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THE IMMUNOBIOLOGY OF RESPIRATORY SYNCYTIAL VIRUS INFECTION

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
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THE IMMUNOBIOLOGY OF RESPIRATORY SYNCYTIAL VIRUS INFECTION

By Imran Raza Hussain

This thesis outlines the role respiratory syncytial virus (RSV) in infantile viral bronchiolitis, it assesses the role of local airway epithelial responses to RSV infection and the role of the alveolar macrophage in the pathophysiology of RSV infection.

The localised immune in-vitro responses of epithelial cells and airway macrophages were studied; protein cytokine protein production as well as cytokine mRNA analysis was performed to investigate the innate immune response to RSV infection. The results were correlated with the infecting viral load and this was analysed by quantitative polymerase chain reaction.

A study of children during their first RSV season was performed, the nasal and peripheral blood cytokine responses during the first week of RSV infection were analysed, and those children who developed bronchiolitis were compared to those that only developed an upper respiratory infection.

The results demonstrate that RSV infection of epithelial cells can stimulate the production of pro-inflammatory cytokines and chemokines, which would recruit and stimulate incoming immune cells. There was evidence of the production of protective proteins such as the defensins and nitric oxide from epithelial cells. In-vitro macrophage infection leads to a skew towards Th2-type responses, with a reduction in interleukin (IL) -18 and interferon gamma (IFN-γ), which could lead to subsequent asthma or atopy.

Analysis of the cytokine responses from children with RSV infection correlated with the in-vitro data, with evidence of an early skew towards a Th-2 type response (IL-4/IFN-γ ratio and IL-10/IL-12 ratio) in both airway secretions and blood of children that developed RSV bronchiolitis.

This study provides evidence of a skew towards a Th-2 type response within 48 hours of the onset of respiratory symptoms. There are no previously published reports that have demonstrated a reduction in IL-18 in association with infection or the production of defensins following viral infection.

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Chapter 1 - Introduction

1.1 Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) was first isolated in 1956 from a chimpanzee with an upper respiratory tract infection (1). It is a negative sense, single stranded RNA virus related to the causative agents of measles, mumps and parainfluenza. The first clinical isolates of RSV were from children with pneumonia and croup (2), but a further four years elapsed before RSV was associated with bronchiolitis (3-5).

RSV infection affects infants, with the majority of serious infections occurring in the first few months of life. This virus is the major cause of bronchiolitis (accounting for 75% of the cases), which is the most common cause of hospital admission in the UK during the first year of life. The virus can also cause pneumonia (6), croup (7) and upper respiratory tract infections. Population studies that have been conducted in the UK suggest that up to 2% children infected by may require hospital admission for supportive care (8), resulting in acute hospital accommodation problems during the winter months. Cost estimates have been produced to highlight the significance of RSV infection and the burden it places on healthcare. The most recent estimates have come from the USA and suggest that the cost of RSV infection is in the order of $300 million per annum (9).

