how the lung repairs (see 2.2.7.3). Theses cells are not thought to be fully functional AETII cells however as Clegg *et al* showed that they were also expressing the T1α protein, which is not expressed by AETII cells under normal conditions.

2.5. Biomarkers

2.5.1. What the law says about their use

Prior to 1992 for a drug to be approved it was evaluated via randomized, well controlled, double blind studies. This enabled evaluating bodies and the public to be sure that the drug/device being tested was thoroughly and scientifically evaluated, ensuring safety. However, reliance on true endpoints (such as mortality or morbidity) caused potential life saving drugs to be stalled in development and clinical trials for long lengths of time, increasing the cost for drug companies and preventing treatments from reaching the consumer. In 1992 this changed when the FDA added previsions for accelerated drug approvals, for treatments of life threatening diseases (Food and Drug Administration, 1992).

In 1997 these powers were extended under Federal Law to allow the FDA to 'grant marketing approval for a new drug product on the basis of adequate and well-controlled clinical trial establishing that the drug product has an effect on a surrogate endpoint that is reasonably likely, based on epidemiological, therapeutic, pathophysiological or other evidence, to predict clinical benefit or on the basis of an effect on a clinical endpoint other than survival or irreversible morbidity'(US Congress, 1997). This regulation was the first that allowed surrogate endpoints to be used across all areas of research as indicator of the true endpoint, however the key words here are the surrogate has to be *reasonable likely*, based on various evidence, *to predict clinical benefit*. If a disease is well understood, with a definite pathway of injury to disease to outcome, it is reasonable to say that a biomarker is a good surrogate endpoint, such as high CD4 cell counts in patients with HIV/AIDS corresponds to a decreased survival rate (Baker, 2005). If, however, as more often occurs, the disease pathway is poorly understood, or it is suspected that there is more than one pathway that leads to the disease state, or from disease state to death, then saying that a potential biomarker is a good surrogate

endpoint can be hard. Often this causes surrogate endpoints to be validated along side the drug it is validating.

Despite problems with validation of biomarkers, between 1990 and 2002, 53 out of 71 oncology drugs approved by the FDA gained approval on the basis of surrogate endpoint data, the remaining 18 relied upon traditional survival data. The most common surrogate end point is tumour size reduction (Pien, 2005). Tumour reduction is not a key biomarker in all types of cancer however; in colorectal cancer the correlation coefficient between tumour response and survival was found to be 38% (Pien, 2005), suggesting that in this cancer, using tumour size as a surrogate endpoint would be a bad idea.

2.5.2. Type of biomarkers

A biomarker is 'any substance or its products, structure or process that can be measured in the body and that can influence or predict the incidence of outcome or disease' (Gundert-Remy, 2005). Biomarkers are split into different types by the FDA. Type 0 biomarkers mark the natural history of a disease and correlate longitudinally with known clinical indices, such as symptoms over the full range of the disease states. Type I biomarkers capture the effects of an intervention in accordance with the mechanism of action of the drug, *even though the mechanism might not be known to be associated with clinical outcome*. Type II biomarkers are considered to be surrogate endpoints because a change in that marker predicts clinical benefit. (Frank, 2003). Type 0 and type I biomarkers, while useful in understanding a disease are not accepted as surrogate endpoints, and are therefore not acceptable for drug approval.

Biomarkers can be further split into either different modes of measuring change or into stages of clinical usefulness. If classifying biomarkers by method of measuring change then most biomarkers fit into one of four groups: imaging biomarkers, DNA biomarkers, RNA biomarkers and protein biomarkers. However assuming all biomarkers are

eventually intended for use in the clinical setting, than a better method for classification would be by their functional use, i.e. diagnostic, prognostic and drug selection.

2.5.2.1. Imaging biomarkers

Imaging biomarkers are mainly used with regard to tumours or diseases that cause a large change to the architecture of an organ. The oldest technique for imaging diseases is X-ray, which is still in use, both as straight images and as 3D imaging via computed tomography (CT scans). X-ray is mainly used for 'hard' tissues such as bone scans or when an image is required quickly to asses an injury (Pien, 2005). Magnetic resonance imaging (MRI) is a preferred technique for soft tissues as it gives higher definition, such as in cardiology where MRI scanning is used to asses plaque vulnerability (Frank, 2003). MRI can also be used in areas of the body where X-ray is not recommended, however repeatability and standardisation of MRI machines have been found to be challenging in multi centre trials (Pien, 2005). Another widely used form of image analysis is Positron emission tomography (PET). Depending on the radioactive isotope used PET scans can give information about cellular metabolism (^{18}F-flurodeoxyglucose), cell proliferation (^{18}F-fluro-L-thymidine) or apoptosis (^{99m}Tc-annexin) (Park, 2004). PET scans are also in use in hospitals to image the inflammatory component of vulnerable plaques in the heart (Frank, 2003) and in cancer research to measure resistance to chemotherapy and angiogenesis (Park, 2004).

2.5.2.2. DNA biomarkers

DNA biomarkers work on the basis that people have a pre-determined susceptibility to a disease (such as cystic fibrosis), that their body has particular polymorphs of a gene that means they are more/less susceptible to a drug/disease or that a sub-population of cells have had a mutation in the genome to cause a disease (I.E. cancer). DNA biomarkers usually fall into one of four categories: changes in gene hypermethylation (which can be measured in sputum) (Park, 2004), variations in single nucleotide repeats, microsatellite

instability (Frank, 2003) or gross chromosomal aberrations. In December 2004 however a DNA microarray looking at different types of P450 genes a person has was approved by the FDA as a method of working out the speed at which a person will break down specific drugs (Baker, 2005).

2.5.2.3. RNA biomarkers

RNA biomarkers work by looking at the mRNA that is produced by an individual/group of individuals after an assault, compared to those with no assault. The theory is that mRNA will be produced to overcome the injury, or in the case of cancers mRNA will be being produced that would not be found in either healthy individuals or in non-affected areas of the same individual. By profiling mRNA that has been up/down regulated by an assault it would be possible to either a) screen people of whose status is unknown and assign them to a group (affected/ not affected), or b) work out the best drug/therapy regime to treat the individual based of the pathway their disease is following. RNA biomarkers caused a big stir when they initially came on the market, but interpretation of the results has caused many problems in laboratories around the country due to the large number of genes that are found to change, and the inability to asses which of these are due to outside factors. Generally, most laboratories have found that the mRNA that changes the most are markers of either general poor health and not changing due to the specific disease being studied (Ilyin, 2004).

2.5.2.4. Protein biomarkers

The main advantage of protein biomarkers is that they usually use fluids that are easy to access from the body, such as urine (diabetes), blood, sputum or bronchi-alveolar lavage fluid. Most of these biomarkers are found by large screening of all protein in the fluid via 2D electrophoresis (where the proteins are first separated by charge on a pH gradient and are then separated via weight by traditional electrophoresis). Comparison of proteins found in control and test subjects then allows candidate proteins to be found for

biomarkers. This technique does however only work if the diseased state has a large affect on one or more proteins, as the 2D gels only pick up big differences in levels. Once a candidate has been identified an assay for that particular protein is then devised. Some examples of biomarkers found in this way are: troponin C-reactive protein and lipoprotein-associated phospholipase A2 (both associated with risk of heart disease/stroke (Baker, 2005)). More recently protein arrays have started to be used instead of 2D gels (Gundert-Remy, 2005). Other areas looked at have been protein location within cells, modification of proteins (glycans, truncated forms of the protein etc) and protein-protein interactions; however many of these techniques are only currently being used in animal models to better understand the diseases as they require biopsies (Ilyin, 2004).

2.5.2.5. Exhaled biomarkers

Many laboratories looking into lung disease are using exhaled gases as a way of measuring the extent of specific diseases. Examples of these are nitric oxide, carbon monoxide and hydrogen peroxide. Nitric oxide is produced by many of the cells within the lung including macrophages and is up regulated by inflammatory cytokines and endotoxin (Kharitonov, 2001). In viral infections, asthma and IPF nitric oxide levels are found to increase, while in cystic fibrosis and ARDS it is found to decrease. Levels of exhaled nitric oxide, interestingly, remain the same as for normal lungs in COPD (Kharitonov, 2001). Carbon monoxide and hydrogen peroxide also give distinct patterns as to how the gases levels increase/decrease. Interesting as this data is, as many different diseases cause increases and decreases and as the affect of smoking (past and present) has a large affect on the results, I feel that the possibility of using exhaled gases as a reliable method of quantifying lung injury is slim.

2.5.3. Biomarker uses in each stage of drug development

Potentially biomarkers can be used throughout all stages of drug development. In cancer research biomarkers such as human epidermal growth factor and receptor-tyrosine kinases, VEGF, ras and many more have been found to be linked to clinical out come for specific cancers (Park, 2004). These proteins could therefore be used as drug targets to reduce the proteins effect on the system. Biomarkers can help in optimising a drug during development, for example by checking the percentage of the drug administered reaches the organ of interest via PET scans. In pre-clinical studies, where typically animal models are used (with the well documented limitations of diseases not performing the same as they would in humans), markers that can be found to correlate with the human disease can greatly assist in authenticating the model (Lee, 2004), and therefore the drug being trialled. Once a drug is in phase I/II trials appropriate biomarkers can help with assessing correct dosing (such as the P450 chip for drug metabolism), again PET scans are useful to check that the drug is finding the correct target and mechanistic biomarkers could also help select people to by put on the trial regime (I.E. when there are diseases with similar symptoms, but caused by different mechanistic pathways). In Phase III trials the development of a surrogate end point, so that accelerated or full drug approval and registration can occur, is a great benefit as it saves the drug company from having to wait for traditional endpoints of the disease.

2.5.4. Markers in the clinical setting

Biological markers are used right through clinical medicine, from diagnostic markers to prognosis markers to selection of patents to particular drug regiments. Diagnostic markers are used to identify diseases at early onset before clinical symptoms are readily visible, or when two different conditions have similar morphology (e.g. free PSA in the sera differentiates between prostatic hyperplasia and cancer (Ludwig, 2005)). The advantage of biological markers in this setting is it allows for quick identification of the disease which therefore allows treatment to start earlier. Prognostic markers predict the

likelihood of survival from a disease, or the likelihood of reoccurrence of a disease. In December 2004, the FDA approved the first DNA microarray test for gauging speed of drug metabolism, which is based on the number of SNP and haplotypes of Cytochrome P450 (Frank, 2003, Baker, 2005). Markers like this allow physicians to select drug regimes and dosages based on the likely reaction of a patient to the treatments.

2.5.5. Why biomarkers should be thoroughly investigated

Biomarkers, while proving useful in reducing the length of time required for drugs to go forward to clinical trials, also can produce problems. If it is not thoroughly understood what a biomarker is measuring (and how this relates to the disease) then any decrease/increase in the levels of the marker can not be accurately interpreted. Most biomarkers that do fail fall into one of four categories:-

1) Those that recognise one pathway of a disease but not all possible pathways (false negative)
2) Marker recognises a molecule which is increased as an effect of the disease, but the molecule is not part of the disease pathway.
3) Marker recognises more than one molecule which can cause false positive/ negative results.
4) Marker does not recognise an extra effect caused by the treatment.

Examples of trials that have used biological markers to assist in drug validation where the results from the biomarker have given false positive or false negative results include the Cardiac Arrhythmic Suppression Trail (CAST) (Baker, 2005), fluoride treatment for postmenopausal osteoporosis and Rifaximin treatment of Diarrhea (Steffen, 2003). In the Cardiac Arrhythmic Suppression Trail people who had a heart attack and subsequently diagnosed with ventricular arrhythmia were given trial drugs which reduced the arrhythmia. These drugs had been shown to work based on electrocardiograms on a few hundred patients. The drugs reduce the arrhythmia within a

few weeks, and based on this the drugs were put forward as a treatment that reduces the mortality rate from this condition. However, after a year long study into these drugs, it was found that patients that were being treated with the drugs had a 2.5 fold greater chance of dying than the control group (Baker, 2005). In this case exactly why the drug caused such a different result from that expected has not be reported in the literature, however it is most likely that this trial failed because the proposed marker (regularity of heart beats) is not a marker for all possible pathways that could result in a heart attack.

Fluoride treatment for postmenopausal osteoporosis showed that the treatment, which increased bone density, but did not decrease the rate of fractures. This was found to be due to the decrease in bone quality in these patients. This marker appears to have failed as it did not recognise an alternative effect caused by the treatment (type 4 fail)(Baker, 2005).

Other studies, such as Rifaximin, produced a positive result with regards to symptoms (the traditional method of measuring a drugs success), however the Biomarker being used to test the effectiveness of the treatment (pathogen levels in stools) remained constant (Steffen, 2005). This suggests that Rifaximin is affecting more than one pathway within the body and 'masking' the symptoms of the pathogen while not eradicating the pathogen. Studies like these highlight the importance of detailed investigations into how biomarkers are linked to disease pathways and how diseases progress to the clinical endpoint.

3. Purpose of my study

As the alveolar epithelia is affected in many lung diseases and can affect the outcome of the disease (resolution of fibrosis) a better understanding of how AETI and AETII cells are affected and respond to injurious agents could help in producing drugs to aid normal lung resolution. Biomarkers of the epithelial cells would allow researchers to follow the progression of an injury via secreted biomarkers (in BAL fluid of sputum for example) and by staining lung sections (from biopsy or tissue taken at death). Proteins, which could be biomarkers, are already known for both AETI and AETII cells, however, only one of the AETI cell proteins is specific for AETI cells within the lung (RTI_{40}), and it recognises a protein to which no know function has yet been assigned. As such this is not very useful as a biomarker as, with no known function, explaining increased and decreases in its levels after injury is difficult as it is unknown if the injury is causing the affect to the protein specifically, or to the number of AETI cells. SP-A and SP-D have been proposed as markers for AETII cells, however as these proteins are also expressed by Clara cells the changes in levels in the BAL/sputum can not be assumed to AETII cells meaning that a biopsy would be required to make this an effective biomarker.

In my research I therefore planned to see if I could identify potential markers of AETI/AETII cells by looking at proteins that are not currently considered to be expressed in the lung. I also planned to further investigate two potential biomarkers that had been made in our laboratory group, to asses their usefulness as biomarkers of the alveolar epithelium.

4. Hypotheses

1a) Global gene analysis of RNA from *Staphylococcus aureus* injured lungs can further characterise the 72 hour rat model of pneumonia.

1b) An animal model of 72 hour *Staphylococcus aureus* injury to the lungs could be used to identify new biomarkers of the alveolar epithelium using Affymetrix U34 gene chips.

2a) A potential biomarker in the literature (MMC4 monoclonal antibody) (Boylan, 2001) recognises a novel protein on AETII and Clara cells.

2b) MMC4 monoclonal antibody is a useful biomarker of AETII and Clara cell injury in the rat.

3a) A potential biomarker of AETI cells (MMC6 monoclonal antibody) recognises a protein novel to AETI cells.

3b) MMC6 monoclonal antibody is useful as a biomarker of AETI cell injury in the rat.

5. Materials and Methods

All reagents, unless otherwise stated, were supplied by Sigma

5.1 General techniques

5.1.1. Organ isolation and storage

All organs (except ovaries and placenta) were isolated from healthy, adult, male, specific pathogen free (SPF) Sprague Dawley (SD) rats (Harlow UK) over 300g (n=32). Ovaries and placenta were isolated from healthy, adult, female SD rats (ex-breading stock) (n=4). Rats were euthanized via a lethal dose of anaesthetic (Sagital (60mg/Kg) or Ketermine/Hypoval (133mg/Kg and 13.3 mg/Kg respectively) injected into the intraperitoneal cavity.

Once animals had stopped breathing the abdomen and rib cage were opened. A tracheoscopy was preformed and a shortened wide boor syringe was inserted into the trachea. The lungs were lavaged twice with tris buffered saline (TBS) (containing NaCl (0.154M), Trizma base (0.02M), pH8.2) or phosphate buffered saline (PBS) (with Ca^{2+}/Mg^{2+}). The lavage was preformed by using a 10ml syringe to add 10ml of TBS/PBS to the lungs via the tube inserted in the trachea. The saline solution plus lung contents were then removed from the lungs via suction using the syringe. Lavage fluid was stored on ice until needed. Lungs were then perfused with TBS or PBS. Perfusion was carried out by attachment of an empty 10ml syringe to the trachea tube and insertion of a 50ml syringe containing TBS/PBS into the right ventricle of the heart. Simultaneously the lungs were inflated/deflated with the 10ml syringe and the TBS/PBS was added to the heart. Once the lungs went white in appearance they were removed from the rat by cutting down behind the trachea and lifting the lungs away. Lungs were then stored on ice until needed.

A section of the small intestine (just distal to the stomach, approx 10cm), the stomach and a section of the large intestine (approx 10cm) were then removed. The contents of all three were squeezed out gently before these organs were placed on ice. All other organs were removed and stored on ice.

All organs were washed in TBS with Complete© protease inhibitor (Roche) added as per the manufacturer's instructions. The tissues were homogenised in TBS with Complete© protease inhibitor (ratio 1:5 weight to volume) at 13,000rpm using an Ultra Terrax T25 basic homogeniser (Werke) and then spun at 300*g* for 3 minutes to remove larger tissue pieces. (Here after the supernatant is referred to as 'homogenate'.) Aliquots of the homogenates were then stored at -70°C until needed.

5.1.2. Bradford protein assay

Bradford assay (BioRad) was used to measure protein concentrations of all samples initially. Briefly, 40µl Bradford reagent was diluted with 150µl water and 10µl sample was added. The absorbencies of the samples were measured using an ELISA plate reader (MRX, Dynatech laboratories) at 600nm. Each sample was tested at two different dilutions and with three repeats of each dilution. Samples were compared to a standard curve of Bovine Serum Albumin (BSA) (Pierce) (0-1.2µg protein).

5.1.3. Lowry protein assay

A modified Lowry protein assay (Lowry, 1951) (BioRad) was used to measure the protein concentration of samples containing detergents (due to detergents interfering with the Bradford assay). Briefly, 20µl of samples were incubated with copper tartrate and SDS (Sodium dodecyl sulphate) for 5-10 minutes, with shaking, at room temperature in a 96 well plate (NUNC). Folin reagent was added and incubated at room temperature, with shaking, for 15 minutes. The absorbencies of the samples were

measured using an ELISA plate reader at 630nm. Each sample was tested at two different dilutions, and with three repeats of each dilution. Samples were compared to a standard curve of BSA also with detergent added at the same ratio of 1:5 mg protein (determined by Bradford assay before the addition of detergent) to mg detergent.

5.1.4. Enzyme-linked immunosorbant assay (ELISA)-based dot blot

Immobilon-P transfer membrane (a polyvinylidene fluoride (PVDF) membrane) (Millipore) was hydrated via a methanol wash followed by repeated washing in distilled water. Samples, suspended in TBS, were vacuumed onto the hydrated PVDF membrane using a dot blot manifold (figure 2) (Schliecher & Scheuell). The PVDF membrane was then incubated in casein (2.5% w/v in TBS) for 1.5 hours to block the PVDF membrane, preventing any other protein binding to the membrane. Half the blot was then incubated with the primary antibody (see table 5 for a list of primary antibodies used) for 30 minutes, while the other half of the blot remained in blocking buffer. Both halves of the membrane were washed with TBS-Tween (polyethtlene sorbitan monolaurate) (0.5% v/v) for 30 minutes and then incubated with IgM anti-mouse-IgG-HRP (0.1% v/v, casein (2.5% w/v) in TBS) (Rockland c/o Lorne Laboratories) for a further 30 minutes. After washing with TBS-Tween, again for 30 minutes, the membranes were added to electrogenerated chemiluminescence detection reagents (GE Healthcare). Immediately the membrane was exposed to Biomax Light X-ray film for various lengths of time (typical exposure times were 15 seconds, 30 seconds, 1 minutes and 2 minutes). After development in a X-ray development machine (Biorad), the film was attached to the bottom of a 96 well plate and read in an ELISA reader at 630nm (MRX, Dynatech laboratories). To allow for variations between blots, all samples to be compared and controls were loaded onto one membrane.

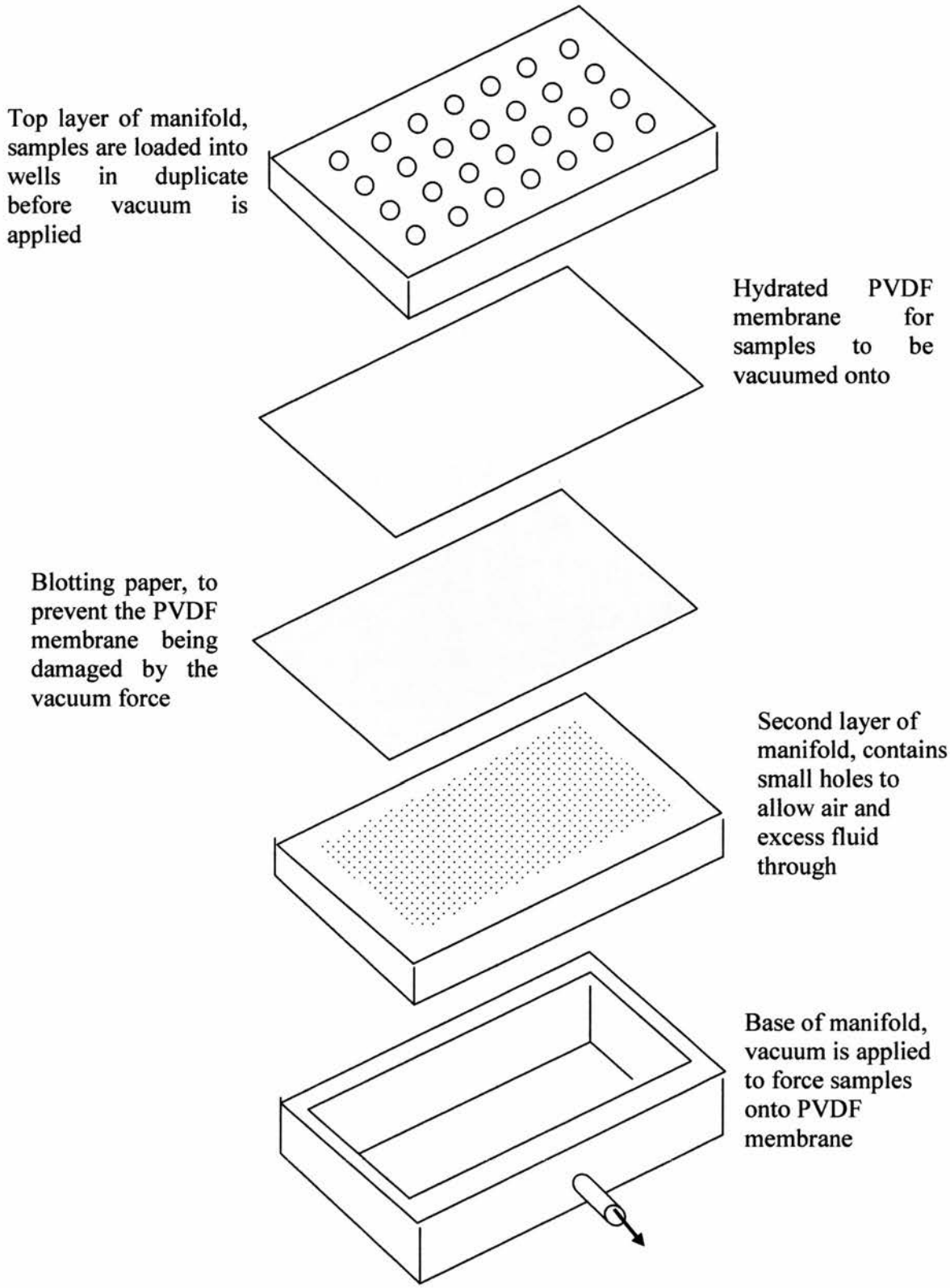


Figure 2: Assembly of the dot blot manifold for ELISA based dot blots. Samples, mouse serum or water was loaded into the wells the vacuumed onto the PVDF membrane.

Antibody	Isotype	Raised in	Dilution in DMEM and 10% foetal calf serum	Antigen	Antibody source
MMC4	IgG2b	Mouse	Hybridoma supernatant	APN	McElroy
RTI_{40}	IgG1	Mouse	1:50 to 1:5000, dilution of hybridoma supernatant	T1α	Dobbs
MMC6	IgG2b	Mouse	Hybridoma supernatant	Unknown	McElroy

Table 5: Antibodies used for ELISA based dot blot analysis of sample.

5.1.5. Sodium dodecyl sulphate –poly acrylamide gel electrophoresis (SDS-PAGE)

Six, eight or ten percent acrylamide separating gels (see table 6 for recipes) were poured into BioRad equipment and allowed to set for 30 minutes. A stacking gel (table 6) was then loaded on top of the separating gel into which a 10 or 15 well comb was placed. Once the gel had set the apparatus was assembled and a running buffer (containing Tris (25mM), glycine (192mM) and SDS (0.1% v/v) at pH 8.3) was added to both upper and lower chambers. Samples and molecular weight markers (GE Healthcare), diluted in sample buffer (without mercaptoethanol), were loaded into the wells. The gel was run at 100-130 volts at 4°C until the dye in the sample buffer ran to the bottom of the gel.

The gels were then stained with Gelcode (Pierce), Rapid silver stain (Gottlieb, 1987) or Proteosilver Plus (Gharahdaghi, 1999, Rabillaid, 1994) as per manufacturer's guidelines. Bands required for further analysis were excised from the gel and stored at -20°C. Gels were then dried either via the heat/vacuum method (equipment from BioRad), or using a TUT's Tomb air drier (Matsvdaira, 1978). Gels and membranes (Promega) for the TUT's Tomb drier were pre-soaked in 5% glycine, 10% ethanol for 30 minutes before apparatus was assembled.

5.1.6. Harvest of lungs for immunohistochemistry

Rats were euthanased via a lethal dose of pentobarbital and 0.7ml heparin via an IP injection. Blood was then extracted from the aorta. A tracheotomy was performed and 2x10 millilitres sterile PBS with Mg^{2+}/Ca^{2+} were used to lavaged the lungs. Lungs were then perfused with 50 millilitres sterile PBS with Mg^{2+}/Ca^{2+} before inflating the lungs with paraformaldehyde (4% w/v in PBS without Mg^{2+}/Ca^{2+}). Lungs were removed from the rat and suspended in paraformaldehyde for 24 hours at room temperature. Small areas of the lung were then dissected out and stored at 4°C in 30% sucrose for a further

Solution components	6% gel (ml)	8% gel (ml)	10% gel (ml)	Stacking gel (ml) (5%)
Water	7.9	6.9	5.9	3.4
Acrylamide/Bis (30% T, 2.67% C) (37.5:1 mixture)	3.0	4.0	5.0	0.83
1.5M Tris-HCl, pH 8.8	3.8	3.8	3.8	0
1.0M Tris HCl, pH 6.8	0	0	0	0.63
10% (w/v) SDS	0.15	0.15	0.15	0.05
10% (w/v) ammonium persulfate	0.15	0.15	0.15	0.05
TEMED	0.012	0.0009	0.0006	0.005

Table 6: Recipes for Tris-glycine SDS-Polyacrylamide gel electrophoresis resolving gels.

24 hours. The tissue was then rapidly frozen and cut into 5 micron sections. These samples were then fixed to glass slides and stored at -20°C until needed.
(Freezing, cutting and fixing to slides was performed by S. Harvey).

5.1.7 Immunohistochemical staining of frozen lung sections

Frozen sections of lung prepared as above were washed in PBS with Mg^{2+}/Ca^{2+} then incubated in blocking buffer containing 0.1% Triton X-100 (0.352% fish gelatine, 20% goat serum, 0.1% Triton X-100 in PBS with Mg^{2+}/Ca^{2+}) for 20 minutes. After washing with PBS, slides were incubated with primary antibodies (see table 7 for antibodies and quantities used) made up to 100µl in blocking buffer for 20 minutes. Slides were washed, again in PBS, and then incubated with the appropriate secondary antibodies (Calbiochem) (in 100µl blocking buffer) for 15 minutes (see table 7). If nuclear stain was to be added then TP3 (Calbiochem) (diluted 1:100 in blocking buffer) was added after a further wash and left for 10 minutes. After a further wash cover slips were mounted using Dako mounting medium. Slides were scanned using wavelengths of 488nm (green), 546nm (red) and 647nm (blue) depending on the secondary antibody used.

5.1.8. Statistical analysis

Values are shown as the mean average with conservative standard deviations. Where significance has been shown first the data was F-tested to ensure that the values had a similar distribution profile, followed by the Student t-test assuming two tailed distribution and equal variance (as shown by the F-test). Statistics were carried out in Microsoft Excel and GraphPad Instat.

Primary antibody	Dilution in blocking buffer	Secondary antibody	Dilution in blocking buffer	Primary antibody source
MMC4	1:2	Anti-mouse IgG2a	1:100	McElroy
MMC6	1:2	Anti-mouse IgG2b	1:100	McElroy
RTI_{40}	1:100	Anti-mouse IgG1	1:100	Dobbs
CD9	1:50	Anti-mouse IgG3	1:100	BD Biosciences
CD68	1:50	Anti-mouse IgG1	1:100	Serotec

Table 7: Primary and secondary antibodies used for immunohistochemical analysis of frozen lung sections. All secondary antibodies were from Calbiochem.

5.2. Chronic *Staphylococcus aureus* model of pneumonia in rats

All bacterial work was carried out using sterile equipment and techniques in a Class II safety hood.

5.2.1 *S. aureus* stock production

Single colony forming units (CFU) of S. aureus strains 8325-4 and 8325-4 PLs grown on blood agar base plates (DB Biosciences) were added to 3ml Todd Hewitt broth (Fisher Scientific) and incubated for 16 hours at 37°C in an orbital shaker at 300 rpm. The resulting bacteria broth was added to 7ml glycerol, split into 500µl aliquots and stored at -70°C. If a stock solution of broth completely thawed out, it was not re-frozen as it was found that repeated freeze-thawing cycles killed the bacteria.

5.2.2 *S. aureus* culture

Scrapings (<10µl) of the glycerol frozen *S. aureus* were streaked out onto blood agar base plates and incubated at 37°C for 16 hours to produce single colony forming units. A single CFU from the plate was then transferred to 3ml Todd Hewitt broth in a 12ml bacteria tube. Tubes were turned to be horizontal and shaken for 30 seconds to maximise the aeration of the broth. Tubes were incubated for 16 hours in an orbital shaker at 300rpm and 37°C.

5.2.3. Instillates

The optical density of the Todd Hewitt broth at 600nm was measured and was compaired to a previously established standard curve to give a rough guide to the colony forming units per millilitre (cfu/ml) of the broth. From this, it was calculated how much broth would be needed to give 7-9x10^8 cfu/ml. After dilution in PBS with Mg^{2+}/Ca^{2+}, 1.5ml of broth was washed 3 times via centrifugation at 13000*g* for 3 minutes, the pellet

was re-suspended between each wash in 1.5ml fresh PBS. The bacteria were finally re-suspended in 1.5ml PBS. Ten microlitres of each instillate was removed, diluted and plated on blood agar plates to confirm cfu/ml and also the antibiotic resistance of the 8325-4 PLs strain.

5.2.4. Instillation

SD SPF rats (300-350g) were stored in groups of 4 in closed bottom cages with free access to food and water. Animals were stored in a separate room to prevent distress due to excessive noise/movement. Sixteen hours before the start of the procedure the required animals were removed to the procedure room to allow them to settle before they were instilled. To prevent stressing the animals, all work was carried out in silence.

Rats were anaesthetised with a Hypnoval/Hypnorm mixture (0.2ml Hypnoval plus 0.2ml Hypnorm plus 0.8ml water) via an IP injection. Once anaesthetised rats were weighed and suspended over the apparatus shown in figure 3. A fine tube was passed down the trachea and through this 0.5ml of instillate or 0.5ml PBS was passed, followed by 2ml of air. Generally, this led to bacteria being instilled into the left lung lobe. The rats were then placed back in their cages on paper to prevent inhalation of the wood shavings. Cages were placed in a Class II safety hood on top of a heating pad. Rats breathing status during and immediately after instillation were recorded. Further recordings of animals breathing, motility, colour and weight were taken every 2 hours post instillation, until 8 hours post instillation and then again at 24 and 48 hours post instillation. Twenty four hours after instillation animals were returned to their usual holding room.

5.2.5. Harvest for protein analysis

Rats, at various time points after instillation, were given a lethal dose of pentobarbital and 0.7ml heparin IP. Blood was then extracted from the aorta, and plated out on blood agar plates to check for bacteria and in the case of 8325-4 PLs, antibiotic resistance. The

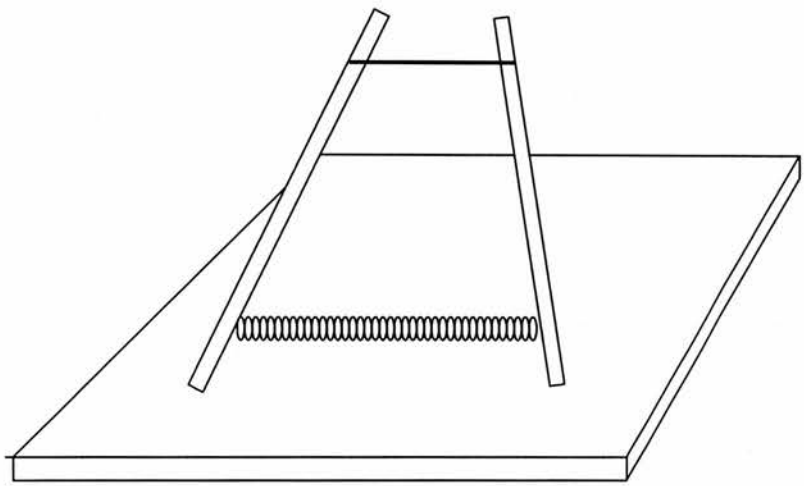


Figure 3: Drawing of the stand used to drape rats over for instillation of bacteria. Rats' front teeth were placed over top wire and front feet were placed on spring at bottom. This ensured that the rats mouth remained open and that the trachea was straight. A fine tube was then passed down the trachea via which bacteria suspended in PBS or PBS was instilled into the lungs.

remaining blood was spun at 2500rpm for 20 minutes and the serum was collected and stored at -70°C. The diaphragm was then perforated and 2ml PBS with Mg^{2+}/Ca^{2+} was used to wash the plural cavity. Plural fluid was plated on blood agar based plates to check for *S. aureus* and the level and type of white blood cells were analysed as described below. Leukocytes were then removed from the plural fluid prior to its storage at -70°C.