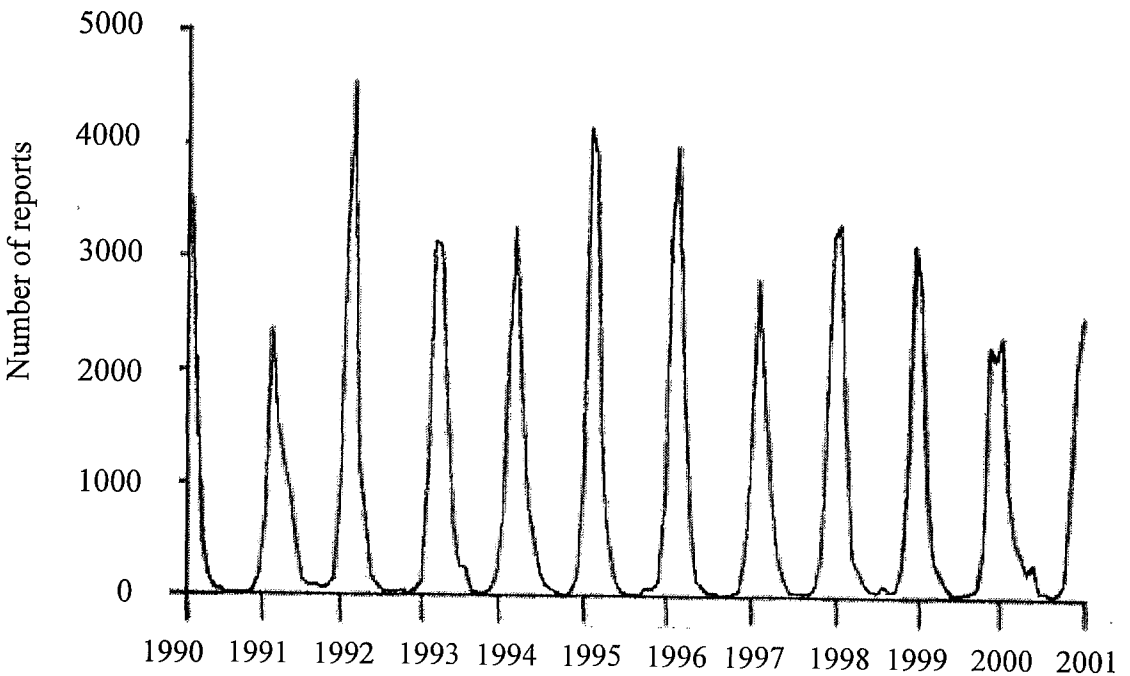
RSV is found throughout the world, and is a significant worldwide healthcare problem. In the Northern Hemisphere RSV infection has a seasonal variation (7,10) (see figure 1.1), the majority of infections occur between November and March, the peak incidence occurs in January, the Southern Hemisphere mirrors this trend, with peak in their winter. In tropical countries RSV outbreaks tend to be associated with the rainy season (11,12). An increase in the incidence of RSV infection has been associated with overcrowding (13); large congregations of people are associated with outbreaks of the virus. The mortality rate associated with RSV infection is higher in the developing world and is related to lack of support care, rather than severity of illness.

Children who have been infected previously do have a lower rate of lower respiratory tract infection, though the rate of URTI is not significantly reduced (14). Thus the immune response in normal children to RSV infection induces partial but not complete protection.

Longitudinal studies have shown that approximately 69% of children will be infected during the first year of life, rising to 83% by the second year and by the age of three years virtually all children will have been infected at least once, with as many as 50% of them having suffered multiple infections (15). The attack rate for the virus is high, during RSV epidemics the attack rate can be as high as 95% (16). The high infectivity of the virus has major health implications as adults also suffer recurrent infections, thus making nosocomial infection a concern. There have been reported cases of large outbreaks of RSV infection in hospital wards, with significant morbidity and occasional mortality (17).

Figure 1.1

The Seasonal variation in the Incidence of RSV infection in the USA 1990 to 2001



Data derived from CDR respiratory archive 2001, vol 11 (5)

1.2 Molecular Virology

RSV has a negative polarity RNA genome, the virus belongs to the order *mononegavirales*, the family *paramyxoviridae* and sub-family *pneumoviriae*. Other members the sub-family include bovine RSV (BRSV), ovine RSV (ORSV) and the mouse pneumonia virus (PVM).

In the virion the genome is tightly associated with the viral nucleocapsid protein for its entire length and has a virally encoded polymerase packaged with it.

The viral genome is composed of 15,000 nucleotides and has an estimated weight of 500 kilodaltons (kDa). The genome codes for 10 separate genes and has 2 overlapping open reading frames.

1.2.1 Viral Replication

Viral replication occurs in the cytoplasm of the infected cell, initially the genomic RNA is transcribed into a positive polarity RNA strand and new negative polarity RSV genome copies are transcribed from this template (18). Progeny virions form when the newly formed nucleocapsid, enclosing the viral genome buds through the plasma membrane of the host cell. The size of the virions (nucleocapsid and lipid envelope) is variable, between 150 and 300 nm, each virion contains one single gene copy. The lipid bilayer of the viral envelope is derived from the host membrane, virally encoded transmembrane surface glycoproteins are intercalated into this.

Approximately 90% of RSV progeny remain associated with the host cell membrane, consisting of near complete virus particles that fail to bud from the cell surface. Large proportions of the released virus particles appear empty and are non-infectious. From one infected host cell about 10 plaque-forming units (pfu) of virus are formed.

1.2.2 RSV Genome Structure

The structure of the RSV genome is shown in figure 1.2.