A tracheotomy was then performed. The rib cage was cut open so that the lungs could be examined for any visible signs of damage. Lungs were lavaged as described previously (see 5.1.1). Again, this fluid was plated to measure the remaining *S. aureus* within the lungs. Leukocyte number and type were measured (see below) prior to the cells being removed from the remaining bronchioalveolar lavaged fluid (BAL). BAL fluid was then also frozen at -70°C. The lobes of the lungs were then removed and homogenised as described previously (see 5.1.1).

5.2.6 Leukocyte cell counting and typing

One millilitre of the BAL fluid from control animals was spun down at 13000*g* for 3 minutes, excess fluid was removed and the pellet was re-suspended in 100µl PBS. Crystal violet was added to either the concentrated BAL (controls) or straight BAL (infected) at a ratio of 1:1 (v/v). Plural fluid was diluted in crystal violet at a ratio of 1:5 (v/v). Ten microlitres of each was added to a haemocytometer. Twenty five squares on the haemocytometer were counted for number of leukocytes. From this the total number of leukocytes in the BAL or plural fluid was calculated.

Five hundred microlitres of BAL or 20µl of plural fluid were cytospun onto glass slides. Once dry, slides were stained with DiffQuik (Gamidor). Slides were then analysed for number of each type of leukocyte using light microscopy.

5.2.7 Harvest of lungs for RNA extraction

S. aureus infected rats, 72 hours after inoculation, were euthanized and blood was extracted as described previously (see 5.2.5). A tracheotomy was performed, the rib cage was opened and the damage to the lungs was assessed. Lungs were lavaged as descried previously (see 51.1). The BAL fluid was plated for remaining cfu of *S. aureus*, leukocytes were removed from the BAL and the remaining fluid was frozen at -70°C until needed. Leukocytes were analysed as described previously (see 5.2.6). The lungs were then perfused with 50ml sterile PBS with Mg^{2+}/Ca^{2+} (as described in 5.1.1) and inflated with RNAlater (Ambion). The lungs were removed whole and stored in RNAlater at 4°C for 24 hours.

5.2.8 RNA extraction

Injured regions of the RNAlater stored lungs were dissected out. One hundred milligrams of this tissue was homogenised in homogenisation buffer (Qiagen) and the sample was then added to an extraction column. Total RNA was extracted using the standard Qiagen protocol with the addition of the DNA removal step (Qiagen). Purified total RNA was removed from the column using 30µl of 70% ethanol. Total RNA collected from 2 columns was then pooled and frozen at -70°C until required. Purity of the RNA was measured via 2100 Bioanalyzer (Agilent Technologies)

5.2.9 Analysis software used

Initial analysis of the raw data was carried out using Affymetrix software to normalise the data (U34 gene chip algorithm, see appendix i for a guide to how this algorithm works). GeneSpring (Agilent Technologies) was used to carryout rough analysis of the data (see appendix i for a guide to how GeneSpring works). This confirmed that the data was normally distributed. All further analysis was carried out using Microsoft Excel.

5.3 MMC4 monoclonal antibody's antigen analysis specific techniques

5.3.1. Solubilization of homogenates in $C_{12}E_8$

Homogenates from kidney, lung, intestine and thymus were incubated overnight in various ratios of mg protein to mg $C_{12}E_8$ (1:1, 1:5 and 1:10). The solubilized homogenate was then ultra-centrifuged at 500,000g for 10 minutes to remove any $C_{12}E_8$ insoluble fragments. Soluble supernatant (here after referred to as the 500,000g supernatent) were then tested for remaining MMC4 reactivity by ELISA based dot blot.

5.3.2 Immuno-precipitation with Dynabeads®

(See figure 4 for an overview) Pan anti-mouse IgG Dynabeads® (Invitrogen) (4 x 10^6 beads) were incubated with either 100µl hybridoma supernatant MMC4 antibody or a control non-specific IgG2a antibody (Stratech Scientific Ltd) for 30 minutes at 4°C with rotation, allowing the antibody to bind to the anti-mouse IgG antibody bound to the beads. The coated Dynabeads® were washed three times with PBS with Ca^{2+}/Mg^{2+} to remove excess unbound antibody. Then the 500,000g supernatants were added to the beads (see table 8 for the amount of protein added to the Dynabeads®). The beads and samples were incubated at 4°C with rotation for 2 hours to ensure maximum antigen binding to the MMC4 antibody. The beads were washed with PBS with Ca^{2+}/Mg^{2+} five times and bound proteins were removed from the Dynabeads® by incubation with 50µl SDS-PAGE sample buffer without mercaptoethanol (containing Tris-HCl (62.5mM pH 6.8), glycerol (20% v/v) and SDS (2%w/v)) at 95°C for 4 minutes. The sample buffer plus proteins were then loaded (20µl per well) and run on 6 or 8% acrylamide gels (see 5.1.5.). A control of Dynabeads® with no antibody bound was also incubated with kidney 500,000g supernatant to check for non-specific binding to the Dynabeads®.

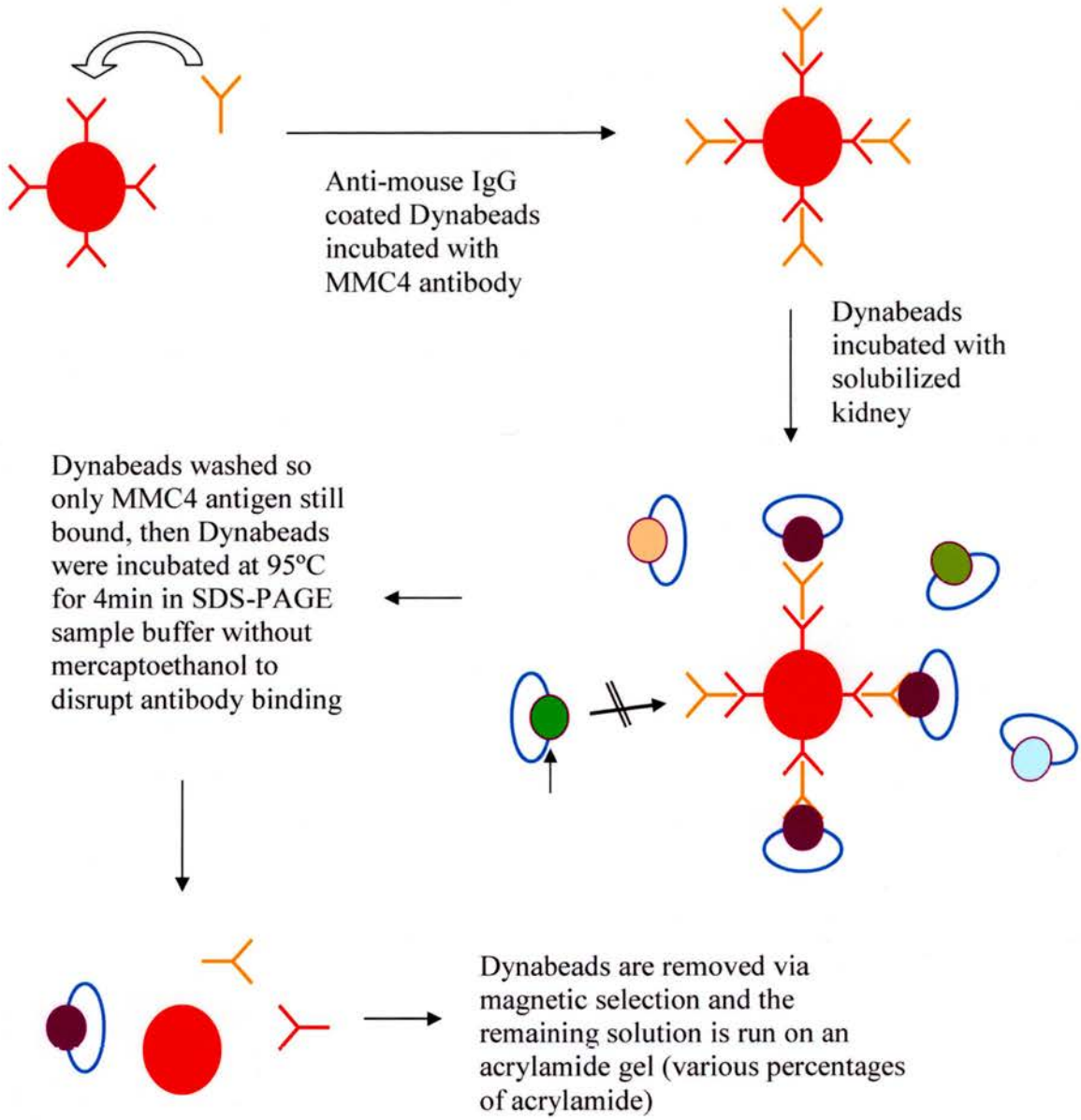
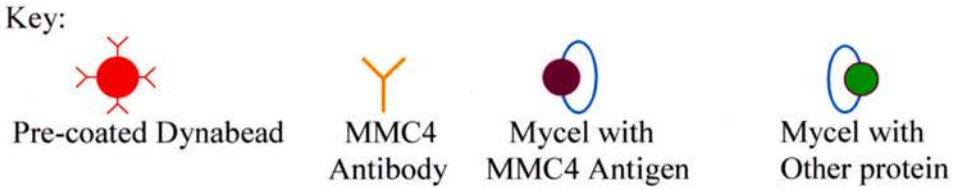


Figure 4: An overview of the selection of the MMC4 antigen using Dynabeads



Organ	Mg protein added to beads
Kidney	1.37
Lung	44.8
Thymus	10.5
Intestine	3.63
Liver	101

Table 8: Amount of protein from various organs required to be added to the Dynabeads® to enable the MMC4 antigen band to be visible via SDS-PAGE electrophoresis.

5.3.3. Immuno-depletion of MMC4 antigen from solubilized homogenates

(See figure 5 for an overview) MMC4 Monoclonal antibody was attached to Pan Anti-mouse IgG Dynabeads® (4 x 10^6 beads) as described above (5.3.2). Kidney 500,000g supernatant was incubated in four successive rounds of Dynabeads®. After each incubation, 100µl of the remaining supernatant was removed and replaced with 100µl PBS (without Ca^{2+}/Mg^{2+}). The supernatants removed were all diluted to the same protein concentration and tested for MMC4 reactivity via an ELISA based dot blot as described previously (5.1.4). Proteins bound to the Dynabeads® were removed as described above and run on 6 or 8% SDS-PAGE gels.

5.3.4. Trypsin digestion

Candidates for the MMC4 antigen were cut out of the gel using a sterile scalpel and stored at -20°C until needed. SDS was removed from the gel piece via 3 incubations with NH_4HCO_3 (0.2M) in 50% acetonitrile (ACN) at 30°C. The protein was then reduced via incubation with DTT (20mM with NH_4HCO_3 (0.2M) and 50% ACN) at 30°C.

After washing with NH_4HCO_3 (0.2M 50% ACN), the cystines were alkalated in iodoacetamide (50mM in NH_4HCO_3 (0.2M) 50% ACN) at room temperature in the dark. The gel piece was then washed in 20mM NH_4HCO_3 and 50% ACN. All water was then removed from the gel pieces via incubation with 100% ACN. Excess ACN was removed and the gel pieces were allowed to dry (increases the probability of trypsin entering the gel and therefore optimises protein cleavage). One microlitre sequence grade trypsin (in 50mM NH_4HCO_3) (Promega) was added to the gel pieces and stored at 4°C until the gel pieces swelled and then pieces were transferred to 30°C overnight to digest the protein. The digestion mixture was then agitated (vortex 1 minute) and spun for 20 minutes at 13000rpm to remove remaining peptides from the gel.

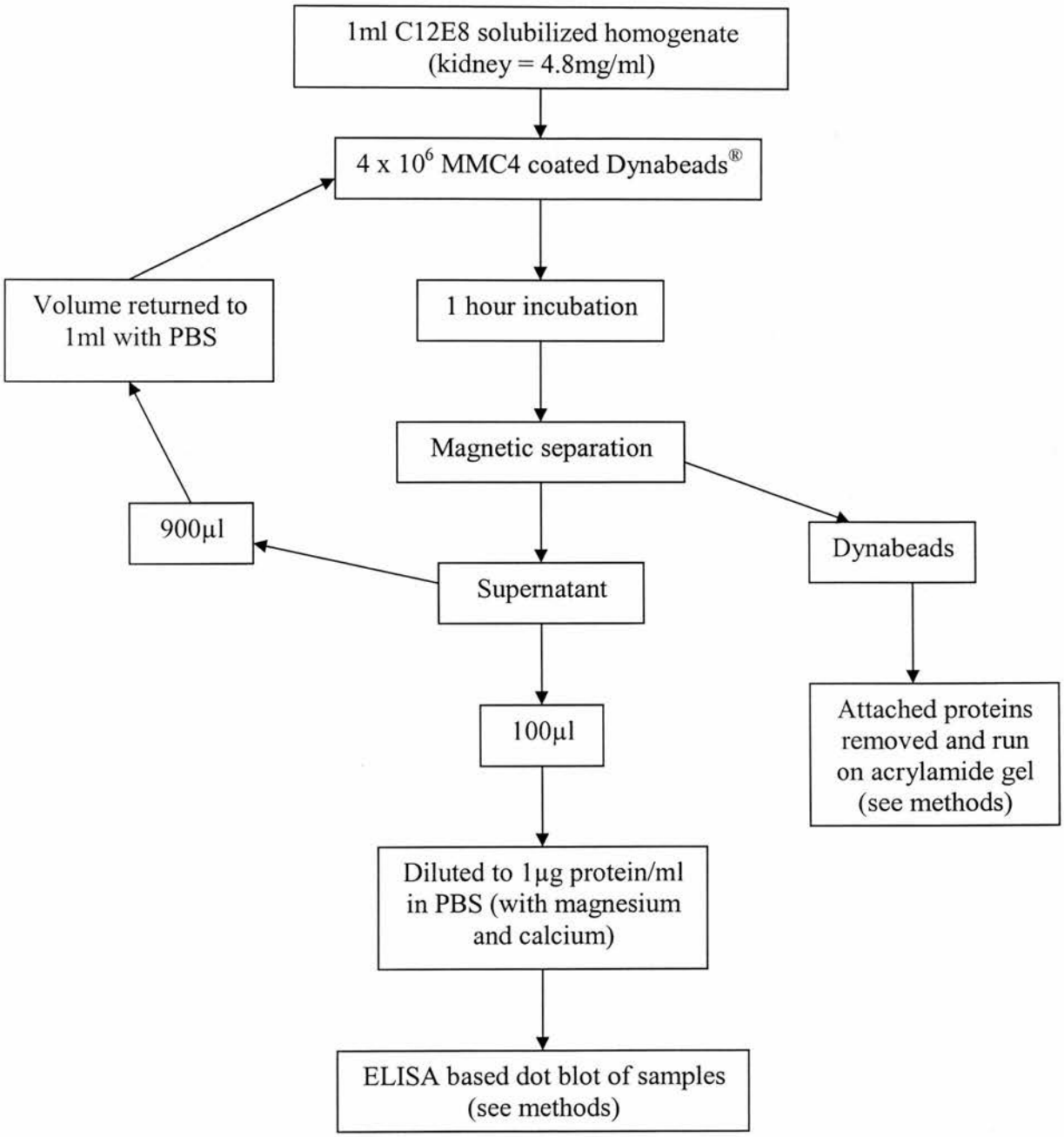


Figure 5: Immuno-depletion of MMC4 antigen from solubilized kidney homogenate. Homogenate was incubated with five successive rounds of Dynabeads® (coated with MMC4 antibody) as described. The same technique was used for other organs; however different initial and final protein concentrations were used for each organ.

Trypsin digested proteins were then processed via a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems) or via liquid chromatography mass spectrometry and molecular weight fingerprints obtained were analysed using SwissProt Tr-EMBL. MALDI-TOF, liquid chromatography mass spectrometry and SwissProt Tr-EMBL analysis were carried out by Dr. A. Cronshaw (see 7.1.1.1, 7.1.1.3 and 7.1.1.2 respectively for a description of how these techniques work).

5.3.5 Aminopeptidase N functional assay

Aminopeptidase N (APN, 2.5µl) (Calbiochem) was incubated with 5µg L-Alanyl-*p*-nitroanlilde (L-Ala-pNA) (Calbiochem) for 6 minutes. At 1 minute time points, the optical density of the mixture was measured at 405nm. APN cleaves the N-terminal alanine from L-Ala-pNA. As this occurs the colour of the solution shifts from yellow to blue, therefore increasing the reading at 405nm. The rate of L-Ala-pNA breakdown by APN was measured with and without the MMC4 Monoclonal antibody, with the non-specific IgG2a antibody (Stratech Scientific Ltd) and without APN. Controls of just APN and just TBS (used to dilute samples) were also tested.

5.3.6. Macrophage isolation

Macrophages were isolated from the lungs of healthy, male, SPF, SD rats between 300 and 350g in weight. Rats were euthanized via a lethal dose of anaesthetic as previously described (5.1.1). Lungs were removed from the rat and lavaged with buffered saline without Ca^{2+}/Mg^{2+}. Macrophages were removed from the lavage fluid via centrifugation at 300g for 6minutes and resuspended in 1ml TBS with Complete® protease inhibitors added. The macrophages were then washed three times by centrifuging at 300g for 6 minutes and re-suspended in TBS with Complete® protease inhibitors added. After the second wash, 10µl of macrophages suspended in TBS was removed to be counted using a haemocytometer and crystal violet to visualise the cells. After the final wash, cells

were re-suspended to a concentration of $10x10^6$ per ml in TBS with Complete® protease inhibitors added.

5.3.7. Deglycosylation of N-linked glycans

Twenty microlitres N-Glycosidase F (Roche) was incubated with 10µg APN for 1 hour at 37°C. Fifteen microlitres was then added to Dynabeads® coated with MMC4 monoclonal antibody as described previously. Dynabeads® were incubated with the N-glycosidase F, APN mixture for 2 hours at 4°C with rotation, the Dynabeads® were washed to remove unattached proteins and bound proteins were removed as described previously. APN, APN plus N-glycosidase F, APN plus Dynabead® incubation and APN plus N-glycosidase F and Dynabead purification, all having had a 1 hour 37°C incubation, were run on a 10% acrylamide gel. The remaining 15µl of the overnight N-glycosidase F, APN mixture was diluted to a total volume of 200µl in TBS and tested for MMC4 reactivity via ELISA based dot blot (5.1.4).

5.3.8 Deglycosylation of O-linked glycans

O-linked glycans were removed (as per the manufacturers' guidelines) using O-glycosidase (Roche). Briefly, O-glycosidase was added to APN and then heated to 37°C overnight. Samples were then tested for remaining MMC4 reactivity via ELISA based dot blot (5.1.4) or Dynabead purification (5.3.2) followed by SDS-PAGE gel separation (5.1.5). Controls of APN with no O-glycosidase and O-glycosidase by itself were also tested.

5.4 MMC6 antigen analysis techniques

5.4.1 Proteinase K treatment of lung homogenate

Lung homogenate corresponding to 9mg protein was incubated with 2mg proteinase K (stock solution is 20mg/ml in 10mM Tris-Cl pH7.5) (Qiagen) for 16 hours at 37°C with rotation. Controls of lung homogenate (37°C and 4°C) and proteinase K (37°C) were also incubated overnight. Half of each sample was spun at 500,000*g* for 10minutes (Optima TLX ultracentrifuge, Bechman) to separate the soluble and insoluble fractions. The pellet was re-suspended in 0.5 ml TBS. The un-separated, the 500,000*g* supernatant and the 500,000*g* pellet samples were all tested for remaining antigenicity to MMC6 monoclonal antibody via an ELISA based dot blot as described above (5.1.4)

5.4.2. Sodium carbonate treatment of lung homogenate

Lung homogenate was tested to see if the MMC6 antigen was a membrane protein via ultracentrifugation at 500,000*g* (which causes membranes to move to the pellet) followed by ELISA based dot blot. The membrane pellet was then treated with 0.1M (pH11) sodium carbonate for 1 hour on ice to remove peripheral proteins from the membrane. The treated pellet was then spun again at 500,000*g*, causing integral membrane proteins to be in the pellet still and peripheral membrane proteins to be in the supernatant. Lung homogenate, and the pellet and supernatant at each stage were all tested so that the location of the MMC6 antigen could be determined.

2.4.3 Solubilization of tissue homogenate for antigen purification

Homogenates from the 300g centrifugation were incubated with various ratios of detergents to total protein concentration for 18h at 4°C (see table 9 for a list of detergents used and at which ratios). Retention of antigenicity was checked via an ELISA based dot blot (as described in 5.1.4). Samples were then spun at 500,000g for

Detergent	Ratios used	With Caproic acid added?	Without caproic acid?
NP-40	1:1, 1:5, 1:10	Yes	Yes
Triton X-100	1:1, 1:5, 1:10, 1:20, 1:50	No	Yes
Dodecyl mactoslate	1:1, 1:5, 1:10	Yes	Yes
SDS	1:1, 1:5, 1:10	No	Yes
Mega 8	1:1, 1:5, 1:10	Yes	Yes
Octyl-β-D-glucopyranoside	1:1, 1:5, 1:10	Yes	Yes
$C_{12}E_8$	1:1, 1:5, 1:10	No	Yes
$C_{12}E_9$	1:1, 1:5, 1:10	Yes	Yes
Cholic acid	1:1, 1:5, 1:10	Yes	Yes
CHAPS	1:1, 1:5, 1:10	Yes	Yes

Table 9: Ratios and conditions of detergents that were used for solubilization of lung membranes without destroying MMC6 antigenicity.

10 minutes to pellet the insoluble fraction The insoluble pellet was re-suspended in an equal volume of TBS and together with the detergent soluble fraction (hereafter called the 500,000g supernatant) was tested for reactivity of the Monoclonal antibody's via ELISA based dot blot. Controls of unsolublised 300g homogenate, 500,000g pellets (no detergent) and 500,000g supernatants (no detergent) were also tested.

5.4.4. Mechanical disruption of lung cell membranes

One hundred microlitres of homogenate were sonicated for five one-second bursts with a one second rest between bursts on ice. Confirmation that the cells were broken was carried out microscopically. Alternatively, organ homogenates were further homogenised at 30,000rpm for 5 minutes on ice.

With both methods, samples were then spun at 500,000g for 10 minutes to pellet the membrane insoluble fraction. Supernatant and pellet were then tested for remaining antigenicity via ELISA based dot blot as described previously (5.1.4). Alternatively the 30,000rpm homogenate was spun at 13,000g for 5 minutes to remove the larger tissue fractions and the remaining supernatant was also measured for remaining antigenicity via ELISA based dot blot.

6. Global gene analysis as a method to identify novel alveolar epithelial markers

6.1 Introduction

As already discussed, to produce a biomarker specific for an injury, first the injury has to be well studied. This increases the chance that the potential biomarker is linked directly to the disease being studied and is not just a general marker of injury. Global gene analysis is one way to do this, either by taking repeated samples at different time points (to monitor the change in gene expression over time), or by taking a snap shot of the change based on previous knowledge of the disease being studied.

It was decided to see how useful global gene analysis would be in a rat model of *S. aureus* induced pneumonia which had previously been established in our laboratory group. This model was chosen for two reasons:

1) The limited budget for Affymetrix chips meant that my sample size would have to be small and it was thought that I would be able to control and limit outside factors better with an animal model.

2) The model had already been partially characterised within our laboratory group, and had shown a decrease in AETI cells, but an increase in AETII cells at 72 hours post-infection.

The established change in AETI and AETII cell numbers meant that we could also analyse the data for potential markers for these cell types.

Hypotheses of this study were:

1a) Global gene analysis of RNA from *S. aureus* injured rat lungs can further characterise the 72 hour *S. aureus* model of pneumonia.

1b) An animal model of 72 hour *S. aureus* injury to the lungs could be used to identify new biomarkers of the alveolar epithelium using Affymetrix U34 gene chips.

6.1.1. U34 Affymetrix gene chip

The U34 Affymetrix gene chip has probes for 8740 different proteins found in the rat. For each protein there are 22 DNA probes, which are paired. Eleven probes are exact matches for the copy RNA and 11 are single base pair mismatches. Figure 6 shows cartoon representation of a chip to explain the probe layout. As the mRNA is copied (via reverse transcription into copy DNA and then transcription into copy RNA) biotin labelled nucleotides are added to the mix so that the resulting copy RNA is biotin labelled. The copy RNA, along with control biotin labelled RNA, is allowed to hybridize to the chip for 18 hours prior to the chip being read. The whole chip is scanned and then using the control markers that were added to the RNA a grid is laid over the whole chip. (The control RNA binds to the outside of the chip making a checkerboard positive/negative pattern allowing the grid to be accurately placed). The absorbance for each probe is recorded and then, using an algorithm designed for the chip the 11 matched probes and the 11 single mismatched probes are compared and a value is assigned for the gene.

6.1.2. Analysis of results

As the model of pneumonia is already known to cause an influx of leukocytes both 24 and 72 hours after instillation of *S. aureus*, at 72 hours it was expected that there would be a change in the levels of expression of many of the genes. Therefore, before starting to analyse these results, a stratagem was required which would allow handling of this data and to draw a finite finishing point for each question to be addressed. Figure 7 is a flow diagram overview of how I intended to manage this problem.

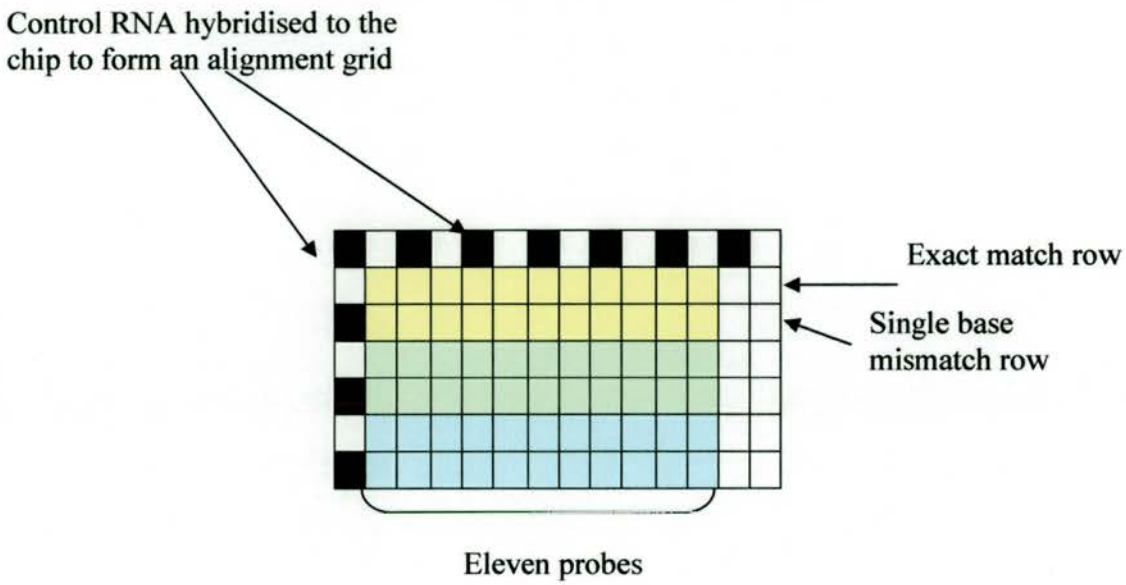


Figure 6: Cartoon representation of part of an Affymetrix U34 gene chip. Control probes are arranged around the edge of the chip to allow a grid to be overlaid after hybridization. This allows the computer to work out which gene is where. The 11 probe pairs for each gene are arranged in blocks with the 11 complete matches of 25 base pairs on one row and the single base pair mismatch on the row underneath. (More recent types of Affymetrix chips have abandoned this format in favour of probes being placed in a random pattern across the whole chip).

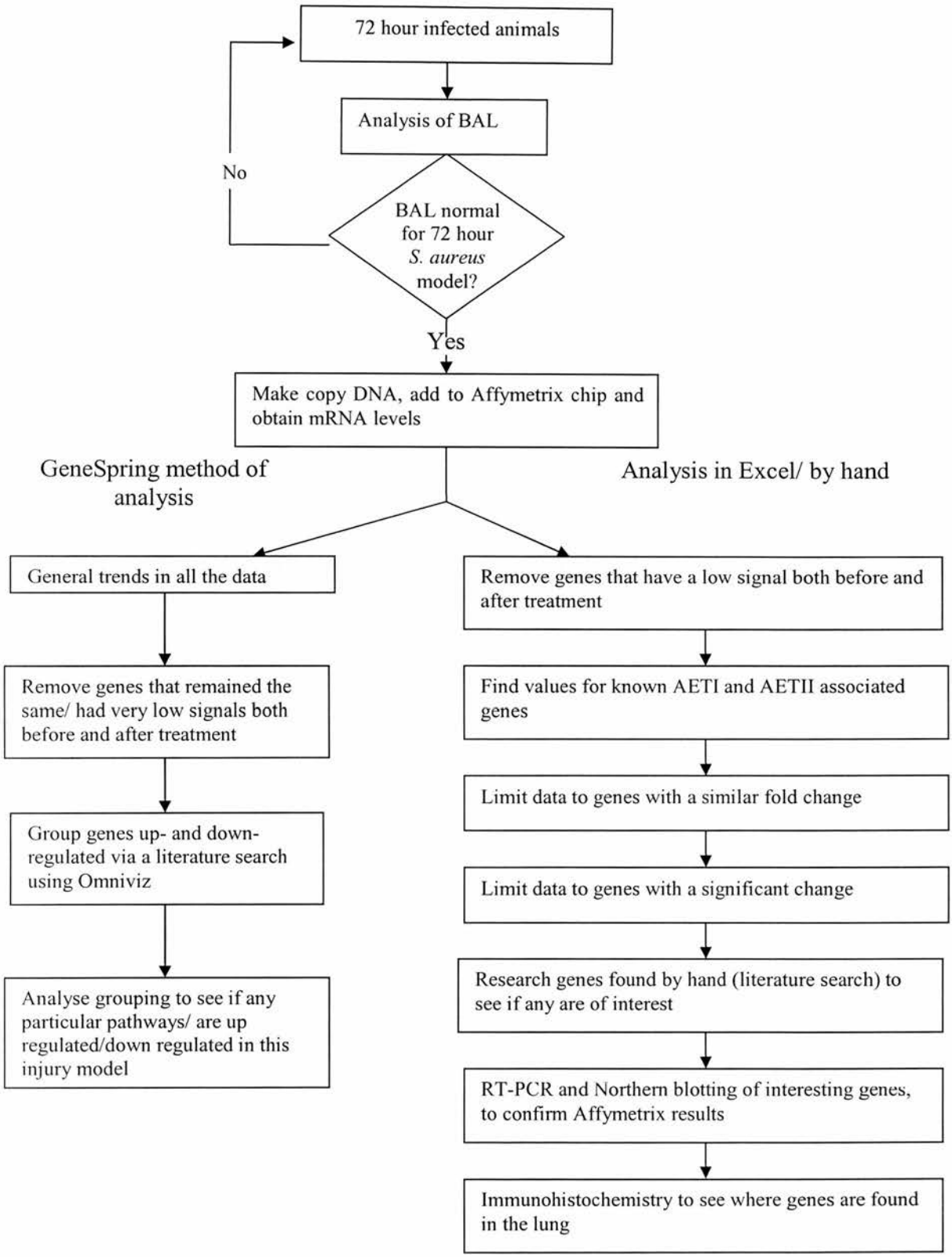


Figure 7: Proposed method for data analysis from the Affymetrix U34 gene chips after injury with *S. aureus*

6.2. Results

6.2.1. Visible injury to the lung

72 hours post *S. aureus* instillation, rats were harvested and the lungs examined for injury. Figure 8 part i) shows a typical set of lungs from an injured animal, showing the visibly grey area typically associated with necrotic tissue. Figure 8 part ii) shows the same set of lungs after perfusion, demonstrating that the injured region of the lungs is still visible, even after perfusion. The injury was typically to the left lung lobe (green circle).

6.2.2. Protein analysis of bronchioalveolar lavaged fluid

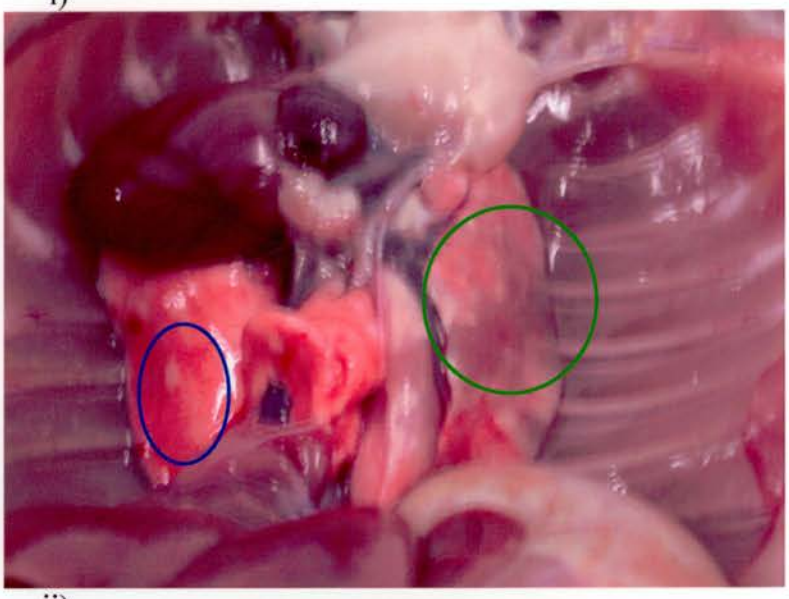
Figure 9 shows that a 1.6-fold increase (P=0.0026, F=0.2614) in protein in the BAL of *S. aureus*-treated lungs compared to PBS-treated lungs 72 hours after treatment. This is the same increase that was found by Clegg *et al* (2005). ELISA based dot blots for RTI_{40} (a proposed AETI cell marker) showed no change in free RTI_{40} in the BAL after treatment (data not shown), as was found by Clegg *et al* (2005).

6.2.3. Leukocyte levels in the bronchioalveolar lavaged fluid

BAL from *S. aureus*-treated lungs had a 2.6-fold increase (P=0.386, F=0.2676) in leukocytes compared to PBS-treated animals. This value is very similar to the 2.5-fold increase that was found by Clegg *et al* (2005).

The above information suggests that the injury obtained is similar to that of Clegg *et al* (2005). From this it can be predicted that if frozen sections of these lungs (not possible as they have been fixed in RNAlater) were studied, similar findings of increased numbers of AETII cells in long lines lining the alveoli and a decrease in the numbers of AETI cells would be observed.

i)



ii)

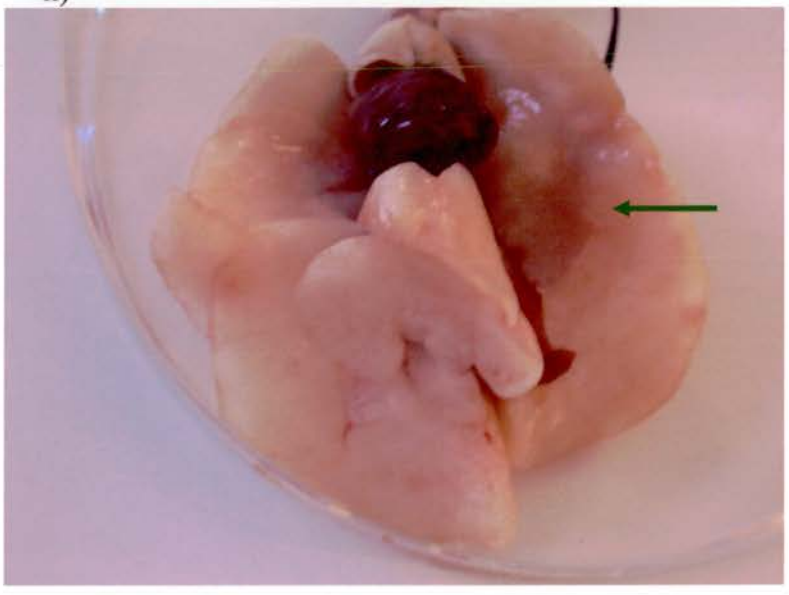


Figure 8: Lungs of a *S. aureus*-treated rat 72 hours after inoculation. i) Lungs before perfusion, with characteristic 'grey' area where lungs are damaged (green circle) in comparison to the healthy lung (blue circle). ii) The same set of lungs after perfusion showing that the injury is still visible after perfusion (green arrow).

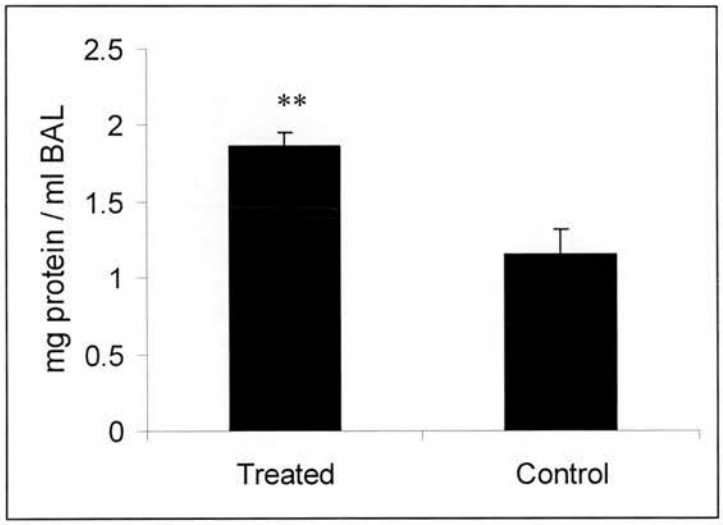


Figure 9: BAL fluid collected from rats 72 hours post instillation with *S. aureus* showed an increase in protein levels of 1.6-fold compared to PBS-treated rats. (P=0.0026, F=0.2414).

6.2.4. RNA extraction

Total RNA was extracted from approximately 100μg sections of the injured regions of the lungs. Control RNA was extracted from tissue samples obtained from the left lobe of the control lungs. This lobe was chosen as this was where *S. aureus* instillation typically caused an injury. Collected total RNA was tested for quantity and quality via measurements of the absorbencies at 260 and 280nm (table 10) and also by continuous measurements of the absorbance of the solution (figure 10). *S. aureus*-treated lungs all had more total RNA than the control PBS-treated lungs; this could be due to increased numbers of cells within the lung or cells within the lungs being more active.

Collected total RNA was used as a template for making copy DNA, which was used as a template for making biotin labelled copy RNA. This labelled RNA was added to Affymetrix U34 Chips as per the manufacturer's guidelines. The making of copy RNA and processing of the Affymetrix chips was carried out by Dr. P. Dickinson (Scottish GTI). Figure 11 shows a section from a typical injured and a control chip. Raw results for all 6 chips are enclosed on a compact disk at the back of this thesis.

6.2.5. General analysis of the data

Initially I used GeneSpring to analyse my data. Figure 12 shows a basic representation of all the genes, showing average control vs. average injured values (log scale). This shows that a) the data is approximately normally distributed (once in log scale) and b) that although the majority of genes do not change (i.e. are between the 2 fold green lines) a large number of genes do have a change in the level of RNA collected. A large proportion of the genes have a low expression (blue dots on figure 12), suggesting that these genes are just background noise and further analysis will have to be done with these removed.

Sample	Total RNA extracted (µg)	Ratio 260:280nm
Control 1	29.88	1.52
Control 2	21.2	1.47
Control 3	15.6	1.53
Treated 1	49.5	1.48
Treated 2	66.2	1.51
Treated 3	81.6	1.48

Table 10: Total RNA extracted from approximately 100µg of each set of lungs. Ratio of absorbencies at 260nm and 280nm is given as an indicator of the purity of each sample.

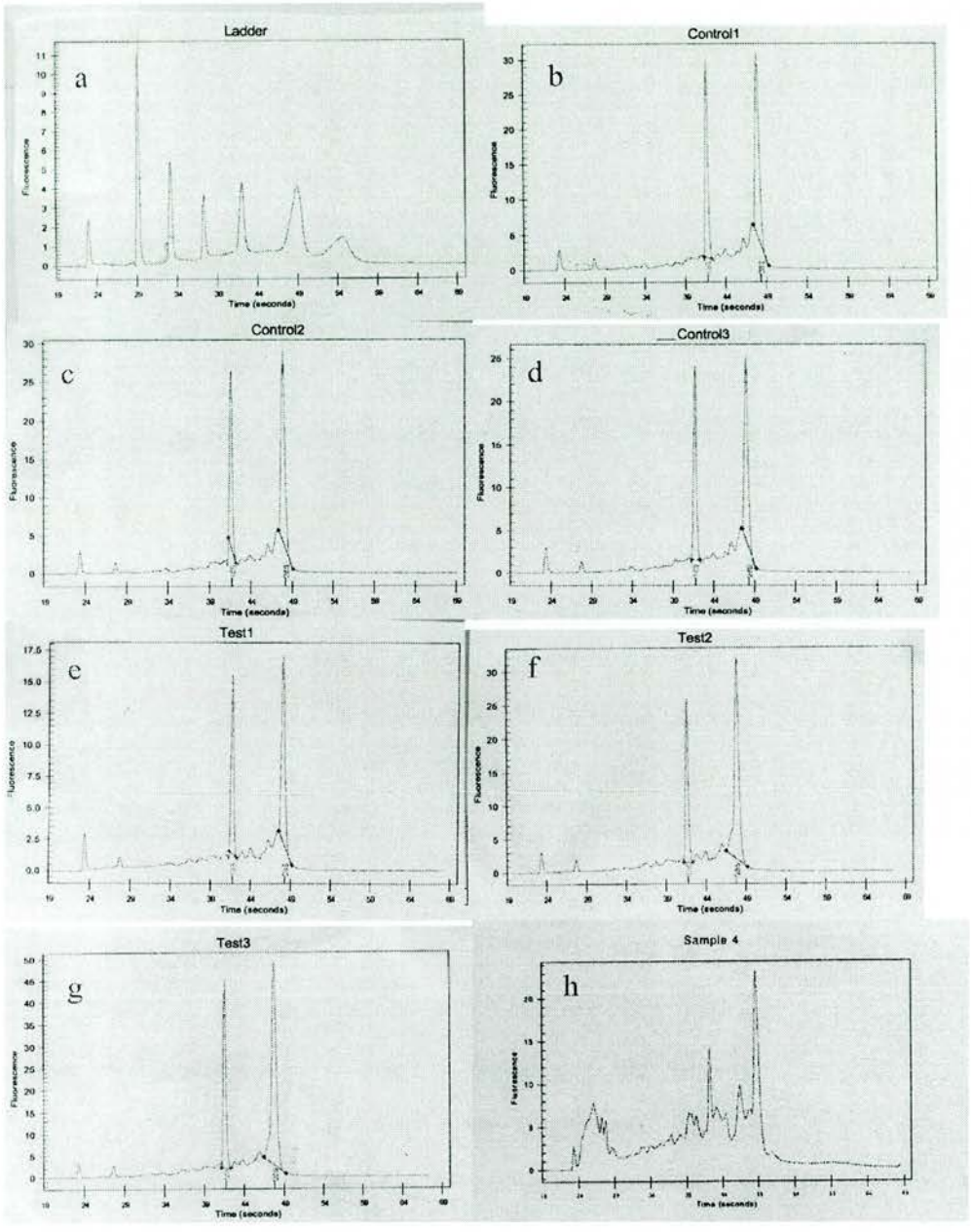


Figure 10: Spectra of RNA absorbencies found using the 2100 Bioanalyser. Machine was normalised using a protein ladder (a). RNA from PBS-treated lungs are shown on (b-d), (e-g) are RNA from *S. aureus*-treated lungs, (h) is an example of RNA where the preparation has gone wrong producing contamination of the sample. (Figure (h) is a kind donation from Dr. P. Lu)

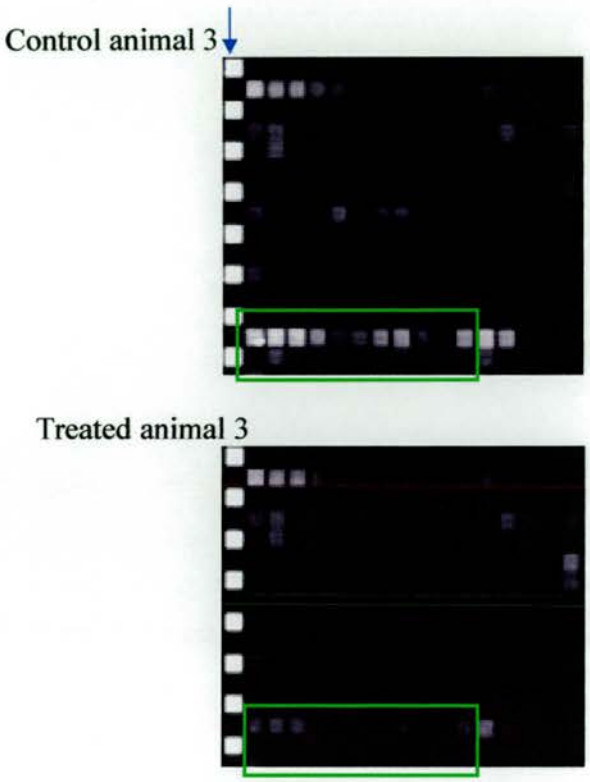


Figure 11: Comparison of the same area of the U34 Affymetrix gene chip which copy RNA from a control lung (top) and 72 hour post *S. aureus* instillation lung (bottom) have been hybridized. Note the control RNA hybridised to the side, which forms the grid for alignment (blue arrow). Green box surrounds a probe set for a gene that is decreased 72 hours after *S. aureus* injury.

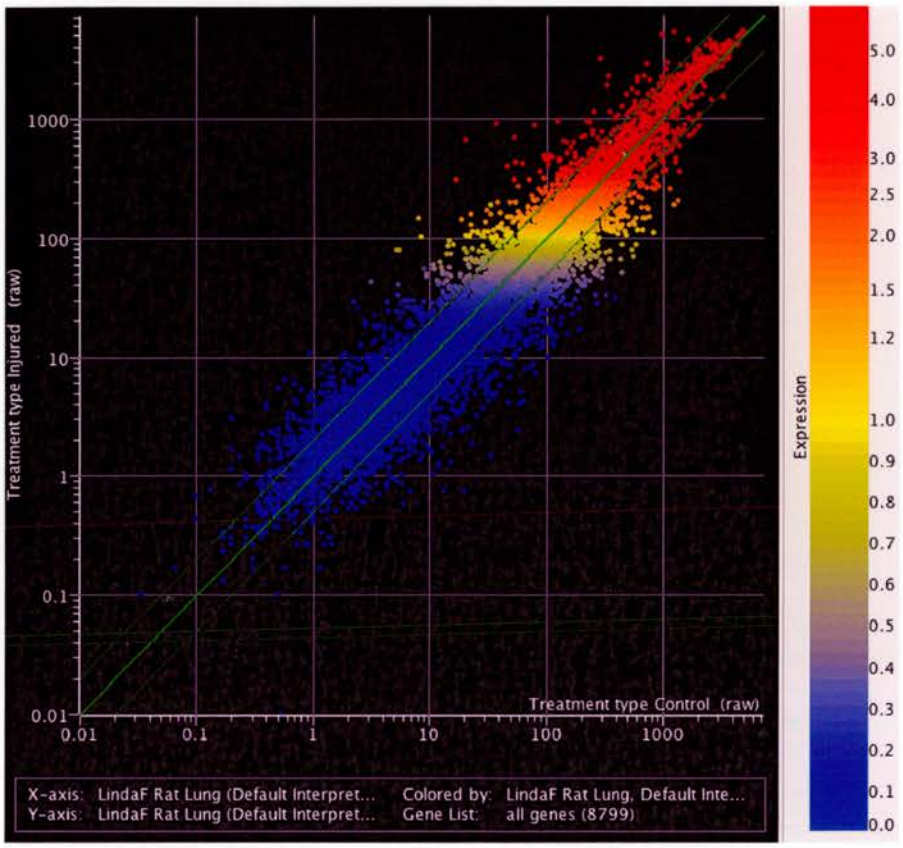


Figure 12: Gene Spring representation of all the genes on the Affymetrix U34 Chip. Graph shows that the majority of genes do not have large changes in the level of expression (between the 2 fine green lines). However, there are still many genes that do have greater than 2-fold changes in the levels of expression (above or below outer green lines). Graph also shows that a large proportion of the genes have low expression (coloured blue) which suggests that these genes are turned off.

For each chip, I found the mean and median reading, the standard deviation (of the mean) for each chip and the skew of each chip (Table 11). This was to ensure that all 6 chips had approximately the same overall results, so any differences found in one gene can be attributed to changes in that gene's expression, rather than being due to the chips having a higher or lower overall expression. In general, I found that there were no big differences between each chip. Therefore, any changes in a genes expression found between the two treatments can be attributed to a specific change in that genes expression, and not general variation between the chips.

Perceived 'housekeeping' genes were investigated, to see if these changed after the injury (Figure 13). Actin, Hexokinase and 5s RNA (except for one reading) remained the same, while GAPDH (another housekeeping gene) changed. This is not unusual in a model such as this (P. Dickinson, personal communication) as there is an influx of different cells into the lung during the course of the treatment and the cells present are also undergoing changes. GAPDH has also been shown to change in other injury models, which raises questions about its perceived use as housekeeping gene.

Overall 680 genes increased in expression 2-fold or greater (7.7% of total number of genes) and 1055 decreased 2-fold or greater (12% of total number of genes). Figure 13 shows that the number of genes that were significantly increased/decreased were 67 and 197 respectively (0.76% and 2.25 % respectively). Approximately a third of both the significantly increased and decreased genes found were for gene sequences that are for predicted/estimated genes based on the rat genome. As no known protein is ascribed to these sequences, it is impossible to find out what functions these potential genes have, therefore I have excluded them from further analysis of gene functions.

Treatment	PBS 1	PBS 2	PBS 3	*S. aureus* 1	*S. aureus* 2	*S. aureus* 3
Mean (rfu)	143.88	141.01	140.64	151.65	156.86	149.70
Standard deviation (rfu)	392.08	369.71	370.27	443.75	485.78	430.80
Median (rfu)	31.30	29.80	27.80	23.00	26.40	25.80
Skew	0.86	0.90	0.91	0.87	0.81	0.86

Table 11: Basic statistics on the individual Affymetrix chips. The skew shows that as predicted, the data is not normally distributed prior to logging of the data. However the amount of skew is approximately equal for each chip. This also shows that a large number of the genes are not turned on. The mean, median and standard deviation are showing that the individual chips are similar with regards to intensity of readings.

Treatment	PBS 1	PBS 2	PBS 3	*S. aureus* 1	*S. aureus* 2	*S. aureus* 3
GAPDH	483.76	498.55	511.31	822.35	1149.86	1194.83
Actin	2218.6	2047.05	2169.23	2004.26	2712.5	2359.18
Hexokinase	56.83	70.86	55.56	33.23	46.86	73.66
5s RNA	38.4	32.7	30.9	50.2	32.3	37.3

Table 12: Table of commonly used 'housekeeping' genes. Table shows that, depending on which gene you chose, either the chips are standardised for loading or they are not. Three of the housekeeping genes are approximately standardized for loading. GAPDH RNA levels increase approximately 2 fold 72 hours after *S. aureus* instillation. Other global gene analysis studies have also shown that levels of GAPDH change after different insults.

	Increase 2 fold	Decrease 2 fold	Remaining genes
Genes on chip	680	1055	7005
F>0.05	478 (343)	919 (618)	
F>0.05 P≤0.05	275 (199)	667 (460)	
F>0.05 P≤0.01	170 (127)	462 (316)	
F>0.05 P≤0.001	67 (49)	197 (125)	

Table 13: Gene changes, as found on the U34 Affymetrix Chips, within the lung 72 hours after *S. aureus* instillation. F values refer to the number of genes that have equal variance. P values refer to the number of genes with a probability less than 0.05,0.01 or 0.001 using a Student t-test, assuming 2 tail distributions and equal variance. As expected large numbers of genes were found to change, interestingly, due to the influx of leukocytes it was expected that there would be more genes increasing in expression than decreasing, when the opposite was found here.

6.2.6. Changes in known alveolar epithelial type II cell markers.

Proteins that are known to be found on Type II alveolar epithelial cells (I.E. table 2) were searched for on the Affymetrix chip. Table 14 shows all the genes that were present on the chip, with their corresponding changes after treatment. Firstly, not many of the genes were on the chip; however, of those that were, all bar three had less than a two-fold increase in mRNA expression. In fact some seemed to decrease in expression, possibly indicating that, although there are more type II cell-like cells within the repairing alveoli, these cells could possibly not be acting as 'normal' type II cells.

The three genes that did have greater than two-fold increase in mRNA were C3, MMP-7 and manganese superoxide dismutase, all of which are also found to be expressed by macrophages, which, at this time point after injury, are already known to be in the lungs in greater quantity than in uninjured lungs, suggesting that this could be a possible reason for these increases.

Surfactant proteins A and D were found to increase slightly, suggesting that there could be an increase in type II cells. However, alkaline phosphatase (an enzyme found in the lamellar bodies of type II cells (Edelson, 1988)) remained constant. Four out of five β-adrenergic receptors (which aid surfactant secretion (Fabisiak, 1987, Ewing, 1992)) actually decreased in expression, which would again suggest that the type II-like cells are not functioning as 'normal' type II cells.

Due to the amount of evidence suggesting that the type II like cells are not expressing the full range of type II cell mRNA (and therefore proteins) and because the fold changes of the known type II cell genes that did increase did not do so by much, I decided to not use this model to identify novel type II cell associated proteins.

				PBS		*S. aureus*	
Protein	**Fold increase**	**F=**	**P=**	**Mean**	**StDev**	**Mean**	**StDev**
SP-A	1.24	0.24	0.03	3256.17	141.39	4045.00	384.42
SP-D	1.71	0.21	0.02	1618.83	180.51	2765.17	524.58
Cathepsin H	1.83	0.01	0.00	844.57	3.55	1543.97	52.45
B-adrenergic receptor	1.48	0.87	0.02	83.20	11.75	123.07	13.38
B-adrenergic receptor	0.93	0.41	0.91	16.17	14.32	15.00	7.25
B-adrenergic receptor	0.37	0.16	0.04	130.77	46.62	48.17	13.86
B-adrenergic receptor	0.18	0.44	0.00	54.03	7.92	9.83	4.20
B-adrenergic receptor	0.37	0.05	0.03	118.33	39.81	43.97	6.23
annexin II	1.28	0.63	0.12	1211.63	167.90	1550.00	248.71
Alkaline phosphatase	0.88	0.29	0.11	301.30	11.53	265.37	28.23
Alkaline phosphatase	1.02	0.53	0.80	2294.60	188.20	2351.90	314.02
APN	1.07	0.08	0.86	151.17	19.73	161.73	94.48
APN	1.48	0.95	0.48	107.20	82.99	158.33	79.26
AQP-1	0.94	0.58	0.91	14.03	6.37	13.17	10.01
C3	3.56	0.01	0.00	161.27	3.76	573.47	45.84
C4	1.00	0.79	1.00	839.97	611.20	839.67	755.84
MMP-7	2.13	0.26	0.23	10.53	5.33	22.47	13.76
TIMP-2	1.65	0.03	0.17	766.27	512.57	1264.40	66.56
TIMP-3	0.19	0.22	0.01	602.53	187.00	116.33	66.16
Mn Superoxide dismutase	2.11	0.05	0.04	61.93	6.33	130.57	37.82

Table 14: Fold increase in mRNA for proteins that are known to be associated with AETII cells. Other proteins known to be associated with AETII cells that are not listed here are either a) not on the chip or b) general cell proteins (such as cyclins, and housekeeping genes)

6.2.7. Changes in known AETI cell specific markers

Proteins known to be found on AETI cells (described previously in table 1) were searched for on the gene chip (table 15). As with AETII cell associated genes, not all known AETI cell associated genes were on the U34 Affymetrix® chip. The one protein known to be specific for AETI cells within the lung, RTI_{40}, is on the chip however. This gene, as expected, did have a reduction in the levels of expressed mRNA, but the decrease was only of 1.4-fold, and the result was not significant. This brings into question the use of this method to assess the number of the cells within the lung. Caveolin, AQP-5, RAGE and eotaxin did however all decrease by over 2-fold (eotaxin and caveolin decreased greater than 5-fold). Ideally, as RTI_{40} decreased by 1.4-fold, I would carry on searching for novel proteins associated with AETI cells by using a decrease in mRNA expression of 1.4-fold or greater. However, using this thresh hold produced a vast number of proteins to be investigated. I therefore decided to look at proteins with a mRNA expression decrease of 2-fold or greater, as these would also give me proteins where the decrease in expression was more likely to be detected via immunohistochemistry and also gave me a more manageable number of proteins to investigate.

6.2.8. Potential AETI cell markers

Genes that could potentially be AETI cell markers were then searched for from the entire U34 Affymetrix gene chip results using the following criteria:

1) The level of expression in the normal lung was over 500 units of absorbency (i.e. the gene was turned on in normal conditions)
2) The level of expression decreased by 2-fold or greater in animals 72 hours after the *S. aureus* insult
3) The decrease in the expression was significant using Student t-test, after checking that the standard deviations were similar (F-test).

Treatment				PBS		*S. aureus*	
	fold decrease	**F=**	**P=**	**mean**	**Stdev**	**mean**	**Stdev**
RTI40 precursor	1.40	0.946	0.09	700.57	110.62	501.17	104.84
Caveolin	6.54	0.086	0.00	1224.90	159.93	187.20	34.01
Na,K-ATPase a2	0.48	0.377	0.06	12.47	3.96	26.17	8.25
Na,K-ATPase a2	0.23	0.373	0.17	0.60	0.10	2.57	2.06
Na,K-ATPase a2	0.45	0.227	0.06	29.07	3.97	65.07	23.82
Aquaporin 5	2.11	0.08	0.01	688.00	137.66	326.23	29.63
ICAM-1	1.73	0.65	0.01	357.87	51.52	206.73	35.78
ICAM-1	1.62	0.231	0.00	1231.30	70.57	758.97	25.52
connexin 43	0.80	0.564	0.52	4.70	1.57	5.90	2.51
p-glycoprotein	0.25	0.256	0.09	5.90	5.02	23.67	13.10
RAGE	2.23	0.84	0.00	3317.87	325.97	1490.73	68.52
PAI-1	0.18	0.145	0.00	31.10	5.35	169.23	19.08
eotaxin	5.21	0.569	0.01	30.07	7.23	5.77	4.56
GGT	0.22	0.081	0.00	12.73	2.70	56.67	13.13
osteonectin	1.72	0.237	0.02	1515.77	269.04	880.17	98.65

Table 15: Known AETI cell associated proteins decreased in the lung 72 hours after *S. aureus* treatment. Other AETI associated proteins not on this list were not on the Affymetrix U34 chip.

Using this selection criteria, 89 genes were selected for further investigation (appendix ii). All these genes were then researched by hand to find main functions of the protein product, which cells were known to produce the protein and where on the cell the protein was commonly located (appendix ii).

A large proportion of the genes were found to be linked to the endothelium (endothelial cells, smooth muscle cells), suggesting that this model of *S. aureus*-induced pneumonia also damages the endothelium. (However, bacteria were not found in the blood of any of the animals, suggesting that the lung air-blood barrier remains reasonably intact).

6.2.9. CD9

Interestingly, CD9, known to be found on macrophages, giant cells, bronchiolar epithelial smooth muscle cells and found in the alveoli (cell type not specified) decreased (2.41-fold, p=0.00002, F=0.79). As there was a 2.6-fold increase in the number of leukocytes (most of which being macrophages, (data not shown)) found in the BAL from lungs at this time point, I would have expected the levels of CD9 within the lung to increase. I therefore decided to investigate where in the alveoli CD9 is expressed to see if this could explain the decrease in CD9 mRNA expression. Frozen sections of normal lung were stained with an anti-CD9 antibody, MMC4 (a potential AETII cell biomarker) and RTI_{40} (a potential AETI cell biomarker). Figure 13 shows that the anti-CD9 antibody is co-localising with the RTI_{40} antibody and not the MMC4 antibody, suggesting that CD9 is expressed by AETI cells and not AETII cells.

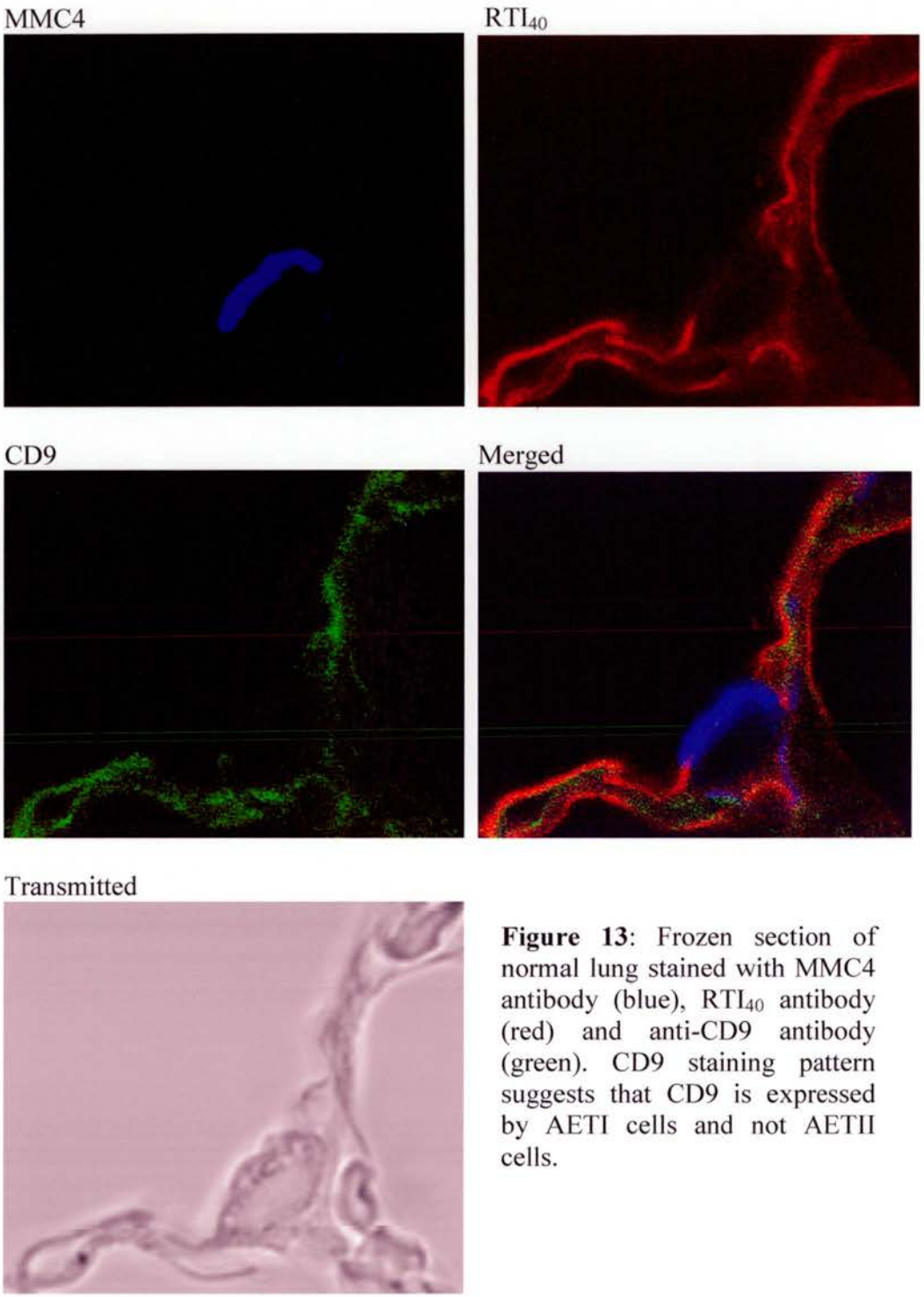


Figure 13: Frozen section of normal lung stained with MMC4 antibody (blue), RTI_{40} antibody (red) and anti-CD9 antibody (green). CD9 staining pattern suggests that CD9 is expressed by AETI cells and not AETII cells.

6.3. Discussion

The animals that I treated with *S. aureus* bacteria 72 hours post instillation did have similar biochemical analyses to those done previously by Clegg *et al* (2005). This suggested that I had indeed caused a similar pneumonia injury within the rats via the instillation of *S. aureus* as had been previously characterised. The Affymetrix analysis of the RNA from the injured lungs did, as expected, show a large number of genes to be either up and down regulated after the injury. Despite the increase in AETII cell-like cells found within the lung 72 hours post inoculation via electron microscopy and immunohistochemistry (Clegg, 2005), a corresponding increase in mRNA from AETII cell associated genes was not found. This further supports the hypothesis proposed by G Clegg (2005) that the AETII cell-like cells within the lung at this time point are not functioning as conventional AETII cells. However, the lack of change could also be due to the proteins having a auto-regulatory loop with themselves to prevent levels of expressed protein from increasing dramatically, an example of this is surfactant protein-A. Although auto-regulation could explain why some genes did not have as high mRNA expression as expected, the number of genes that remained constant suggests to me that it is more likely that the AETII-like cells within the lung at this point are not all functioning like AETII cells would be in the normal lung.

Within the group of genes that decreased after *S. aureus* instillation there were a large number of genes that are associated with the endothelium (31 out of 89). This was unexpected as all previous studies of this model of *S. aureus* induced pneumonia have shown no injury to the endothelium (as shown by lack of blood serum proteins in the BAL and the fact that no bacteria were found in the blood). This suggests that either there is minor damage to the endothelium that the body is able to compensate for or that, although the endothelium is intact, that these cells have also changed their gene expression profile due to the *S. aureus* injury. Further investigation into the extent and type of damage to endothelial cells caused by the *S. aureus* induced pneumonia is therefore required to fully understand these results.

Within the down regulated group of genes there were also some genes associated with AETI cells, suggesting that this data set could be used to study AETI associate proteins. However, there were also some proteins that were found to decrease that were not expected to. CD9 is known to be associated with most cell types including macrophages which express CD9 at high levels. As there was an increase in macrophages within the lungs at 72 hours post-instillation it was surprising to find this protein decreasing in my data set. Due to this, we investigated the protein further. CD9 has been found to be on the alveolar surface, but cell specificity for either AETI or AETII cells has not been shown before. Immunohistochemical analysis of frozen lung sections showed that an anti-CD9 antibody co-localises with RTI_{40} antibody but not MMC4 antibody in the normal lung. This suggests that CD9 is expressed on AETI cells but not AETII cells.

CD9 is a tetraspanin protein (Takeda, 2003) known to have roles in reducing cell proliferation by binding HB-EGF (a potent soluble mitogen which affects epithelial cells, fibroblasts and vascular smooth muscle cells) (Makamura, 2000), reducing cell motility by binding to β1 intergrins (Funakoshi, 2003), affects cell activation, proliferation, fusion of gametes and apoptosis (Takeda, 2003). CD9 also has an effect of cell signalling via interactions with PKC and PI4K (Yunta, 2002). Based on this information CD9 expression by AETI cells is not un-expected as these cells are known to not be motile, terminally differentiated and non-proliferative, all characteristics that CD9 aids within the cell. However the lack of CD9 on AETII cells does seem odd based on the wide spread distribution of the protein. The sections that I stained with the CD9 antibody were from control normal lung were there is not a large turn over of cells. As the AETII cells are not therefore proliferating or moving as much as they do after injury I would have therefore expected these cells to have also been producing CD9. One explanation for this however could be linked to how AETII cells react in the absence of AETI cells. *In vitro* freshly isolated AETII cells, over a period of days, move and differentiate into AETI-like cells. *In vivo* the lack of neighbouring AETI cells also cause AETII cells to start to proliferate, become more motile and differentiate to AETI cells. From this it appears that AETII cells only retain their morphology when neighboured by

AETI cells. This suggests that AETI cells produce a molecule that prevents these characteristics in AETII cells. CD9, with its ability to bind HB-EGF explains one of these methods. It would be interesting to see if CD9 could cause a decrease in differentiation and motility of AETII cells in culture.

Despite CD9 being found on macrophages, making it unsuitable for use as a marker of AETI cells in BAL from the lung, the specificity of CD9 for AETI cells and not AETII cells that was found does show that, at least in principle, this method for looking for novel markers of AETI cells does work. By looking at the proteins that were found to be down regulated, but are not known to be in the lung, vascular or nervous system it may be possible to find other proteins that are currently not known to be associated with AETI cells. Even if, as in the case of CD9, they prove not to be specific for AETI cells within the lung, due to the lack of knowledge about proteins that are found associated with AETI cells', and therefore the cells' functions, any further proteins found to be associated with AETI cells will increase the knowledge about this cell population.