The genes coding for the non-structural proteins NS1 (532 nucleotides) and NS2 (503 nucleotides) have unknown function. The protein product of the nucleocapsid gene, N, forms part of the nucleocapsid core of RSV, when associated with P (coded for by a gene of 914 nucleotides) the phosphoprotein (gene size 6579 nucleotides) and L the RNA polymerase gene (19). The phosphoprotein is important in forming replication complexes with the RNA polymerase. The matrix protein M (958 nucleotides) is associates with the host cell membrane and is involved in targeting viral proteins and nucleocapsids to the site of viral budding. The second matrix protein, M2 or 22KDa (961 nucleotides) and may be important in the cytopathic effect seen with RSV infection.

There are three surface proteins coded for by the RSV genome, the SH protein (gene - 410 nucleotides), the G or glycoprotein (gene - 923 nucleotides) and the F or fusion protein (gene - 1903 nucleotides). These surface proteins are important in RSV immunology and will be discussed further.

There are 2 sub-types of RSV, the A and B of the key differences between the sub-types occur in the surface proteins. NS1 and NS2 proteins share 78% sequence homology and there are virtually no differences between the phosphoprotein, matrix proteins and polymerase genes. However there is only 40% sequence homology in the G protein between A and B strains (20), although the SH protein (75%) (21) and the F protein (92%) (22) are much less variable.

1.2.3 Surface Proteins
G or Glycoprotein

The gene coding for the G protein of RSV is 923 nucleotides long, giving a relatively small protein of 298 amino acids. However, post-transcriptional modification with heavy glycosylation of the protein backbone raises the molecular weight of the protein from a theoretical 32.5kDa up to 90kDa (23). The structure of the protein backbone is such that the majority of the glycosylation is via O-linked glycosidic bonds. The acceptor sites for these O-linked sugars are serine and threonine, as these account for 30.6% of the composition of the G protein backbone, this allows a large amount of modification to occur (24). There are only 4 sites for N-linked oligosaccharides to attach to the G protein backbone.

A sequence of amino acids between residues 38 and 66 function as the G protein anchor domain. Approximately 15% of the G protein produced is soluble, lacking the anchor domain, this soluble G protien is slightly smaller at 82kDa (25). The cleavage site to form the soluble form of G is between residues 65-66 or 74-75 (26). Mutation analysis of the anchor domain reveals that if significant deletions are made this blocks glycosylation of the G protein. The implication is that the G protein anchor domains target the host Golgi apparatus, without the anchor domain glycosylation cannot occur. The removal of a few carbohydrate residues can alter the viral infectivity dramatically, despite not altering the weight of the G protein significantly (27).

The G protein is involved in the initial virus-host cell interaction and attachment. The host cellular receptor for the G protein is not known, but the evidence suggest that a fully glycosylated protein is critical for this step to occur. There is recent evidence that the G protein has homology with Fractalkine a cell surface associated chemokine (28). Antibodies against the G protein can be used to interfere with the adherence of RSV to the cell surface (29). The G protein is not involved in syncytia formation, as antibodies against the G protein do not disrupt syncytia formation during RSV infection. The variation in the G protein accounts for the majority of the differences between the 2 sub-types of RSV.

F or fusion protein

The F, or fusion protein has a coding sequence of 1899 nucleotides, this codes for a protein of 574 amino acids in length, which has a molecular weight of 63kDa. The fusion protein is synthesised as a single strand, F0, which undergoes post-transcriptional modification, cleavage into two sub-units F1 and F2. The sub-units are linked together by disulphide bonds (30). The F0 protein also undergoes post-transcriptional glycosylation, though this is much smaller than the modification to the G protein. Initially N-linked sugars are added to the protein backbone, the protein is then targeted to the rough endoplasmic reticulum, from here it moves to the trans-Golgi apparatus. While the F0 protein is associated with the Golgi apparatus it is cleaved into the F1 and F2 proteins by cellular proteases (31). The F protein is expressed at the host cell surface as a tetramer mediating cell-cell fusion and syncytia formation. The F1 sub-unit is hydrophobic, while the smaller F2 sub-unit is hydrophilic (30). The region of the F gene that codes for the F1 region is identical between sub-types of RSV, the variability in F protein between the A and B subtypes is due to differences in the F2 sub-unit. Overall there is 92% homology between the two sub-types, with the majority of the sequence variation to be found at the 5' end of the F2 protein (22).

SH Protein

Little is known about the small hydrophobic (SH) protein, which is the third membrane protein and coded for by a 410-nucleotide sequence, though it is though to have a role in cell fusion (32). SH undergoes little post-transcriptional modification. There is some variation between the 2 sub-types, with homology between the 2 strains of 75% (33).