Global gene analyses in models of injury are very hard to analyse due to the large number of different genes that change within the organism. Here we used an animal model in the hope of reducing the number of genes that would change as the animals would all be in a controlled environment, eating the same food and be given identical medication, drugs etc. However, even with these precautions there were still a large number of genes that changed. Many existing packages for sorting data from gene chips rely on grouping data based on known function/cell type. While these methods may be useful in interpreting data where the disease is being investigated, when the investigation is instead into the genes found to be produced by the different cells types many of these packages fall down. This is due to the packages assigning the protein to a cell type regardless of the RNA having an increase or decrease in expression. This meant that if a gene was known to be expressed on one cell type, for example macrophages, then the gene was assigned to that cell type, regardless of the fact that the gene expression has decreased, while the number of these cells has increased. I found

using these databases both time consuming and ultimately a waste of time. This is why I proposed and eventually used a simpler technique, even though it did in the long-run prove to be more time consuming to search the literature by hand.

7. Purification of a rat TII and Clara cell antigen

3.1 Introduction

MMC4, a monoclonal antibody (isotype IgG2a), recognises alveolar epithelial type II and Clara cells immunohistochemically within the lung (Boylan, 2001). It has therefore been proposed as a potential marker of injury to these cell types (Boylan, 2001). Despite proposing the MMC4 monoclonal antibody as a marker of these cell types, the actual antigen for the antibody is unknown.

The objectives of this study were therefore:

a) to identify the antigen of the MMC4 monoclonal antibody

b) to assess the usefulness of MMC4 monoclonal antibody as a marker of AETII and Clara cell injury

MMC4 monoclonal antibody was raised via 2 injections of an enriched AETII cell preparation from SD rats into female BALB/c mice. The resulting sera were screened for reactivity to rat AETII cells on frozen sections of the lung. Hybridomas were then re-cloned three times to ensure a single clone was in the preparation. Reactivity to the MMC4 monoclonal antibody was also found on proximal tubule epithelial cells of the kidney, on villus epithelial cells of the intestine (Boylan, 2001) and in the thymus (cell type undetermined (Boylan, thesis)). Within the lung, the MMC4 monoclonal antibody binds to the apical surface of both AETII cells and Clara cells (Clegg, 2005, Boylan, 2001) via immunohistochemistry. The MMC4 antigen is an integral membrane protein as shown by Triton X-114 phase separation and the ability of Proteinase K to obliterate antibody binding to lung tissue (Boylan, 2001). Boylan *et al* were unable to purify the MMC4 antigen via Western blotting, and they showed that this was due to the SDS in the gel buffers, as SDS was shown to reduce MMC4 monoclonal antibody binding to lung homogenate via ELISA based dot blot (Boylan, thesis).

Clegg *et al*(2005) recently showed that the MMC4 antigen is expressed on freshly isolated type II cells *in vitro* but over time the expression is lost as the type II cells trans-differentiate into type I-like cells. However, MMC4 monoclonal antibody binding is retained after some other type II cell markers ($RTII_{70}$) are no longer recognising the cells, and after some type I cell markers are expressed (RTI_{40}). Due to the binding pattern of the MMC4 monoclonal antibody (with regards to organ distribution and also within the cell), it is thought that MMC4 monoclonal antibody recognises a novel protein (Boylan, 2001).

7.1.1. Investigation method.

Validating the MMC4 antibody as a biomarker required investigations into two different areas of the antibody:

1) How useful is the MMC4 monoclonal antibody as a marker in an injury model? (Including is an invasive technique required to obtain a measurable sample?)
2) What is the MMC4 antigen?

To investigate the usefulness of MMC4 antibody as a marker it was decided to look at MMC4 levels in the BAL fluid as well as in the lung. To cause an injury to the lungs it was decided to use two different strains of *Staphylococcus aureus*, 8325-4 and 8325-4 PLs. As previously described (see 6.1.1) *S. aureus* was known to cause an injury to the alveolar epithelium 24 hours post instillation, typically a localised denuding of the basement membrane of AETI and AETII cells. An *in vitro* study into different fibronectin binding proteins of *S. aureus* showed that strains containing the PLs vector were less likely to be phagocytosed than those that did not have this vector (Franklin, thesis). As the *S. aureus* 8325-4 PLs bacteria would therefore survive longer within the lung it was predicted that there would be greater damage to the epithelium with this strain than the 8234-5 strain. It was therefore decided to see if the MMC4 monoclonal antibody could firstly recognise a change in its antigen in the BAL fluid and secondly if

the antibody was sensitive to recognise a difference in the levels of MMC4 antigen within the BAL between the two treatments.

As Boylan had already been unable to purify the MMC4 antigen via western blot (due to SDS denaturing the protein), it was decided to try to purify the antigen without the addition of any reducing agent. Dynabeads® were suggested as a method of separating the antigen from other proteins of the cell. Dynabeads® are antibody coated beads, which hold no charge. Antibodies of choice can be attached to the beads and then they can be used to bind proteins. Once a magnet is placed near the tube containing the beads the beads become charged and move towards the magnet, enabling any liquid in the tube to be removed. Attached proteins and antibodies can be removed from the Dynabeads® via heating in the presence of SDS-PAGE sample buffer.

Once the protein has been purified and separated from the antibody via SDS-PAGE, I planned to analyse the protein via MALDI-TOF mass spectrometry (MS) analysis.

7.1.1.1. MALDI-TOF Mass Spectrometry

MALDI-TOF stands for Matrix-Assisted Laser Desorption/Ionization-Time of Flight. Figure 14 shows a general diagram of how MALDI-TOF MS machines work. Sample proteins to be tested are first broken down into smaller peptides via enzymatic digestion (usually with trypsin). The peptides are then co-crystallised onto a surface with a compound of high molecular weight (normally an organic acid which weakly absorbs UV rays) on a cassette. The cassette is loaded into the MALDI-TOF machine and a UV laser is then used to vaporise the matrix and therefore the peptides of interest as well. At the same time the matrix compound becomes an ion donor/acceptor allowing the peptides to become charged. The vaporised peptides then travel down a tube to a plate reader. The time it takes for a peptide to travel to the reader is dependent on the size and charge of the peptide. As proteins when broken down by trypsin digestion will form peptides of various lengths and number the resulting MALDI-TOF spectra obtained

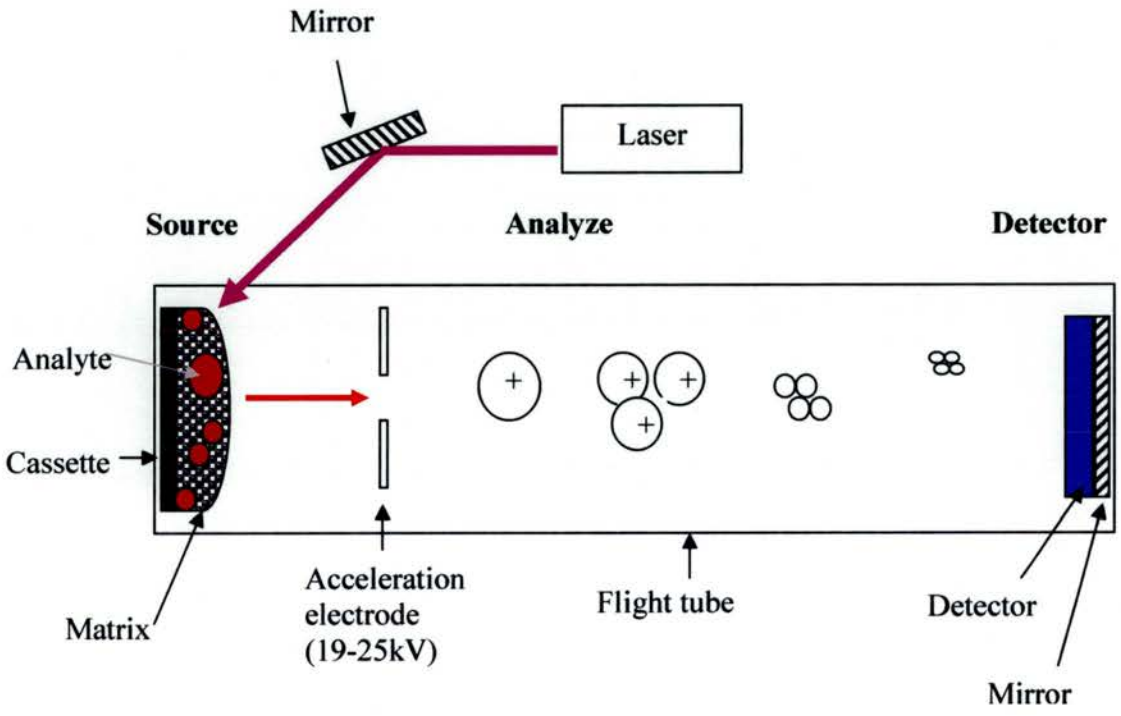


Figure 14: Principle of the MALDI-TOF mass spectrometer. Peptides (analyte) are mixed with a matrix medium and dotted onto a cassette. UV irradiation ionises the matrix, causing the peptides to become charged. Charged peptides move down the fight tube at different speeds based mainly on weight, but also charge. (Figure adapted from Marvin, 2003)

should be unique for the protein in question. Comparison of the peaks obtained from the spectra can then be compared to other known protein's spectra (through packages such as SwissProt-TrEMBL, see 7.1.1.2 for how these packages work) to discover what the protein in question is.

7.1.1.2. MOWSE scores

Databases such as SwissProt-TrEMBL cannot determine exactly what a given protein is, however they can give a best guess based on the fingerprint caused by the peptides found during the MALDI-TOF MS. As knowledge of the probability of a 'hit' occurring is useful in determining if the result is believable or a result of many contaminating proteins a probability score called MOWSE scores are used (Pappin, 1993). MOlecular Weight SEarch fragment database scores (MOWSE scores for short) are produced by a complex algorithm.

To obtain a MOWSE score, the molecular weight of individual fragments after digestion are required as well as the full molecular weight of the protein being examined. If the researcher knows the molecular weight of the protein being examined then proteins are excluded from the search if the database protein weight is more than the specified molecular weight plus R or if the database protein is less than the specified molecular weight minus R. R is the specified molecular weight multiplied by the molecular weight filter percentage (which the researcher specifies) divided by 100. This system allows for error in the molecular weight found from the actual molecular weight of the amino acid backbone, for example if the protein is glycosylated.

The MOWSE database therefore has a list of possible proteins based on the protein's overall weight. The database then compares fragments of these proteins one by one to the fragments found by the MALDI-TOF MS analysis. If a fragment is found to be of

similar length to the expected fragment then a peptide score value is assigned to the fragment. Fragments that are not found in a protein are considered neutral and not assigned a peptide score value. Peptide score values are assigned based on the probability of a peptide fragment of that length being found for a protein of that molecular weight (all values are between 0 and 1). When more than one fragment is identified for a protein (such as in MALDI-TOF MS analysis) individual peptide score values are multiplied together. Finally to allow for skew due to large proteins having many fragments while small proteins do not, the product of the individual peptide scores (Ps) is multiplied by the molecular weight of the found protein (Fmw), then dividing 50,000 by this value (I.E. MOWSE score = 50,000/(Ps x Kmw)).

This scoring system means that greater weight is given to peptides of unusual weight (normally the heavier peptides) as they have low initial peptide score values (Pappin, 1993). If however a protein does fragment into many small peptides this technique will give a possible peptides back, but the MOWSE scores will be lower. In this case looking at the comparible difference between the top MOWSE score and the next most likely 'hit' gives a better idea as to the probability of the top 'hit' being correct. Overall, the larger the MOWSE score, the more probable that the protein being proposed by the database is the same as the protein that you are trying to identify.

7.1.1.3. Tandem mass spectrometry

Tandem mass spectrometry (also called MS-MS spectrometry) covers a wide variety of different techniques. Basically one type of mass spectrometry is linked to a second type to give more detailed results than using one method alone would. Figure 15 shows how the method used in this thesis works. In brief, trypsin digested peptide fragments of the protein to be identified entered the initial mass spectrometry chamber via nanospray and then hit by a lazar to cause the particles to become charged. These pass through magnets which cause a deviation in the particle based on charge. As with the MALDI-TOF mass

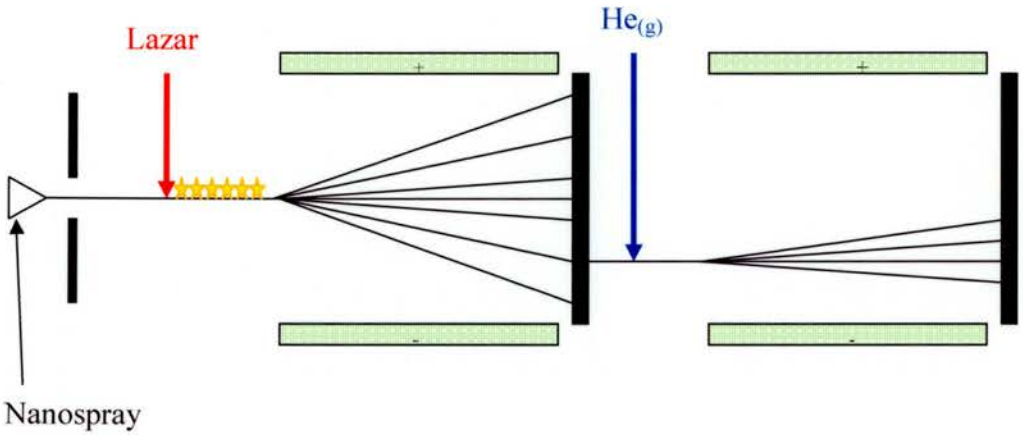


Figure 15: Overview of how the tandem mass spectrometry machine used works. The lazar excites the incoming peptides, causing them to become charged. The charged peptides move in different directions due to the magnets. Selected peptides move into the second chamber where they become further charged due to bombardment with helium gas. The change in charge is then recorded by a plate reader after, again, passing though magnets.

spectrometer the particles also move at different speed based on size. The peptides reach the first plate reader over a period of time. Every second the computer takes a reading of the mass, charge and intensity of peptides hitting this plate reader. The three peptides with the highest intensity are filtered through to the next chamber IF they have a charge greater then one (I.E. -1, 0 and +1 are not allowed through). These fragments then are further bombarded with helium gas which causes disassociation of the peptides along the N-C bonds, further charging the peptides. After passing through magnets again the new charge to mass ration is measured. As with the MALDI-TOF data, the data from the tandem mass spectrometer is analysed via SwissProt-TrEMBL and MOWSE score are assigned based on the likelihood of the protein being investigated being the known protein.

7.2 Results

7.2.1 MMC4 as a marker of AETII cell injury

SD rats were instilled with either *S. aureus* strain 8325-4, *S. aureus* strain 8325-4 PLs (both suspended in PBS) or just PBS. 24 hours after instillation rats were euthanized and BAL fluid and lungs were removed for protein analysis. Table 16 shows the average inoculums used, which are approximately equal for the two *S. aureus* treatments, and the total protein, colony forming units and leukocytes recovered in the BAL fluid. There was no significant difference found between all three treatments with regards to protein levels recovered. This was slightly unexpected as a previous study by McElroy *et al* showed that at inoculums 6×10^8 and 4×10^9 there was a 3.5 fold and 12 fold increase in protein levels respectively (McElroy, 1999). The levels of inoculation used were slightly less than the 6×10^8 used by McElroy *et al* however, similar results were expected at this inoculation level.

As expected most of the 8325-4 strain of *S. aureus* infection had been removed 24 hours post-instillation. Interestingly there was no significant difference in the levels of colony forming units of 8325-4 and 8325-4 PLs strains recovered from the lungs; this suggests that despite the *in vitro* differences in phagocytosis, there is no difference between these two strains *in vivo* with regards to bacterial clearance times. Despite this there was almost 3 fold more leukocytes recovered from the lungs of rats treated with 8325-4 PLs *S. aureus* compared to rats treated with the 8325-4 strain, indicating that the host response was larger in these animals.

ELISA based dot blots were used to analyse the levels of MMC4 and RTI_{40} reactivity within the BAL fluid and lung homogenate. MMC4 and RTI_{40} levels did not change significantly within the lung homogenate, however levels did change in the BAL (figure 16). In both cases levels of reactivity did not change significantly between PBS treated and 8325-4 *S. aureus* treated animals. Within the BAL samples collected, MMC4

Instilled	Innoculum size (CFU)	n	Protein in BAL (mg)	remaining CFU in BAL	leukocytes in BAL
PBS	0.00E+00	3	2.217 ± 0.907	0	1208000
8325-4	4.84E+08	4	2.904 ± 0.653	1595	9390000
8325-4 PLs	5.32E+08	5	3.061 ± 1.095	2217.5	29210000

Table 16: Levels of bacteria instilled into rats and levels of protein, colony forming units (CFU) and leukocytes recovered in the BAL. Levels of protein recovered increased after both *S. aureus* 8325-4 and 8325-4 PLS treatments compared to the PBS control . There was however no significant difference with regards to the levels of protein or remaining cfu found between the two *S. aureus* strains. There was however an almost 3 fold increase in the number of leukocytes found in the BAL of the 8325-4 PLs infected animals compared to the 8325-4 infected animals.

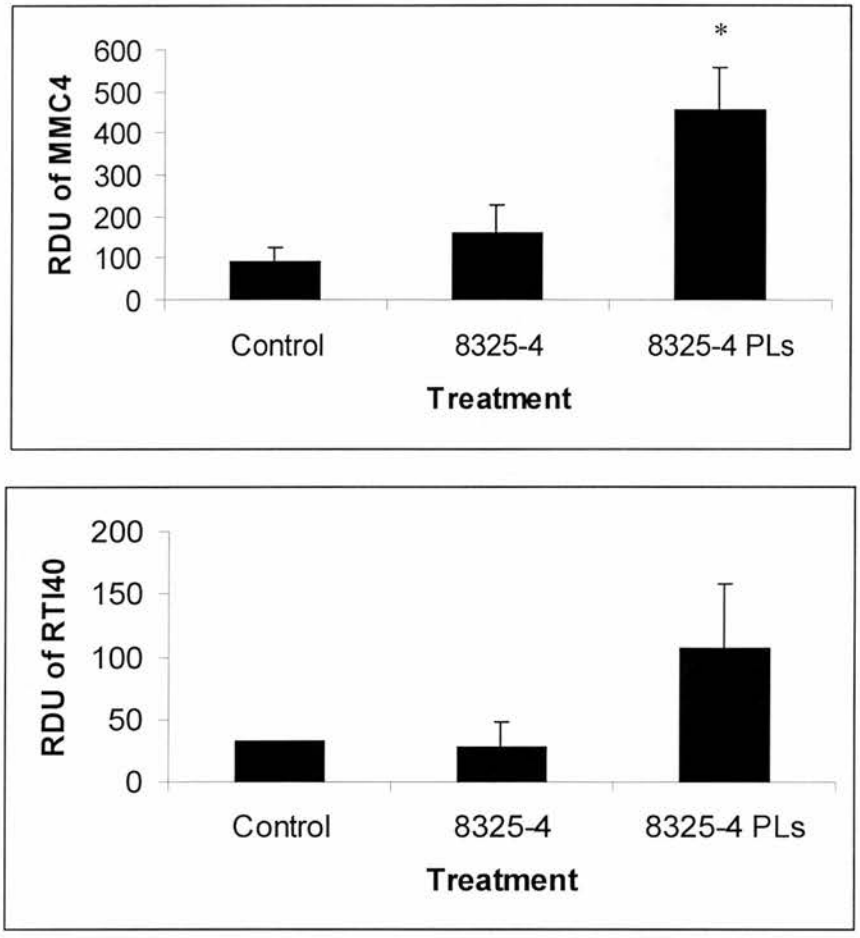


Figure 16: Relative densitometry units (RDU) of MMC4 and RTI_{40} recovered from the BAL via ELISA based dot blot 24 hours after inoculation of *S. aureus* strains 8325-4 and 8325-4 PLs

reactivity increased 4.9 fold (P=0.0012) after *S. aureus* 8325-4 PLs treatment compared to PBS alone. RTI_{40} reactivity also increased in the BAL (3.3 fold), however this result was not significant (P=0.0537). This suggests that AETII cells are more damaged by S. aureus induced pneumonia than AETI cells. This was unexpected as AETI cells are known to be more venerable to damage than AETII cells in general lung injury.

This therefore shows that MMC4 reactivity is detectible in the BAL. It is also possible to detect changes in the MMC4 reactivity levels after *S. aureus* injury suggesting MMC4 could be used in a non-invasive manor to measure changes in AETII and Clara cell numbers within the lung.

7.2.2. Relative amounts of MMC4 per mg protein

The proportion of the protein that is the MMC4 antigen in each organ was measured as optical densitometry units (ODU, n=4 with samples run in duplicate) per milligram protein ($n \geq 7$, depending on organ) (Figure 17).

The kidney has the highest MMC4 reactivity per milligram protein. This was unexpected as the antibody was raised against alveolar epithelial type II cells from the lung and, therefore, it was expected that the lung would have the highest antibody reactivity. This result does however confirm G. Boylan's earlier finding (Boylan, 2001). MMC4 antigen was also found in the intestine, thymus and placenta. ELISA based dot blot shows the liver to have no MMC4 antigen.

7.2.3 Optimisation of $C_{12}E_8$ solubilization

As the MMC4 antigen was known to be membrane associated (Boylan, 2001) the cellular membranes needed to be solubilized prior to the MMC4 antigen being purified. It had previously been shown that $C_{12}E_8$ solubilized cell membranes whilst conserving the MMC4 antibody's reactivity (Boylan, thesis). Intestine, kidney, lung and thymus

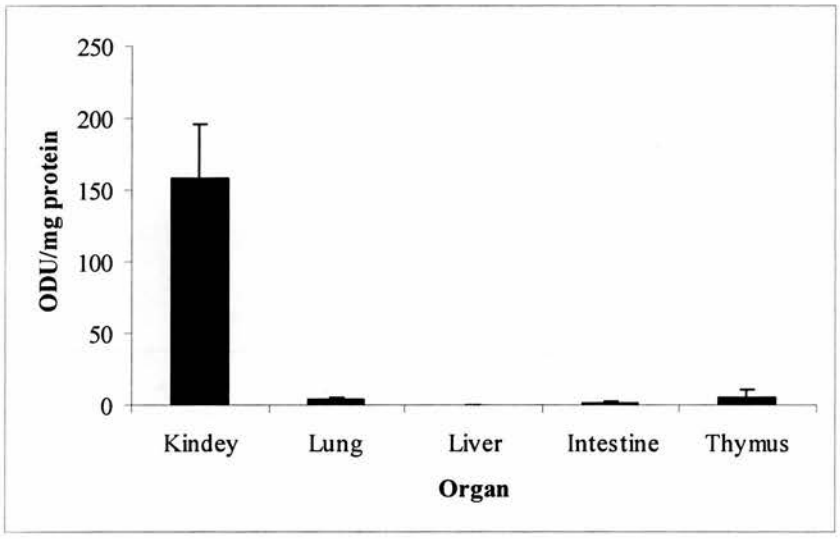


Figure 17: Relative binding of MMC4 Monoclonal antibody to homogenates of various tissues. Data expressed as MMC4 binding (represented as Optical densitometry units (ODU)) per mg protein.

were solubilized in various ratios of protein to $C_{12}E_8$ (mg protein to mg $C_{12}E_8$) overnight to find the optimum ratio of protein to detergent for solubilization. Insoluble cell contents were removed from the samples via ultra-centrifugation. The soluble supernatants were then tested for MMC4 reactivity via an ELISA based dot blot (Figure 18).

In the thymus (Figure 18c) there appears to be some soluble MMC4 antigen before solubilization with $C_{12}E_8$, as the negative control (supernatant from the 500,000g spin, where no $C_{12}E_8$ had been added) shows 16% of the MMC4 reactivity of the positive control, suggesting that although the majority of the MMC4 antigen is membrane bound there is a small proportion that can be found free of membranes.

Overall, it was found that a ratio of 1 mg protein: 5 mg $C_{12}E_8$ had the maximum recovery of MMC4 after solubilization for the intestine, kidney, lung and thymus. As the liver showed no MMC4 activity before solubilization (figure 17) it was not possible to find which ratio of $C_{12}E_8$ solubilized this organ best. However, as all other organs were being solubilized with a ratio of 1:5 (mg protein: mg $C_{12}E_8$) it was decided to use this ratio for the liver as well.

7.2.4 Immuno-precipitation of the MMC4 protein from all organs.

Dynabeads® pre-coated with MMC4 monoclonal antibody were used to select the MMC4 antigen from $C_{12}E_8$ solubilized homogenates of the intestine, kidney, liver, lung and thymus. Precipitated antigen was removed from the Dynabeads® via heating in SDS-PAGE sample buffer, which was then run on 6 or 8% acrylamide gels. Controls of Dynabeads® with a non-specific IgG2a and no secondary antibody were also incubated with kidney $C_{12}E_8$ soluble homogenates (Figure 19a).

For all 5 organs, once the gel was stained with Gelcode there were 2 protein bands clearly visible (Figure 19b). The heavier of these, approximately 150kD, are the MMC4

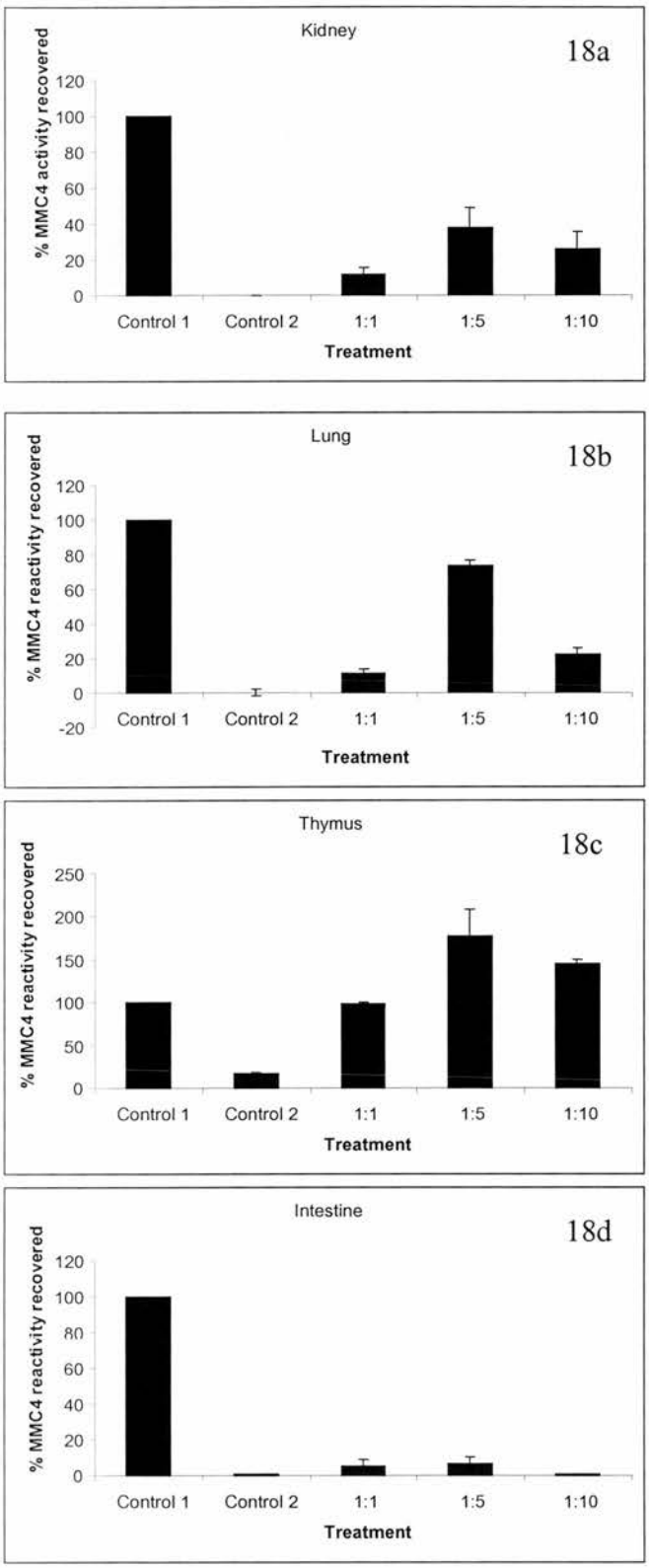
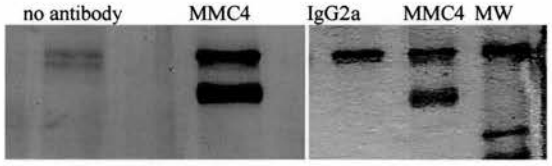


Figure 18: MMC4 reactivity remaining after solubilization of organs with various ratios of $C_{12}E_8$. Data is shown as a percentage of the MMC4 reactivity recovered for the unsolubilized positive control (raw homogenate). The level of MMC4 antigen already soluble (without adding any detergent) is also shown (negative control). 18a and 18b (kidney and Lung) both show the best recovery of MMC4 antibody reactivity with the 1:5 ratio of $C_{12}E_8$. 18c (Thymus) shows an increase in the amount of reactivity recovered after $C_{12}E_8$ solubilization compared to the positive control, but this can be explained by some of the MMC4 being already soluble within the cells' cytoplasm (control 2). The intestine does not have good recovery of MMC4 reactivity after $C_{12}E_8$ solubilization (18d) and the liver does not show any MMC4 reactivity under any of these conditions (data not shown)

19a)



19b)

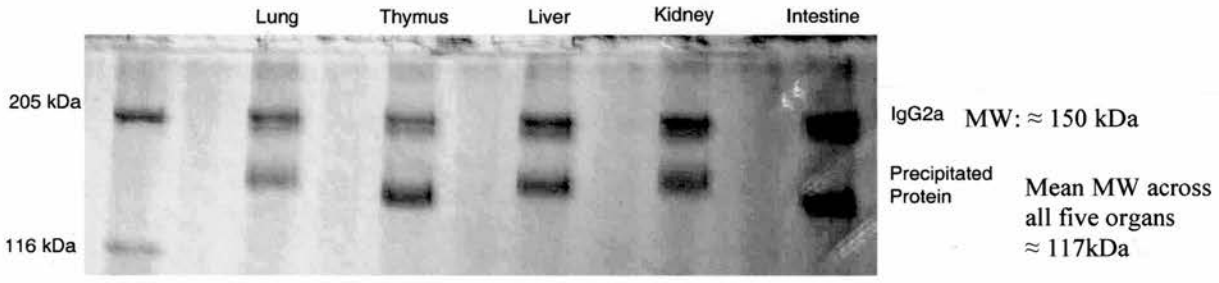


Figure 19: Six percent acrylamide SDS PAGE gel of immuno-precipitated putative MMC4 antigen and IgG bands for all five organs (19b) and controls using kidney homogenate (19a). Samples were purified using MMC4 coated Dynabeads. All protein was removed from the Dynabeads via SDS PAGE sample buffer without mercaptoethanol and heating and then run on acrylamide gels.

antibody and the anti-mouse IgG antibody (which comes pre-coated on the Dynabeads®). The 150kDa band was also found on both the controls. The second band (approximately 117kDa) found from the samples that had been incubated with MMC4 monoclonal antibody coated Dynabeads® was not, however, found on either the controls (Figure 19a). This strongly suggests that the second band found is specific binding. It was noticed that the band was running at slightly different molecular weights in different organs and that this pattern was repeatable (n=3).

Immuno-precipitation of the MMC4 antigen was also tried from TII alveolar epithelial cells; however only one band (150kDa, therefore immunoglobulin) was recovered (data not show). This is thought to be due to there not being enough type II cells added to the beads. Various numbers of AETII cells were tried up to the entire number recovered in an isolation. Due to the time, cost in materials and rat and difficulty in isolating AETII cells from the rat lungs it was decided not to carry on trying to isolate the MMC4 antigen from enriched AETII cells.

7.2.5 Immuno-depletion of the lung and kidney via successive rounds of MMC4 coated Dynabeads®

To confirm that the 117kDa band found was linked to the MMC4 antigen, a sample of solubilized homogenate was put through successive rounds of Dynabeads® coated with the MMC4 monoclonal antibody (kidney and lung only). At each stage a small sample of supernatant was removed for ELISA based dot blot. The supernatants that were removed were all diluted to have the same protein concentration before being loaded onto the dot blot manifold. The proteins that were attached to the Dynabeads® were also removed and run on a 6 or 8% down to acrylamide gel to confirm that there was still only one unknown band being found and that is was at the correct molecular weight (kidney only). Figure 20 shows that as the unknown 117kDa protein is removed from the supernatant (as shown by it running on the gel (figure 20a)) there is a resultant

Figure 20a:

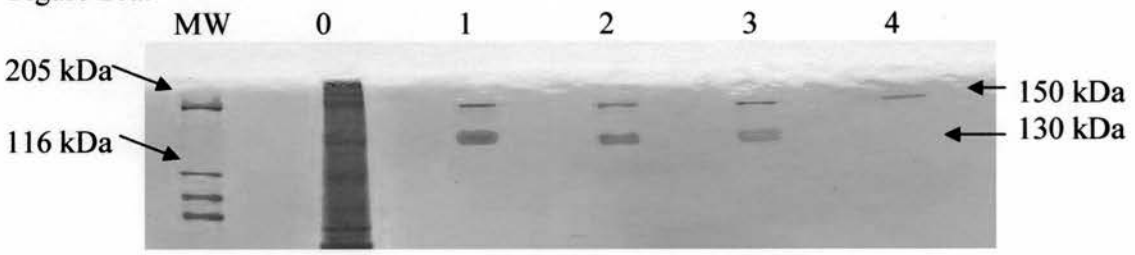


Figure 20b:

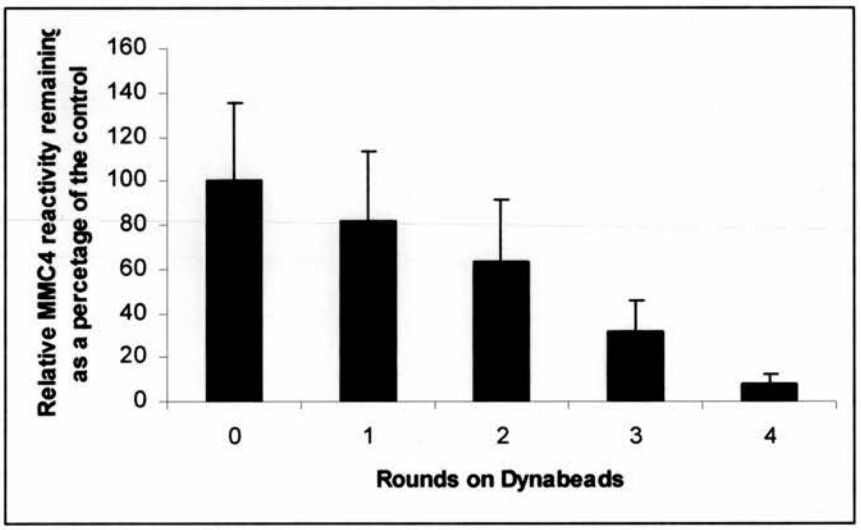


Figure 20: Immunodepletion of MMC4 antigen from kidney homogenate.

20a) 8% acrylamide gel showing the IgG band (150kDa) and the putative MMC4 band (130kDa) after 0-4 rounds of Dynabeads purification. The decrease in the amount of putative MMC4 attached to the beads is probably due to there being less MMC4 remaining in the sample.

20b) Graph of the results from the ELISA based dot blot showing that the level of MMC4 reactivity is also going down after each round of Dynabeads, suggesting that the MMC4 antigen is being removed from the sample.

decrease in the level of MMC4 reactivity on an ELISA based dot blot (Figure 20b) (n=3). This suggests that the unknown protein is either a) the MMC4 antigen or b) a protein closely associated with it.

7.2.6 Identification of the Putative MMC4 band

The putative MMC4 antigen (from each organ) was excised from the acrylamide gel and trypsin digested. The resulting peptide fragments were analysed by MALDI-TOF mass spectrometry (figure 21 shows the raw MALDI-TOF data for each organ) and SwissProt-TrEMBL was used to find the most likely candidates for the MMC4 antigen. In all five organs, there was one protein with a high MOWSE score and many proteins with lower MOWSE scores (figure 22) suggesting that the strong hit is the correct protein. The protein with the high MOWSE score in each case was rat aminopeptidase N (APN). Table 17 gives the actual MOWSE scores and the number of masses matched for each organ. The intestine also had a high MOWSE score for a second protein which was CD13, the mouse homolog of APN. This result is not unexpected due to the high degree of homology between APN in the two species.

Peptides from the trypsin digestion of the kidney were also analysed via tandem mass spectrometry. The resulting spectra were again analysed in SwissProt-TrEMBL. Again APN was found to be the most likely candidate for the MMC4 antigen (MOWSE score 1.9×10^7) with 6 of the peptides analysed being from APN. The 6 peptides found are from various regions of the APN protein (figure 23), suggesting that the protein analysed is APN and not an unknown protein with a similar domain to APN.

7.2.7. Confirmation that the MMC4 antibody is recognising APN

To confirm that the MMC4 antigen is APN, decreasing concentrations of purified (>90%) rat APN were tested for MMC4 reactivity on an ELISA based dot blot. Figure 23 shows that the MMC4 antibody is binding to the APN protein, confirming that this protein is an antigen for the MMC4 monoclonal antibody. The distribution of APN

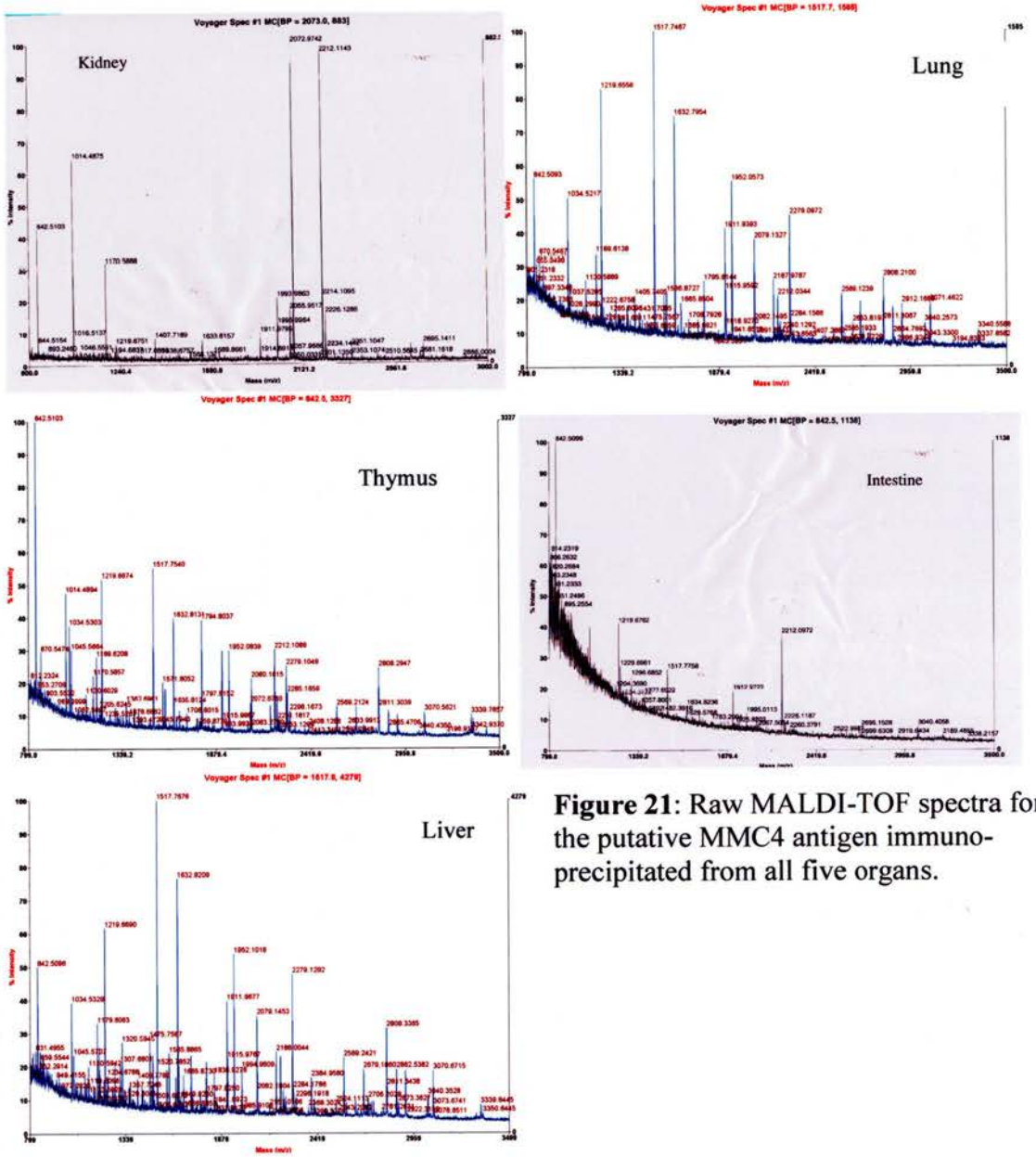


Figure 21: Raw MALDI-TOF spectra for the putative MMC4 antigen immuno-precipitated from all five organs.

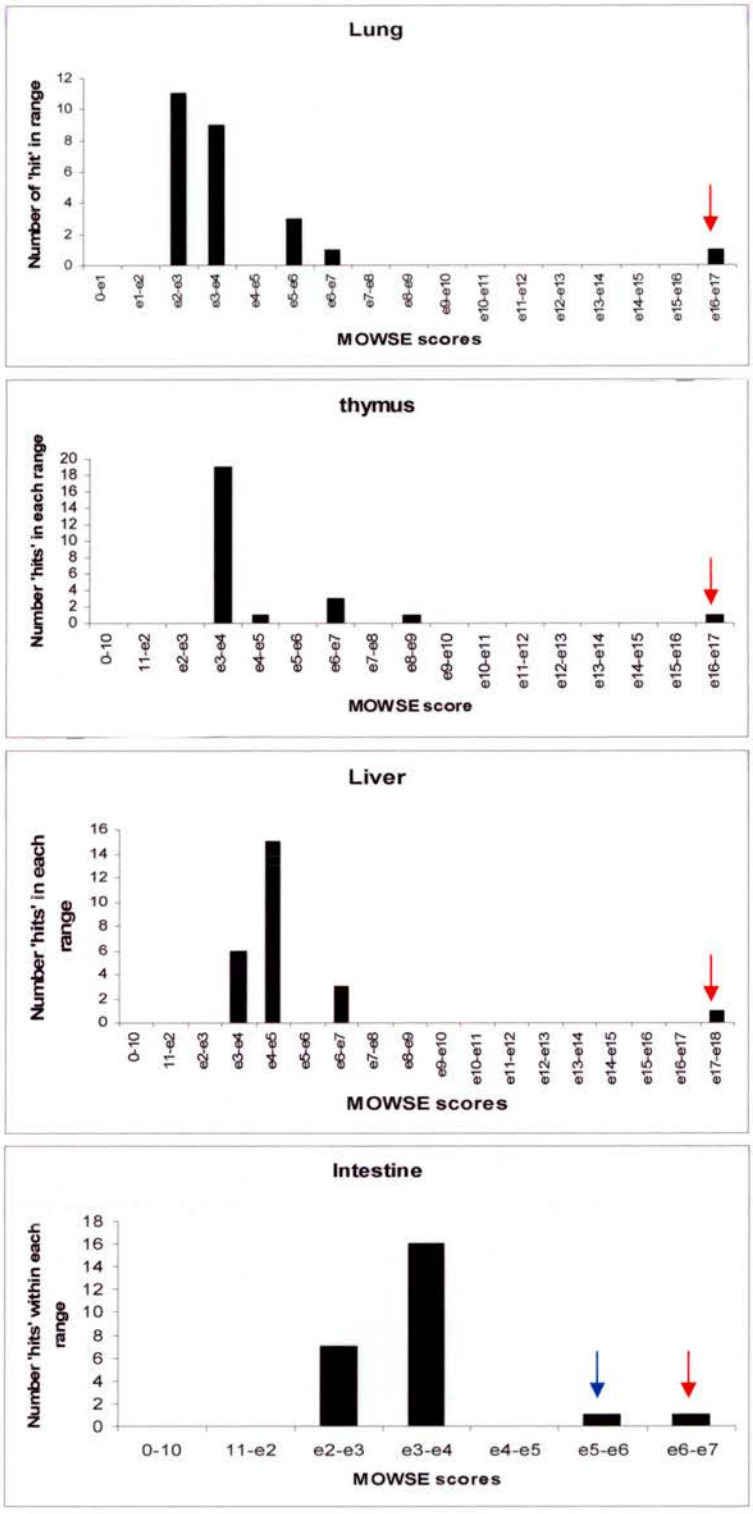


Figure 22: MALDI-TOF MOWSE scores found for the MMC4 unknown protein using SwissProt-TrEMBL search engine. Rat APN MOWSE score is shown by red arrow on each graph. Lung, liver and thymus searches were limited to rat proteins only. The intestine search was for all mammals, mouse APN (blue arrow) is therefore also a high hit, due to the level of homology between the two species.

Organ	MOWSE score	Masses matched/ total number masses entered into SwissProt-TrEMBL
Lung	1.76×10^{16}	28/50
Liver	3.6×10^{17}	30/82
Intestine	5.3×10^{6}	11/31
Thymus	4.24×10^{16}	28/68

Table 17: Actual MOWSE scores and number of masses matched to aminopeptidase N from the raw MALDI-TOF mass spectrometry data for the putative MMC4 antigen from each organ.

makgfyiskt lgilgillgv aavctiials vvyaqeknrn aensaiaptl pgstsattst
tnpaideskp wnqyrlpktl ipdsyqvtlr pyltpneqgl yifkgsstvr ftcnettnvi
iihskklnyt nkgnhrvalr **algdtpapni** **dttelver**te ylvvhlqgsl vkghqyemds
efqgeladdl agfyrseyme ggnkkvvatt qmqaadarks fpcfdepamk asfnittlihp
nnltalsnml pkdsrtlqed pswnvtefhp tpkmstylla yivsefkyve avspnrvqir
iwarpsaide ghgdyalqvt gpilnffaqh yntaypleks dqialpdfna gamenwglvt
yresalvfdp qsssisnker vvtviahela hqwfgnlvtv dwwndlwlne gfasyveflg
adyaeptwnl k**dlivlndvy** **r**vmavdalas shplsspane vntpaqisel fdsityskga
svlrmlssfl tedlfkkgls sylhtfqysn tiyldlwehl qqavdsqtai klpasvstim
drwilqmgfp vitvntstge iyqehflldp tskptrpsdf nylwivpipy lkngkedhyw
leteknqsae fqtssnewll lninvtgyyq vnydennwr**k** **iqnqlqtdls** **vipvinraqi**
ihdsfnlasa **gk**lsitlpls ntlflasete ympweaalss lnyfklmfdr sevygpmkry
lkkqvtplfa yfkiktnnwl drpptlmeqy neinaistac ssgleecrdl vvglysqwmn
nsdnnpihpn lrstvycnai afggeeewnf aweqfrkatl vneadklr**sa** **lacsnevwil**
nrylsytlnp **dyir**kqdats tivsiannvv gqtlvwdfvr snwkklfedy gggsfsfanl
iqgvtrrfss elelqqleqf kednsatgfg sgtraleqal ektkanikwv kenkdvvlkw
ftens

Figure 23: Amino acid sequence of aminopeptidase N. Fragments shown in red represent the peptides identified by tandem mass spectrometry. These peptides are distributed throughout the entire proteins, decreasing the probability that the SwissProt-TrEMBL search engine has found a protein with a similar domain to the unknown MMC4 antigen rather than the MMC4 antigen.

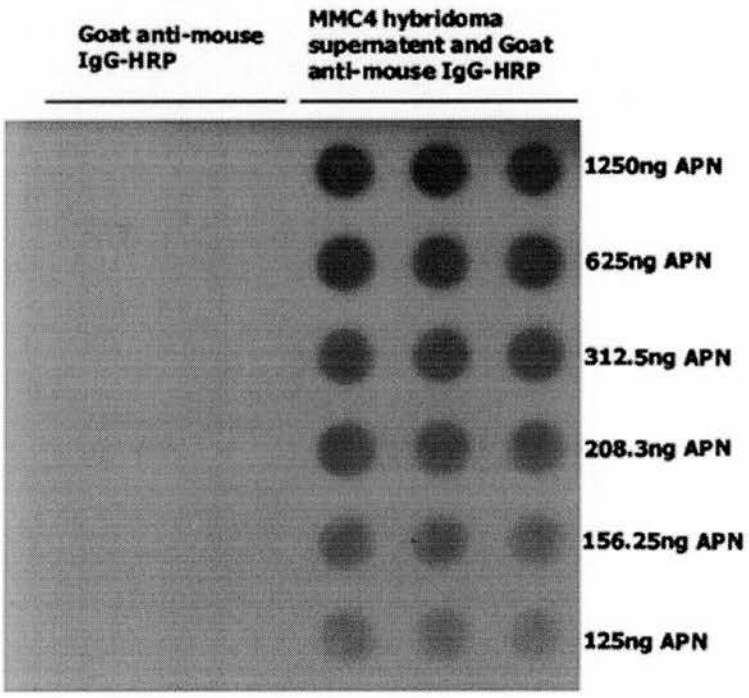


Figure 24: ELISA based dot blot of commercially purified aminopeptidase N. The MMC4 monoclonal antibody does bind to purified aminopeptidase N, confirming that this is an antigen for the antibody.

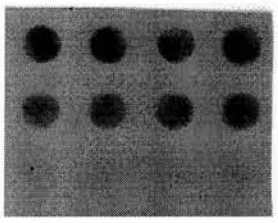
between organs is close to that which I found with the MMC4 antibody with the kidney having a far higher level than elsewhere. However, the ELISA based dot blot does not show MMC4 antigen in the liver (although subsequent analysis via immuno-precipitation does show it to be present) or brain (data from Boylan, thesis), where APN has been much studied (Gillespie, 1992, Kunz, 1994). APN has also been found on macrophages (Ashmun, 1990).

7.2.8. Aminopeptidase N presence on macrophages

Leukocytes were removed from the BAL of SD rats, diluted to contain an equal number of cells per millilitre and stored at -70°C. Samples were then tested via ELISA based dot blot for MMC4 reactivity, with varying success (figure 25). To further confirm whether macrophages express APN in a form recognisable by MMC4 Monoclonal antibody, frozen sections of lung were stained for MMC4 and CD68, a glycoprotein strongly expressed in the cytoplasm of macrophages. Tissue from four rats was tested. Elevenor twelve fields from each animal were captured and the number of macrophages staining positive for APN was analysed using the MMC4 antibody. (figures 26a and 26b show typical pictures of macrophages both with and with out MMC4 reactivity). Overall 8.45% (SD=6.94) of macrophages counted had MMC4 reactivity. The fact that there are very few macrophages per tile, and only a small proportion of these are MMC4 positive, could explain why G. Boylan reported that macrophages were negative for MMC4 reactivity (Boylan, thesis).

7.2.9. MMC4 is not a functional inhibitor of aminopeptidase N

APN is an enzyme that cleaves the N-terminus from other proteins. An artificial substrate for APN is L-Ala-pNA which becomes blue when the N-terminal alanine is removed. Commercial APN was incubated with L-Ala-pNA in the presence and absence of the MMC4 monoclonal antibody and also with an IgG2a not specific antibody for 0-6 minutes and the absorbencies of each solution was measured at 405nm. Figure 27 shows



APN (1.25μg)

Macrophages 10^7 cells

Macrophages 10^6 cells

Figure 2425: MMC4 monoclonal antibody binds weakly to macrophages from the lung.

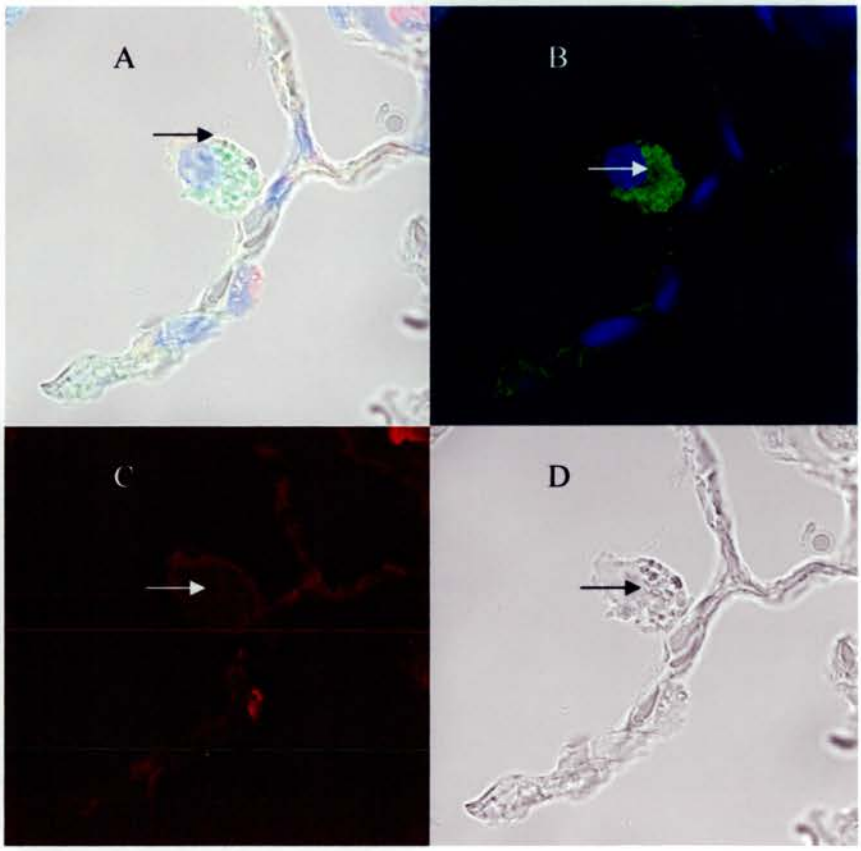


Figure 26a: Frozen lung section stained with Topro (blue), CD68 (green) and MMC4 (red). Section shows a lung that is negative for MMC4 monoclonal antibody staining (arrow)

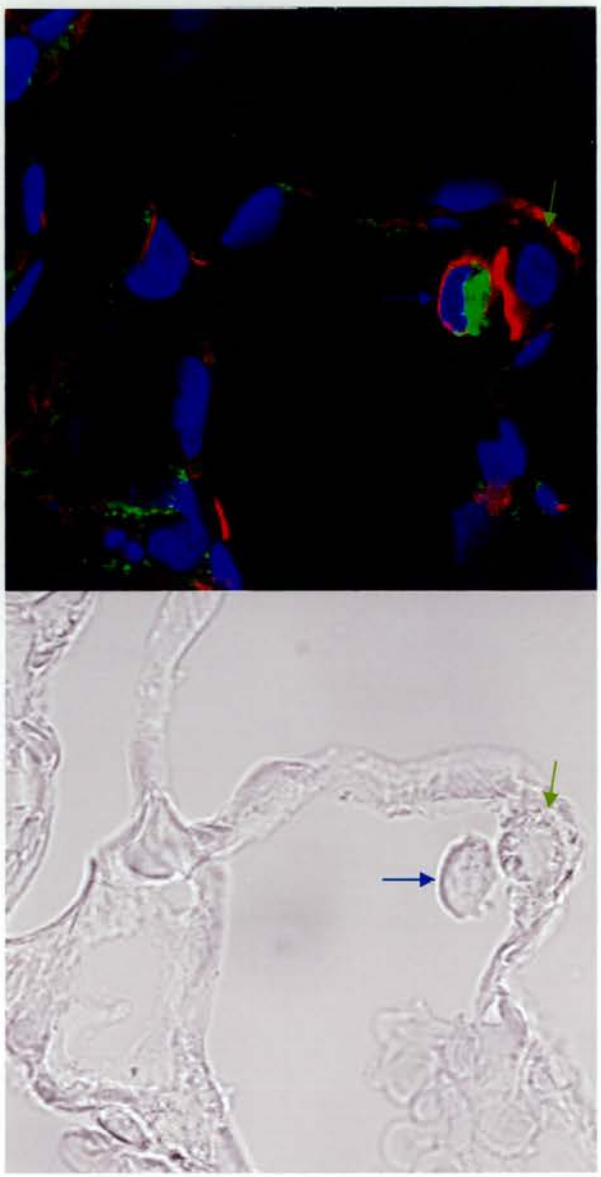


Figure 26b: Frozen lung section stained with topro (blue) CD68 (green) and MMC4 (red). Section shows a macrophage that is positive for MMC4 monoclonal antibody staining (Blue arrow), adjacent to a type II cell (green arrow)

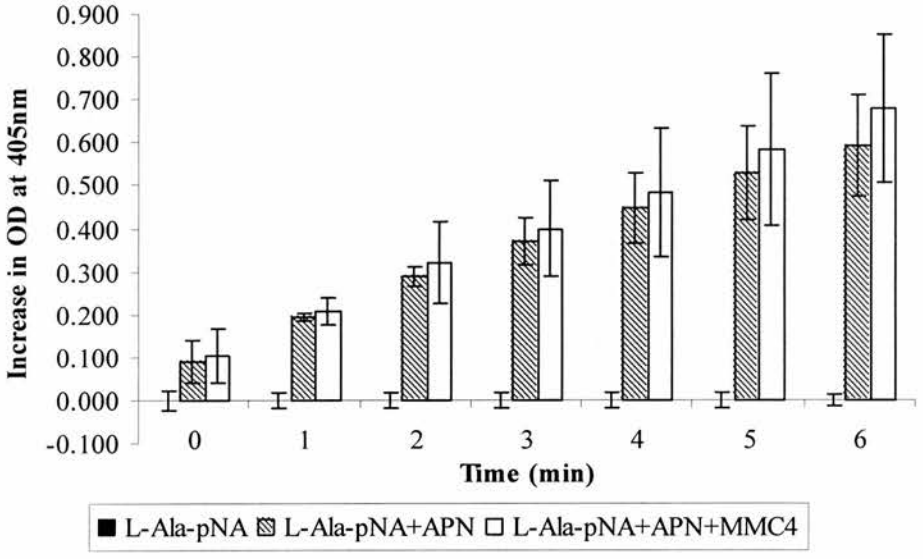


Figure 27: APN hydrolysation of L-Ala pNA is not inhibited by the addition of MMC4 Monoclonal antibody or an IgG2a control, suggesting that the MMC4 binding site is not the catalytic domain of APN.

that the increase in optical density was not affected by the addition of the MMC4 antibody. The IgG2a control antibody also caused no change in rate of hydrolysis (data not shown). The MMC4 monoclonal antibody, therefore, does not affect the removal of N-terminal amino acids from L-Ala-pNA. This suggests that the MMC4 monoclonal antibody does not bind to the active site or L-Ala-pNA's binding site of APN.

7.2.10. MMC4 monoclonal antibody does not recognise glycans attached to aminopeptidase N

To test if the MMC4 antibody was binding to a glycan on APN, APN was incubated with either N-glycosidase F or O-glycosidase to remove N-linked or O-linked glycans respectively.

O-linked glycans were removed from commercial APN as shown by the slight decrease in molecular weight found after treatment (gel not shown). ELISA based dot blot analysis (figure 27) shows that this decrease in molecular weight does not cause a corresponding decrease in MMC4 reactivity. This suggests that the MMC4 monoclonal antibody is not binding to a O-linked glycan.

N-linked glycan removal was not complete, as shown by the SDS-PAGE gel (figure 28). This was due to the reaction conditions not being optimal. Optimal conditions for this reaction, however, involved denaturing the APN protein and therefore the MMC4 antibody would not recognise the APN protein at all. Sub-optimal conditions were used to cause partial cleavage of the N-linked glycans. This caused some APN to be lose N-linked glycans as shown by lane 2 in figure 28. After immunoprecipitation with MMC4-coated Dynabeads®, the APN band minus N-linked glycans is still present, suggesting that the MMC4 Monoclonal antibody is not binding to an N-linked glycan.

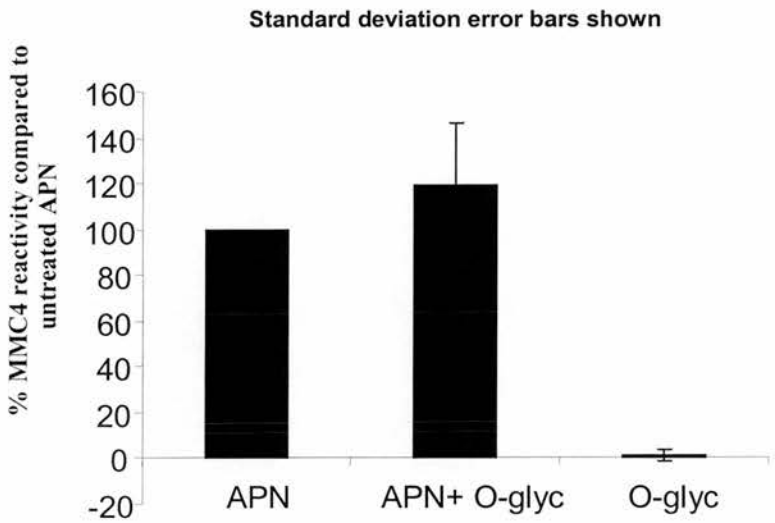


Figure 28: Analysis of ELISA based dot blot results from MMC4 monoclonal antibody recognition of aminopeptidase N both before and after incubation with O-Glycosidase.

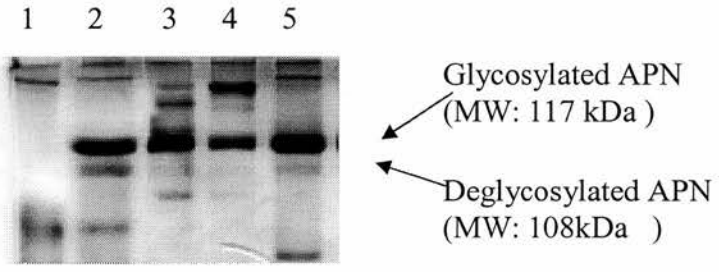


Figure 29: 6% acrylamide gel of aminopeptidase N plus and minus N-Glycosidase F (which removes N-linked glycans from the protein) both before and after immuno-precipitation using magnetic beads bound with MMC4 Monoclonal antibody. The presence of the deglycosydated APN band after immuno-precipitation shows that the MMC4 Monoclonal antibody is not binding to N-linked glycans.

1 = Molecular weight makers
2 = Aminopeptidase N plus N Glycosidase F
3 = Aminopeptidase N
4 = Immunoprecipitated (via Dynabeads coated in MMC4 antibody) aminopeptidase N
5 = Immunoprecipitated aminopeptidase N after incubation with N Glycosidase F

7.3 Discussion

MMC4 reactivity was found in the BAL of both *S. aureus* instilled and non-instilled rats. Levels of reactivity were found to increase after *S. aureus* instillation. These two findings suggested that the MMC4 antibody could be used as a marker of AETII and Clara cell injury within the lung in a non-invasive manor. However until the antigen and its specificity was fully known, it would not be appropriate to say that the increase in reactivity was due to the damage of these two cell types.

The MMC4 antigen was purified from the lung, kidney, thymus, liver and small intestine of SD rats via antibody coated Dynabeads®. In all organs the top 'hit' in the SwissProt-TrEMBL database for the isolated protein was aminopeptidase N (APN). This finding was confirmed via Tandem MS of the kidney isolated protein. Further biochemical confirmation showed that the MMC4 monoclonal antibody does bind to APN using commercially purified APN.

APN had been previously discounted as a possible antigen for the MMC4 monoclonal antibody as macrophages in initial screenings were thought to be negative. Re-evaluation of the MMC4 monoclonal antibody binding status to macrophages showed that, in the normal lung, while most macrophages are negative for MMC4 binding, some macrophages are positive (approximately 8%). ELISA based dot blot revealed that the brain and liver appeared negative for MMC4 reactivity (Boylan, thesis), even though APN is known to be expressed in these organs (Larringa, 2005, Jardinaud, 2004). Repetition of this ELISA based dot blot using liver homogenate still showed the liver to be negative for MMC4 reactivity, however, it was possible to immuno-precipitate APN from the liver using MMC4 monoclonal antibody coated Dynabeads®. However, to do so, very high protein levels (101µg) were required. This amount of protein corresponds to a volume of organ homogenate far greater than that which can be placed on a dot blot manifold. As the ELISA based dot blot therefore is not a sensitive test of the presence of

APN within the organ, the brain could also be positive, but at a level lower than is measurable via the technique being used.

Each organ had a slight different molecular weight on the acrylamide gel, this could be due to differences in glycosylation of the protein. Variable glycosylation states are known to occur, for example in humans there are three known glycosylation states: the unglycosylated 110kDa, partial glycosylated at 130kDa and the fully functional glycosylated form at 150kDa (Van De Velden, 1999). N-glycosidase F and O-glycosidase treatment of the commercial APN showed that the antibody is recognising the peptide backbone of APN. MMC4 monoclonal antibody has also been shown not to recognise the active site of amino acid cleavage, or the binding site of L-Ala-pNA, an artificial substrate for APN.

APN is a membrane bound metallopeptidase. The main known function of APN is to cleave the N-terminal amino acids off small peptides, rather to aid in peptide degradation (for example in the intestine (Riemann, 1999)) or to activate proteins. APN substrates include enkephalins, angiotensins, neurokinins and cytokines (Firla, 2001). Potential functions of APN in matrix degradation (Saiki, 1993), angiogenesis (Pasqualini, 2000) and antigen processing (Dong, 2000, Larsen, 1996) have all also been proposed.

Within the lung APN mRNA production has been shown to be regulated by an alternative promoter to that in many other organs (Gillis, 1998). This alternative promoter is the same as that which regulates CD10, surfactant proteins A, B and C and Clara cell specific protein (Gillis, 1998).

As aminopeptidase N is the antigen for MMC4 this antibody would not be useful as a marker of AETII cell and Clara cell damage via BAL analysis, due to this proteins presence on macrophages. However, the antibody is still useful in studies of lung repair (via immunohistochemical studies) due to the continuance of APN expression after

$RTII_{70}$ expression is lost in the transdifferentiation of AETII cells to AETI cells (Clegg, 2005).

8. Characterisation of the MMC6 antigen

8.1. Introduction

AETI cells already have a range of biomarkers in the literature, however, except for RTI_{40}, these all also recognise other cell types in the lung as well as the AETI cells. RTI_{40} recognises a 40kDa protein of unknown function. Until a function is assigned to the protein (T1α) this antibody can not be used as a biomarker for drug testing as it is unclear what pathway within the cell is being affected.

In the process of making novel AETII antibodies (e.g. MMC4, previous chapter), G. Boylan also produced a marker of AETI cells, MMC6 (isotype IgG2b). Initially it was assumed that this antibody would recognise the T1α protein that RTI_{40} also recognises. However resent work in co-localisation of the RTI_{40} and MMC6 antibody via immunohistochemistry by G. Clegg (unpublished) showed that RTI_{40} and MMC6 monoclonal antibody were not always co-localising on the alveolar membrane. C. Tyrrell in a study of *Streptococcus* pneumonia has found that while RTI_{40} antigenicity levels in the lung decrease, MMC6 antigenicity levels remain the same (unpublished communication). These two studies suggest that the MMC6 monoclonal antibody is not recognising the same antigen as RTI_{40}.

The hypothesis of this study was therefore:

1. MMC6 monoclonal antibody recognises a protein associated with AETI cells
2. The MMC6 monoclonal antibody is useful as a biomarker of AETI cell injury in the rat lung.

MMC6 monoclonal antibody was raised via 2 injections of an enriched type II cell preparation from SD rats into female BALB/c mice. The resulting sera was screened for reactivity to rat AETII cells on frozen sections of the lung. As already mentioned MMC6 monoclonal antibody was however found to recognise AETI cells and not AETII

cells. Hybridomas were then re-cloned three times to ensure a single clone was in the preparation. MMC6 monoclonal antibody recognising AETI cells is thought to be due to either contamination of AETI cells in the enriched AETII cell preparation injected into the BALB/c mice, or due to the AETII cells within the preparation beginning to undergo differentiation into AETI-like cells, which they do in culture.

8.1.1. Strategy

As very little is known about the MMC6 antigen, except that is found on AETI cells in a rough screens of antibody recognition, the strategy for identifying the antigen was going to have to start at first principles. Figure 30 shows an overview of how I intend to characterise the antigen, first of all finding the organ distribution of the antigen, then investigation into what the antigen is (i.e., protein, carbohydrate), followed by where on the cell the antigen is found and finally what exactly the antigen is. Due to the MMC6 antibody being made via the same method as the MMC4 antibody, it is expected that, like the MMC4 antibody, it will not be possible to purify the MMC6 antigen via western blotting. Due to this initial screening for antigenicity will be carried out using the ELISA based dot blot system. At the same time as identifying the MMC6 antigen I also plan to show that MMC6 reactivity can be found in the BAL fluid and that these levels increase after *S. aureus* injury.

8.1.1.1. Disruption of cell membranes

Due to the method used to produce this antibody it is predicted that the antigen will be a membrane associated protein. As with the MMC4 monoclonal antibody to purify the antigen I would therefore have to disrupt the cell membranes so that the protein could be isolated. There is a range of methods to do this. Firstly, I could use mechanical disruption, such as sonication or homogenisation. Homogenisation works by suspending the tissue of choice in a liquid and then rotating blades cause the tissue to be sheared into smaller and smaller pieces. Sonication works by using bursts of sound waves to

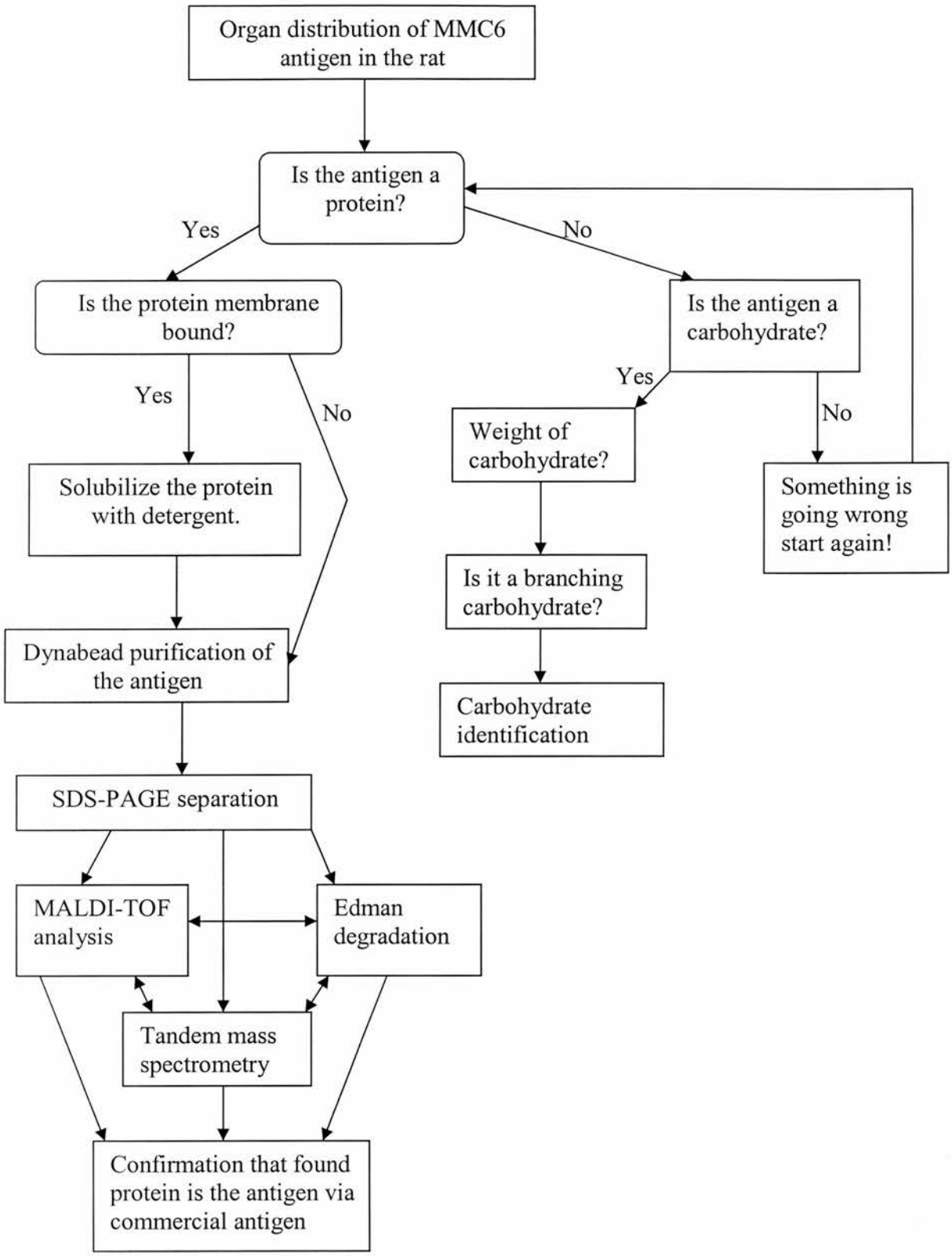


Figure 30: Overview of the planned method for identifying the MMC6 antigen

cause the cell membranes to break up. Both these techniques however cause uneven breaking on the tissue, as tissue nearest the probes end up being affected more that tissue further away. These techniques also cause the tissue to heat up which can in turn destroy antigenicity by denaturing the protein. More recently the preferred technique for disrupting cell membranes has been to use detergents.

8.1.1.2. Detergent solubilization of cell membranes

Detergents break up lipid-lipid and lipid-protein bonds causing the resulting fragments to form micelles. Different detergents have different success in disrupting the cell membrane. For example, Triton X-100 is often used when studying lipid rafts as it does not break these cholesterol rich regions of the membrane into small pieces, while other areas of the cell membrane are fragmented (Schuck, 2003). CHAPS is also used in a similar way, but does partially break up the lipid rafts (Magee, 2003). For studies of the membrane proteins octyl-β-$_D$ gluoside and Dodecyl-β-$_D$-maltoside are often used as both are gentle detergents that do not change the protein from its native state (McGregor, 2003). As the location of the MMC6 antigen within the cell membrane was unknown, a range of detergents were chosen to test to optimise MMC6 antigen recovery.

8.1.1.3. Edman degradation

Edman degradation is a method of finding the amino acid sequence of a protein by continuously cleaving the N-terminal amino acid from the protein and identifying the amino acid based on weight. In basic conditions (pH 9) protein and phenylisothiocyanate in gaseous form react to form a phenylthiocarbamyl derivative at the N-terminus of the protein. Trifluoroacetic acid then cleaves off the first amino acid as the anilinothialinone derivative. The anilinothialinone derivative then passes through the machine aided by addition of N-butyl chloride and converted to a phenylthiohydantoin. The phenylthiohydantoin is then analysed for the molecular weight from which the weight of the amino acid can be determined.

8.2. Results

8.2.1. Screening for MMC6 antigen in multiple organs

Most organs from adult, male SD rats were isolated (ovaries and placenta was harvested from a female breading SD rat) and homogenised in TBS as described in methods. All homogenates were then diluted 1:5 and 1:10 in TBS (except the lung (1:1000, 1:5000)) and vacuumed onto a PVDF membrane. An ELISA based dot blot was preformed, and the X-ray film was analysed as described in methods. I found that all organs (except the lung) were negative for antigenicity to the MMC6 antibody with this method (figure 31). This would suggest that the MMC6 monoclonal antibody recognises a molecule that is specific for the lung.

8.2.2. MMC6 Immuno-histochemistry of the lung

Frozen sections of untreated SD rat lungs were stained with MMC6, MMC4 and $RTII_{70}$ antibodies (Figure 32) or stained with MMC6, RTI_{40} and anti-calveolin antibodies (Figure 33), to test if the MMC6 antigen if found on type I, type II or both alveolar epithelial cell types. Figure 32 shows co-localisation of the AETII cell associated antibodies MMC4 and $RTII_{70}$. However the MMC6 antibody (shown as green) does not co-localise with these two antibodies, suggesting that the MMC6 antigen is not found on AETII cells. However, MMC6 staining does co-localise with the RTI_{40} and anti-calveolin antibodies (figure 33), suggesting that the MMC6 antibody a marker of AETI cells. Freshly isolated type II cells do not express MMC6 antigen, and day 5 Type II cells in culture, which are type I cell like, do express MMC6 (S. McKechnie, personal communication), further suggesting that MMC6 monoclonal antibody is AETI cell specific.

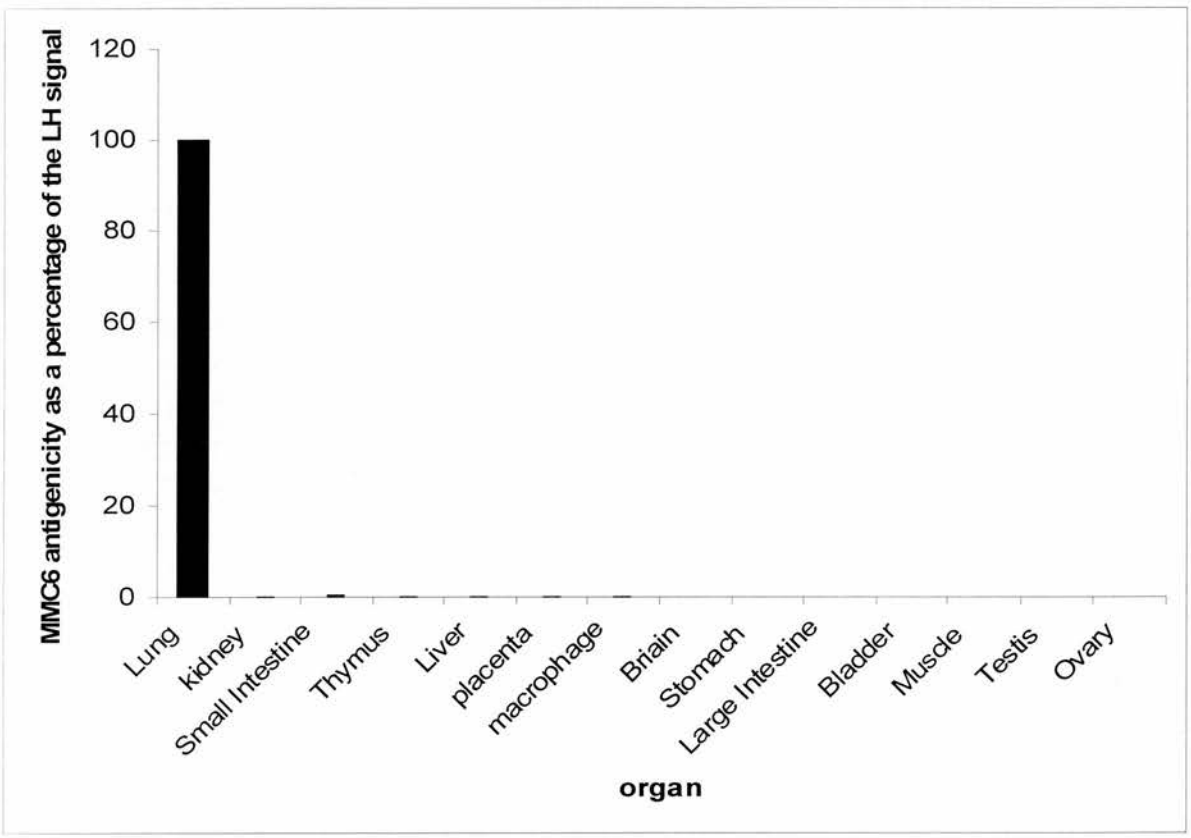


Figure 31: Distribution of the MMC6 antigen in SD rats as found via ELISA based dot blot.

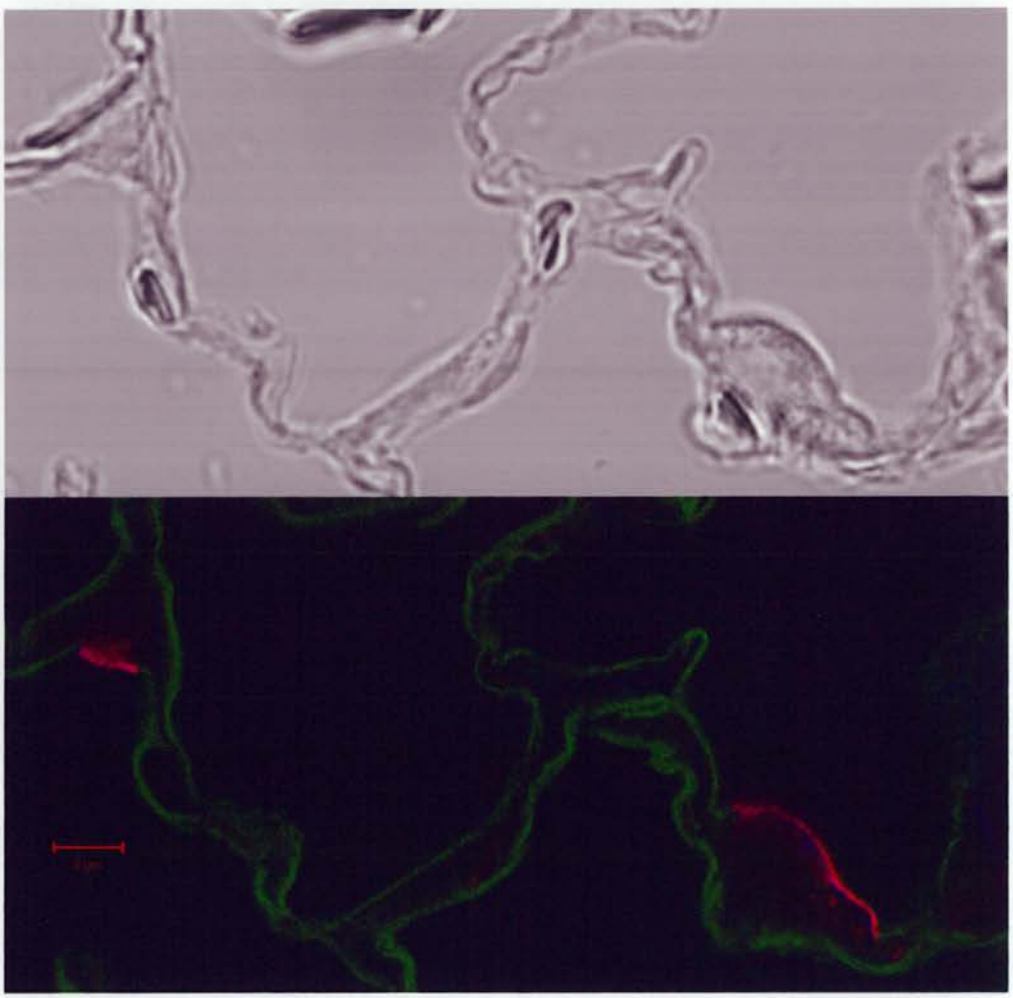


Figure 32: Immunohistochemistry of a frozen normal lung section. MMC6 antibody staining (green) is not co-localising with MMC4 (blue) or $RTII_{70}$ (red) antibody staining. This suggests that the MMC6 antibody does not recognise the same cell type as the MMC4 or RTI_{70} antibodies.

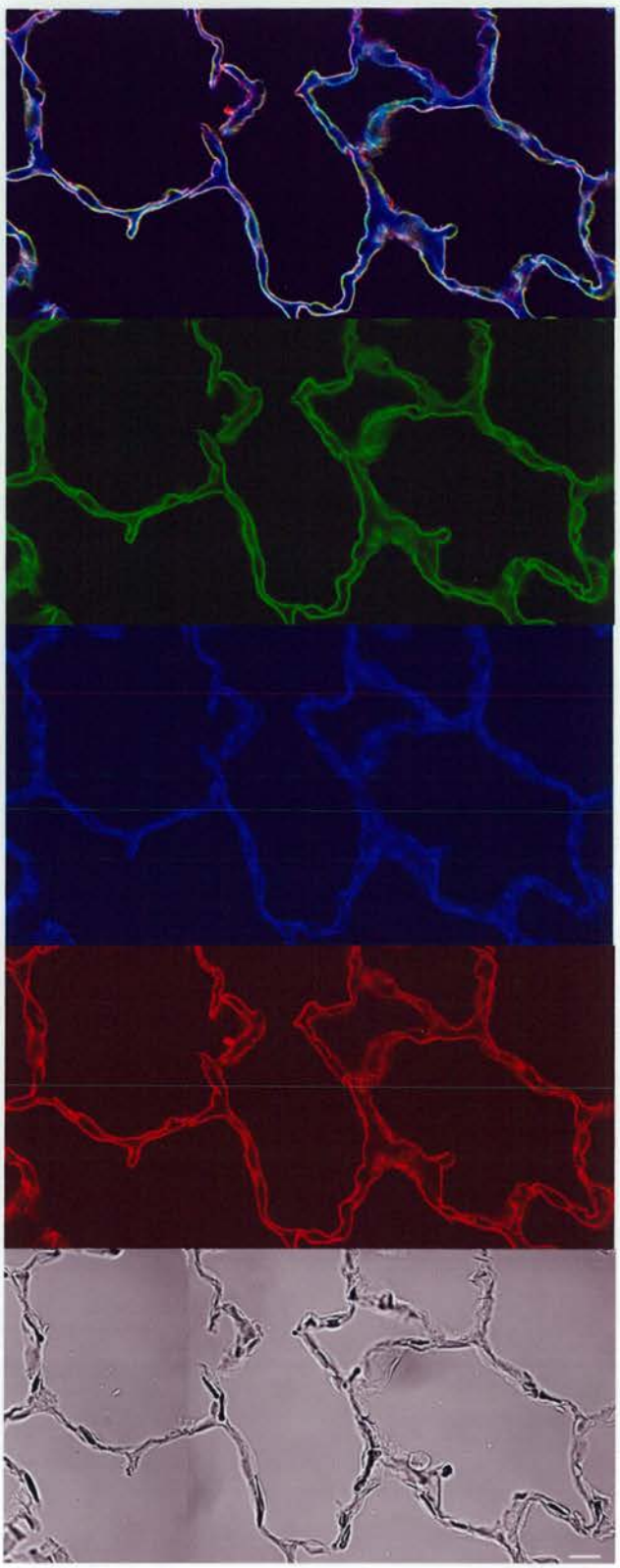


Figure 33: Immunohistochemistry of a normal lung section. MMC6 (Green) is expressed in the same locations as RTI_{40} (red) and Calveolin (blue). This suggests that the MMC6 antibody is expressed by AETI cells

8.2.3. MMC6 monoclonal antibody recognises a protein

Lung homogenate was treated with Proteinase K, an enzyme that degrades most known proteins, overnight at 37°C. The following day samples were tested for remaining MMC6 antigenicity via ELISA based dot blot. Controls of just Proteinase K, lung homogenate incubated at 37°C overnight and lung homogenate stored at 4°C were also tested. Figure 34 shows that little to no antigenicity was lost from the lung homogenate due to heating overnight, however were Proteinase K was added the antigenicity to MMC6 was almost obliterated. This strongly suggests that the MMC6 antigen is a protein.

8.2.4. MMC6 antigen as a membrane protein

From the immunohistochemistry (figures 32 & 33) it appears that the MMC6 antigen is confined to the membranes of the type I cells. To test this, high gravitational forces were used to separate membranes from lung homogenate. Figure 35 shows that after spinning at 500,000g the majority of the MMC6 antigenicity is in the pellet, were all cellular membranes would be found.

The type of membrane binding was further tested by use of a carbonate wash, which causes peripheral proteins to be detached from the membrane. After a further 500,000g centrifugation the MMC6 antigenicity still remains in the membrane pellet (figure 35). This indicates that the MMC6 antigen is either an integral, or a trans-membrane protein.

8.2.5. Solubilization of lung membranes

As the MMC6 antigen is membrane bound the conventional method to purify the antigen would be to initially solubilize the membranes with a detergent. To be considered a successful detergent for our proposes the detergent must a) not cause a confirmation change to the protein resulting in the MMC6 monoclonal antibody being

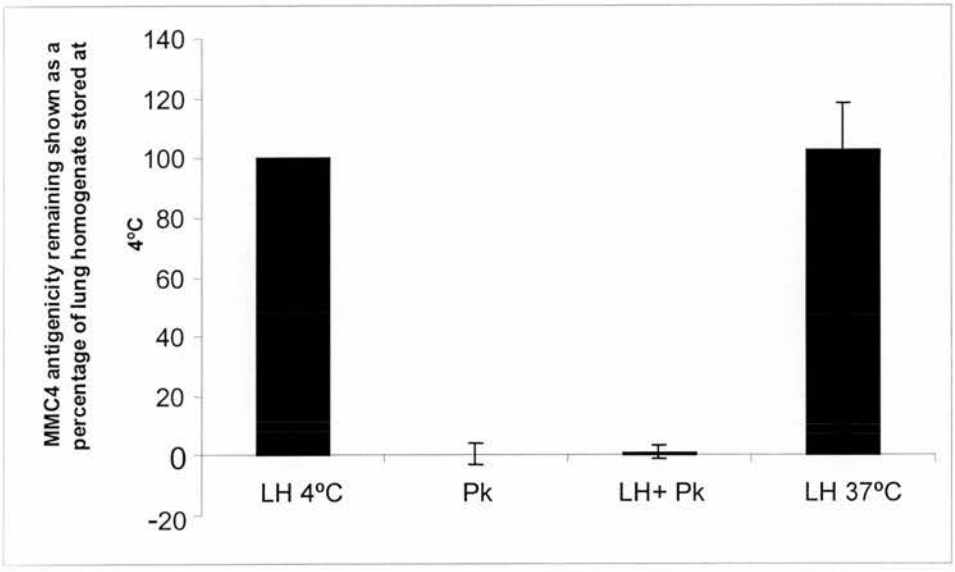


Figure 34: The MMC6 antigen is a protein. LH= lung homogenate, Pk= Proteinase K. Addition of Proteinase K to lung homogenate destroys all antigenicity to the MMC6 antibody, suggesting that the antigen is a protein.

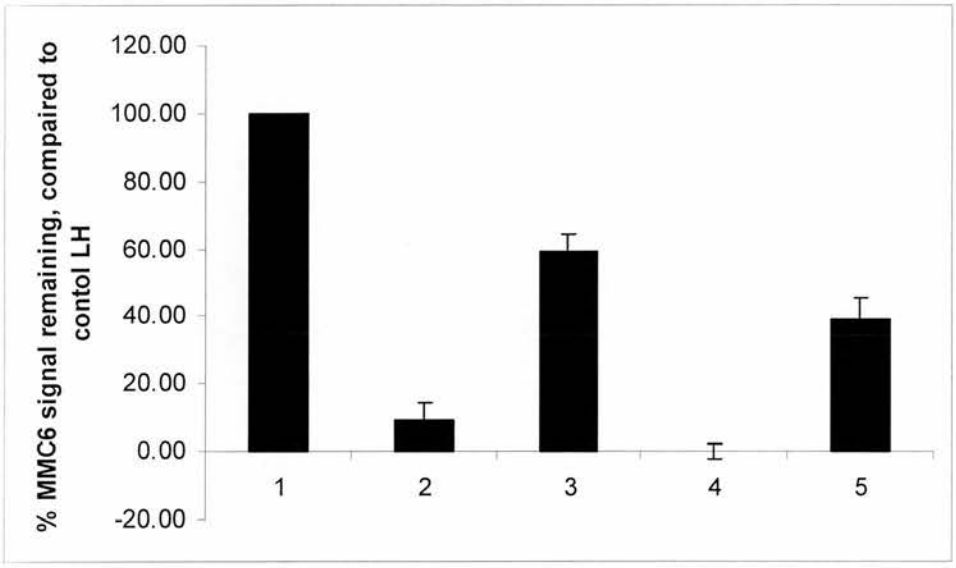


Figure 35: MMC6 monoclonal antibody recognises an integral membrane protein. 1: untreated lung homogenate 2: supernatant after 500,000g ultracentrifugation, 3: pellet after 500,000g ultracentrifugation, 4: supernatant after carbonate wash and then ultracentrifugation, 5: pellet after carbonate wash and then 500,000g ultracentrifugation. Lane 2 shows tat a small amount of the MMC4 antigen is not membrane bound, however the majority (60%) is membrane associated. Lanes 4 and 5 shows that the protein is strongly attached to the membrane, and therefore is most likely to be a trans-membrane protein.

unable to bind to it, b) form micelles of sufficient size to hold the MMC6 antigen, but not so large that thy are separated out with the membranes after a 500,000g centrifugation c) the detergent can not denature or interfere with the MMC6 antibody.

To find a detergent that fulfilled these roles a range of non-ionic, anionic, Zwitterionic and Chaotropic agents were tested both in the absence and presence of Caproic acid (a hydrogen donator/acceptor to maintain the correct pH) to see if they were useful detergents for our purposes. Usefulness was assessed using ELISA based dot blots. Figure 36 gives an overview of the methods used to assess MMC6-detergent compatibility. Initially all detergent, lung homogenate mixtures were assessed to see if the detergent would cause lose of antigenicity. Figure 37 shows a typical result for a detergent that reduced antigenicity and shows a complete list of all detergents that I tested that caused this response. As expected SDS is in this group, this therefore means that purification of the MMC6 antigen via Western analysis would not be possible. I then took lung homogenates with detergents that still showed antigenicity and spun then at 500,000g to pellet the membranes. I again tested un-spun, supernatant and pellet, as well as untreated lung homogenate, via ELISA based dot blot to see were the antigen was found. Figure 38 shows an example of a detergent where the antigenicity for the MMC6 monoclonal antibody was found in the pellet, suggesting that the detergent was not solubilizing the membranes completely. Figure 38 also gives a full list of detergents that I tested that did not cause a loss in antigenicity, but did not solubilize the membranes sufficiently for MMC6 coupled Dynabead purification.

One mixture of detergents at this stage was fulfilling my specifications, a Urea Lysis buffer (0.2M DTT, 7M urea, 50mM HEPES (pH7.6), 0.1M NaCl, 0.05% Triton X-100 (v/v), 10mM NaF). Unfortunately, the presence of DTT and the levels of Urea in this were so high (7M) that it denatured the MMC6 monoclonal antibody, making it unsuitable for Dynabead analysis (data not shown). Removal of the DTT and lowering the urea concentration to a safer level unfortunately caused the buffer to lose its ability to solubilize the MMC6 antigen (figure 39).

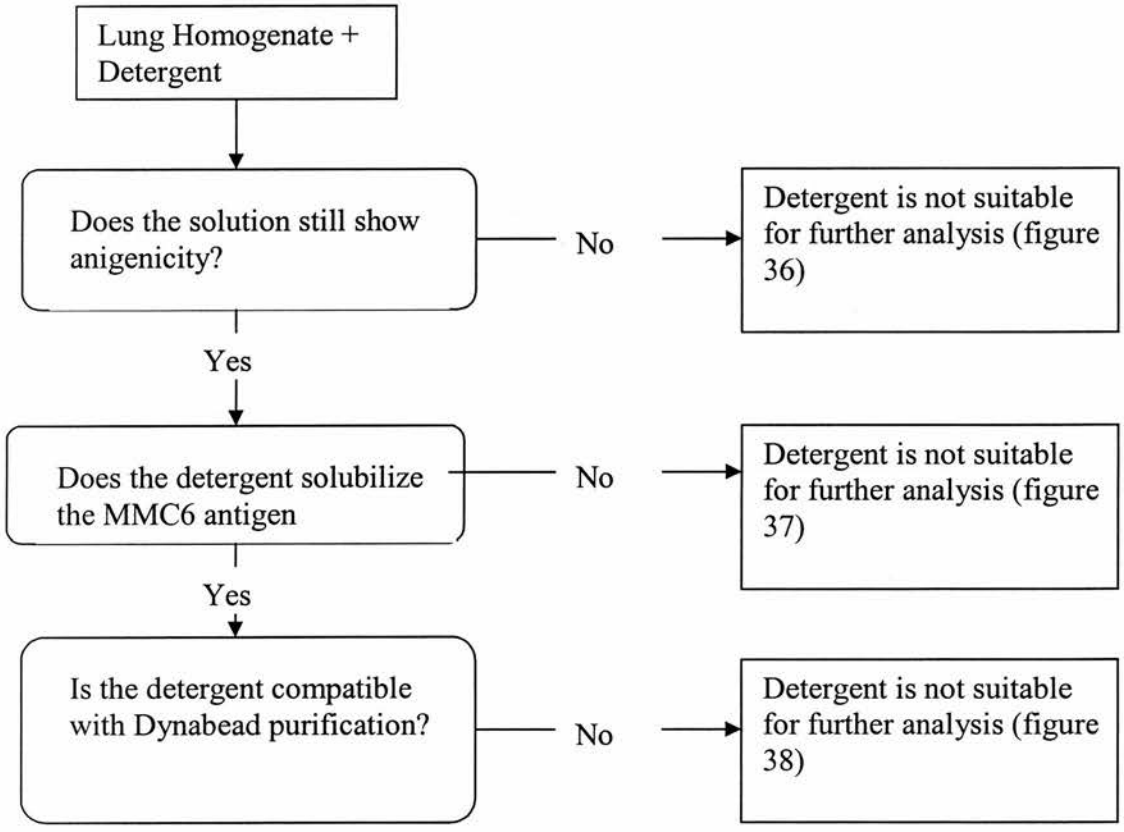


Figure 36: Overview of testing of detergents for ability to solubilize the MMC6 antigen for Dynabead purification

Figure 37: Detergents that caused a loss in Antigenicity after 1 hour 4°C

Detergent type	Detergent name
Non-Ionic	NP-40
	Triton X-100
	Dodecyl mactoslate
Anionic	SDS

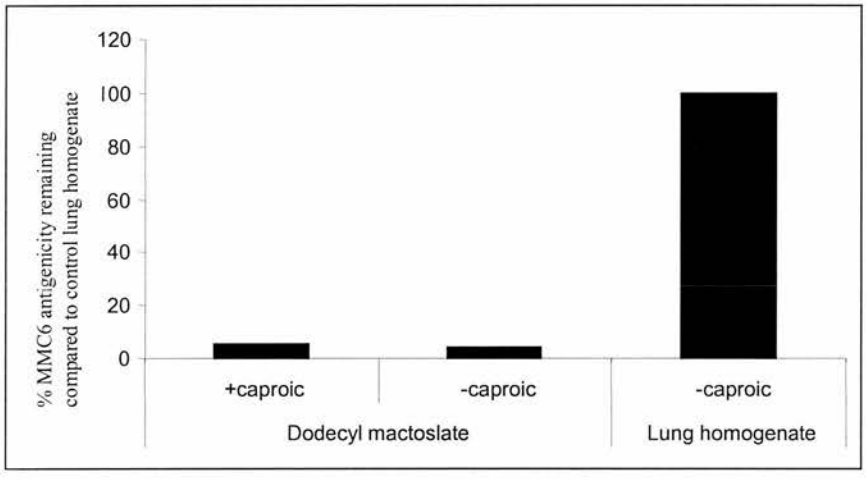
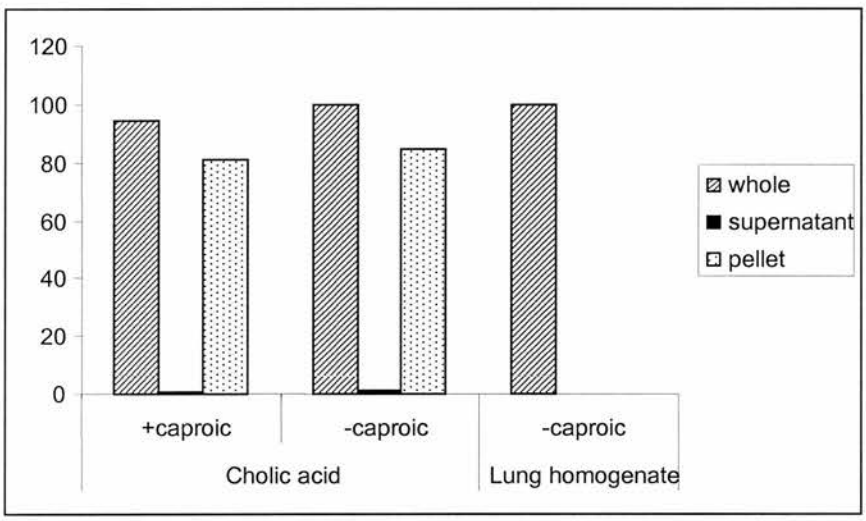


Figure 38: Detergents where antigenicity remains, but no solubilization

Detergent type	Detergent name
Non-ionic	Mega 8
	Octyl-β-D-glucopyranoside
Anionic	$C_{12}E_8$
	$C_{12}E_9$
	Cholic acid
Zwitterionic	CHAPS
Chaotropic agents	Urea
	Guanidine



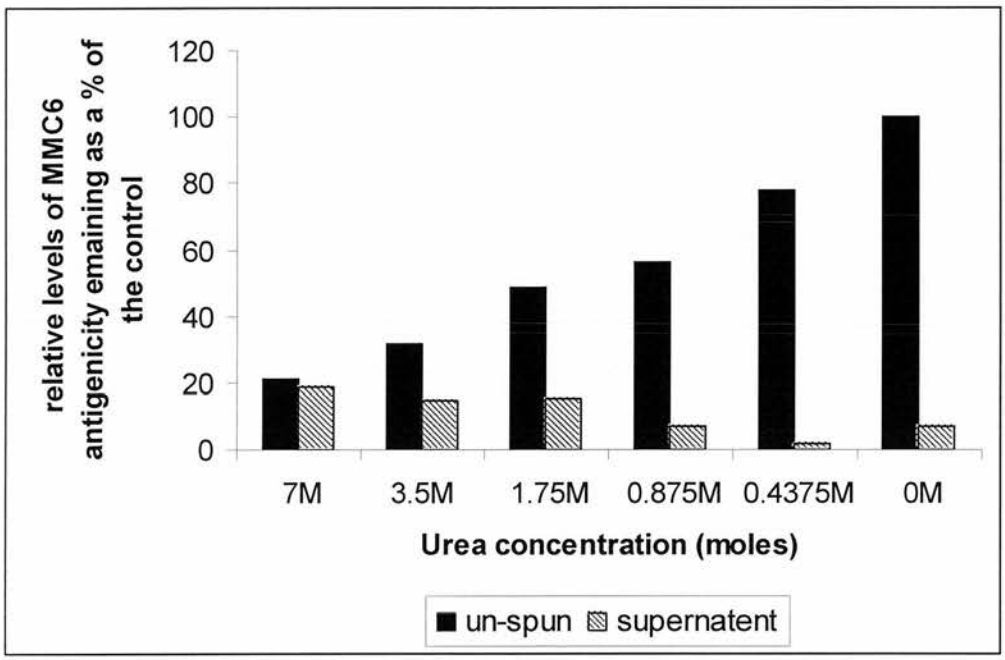


Figure 39: Affect if decreasing the urea concentration on ability of the urea lysis buffer to solubilize the MMC6 antigen.

8.2.6. Mechanical methods of disrupting the cell membranes

As detergents did not appear to be working effectively as a method of disrupting the cell membranes, I decided to try using mechanical methods, such as sonication and harsher homogenisation. Sonication did not cause a loss in antigenicity (measured by ELISA based dot blot), however it also did not solubilize the membranes enough to ensure that the protein was found in the supernatant after a 500,000*g* spin (data not shown). I then tried using a homogeniser at 30,000rpm to brake up the cell membranes (a technique used in breaking cells for RNA extraction) followed by short centrifugation to remove any larger tissue fragments. This did not remove antigenicity, however if the homogenate was treated to a 500,000*g* spin most antigenicity was still lost (data not show).

8.2.7 Purification of the putative MMC6 antigen

Despite this it was decided to use the 30,000rpm homogenate with a short spin to see if we could precipitate any proteins from the homogenate via Dynabead purification (method the same as for MMC4 lung homogenate). Bound proteins were then run on a 10% acrylamide gel. Figure 40 shows that one protein band was found on the gel (mean weight 61 kDa, n=5). As before the putative MMC6 antigen was sent for identification via MALDI-TOF mass spectrometry. Figures 41(a-b) show the probabilities of the top 20 hits for 2 different bands. Please note the low MOWSE scores found both times. The low MOWSE scores indicate that the database was unable to make a definite hit and was probably because these were 'hits' on the lower molecular weight fragments, which are more commonly found in all proteins. This suggests that either something has gone wrong during the trypsin digestion (unlikely as they were preformed by two different people on different days), or that the peptides are not in sufficient levels to be detected via this method, probably due to there being insufficient protein in the initial bands excised from the acrylamide gel (this is the more likely answer as to was difficult to get

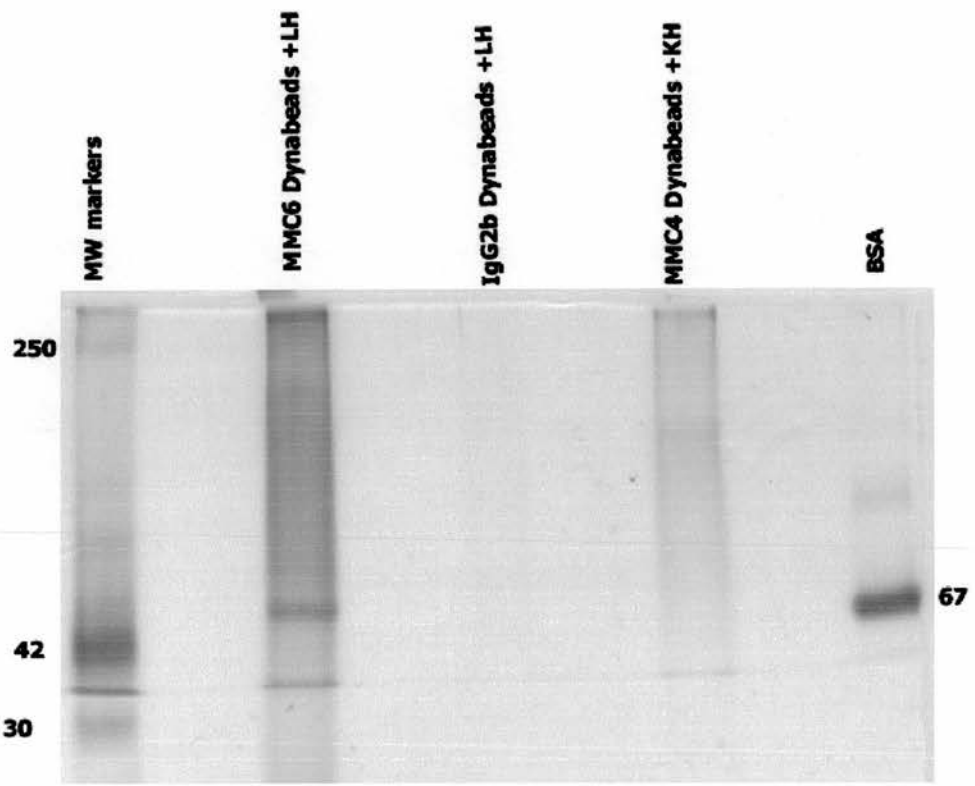


Figure 40: 8% SDS-PAGE gel stained with gelcode showing putative MMC6 antigen band is found when Dynabeads® are coated with MMC6 Monoclonal antibody, but not with an IgG2b control antibody. Putative antigen is approximately 60kDa.

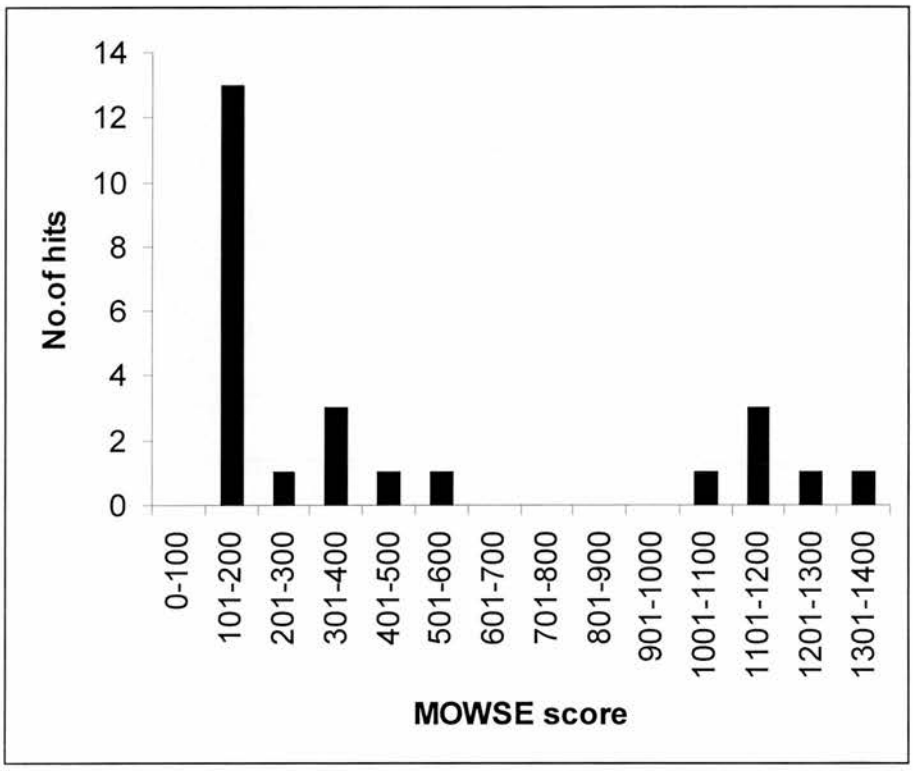


Figure 41a: SwissProt-TrEMBL MOWSE scores from the MALDI-TOF mass spectrometry of the putative MMC6 protein after trypsin digestion.

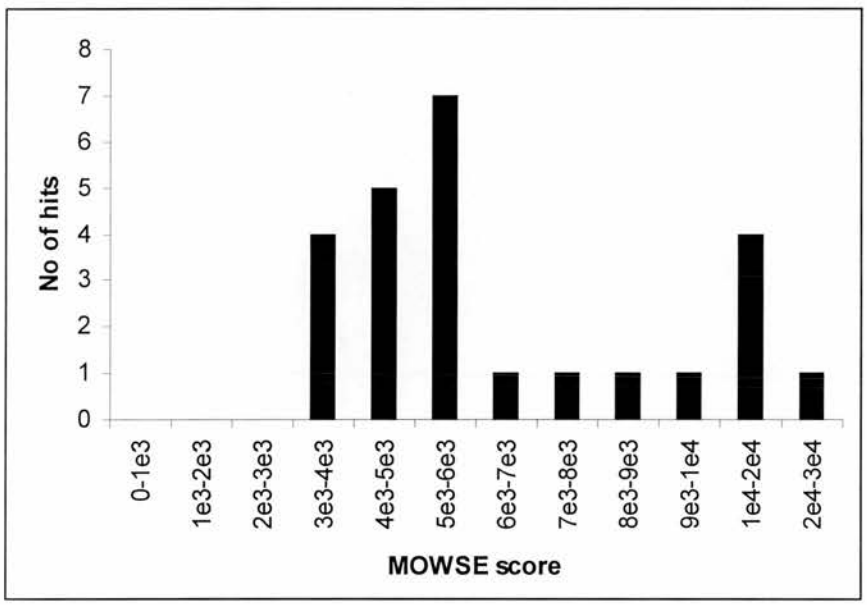


Figure 41b: SwissProt-TrEMBL MOWSE scores from the MALDI-TOF mass spectrometry of the putative MMC6 protein after trypsin digestion.

consistent staining of the gels with Gelcode, and even staining with sliver stain was poor).

8.2.8 MMC6 as a marker of AETI cell injury

SD rats were instilled with either *S. aureus* strain 8325-4 or PBS. 4 hours post instillation rats were euthanized and the BAL fluid and lungs were removed for protein analysis. Table 17 shows the average innoculum used, the total protein found in the BAL fluid and the average number of leukocytes recovered in the BAL fluid. Although there is no change in the protein levels in the BAL at this early time point, there is a 26 fold increase in the number of leukocytes that are found within the lung, showing that there is already an inflammation response occurring due to the inoculation of *S. aureus* into the lungs. This also suggests that at 24 hours post instillation the neutrophil response to the *S. aureus* injury is decreasing as there is only an 8 fold increase in neutrophils at 24 hours post instillation (see 7.2.1, table 17).

Figure 42 shows that levels of MMC4, MMC6 and RTI_{40} reactivity found in the BAL fluid of PBS and *S. aureus* treated rats. RTI_{40} has previously been shown to be increased in the BAL 24 hours post instillation (McElroy, 1999). Here it is shown that a 5 fold increase in RTI_{40} levels in the BAL can be detected within 4 hours of injury. However this increase is not statistically significant. No significant change was found in MMC4 levels, further confirming earlier observations that the initial leukocyte infiltrate into the lungs is mainly neutrophils (which do not express aminopeptidase N) and not macrophages (aminopeptidase N positive). MMC6 levels within the BAL were found to increase 4 fold; however, this again was not a statistically significant result. The similarity between the fold increases in MMC6and RTI_{40} detection within the BAL suggests that the MMC6 antibody could be useful as a marker of damage to the alveolar epithelium and shows that MMC6 reactivity can be measured within the BAL showing that the antibody has potential as a non-invasive biomarker.

Instilled	Innoculum size (cfu)	n	Protein in the BAL (mg)	Leukocytes in BAL
PBS	0	3	1.67 ± 0.747	1.73×10^{7}
8325-4	1.07×10^{8}	3	1.19 ± 0.730	6.46×10^{5}

Table 17: Levels of bacteria instilled into rats and levels of protein and leukocytes recovered in the BAL of rats harvested 4 hours after *S. aureus* strain 8325-4 was instilled.

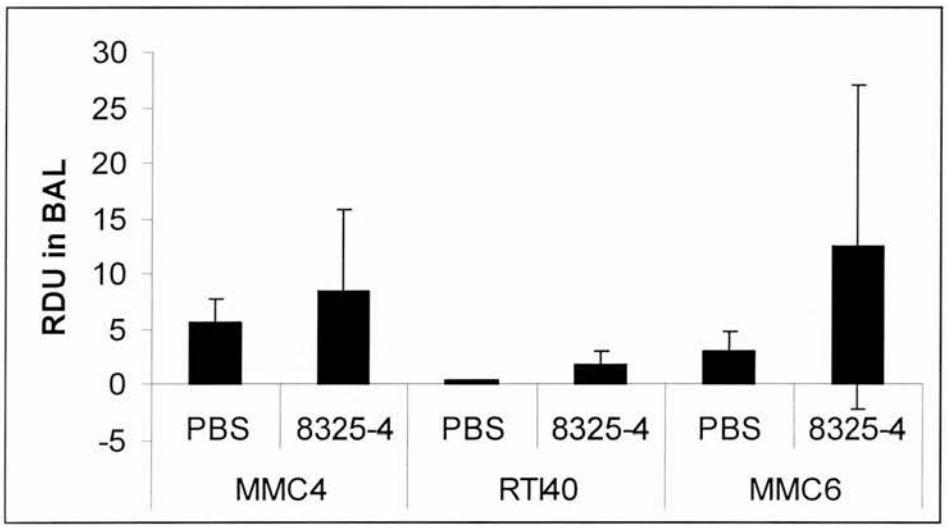


Figure 42: Relative densitometry units (RDU) of MMC4, RTI_{40} and MMC6 recovered from the BAL fluid via ELISA based dot blot from rats harvested 4 hours after either *S. aureus* strain 8325-4 or PBS instillation.

8.3 Discussion

While not managing to identify the MMC6 antigen I have shown that the antigen is an integral membrane protein expressed exclusively on AETI cells within the lung. Currently there are no other proteins known to be completely specific for AETI cells. Although I was unable to solubilized the cell membranes using detergents in a manner that would enable immuno-precipitation to work, a potential MMC6 associated protein was isolated via mechanical disruption of the cell membranes. This protein has a molecular weight of approximately 60kDa. Initially I had reservations about this protein as it is very similar molecular weight to albumin, however MALDI-TOF mass spectrometry followed by SwissProt-TrEMBL analysis did not return albumin as a high 'hit'. However as no known protein was returned by this method there is still a possibility that the isolated protein is albumin contamination.

Attempted solubilization of the cell membranes to isolate the MMC6 antigen has shown that the antigenicity of the MMC6 monoclonal antibody is conformation dependent (as shown by the inability of the antibody to recognise the antigen after SDS treatment). Due to this western blot analysis to find the protein's molecular weight was not possible. An alternative method to isolate the MMC6 antigen would be to try a gene expression library. Alternatively, traditional affinity columns, Triton X-114 separation or western blotting without reducing agents could also be tried if the gene expression library was not successful.

The pilot study into MMC6 being used as a marker of AETI cell damage via measuring MMC6 reactivity within the lung showed that MMC6 can be measured within the BAL fluid, and that differences in the levels of MMC6 expressed were similar to those found with RTI_{40}. This suggests that further work into this antigen is advisable as it does have the potential to be a marker of AETI cell damage if the antigen can be identified.

9. Discussion

The US Congress ruling in 1997 giving the FDA authority to grant marketing approval of devises to aid in the diagnosis, prognosis and selection of participants to drug regimes has greatly increased the number of potential biomarkers that are being converted from laboratory based tests to assays being used in the medical arena. However, considering the wide use of biomarkers within laboratories only a small fraction of these are making it through to clinical practice. Part of this is due to the criteria set down by the FDA which mean that the biomarker has to be more rigorously tested before it can be approved for human use than the testing is on many laboratory markers.

Weir *et al* (2006) argue that a biomarker should not just correlate to a clinical endpoint, but rather the effect of a treatment on the surrogate endpoint being measured by the biomarker should also correlate with the treatments' effect on the final endpoint. This thesis, instead of looking for biomarkers of a specific disease, has investigated markers that indicate a change to the alveolar epithelium which is often an early site of change in lung diseases. As a return to 'normal' levels of AETI and AETII cells after a injury is linked to the lungs returning to normal function the markers I have investigated would fulfil Weir *et al*s' recommendation of a role of a biomarker.

Potential biomarkers are made by a variety of mechanisms. Some are made deliberately, based on known biological mechanisms of a disease producing likely candidates (e.g. CD4 levels in the blood of people infected with HIV). Other markers are however made by random screening of the changes that take place within an organism after infection/development of the disease.

Based on the finding and pitfalls found during this thesis I would like to propose a method of identifying and screening the biological usefulness for potential biological markers.

9.1 Framework of biomarker development

Biological marker development can be split into distinct stages: development of a potential marker, producing a reliable assay system for the marker, biomarker characterisation, determining the biomarker's target population, compare and contrast the marker to existing markers and finally validation of the biomarker. Most of these areas overlap so for ease of explanation I have grouped these areas into:

a) Biomarker development and characterisation,

b) Assay development and compare and contrast to existing biomarkers and

c) Biomarker validation and determining target populations.

9.1.1 Biomarker development and characterisation

Methods for developing biomarker candidates depend greatly on a number of factors: the nature of the disease, availability of information on the disease, presence of biomarkers/ potential biomarkers already in existence, the preferred techniques of the researching group and also the eventual target user group (i.e. biomarkers intended for laboratory use only, un-linked to human diagnostics, tend to be less well researched than those intended for human diagnosis).

Figure 43 shows an overview of the method of identifying candidate biomarkers that I am proposing.

Initially research into the area to discover if there are any known biomarkers for the disease being investigated is required. In my area of alveolar epithelial injury there were proposed markers of both AETI and AETII cells (RTI_{40}, HTI_{56} and aquaporin 5 being the main candidates for AETI cells and Surfactant proteins and Alkaline phosphatase for AETII cells), however, all these markers have factors that make them undesirable as a biomarker. RTI_{40}, recognises the T1α protein (Kato, 2003), which currently has no known function, making linking changes in its levels of expression/ levels in BAL fluid

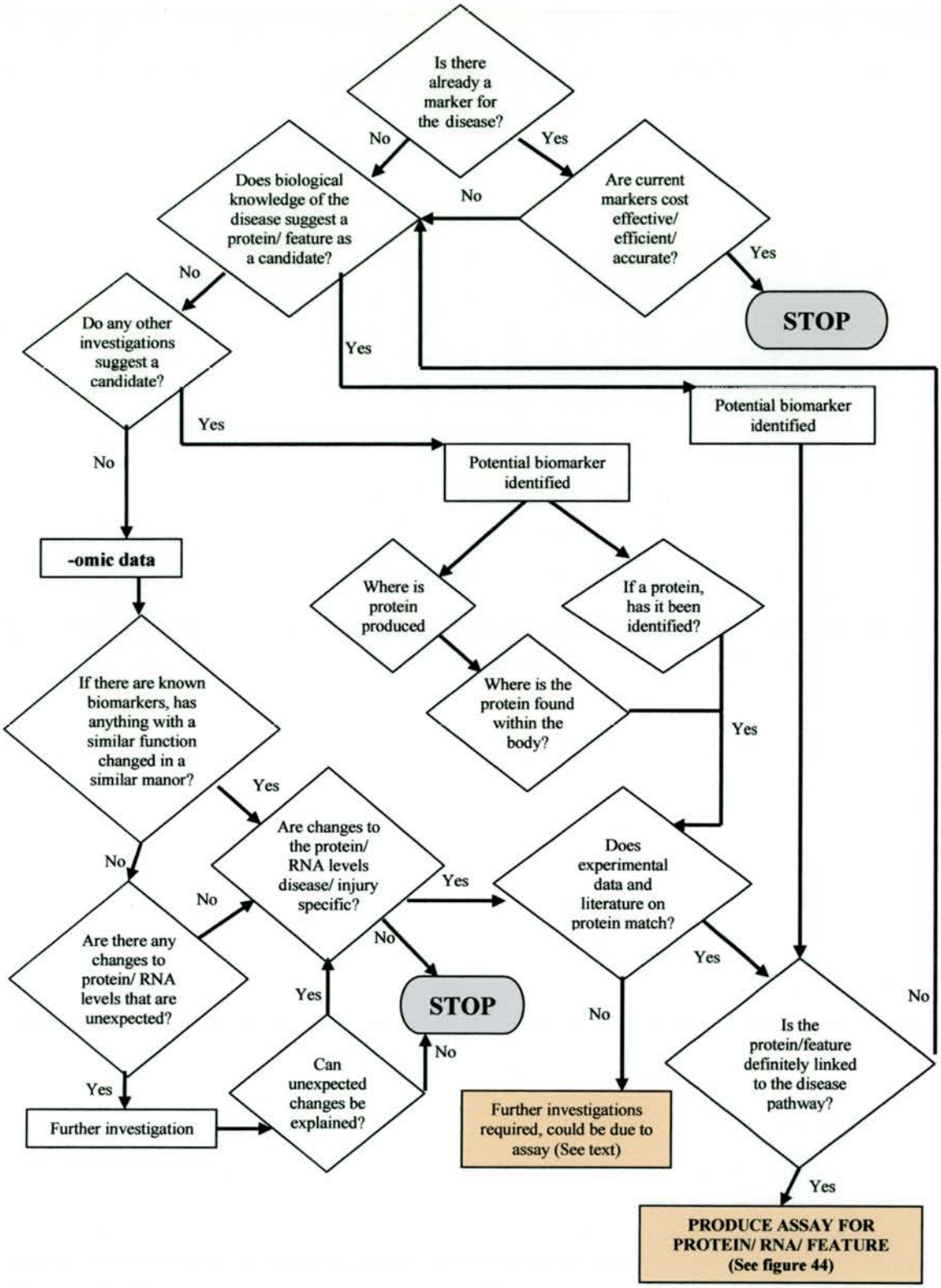


Figure 43: Overview of proposed method of identifying a candidate for investigation as a biomarker. Salmon boxes = see further figures/text

to functional/ physiological changes within the body difficult. The antigen for the HTI_{56} antibody has still to be identified (Dobbs,1999), meaning it has similar difficulties to the RTI_{40} antibody. The functions of aquaporin 5 have been well characterised, however, the protein is also found in bronchial epithelial cells (Nielson, 1997) so any changes in the expression of the protein can not be contributed entirely to changes in the levels of AETI cells. The AETII cell associate proteins mentioned above also have similar difficulties as the AETI associate proteins. This therefore means that the current markers can not be considered to be accurate. Due to the known inaccuracies/limitations of existing markers of the alveolar epithelium investigations into new markers would be appropriate.

The actual numbers of AETI and AETII cells lining the alveoli would be the ideal feature to examine in lung diseases. However getting samples of the lung from living patents to investigate is not recommended and therefore direct looking at the lung surface is not feasible as a method of predicting the likely outcome of lung injury. Investigations into phenotyping the alveolar epithelium using immunohistochemistry within our research group produced new two antibodies which bind to the alveolar epithelium. The MMC4 antibody had already been shown to bind to the apical surface of AETII cells and Clara cells and the MMC6 antibody was suspected to recognise a antigen associated with AETI cells. Both these antibodies were therefore considered as potential biomarkers during this thesis. Once a potential biomarker is identified further characterisation is required to find which cell types express the biomarker, where the active protein is located, what the protein is and what the functions of the protein are. Information from literature searches should be used at this stage to ensure all possible functions/ locations of the biomarker are investigated as assays are often limited to one functional characteristic of a protein, and also do not recognise all forms of a protein.

As already discussed, the MMC4 antigen was found to be aminopeptidase N via mass spectrometry and biochemical analysis. Previous work by Boylan *et al* (2001) had already shown the distribution of MMC4 antibody biding throughout the rat. Once the antigen was known to be aminopeptidase N, a comparison of the distribution of

aminopeptidase N found in the literature and by Boylan *et al* experimentally was carried out. This found that aminopeptidase N was expected to be expressed by macrophages, which Boylan *et al* had found to be negative for MMC4 antigen expression via immunohistochemical analysis of frozen lung sections (Boylan, 2001). Further investigation into MMC4 reactivity via ELISA based dot blot and immunohistochemistry found that a small percentage of macrophages were positive for aminopeptidase N, suggesting that the original screening assay was not rigorous enough. The MMC6 antigen was found to be expressed by AETI cells exclusively. However, I was unable to purify the antigen via its affinity to the MMC6 antibody attached to Affymetrix beads. The possibility of the MMC6 antibody being a biomarker is therefore still unanswered.

Up and down regulated genes were compared to the change in expression of genes known to be associated with AETI/AETII cells. Most known AETII cells associated genes did not change in expression, therefore, this model is not appropriate to investigate potential AETII cell markers. Some AETI cell associated genes did decrease in expression significantly. Genes with a similar decrease in expression were therefore investigated for association with AETI cells. At the same time, down-regulated genes were screened to check for 'unusual' results, which could cast doubt on this method for identifying AETI cell associated markers. Gene expression of CD9, a protein known to be associated with macrophages, was noticed to decrease. This was not expected as macrophage levels had increased within the lung. Further investigation however showed that CD9 is also expressed by AETI cells and the decrease in this cell type could have been the cause of the decrease in CD9 expression. The remaining genes with decreased expression comparable to known AETI cell associated proteins were investigated to find potential novel markers. Investigations are still ongoing to see if any of these genes are useful as markers of AETI cells.

9.1.2 Assay development and compare and contrast to existing biomarkers

A biological marker of injury/ disease is only as good as the assay that is used to detect it. A realisable system of producing an assay is required to a) keep the costs of producing the assay to a minimum b) to ensure that the assay will produce statistically reproducible accurate results. Figure 44 shows an overview of my proposed method of assay development.

If there already and assay for the biomarker available this should be considered first as this would drastically reduce the cost of developing a new assay. If the biomarker being examined is a physiological feature, such as in cancers, then imaging techniques would give the most obvious starting point for assays.

One consideration, which is often missed out in other proposed methods of assay development (Hammond, 2002), is the comparison between the newly developed assay and those already on market with regards to how invasive the collecting of samples for the assay/ the assay itself is. Highly invasive methods of obtaining samples (such as biopsy) are less popular now. If an alternative less/non-invasive technique could be developed then this is more likely to gain FDA approval than producing an assay for a disease that already has a non- invasive method of identification/prognosis.

In the case of the MMC4 and MMC6 antibodies that I was investigating, both had been designed for immunohistochemical investigations on frozen lung sections. This would require lung biopsy to obtain samples. ELISA based dot blots had already been used to measure the levels of MMC4 reactivity in tissues within our laboratory group. This was extended to measuring the levels of MMC4 and MMC6 reactivity within the BAL fluids recovered from the lungs. I have shown that this technique is successful in recognising changes in the lung caused by *S. aureus* instillation into the lungs of rats. ELISA based dot blot measurements of the MMC4 antigen were however shown to be unreliable (it

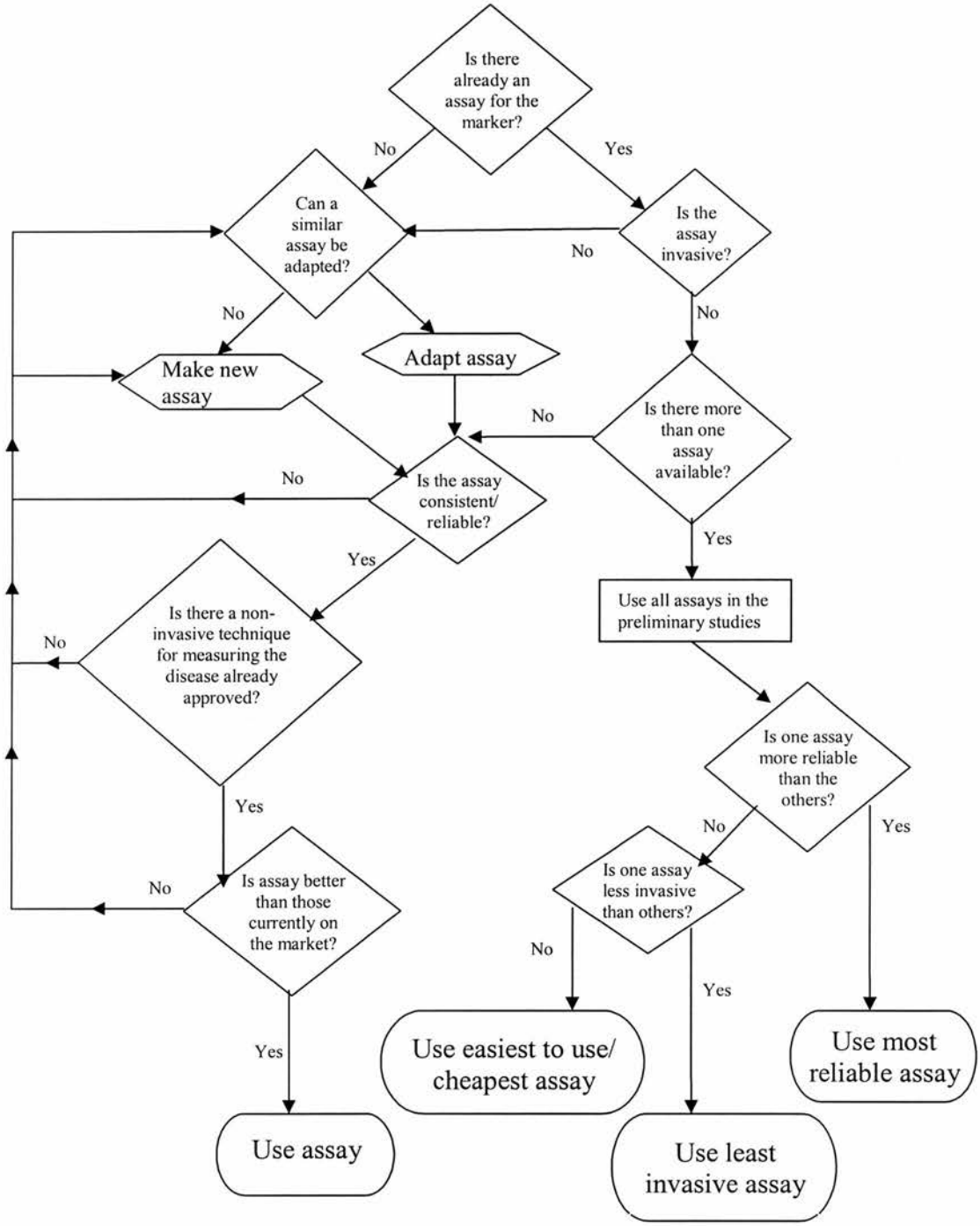


Figure 44: Overview of proposed method of choosing an assay system for a new biological marker.

was possible to purify the antigen from the liver which the ELISA based dot blot assay had shown to be negative for the antigen). If the MMC4 antibody had continued to be useful as a biomarker after the antigen was identified a different assay system would have had to be developed as current assay was not accurate enough.

In some cases more than one assay may be already available for a biomarker. In these cases I recommend that all available assays are used initially. This will enable a comparison of which assay is the most reliable to be carried out, improving the quality of the biomarker. If two or more assays are equally accurate then other factors, such as how invasive sample collection is, cost to produce the assay, ease of performing the assay should be considered. In my field of lung injury, sample collection is via biopsy (rarely), BAL fluid collection or by exhaled breath condensation. As biopsy should be avoided if possible and samples from exhaled breath are not always possible to acquire (such as from patients with reduced lung capacity), currently I feel that BAL fluid analysis is the best method for obtaining samples to use for diagnostic and prognostic analysis.

Once an assay system has been selected conditions (such as type of specimens (fresh, frozen or fixed), physical conditions (pH, temperature, antibody concentrations, reagents)) and a scoring system for the assay need to be developed. Most assays developed do not have just a positive and negative result, but rather are measured on a scoring system of how much the biomarker is present. A 'cut off' point is therefore required from positive and negative results, with a 'at risk'/'result inconclusive' area in between. Detailed statistical analysis (such as that described by Weir *et al* (2006)) is required to ensure that these 'cut off' points are accurately placed.

9.1.3 Biomarker validation and determining target populations

Once a potential biomarker has been identified and an assay system developed, the biomarker needs to be validated. This includes the following tests:

(a) Diseased vs. control samples
(b) Controls from different ethnic groups
(c) Diseased from different ethnic groups
(d) Diseased vs. those with similar symptoms
(e) Diseased on treatment vs. no treatment (at different time points after treatment started)
(f) Diseased on medication for other illnesses

The overall bases of tests (a-c) are to confirm that the biomarker can distinguish between healthy and disease samples in all populations. (d) Shows the ability of the biomarker to differentiate between diseases showing the accuracy of the biomarker. Tests together confirm the biomarkers usefulness as a diagnostic marker. Test (e) shows the possibility of the biomarker as a prognostic biomarker. Test (f) is useful for validating both diagnostic and prognostic biomarkers as it shows how reliable the results are when there are multiple diseases, with multiple treatments. However, picking samples for (f) can be difficult as not only do the diseases have to be relevant to each other, but enough patients are required to ensure that statistical tests can be carried out. For example groups that should be tested for bacterial biomarkers should include people with suppressed immuno systems (either through drugs or illness). Those who are prone to infections for other reasons (I.E. in cystic fibrosis patients and asthmatics) should also be tested.

9.2 Characterisation of *S. aureus* induced pneumonia in rats

As well as answering the main questions of my thesis, regarding alveolar epithelial biomarker development, I have also further characterised the *S. aureus* model on pneumonia used within our laboratory group.

4 hours post inoculation data had not previously been collected. I have shown that although there is no significant damage to epithelium at this time point there is a large

influx of neutrophils to the site of infection. This influx of neutrophils could therefore be a contributing factor to the injury to the alveolar epithelial surface. At 24 hours post instillation there are still elevated levels of neutrophils within the lung, but not as high as at 4 hours, suggesting that production of molecules which are chemotactic with regards to neutrophils has decreased. By 72 hours there are few neutrophils remaining within the lungs; however, there are a large number of macrophages/monocytes, possibly removing damaged neutrophils/cells from the lung.

Global gene analysis at 72 hours post instillation suggests that the endothelium could also be damaged, due the large number of endothelial cell associated genes being down regulated. Previous biochemical analysis preformed by G. Clegg *et al* had found no damage to the endothelium (measured as presence of cfu into the blood and CC10 levels in the BAL). However the decrease in expression of genes that are endothelial associated does cast doubt on these previous findings.

10. Further work

Although I still have questions that I would like to answer with regards to all three of my research areas, these are all surplus to the problem I set out to solve in my thesis. The characterisation of the cellular and molecular response to injury within the lung still requires further investigation and I feel that biomarkers have a strong role within this area, both in the clinical setting and as a research tool. I would therefore like to see more research into cell associated proteins as a method of identifying candidates for biomarkers. I would also like more collaboration between researches using proteomic and genomic platforms as a method of identifying markers that area commonly increased in lung injury as well as those that are disease specific.

Currently the FDA produces guidelines into how a biomarker should be validated. I would welcome a structured approach to biomarker development and validation and feel that a framework, such as the one I have suggested, would make it easier to identify gaps in knowledge required in drug approval applications. This would be due to oversights/missing information with regards to the surrogate endpoint being easier to identify if there are strong guidelines/recommendations detailing exactly what information is the base line requirement for a characteristic to qualify as a surrogate endpoint.

However, testing of this framework is required with a larger range of types of biomarkers and number of biomarkers before it could be widely accepted.

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Appendix i

Computer programs used in the analysis of Affymetrix data.

On the Affymetrix U34 gene chips there were11 exact mach probes and 11 one base mismatch probes for each gene. Gene chips were prepared as described previously (see 6.1.1). Te U34 chips were scanned and the raw data was analysed in MicroArray Suite by Affymetrix to assign a value to each probe set.

Affymetrix MircoArray Suite

He Affymetrix program first of all reads the outside of the scanned chip where the positive and negative controls form a checker board effect. This allows the software to align a grid over the chip so that it can calculate which 'spots' are assigned to which gene. The right hand side of figure 10 shows part of these control probes that were used to align the grid. Readings from the centre of each spot (8x8 pixels) are then used to give a positive reading of each spot. The background reading for the chip (calculated as the average of the lowest 2% of the spot readings on the whole chip) is subtracted from all 'positive' readings This would allow for any variation between chips with regards to the hybridization of the phycoerithin stain, or variation in washing off the excess stain. The program also measures the variation between pixel intensity for each spot, reducing the effect of any minor changes in hybridization.

After 'normalization' across the chip the 11 exact match and 11 one base mismatch readings were collated to give a single reading for the intensity of the gene. As well as this reading the algorithm also gives a probability of this intensity being a true reading (based on the variation in the 22 spot readings).

The data from the chips was then loaded into GeneSpring.

GeneSpring

GeneSpring is a software package widely used in conjunction with gene expression profiling. This is due to the programs ability to filter large quantities of data via a wide range of parameters which the research can set. The program also allows the researcher to perform a wide range of statistical analysis on the data using one /two way ANOVAs, Turkey and Student-Newman and Keults post hoc tests. The software also allows for analysis of false discovery rates and multiple testing corrections. Despite all the statistical analysis that can preformed using GeneSpring most papers citing GeneSpring have used the pictorial representations of the data that can be obtained.

This package allows the researcher to view the genes intensity values as a colour ranging from blue (lowest) to red (highest). Pictorial representation, such as figure 11, of the genes can show multiple sets of information at once. For example, figure 11 shows the intensity of the gene as well as the fold change in the gene. Other pictorial representation that the software can perform include looking at all known gene that encode for a specific pathway and showing if the increase/ decrease in expression, again using the colour to show change. This is also useful if multiple repeats of the same experiment were analysed as it shows any variation between repeats easily.

Using GeneSpring it was possible to isolate a list of genes that have either increase or decreased greater than 2 fold after treatment. However I found that when I compared this to the list I had in excel of genes that had increased/decrease 2 fold these lists were not identical. As it is possible to see the raw numeric data in excel and not in GeneSpring, this finding raised questions as to how GeneSpring was processing the data.

The majority of GeneSpring's data analysis works on the basis of fold change, which has also been used in this thesis. This carries the assumption that a change in expression of 10 to 100 would have a similar biological impact as a change from 500 to 5000. Fold changes however do not work accurately when looking for genes that are either initial

turned off and then turned on by the treatment, or visa versa; or does fold change work well on genes that either start or end with very low expression. To overcome these problems in analysis the data tall genes with an mRNA reading of less than 500 units absorbance were discounted in my analysis. This does however mean that I may have missed out any genes that were switched on or off.

Name	Function	Produced by	Cellular location	Fold change	P=	F=	References
Receptor of vascular intestinal polypeptide	Neurotransmission, endocrine functions, regulation of cell proliferation, suppresses apoptosis	Neurons, Lungs		8.04	0.0002	0.47	Filippatos (2001) Said (2000)
Vascular endothelial growth factor	Alveoli formation, capillary network formation	Endothelial cells	Secreted	6.77 5.45 3.26	0.0002 0.0001 $3x10^{-5}$	0.30 0.58 0.51	Kumar (2005)
Flavin-containing monooxygenase 1 (FMO-1)	Catalyses FAD-, NADPH- and O_2-dependent oxidisation	Most cell types	Intracellular	6.69	0.002	0.11	Lattard (2001)
Caveolin	Structural protein of calveoli	Fibroblasts, endothelial cells, adipocytes, muscle cells	Plasma membrane and calveoli	6.54	0.0003	0.08	Razani (2001)
Carbonic anhydrase IV	Catalyses the hydration/dehydration of CO_2/HCO_3^-	Lung, kidney, pancreas, heart	Ectopeptide of the plasma membrane	6.53 4.53	0.0033 0.0045	0.05 0.09	Alvarez (2003)
Calcitonin receptor-like receptor (CRLR)	Receptor for vasodilator peptides: calcitonin gene-related peptide and adrenomedullin	Endothelial cells	Plasma membrane	5.75	0.0003	0.99	Pondel (2000) Chauhan (2005) Cueille (2005)
Vascular alpha-actin	Muscle contraction	Smooth muscle cells	Cytoplasmic	5.34 3.04	0.0021 0.0088	0.22 0.10	Strauch (1997)
Periaxin	Stabilizing the myelin sheath	Schwann cells	Plasma membrane	5.26	0.0005	0.07	Straub (2003) Parkinson (2003)

Name	Function	Produced by	Cellular location	Fold change	P=	F=	References
Tissue inhibitor of metalloproteinase 3 (TIMP3)	Inhibits inflammation	Most cell types	Secreted	5.17	0.0132	0.22	Black (2004), Federici (2005)
Kininase II (also called angiotensin converting enzyme (ACE))	Blood pressure, water and salt metabolism	Vascular endothelial of the lung, intestitinal and renal tubular epithelial cells neuroepithelial cells, monocytes, macrophages, T-lymphoctes	Ectopeptide of the plasma membrane also secreated	5.17 2.87	0.0001 0.064	0.77 0.15	Oliveri (2001) Van der Velden (1999) Costerrousse (1994)
Bone morphogenetic protein 6 (BMP6)	Chemotaxis, proliferation, differentiation	Bone, vascular smooth muscle cells	Secreted	4.99 3.8 3.4	0.0005 0.0069 0.0083	0.40 0.30 0.07	Wozney (1989), West (2005)
Thrombomodulin	Anti-coagulant, anti-fibrinolytic and anti-inflammatory	Vascular and lymphatic endothelium	Plasma membrane and secreted	4.84	0.0005	0.26	Hanly (2005)
G-protein-coupled receptor (CXCR2)	IL-8 receptor		Plasma membrane	4.81	0.0013	0.19	Ishii (2003)
WAO four-disulfide core domain protein (ps20)	Growth inhibition in the embryo	Mesenchyme cells, smooth muscle cells	Secreted	4.69	0.0002	0.24	Larsen (1998)

Name	Function	Produced by	Cellular location	Fold change	P=	F=	Reference
SOD	Antioxidant enzyme (change could be due to heparin)	Alevolar cells, fibroblasts, vascular smooth muscle cells, sertoli cells	Secreted	4.64	0.0002	0.61	Van der Vliet (2000) Fattman (2003) Zelko (2002)
Plasma glutathione peroxide (EC 1.11.1.9)	Antioxidant enxyme	Lungs, epithelial cells	Secreted	4.49	0.0001	0.20	Van der Vliet (2000) Maccarrone (2004)
Serine-threonine kinase receptor type I	Signal transducers, receptors for TGF-β and BMPs	Most cell types	Membrane	4.37	$3x10^{-6}$	0.82	Josso (1997)
Tensin	Links actin filaments to intergrin receptors	Most cell types	Focal adhesions in plasma membranes	4.3	0.0087	0.21	Lo (2004)
Peptidylglycine alpha-amidating monooxygenase (PAM)	Activation of peptides (usually hormones) via amidation	Atrial myocytes, endocrine cells, neuron airway epithelial cells, endothelial cells, condrocytes, bronchiolar cartilage, smooth muscle cells	Intracellular	4.10 3.09	0.0013 0.0001	0.65 0.33	Prigge (2000)

Name	Function	Produced by	Cellular location	Fold change	P=	F=	Reference
Cytochrome P-450 isozyme 5 (P450 IVB2)	Catalyzes activation of lung toxins	Lung	Microsomes	4.04	0.0004	0.39	Carr (2003)
Ssecks 322	Tumour suppressor, regulates mitogenesis, inflammation response development and differentiation	Most cell types	Actin cytoskeleton, plasma membrane	4.04	0.0004	0.39	Gelman (2002) Streb (2005)
NADH-cytochrome b5 reductase	Desaturation of fatty acids	Most cell types	microsomes	3.94 2.75 2.72	0.0012 0.0025 0.0006	0.70 0.85 0.09	Nishida (1995)
Platelet-endothelial cell adhesion molecule 1 (CD31, PECAM-1)	Cell adhesion	Lungs, endothelial cells	Cell membrane	3.86	0.0377	0.08	Muro (2005)
Fibroblast growth factor receptor 1 beta-isoform	Regulation of cell growth, differentiation, migration and survival	Most cell types	Transmembrane	3.84	0.0005	0.16	Burke (1998)
Aldehyde dehydrogenase (ALDH)	Oxides aldehydes for removal from the body	Bronchiolar cells	Cytoplasm	3.79	0.0008	0.07	Vasiliou (2000)

Name	Function	Produced by	Cellular location	Fold change	P=	F=	References
Cholesterol esterase	Hydrolyzes various glycerols	Adipose tissue, steroidogenic tissue, cardiac muscle, skeletal muscle, macrophages, islets	Intracellular	3.64	0.0094	0.47	Kraemer (2002)
Cyclin D1	Cell proliferation	Most cell types	Intracellular	3.58	0.0010	0.24	Huang (1999)
Carboxylesterase (Es-HVEL)	Detoxify organic-phoshorous compounds	Airway epithelial cells	Endoplasmic reticulum	3.36 2.62	0.0022 0.0043	0.27 0.05	Medda (1992) Wallace (1999)
Connexin 40 (GJA5)	Allow electrical and chemical signals to transfer between cells	Vascular endothelium	Gap junctions	3.35	0.0011	0.10	Hill (2002)
SM22 (transgelin, WS3-10, p27)	Binds to actin cell cycle inhibitor	Fibroblasts, smooth muscle cells	25kDa form membrane associated, 22kDa form cytosolic	3.33 2.66	0.0034 0.0096	0.08 0.65	Morgan (2001) Camoretti-Mercado (1998) Toyoshima (1994)
Lipoprotein lipase	Converts lipoproteins to fatty acids, aids lipoprotein uptake into cells	Endothelial cells, alveolar macrophages, possibly AETII cells	Plasma membrane	3.24	$3x10^{-5}$	0.31	Ryan (2002)
Monoamine oxidase A	Hydrolyses metabolites, respiration	Most cell types	mitochondria	3.32	0.0008	0.56	Cohen (1997) Schnaitman (1967)

Name	Function	Produced by	Cellular location	Fold change	P=	F=	Reference
VGR	Morphogenesis	Oocytes, epithelial cells, cartilage	secreted	3.19	0.0001	0.45	Jones (1991)
Glutathione S-transferase Yb	detoxification	ependymal cells,	secreted	3.07 2.88 2.68 2.46	$8x10^{-5}$ 0.0027 0.0005 $3x10^{-5}$	0.26 0.58 0.40 0.53	Abramovity (1988), Thyagaraju (2005)
Tryosine phosphatase	Phosphorylation	Most, high expression in neurones	cellular	3.04 2.90	0.0130 0.0029	0.81 0.46	Zhang (2005), Stoker (1998)
Myelin protein SR13 (homolg to GAS3)	Growth arrest	Sciatic nerve		2.97	$1x10^{-5}$	0.62	
Alpha B-crystallin		Ocular lens tissue		2.90 2.39	0.0005 0.0058	0.58 0.93	
Channel integral membrane protein 28 (CHIP28)	Water channel (AQP-1)	Proximal tubule kidney, AETII cells	membrane	2.74	$4x10^{-5}$	0.44	Verkman (1995) Wintour (1997) Fehrenbach (2001)
Developmentally-regulated cardiac factor (DRCF-5)	UNKNOWN	Neonatal heart, brain and kidneys	Unknown	2.74	0.0015	0.17	Pak (1999)
Developmentally-regulated intestinal protein (OCI-5)	Induces apoptosis	Intestine, endoderm, mesenchyme cells	Cell surface	2.73	0.0001	0.88	Gonzalez (1998) Madeline (1997)

Name	Function	Produced by	Cellular Location	Fold Change	P=	F=	References
Epithelial membrane protein 1	Component of compact myelin	Lung, ileum, rectum, colon, Schwann cells	Membrane bound	2.71	0.0020	0.85	Lobsiger (1996) Taylor (1995)
Endothelin-converting enzyme	Activates endothelin (needed for blood vessel formation)	Endothelial cells	ECE1a and c are membrane bound ectoenzymes, ECE1b is intracellular	2.70	0.0017	0.24	Ahmed (2000)
BTE binding protein	Transcription factor	Most cell types	Nuclear	2.67	0.0277	0.58	Zhang (2001)
Tissue-type plasminogen activator (t-PA)	Prevents intravascular thrombosis	Endothelial cells	Secreted	2.63	0.0041	0.10	Oliver (2005)
Hyaluronidase (Hyal2)	Breaks down hyaluronic acid, which maintains proteins within the extracellular matrix, angiogenesis (?)	Most cell types	Secreted	2.60	0.0437	0.08	Liu (1996)
FGF receptor-1	Proliferation, differentiation	Epithelial cells of large airway	Plasma membrane	2.54	0.0001	0.89	Powell (1998)
Connexin (CXN-37)	Allows electrical and chemical transfer between cells	Vascular endothelium, bronchioles, and trachea smooth muscle cells	Gap junctions	2.53	0.0122	0.36	Hill (2002) Nakamura (1999)

Name	Function	Produced by	Cellular location	Fold change	P=	F=	References
Prion protein	Replication of prions development,	Brain	Secreted	2.49	0.0126	0.29	Prusiner (1998)
Lysyl oxidase	Crosslinks cologne and elastin in the extracellular matrix	Fibrogenic cells	Secreted	2.47 2.37	0.0119 0.0079	0.63 0.56	Kagan (2003)
Protein kinase Cδ-binding protein	Growth inhibition, differentiation, apoptosis, tumour suppression	Most cell types	Transmembrane	2.45	$7x10^{-6}$	0.68	Gschwendt (1999)
CD9	Differentiation, cell adhesion	Macrophages, oocytes, giant cells of the lung, bronchiolar epithelium, smooth muscle cells	Plasma membrane	2.41	$2x10^{-5}$	0.79	Nakamura (2001) Takeda (2003)
Alpha-tubulin	Cytoskeleton	All cell types	Mitochondria, nucleus, cytoplasmic	2.37	0.0006	0.78	Ren (2003)
Tropoelastin				2.37	0.0012	0.93	
Minoxidil sulfotransferase	Metabolism of toxins/carinogens	Liver	Cytosolic	2.37 2.10	0.0427 0.0102	0.06 0.47	Yshinari (2001)
Amyloid precursor-like protein 2	Unknown, thought to be immune related as associates with HMC class I molecules	All cell types	Cell surface	2.30	0.0070	0.07	Morris (2004) Morris (2003)

Name	Function	Produced by	Cellular location	Fold change	P=	F=	Reference
RB109 (Brain specific protein) (ADAM12)	Cell/cell and cell/ matrix interaction, therefore assumed roles in cell adhesion, migration, survival and proliferation	Most cell types	Cell membrane	2.27 2.03	0.0005 0.0004	0.81 0.77	Iba (1999)
Agrin	Formation of neuromuscular synapses	Motor neurons, synaptic Schwann cells	Secreted	2.26 2.20	0.0050 0.008	0.14 0.33	Martin (2003) Fallon (1994)
Recetor for advanced glycosylation end produces (RAGE)	Immunoglobulin family, activates NF-κB	Endothelial cells, AETII cells	Cell membrane	2.22	0.0006	0.08	Fehrenbach (1998), Shirasawa (2004) Katsuoka (1997) Ding (2005) Bierhaus (2005)
Amyloidogenic glycoprotein (rAG)	Linked to amyloid deposits in Alzheimer's disease (how is unknown)	Brain (neurons)		2.22 2.04	0.0008 0.0043	0.61 0.76	Shivers (1988)
Eosinophil cationic protein	Anti-parasitic, antibacterial, neurotoxic and ribonuclease properties	Eosinophils	Secreted	2.19	0.0030	0.42	Rosenberg (1995)
Vesicle associated membrane protein (VAMP-2)	Transports AQP2 from cytoplasm to apical membrane	Kidney	Cytoplasm and cell membrane	2.17	0.0004	0.05	Wintour (1997)

Name	Function	Produced by	Cellular location	Fold change	P=	F=	References
Moesin	Cell motility (binds actin) cell polarity	Most cell types	Cytosolic	2.13	0.0436	0.06	Doevendans (2002) Miller (2003)
RGC-32	Possibly cell cycle promotion via regulation of $p34^{CDC2}$	Smooth muscle cells		2.12	0.0260	0.71	Badea (2002)
Cytochrome P450b	Catalyzes activation of lung toxins	Clara cells, AETI cells	Microsomes	2.12 2.06	0.0010 0.0016	0.43 0.54	Aida (1992) Carr (2003)
Aquaporin 5 (AQP-5)	Water transport	AETI cells	Plasma membrane	2.10	0.0112	0.08	Funaki (1998)
D6.1 A	Cell motility, liver metastasis, related to CD9	Liver	Cell membrane	2.08	0.0003	0.05	Herlevsen (2003)
Tumor-suppressive gene	(p53) transcription factor,	Most cell types	nuclear	2.03	0.0003	0.62	Vogelstein (1992)
Myosin I heavy chain	Differentiation, cell division, motility	Most cell types	Cytoplasm	2.02	0.0054	0.28	Kiehart (1990)
SPARC	Facilitates cell-matrix interactions, tissue remodelling, cell movement, proliferation	Blood, smooth muscle cells, (endothelium?), gut, kidney	Secreted	2.01	0.0230	0.19	Lane (1994)

Appendix iii

Agilent Technologies South Queensferry West Lothian EH30 9TG	Ambion Ermine Business Park Spitfire Close Huntingdon Cambridgeshire PE29 6XY
Applied Biosystems Lingley House 120 Birchwood Boulevard	BD Biosciences 21 Between Towns Road Cowley Oxford OX4 3LY
Bechman Oakley Court Kingsmean Business Park London Road High Wycombe Bucks HP11 1JU	BioRad Laboratories Ltd BioRad House Maxted Road Hemel Hempstead Hertfordshire HP2 7DX
Calbiochem c/o Merck Biosciences Ltd Padge Road Beeston Nottingham NG9 2JR	Fisher Scientific Bishop Meadow Road Loughborough Leicestershire LE11 5RG
GE Healthcare Amersham Biosciences Ltd Amersham Place Little Chalfont Bucks HP1 9NA	Harlow UK
Invetrogen Ltd 3 Fountain Drive Inchinnan Business Park Paisley PA4 9RF	Millipore (UK) Units 3+5 The Courtyards Hatters Lane Watford WD18 8YH

MRX Dynatech Medical Products West Sussex	NUNC c/o Fisher Scientific UK Ltd Bishop Meadow Road Loughborough LE11 5RG
OmniViz Two Clock Tower Place Suite 600 Maynard MA 01754	Pierce Biotechnology Inc Rockford IL USA
Promega Delta House Chilworth Science Park Southampton SO16 7NS	Qiagen Qiagen House Fleming Way Crawley W. Sussex RH10 9NQ
Roche Diagnostics Ltd Bell Lane Lewes E. Sussex BN7 1RG	Rockland Immunochemicals Inc PO Box 326 Gilbertsville PA19525
Schleicher & Scheuell c/o Whatman International Ltd Springfield Mill Maidstone Kent ME14 2LE	Serotech 22 Bankside Station Approach Kidlington Oxford OX5 1JE
Sigma Aldrich Company Econ Avenue Heatherhouse Industrial Estate Irvine KA12 8NB	Stratech Scientific Ltd Unit 4 Northfirld Business Park Northfield Road Soham Cambridgeshire CB7 5UE

Appendix vi

European Respiratory Society Conference 2004 (Glasgow) Poster and oral presentation. *Eur Respir J;* 24: suppl. 48, 719s.

The type II and Clara cell specific monoclonal antibody (MMC4) recognises aminopeptidase N

Linda Franklin[1], Andrew D Cronshaw[2], David J Harrison[3], and Mary C McElroy[1].

[1] MRC Center for Inflammation Research, University of Edinburgh, Edinburgh. [2] Edinburgh Protein Interaction Centre, University of Edinburgh, Edinburgh and [3] Division of Pathology, University of Edinburgh, Edinburgh.

Abstract:

We have previously described a new monoclonal antibody (MAb), MMC4, which specifically recognises an integral membrane protein localised on the apical surface of rat alveolar epithelial type II and clara cells in the lung. It has also been shown that expression of MMC4 antigen may be associated with type II cells in transition to type I cells (G. Clegg *et al.*, Thorax 2003; 58 (Supp3): 18). The objective of this study was to determine the identity of the MMC4 protein.

The MMC4 antigen was immunoprecipitated from octaethyleneglycol mono-*n*-dodecly ether ($C_{12}E_8$) soluble fractions of lung and kidney homogenates using magnetic beads bound with the MMC4 MAb. One non-IgG associated protein was obtained, which was at a slightly different molecular weight in each tissue (Table 1)(putative MMC4 antigen). The putative antigen from each organ was trypsin digested and the fragments sequenced by MALDI-TOF mass spectrometry (MS) and Tandem MS. In all organs the top 'hit' was for aminopeptidase N (APN) (MOWSE scores in table 1). The MMC4 MAb also recognises a commercial purified rat APN protein. Our data suggests that the lung may have specific post-translational modifications to APN and that APN may be specifically involved in the transition from type II to type I alveolar epithelial cells.

Organ	Lung	Kidney	Liver	Thymus
Molecular weight (kDa)	123	126	113	108
MOWSE score for APN	1.7e16	5e19	3.6e17	4.2e16

The type II and clara cell specific monoclonal antibody (MMC4) recognises aminopeptidase N (APN)

L. Franklin[1], A.D. Cronshaw[2], D.J. Harrison[3], M.C. McElroy[1]

[1] MRC Center for Inflammation Research, University of Edinburgh, [2] Edinburgh Protein Interaction Centre, University of Edinburgh, [3] Division of Pathology, University of Edinburgh

Introduction

We have previously described a new monoclonal antibody (MAb), MMC4, which specifically recognizes an integral membrane protein localized on the apical surface of rat alveolar epithelial type II and clara cells in the lung (Boylan *et al*, 2000). It has also been shown that expression of MMC4 antigen may be associated with type II cells in transition to type I cells in vitro (G. Clegg *et al*, 2003) and in vivo in a rat model of alveolar epithelial repair (McElroy *et al*, 2002). The MMC4 MAb has also been shown to react with the proximal tubule epithelial cells in the kidney and micro villi of the small intestine (Boylan *et al*, 2000).

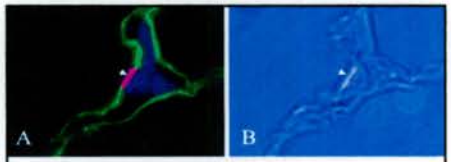


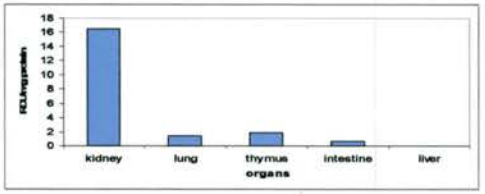
Figure 1: MMC4 MAb staining of a Type II alveolar epithelial cell (red) surrounded by type I cells (green) (picture from Boylan *et al*, 2000)

Aim: To purify and identify the MMC4 antigen.

Methods:

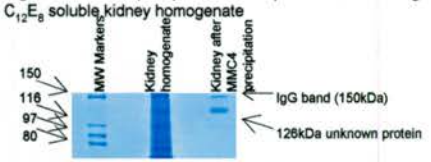
- Lung, kidney, intestine, thymus and liver homogenates were solubilised with $C_{12}E_8$ (1mg protein: 5mg detergent)
- $C_{12}E_8$ soluble homogenates were incubated with Pan IgG Dynabeads, which had been pre-coated with the MMC4 MAb
- Immuno-precipitated proteins were removed from the beads and run on SDS-PAGE gels
- Protein bands were cut out of the gel and analyzed via trypsin digestion and MALDI-TOF mass spectrometry
- The protein band from the kidney was also analyzed via tandem mass spectrometry after trypsin digestion
- MMC4 MAb activity was tested against purified commercial affinity purified APM (Calbiochem) (*in vitro*)
- Ability of MMC4 MAb to inhibit APN activity was tested using an ELISA with L-Ala-pNA as the substrate
- Protein assays were carried out using a modified Lowry protocol

Figure 2: Relative levels of MMC4 in different tissues. Data shown as relative densitometry units (RDU) (n = 4) per mg protein in the organ (n ≥ 7).



- Confirmed MMC4 antigen is in the kidney, lung and other organs

Figure 3: Immuno-precipitation of the putative MMC4 antigen from $C_{12}E_8$ soluble kidney homogenate



- Single unknown protein precipitated, protein was cut out and analysed via trypsin digestion and MALDI-TOF spectrometry

Figure 4: Molecular weight and MOWSE scores for APN from all organs after Dynabead immuno-precipitation.

Organ	Lung	Kidney	Liver	Thymus
Molecular weight (kDa)	123	126	113	108
MOWSE score for aminopeptidase N	1.7e16	5e19	3.6e17	4.2e16

Figure 5: Unknown kidney protein's peptide fragments that were found by tandem mass spectrometry. All peptides shown are from Aminopeptidase N

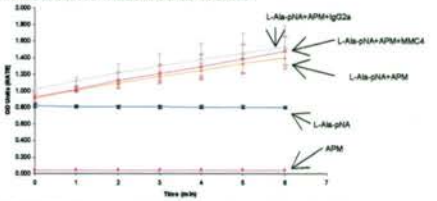
Sequence	MOWSE score
(R) SALACSNEVWILNR	4.1e6
(R) AQIIHDSFNLASAGK	8.1e6
(R) ALGDTPAPNIDTTELVER	4.1e6
(R) KIQNQLQTDLSVIPVINR	3.8e5
(K) DLIVLNDVYR	1.9e6
(R) YLSYTLNPDYIR	9.1e5

- Tandem and MALDI-TOF mass spectrometry results agreed that the unknown protein was APN

Figure 6: MMC4 antibody bound to various concentrations of commercial purified APM via ELISA based dot blot

- An antigen for MMC4 MAb is APN

Figure 7: ELISA assay of functional inhibition of APM by MMC4 MAb. Absorbance measured at 405nm.



- MMC4 MAb does not prevent the cleavage of L-Ala-pNA by APN

Summary:

- MMC4 antigen is aminopeptidase N (also called APM and CD13)
- APN has different molecular weights in different tissues
- MMC4 antibody does not inhibit the activity of APM on the substrate L-Ala-pNA

Discussion:

- Our finding confirms previous finding that APN is found on AETII cells (Funkhouser *et al*, 1991)
- Role of APN in Type II cells is unknown
- In the repairing lungs MMC4 and RTI40 (a Type I cell marker) are co-expressed on the same cell, the same phenomenon is found in vitro when isolated type II cells become type I cell-like (G. Clegg *et al*, 2003)
- We are planning to determine if APN is required for the trans-differentiation of alveolar type II to alveolar type I cells *in vitro*.

References:

G. Boylan *et al.*, Am. J. Physiology 2000; 280(6): L1318-L1326

G. Clegg *et al.*, Thorax 2003; 58(Supp3):18

J. Funkhouser *et al.*, Am. J. Physiology 1991; 260(4): L274-279

M.C. McElroy *et al.*, Infect. Immunology 2002; 70(7): 3865-3873

European Respiratory Society Conference 2004 (Glasgow) Poster presentation

Eur Respir J; 24: suppl. 48,106s.

Gene expression analysis of lungs following *Staphylococcus aureus* induced acute lung injury

Linda Franklin[1], Paul Dickinson[2], David Harrison[3] and Mary C McElroy[1]. [1] MRC Centre for Inflammation Research, University of Edinburgh, Edinburgh; [2] Scottish Centre for Genomic Technology and Informatics, University of Edinburgh, Edinburgh; [3] Division of Pathology, University of Edinburgh, Edinburgh.

Abstract:

Staphylococcus aureus is an important cause of community and hospital acquired pneumonia. We have used a previously established rat model of *S. aureus* pneumonia (McElroy, M.C. *et. al.* Infect. Immunol. 2002;70:3865-3873) to study the changes to the host's lungs during injury and repair. Previous work has shown that 72 hours post *S. aureus* inoculation RTI_{40}, an alveolar epithelial type I cell selective protein is decreased 10 fold by biochemical analysis and this decrease is associated with a decrease in the number of type I cells (unpublished data). The objective of this study was to determine whether we could identify genes specific to type I cells by investigating genes which are down-regulated after *S. aureus* injury. Injured regions of the lung were isolated and the total RNA was extracted. The RNA was amplified and analysed using an Affymetrix system. We found 99 genes were significantly up-regulated 2 fold or greater and 136 genes were significantly decreased 2 fold or greater. Within the decreased genes there were genes known to be associated with alveolar epithelial type I cells e.g. caveolin I (7 fold decrease (P=0.017, n=3)) and Aquaporin 5 (2 fold decrease (P=0.01, n=3)). There are 92 other genes that were significantly decreased between 2 and 7 fold. We are currently investigating these genes to see if they are expressed by type I alveolar epithelial cells.

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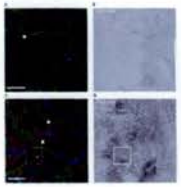
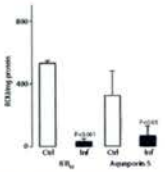


Figure 1: Type I cells staining with $RT1_{40}$ MAb (green) in control (A,B) and 72hours post *S. aureus* inoculation (C,D). The proportion of alveolar wall stained with RTI_{40} is decreased, suggesting a reduction in the number of type I cells after injury (G. Clegg *et al*, 2004)

Figure 2: Type I cell markers in control and *S. aureus* treated lungs. RTI_{40} and aquaporin 5 are both down regulated 10 fold and 3 fold respectively (G. Clegg *et al*, 2004)



Aim:

To identify genes specific to type I cells by investigating genes which are down regulated after *S. aureus* injury

Methods:

- Rats were inoculated with ≈10^8 colony forming units of *S. aureus* strain 8325-4
- 72 hours post inoculation lungs were harvested, lavaged and perfused with sterile PBS and fixed in RNAlater (Ambion)
- RNA was extracted from the injured areas of the lungs (Quigen)
- Collected RNA was amplified and run on U34 rat Affymetrix chips
- Collected data was initially processed in GeneSpring
- Shortened list of genes was manually sorted

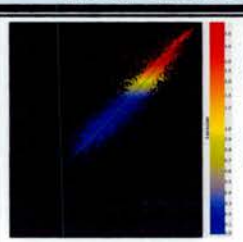


Figure 3: Change in levels of expression of all genes on the U34 chip.

Thick green line = no change

Thin green lines =2 fold change in either direction

Overall 136 genes were significantly decreased 2 fold or greater

Results:

Known type I cell selective proteins on chip

Gene name	Fold decrease	P value	Control (rfu)		*S. aureus* treated (rfu)	
			Mean	St dev	Mean	St dev
Caveolin I	7	0.017	1224.9	159.9	187.2	34.0
Aquaporin 5	2	0.01	667.6	76.7	276.1	8.18
RAGE	2	0.011	2941.2	684.8	1470.7	85.7

Figure 4: Known type I cell proteins data from the chips. P value is from Welsh corrected t-test. Rfu= relative fluorescent units

- Known type I cell selective markers all decreased 2 fold or greater
- Known type I cell selective markers control values all start above 500 rfu

Screening method 1:

- Control value > 500 (487 genes selected)
- Limited to significantly 2 fold or greater down regulated genes only
- 36 potential type I cell specific genes found, including CD9

Dahlin *et al* (2004) found 7 new type I cell markers via array analysis of type II and type I-like cells *in vitro*. 4 of these genes are on the U34 chip, 3 were found to decrease, one did not change (Figure 5)

- Under screening method 1 all four of these genes would have been excluded.

Gene name	Fold decrease	P-value	control		*S. aureus* treated	
			mean	St Dev	mean	St Dev
TIMP3	5	0.051	602.5	187.0	116.3	66.1
CryAB	3	0.002	456.7	27.6	157.3	42.8
sPLA2 IIA	1.19,	0.748	9.6	1.73	7.76	8.45
SEMA3F	2	0.054	14.06	2.515	6.73	3.29

Figure 5: Data from *S. aureus* injured data set for type I cell markers found by K. Dahlin *et al* 2004. P-values found using Welsh corrected t-test

Screening method 2:

- Control values >400 (616 genes selected)
- Fold decrease greater than 2
- 17 additional potential type I cell selective genes found

The CD9 gene was identified in screen 1 as a potential type I cell selective gene.

CD9 is not known to be present within the lung

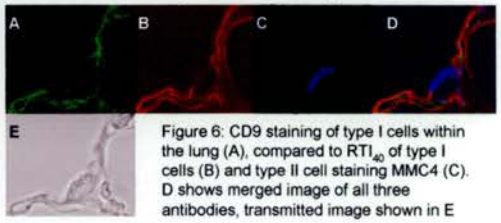


Figure 6: CD9 staining of type I cells within the lung (A), compared to RTI_{40} of type I cells (B) and type II cell staining MMC4 (C). D shows merged image of all three antibodies, transmitted image shown in E

MAb against CD9 has bound to type I cells but not type II cells within the lung.

Therefore our analysis has identified a new alveolar type I cell selective protein

Conclusion:

We have established a novel method for finding new type I cell genes and proteins

References:

G. Clegg *et al*, 2004. *submitted*

K. Dahlin *et al.*, Am J Respir Cell Mol Biol 2004, 31(3): 309-16

McElroy et al., Infect. Immunology 2002, 70(7): 3865-3873

Scottish Thoracic Society Winter Meeting 2004 (oral presentation)

The type II and Clara cell specific monoclonal antibody (MMC4) recognises aminopeptidase N

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Organ	Lung	Kidney	Liver	Thymus
Molecular weight (kDa)	123	126	113	108
MOWSE score for APN	1.7e16	5e19	3.6e17	4.2e16