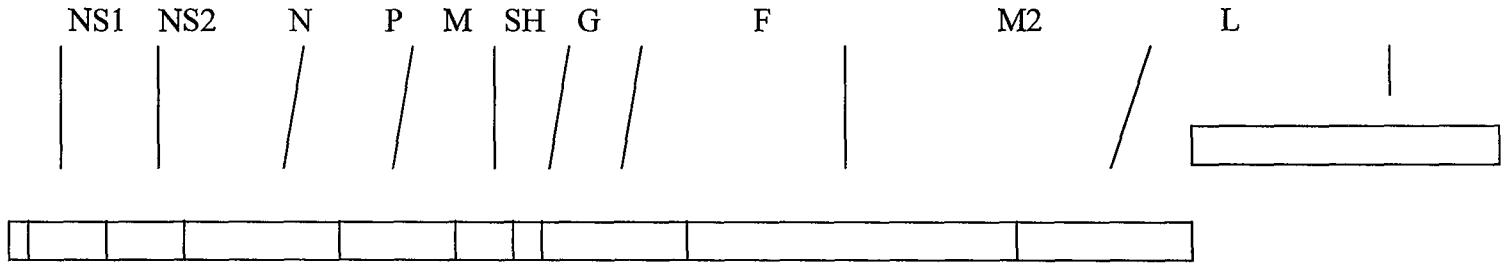


Figure 1.2
RSV genome indicating both open reading frames

1.2.4 Virus Biology

A virion will infect a susceptible cell by initially gaining entry into the cell. The surface glycoproteins of RSV are important in this process (34). The G protein has been shown to be critical for the entry of RSV into the host cell. The cellular receptor for RSV is unknown, despite extensive efforts to define it. The viral membrane fuses with the host cell membrane and the viral nucleocapsid is released into the host cell cytoplasm. With the RNA polymerase being closely associated with the nucleocapsid, this ensures transcription can take place even in enucleated cells.

The RSV genome acts as a template for the production of viral mRNA, with RSV mRNA detected in the cytoplasm within 4 to 6 hours of infection. RSV protein synthesis rapidly follows mRNA production. Peak RSV mRNA production occurs 16 hours post-infection and peak protein production occurs between 18 and 20 hours following infection of the host cell (35).

The genomic RNA is copied to give a positive polarity template from which further viral progeny are manufactured (36). The quantity of this positive strand copy is 10 to 20-fold less than the numbers of negative strand genomic RNA copies. The new RNA strands are linked to the nucleocapsid and polymerase proteins, this is then targeted towards the cell surface by the M2 protein. When the complex is at the host cell surface it buds off from the host cell to release new virions, but as a result of faulty packaging up to 90% of the progeny remain associated with the host cell membrane.

RSV proteins are produced in amounts, decreasing in an inversely proportional manner to the distance the gene is from the 3' end of the genome (37). The timing of gene transcription also follows this behaviour, thus the F mRNA and thus protein is produced relatively late in the infection cycle. The F protein causes the infected host cell to fuse with adjacent cells, thus spreading infection without the need for budding off of viral progeny. This speeds up the process of viral infection.

1.3 Clinical Features

1.3.1 RSV Infection of Children

The incubation period for RSV infection is between 2 and 8 days, with the average being 5 days (38,39). Recovery from the infection often takes up to 14 days from the time of peak symptoms. Transmission is via large droplet and infection is via the nose or eye, with oral administration less likely to lead to infection (40). The peak incidence of RSV infection occurs between 6 weeks and 9 months of age and is normally upper respiratory tract infection (URTI) with rhinorrhea and anorexia (41). RSV infection of the URTI can be complicated by secondary otitis media (42,43). In recent years, the use of PCR technology has helped to establish the importance of respiratory viruses in the aetiology of culture negative otitis media, RSV has consistently been found to be one of the most common aetiological agents (44). In a significant proportion of children URTI progresses and leads to lower respiratory tract (LRT) involvement in approximately 25 to 40% of cases (39). Although the majority of children who develop LRT infection (LRTI) have mild symptoms, 2% of children with RSV infection do require hospital admission (8).

During lower respiratory tract infections there is a prodromal phase consisting of URTI with rhinorrhea and anorexia, this progresses to produce a cough in the subsequent 24 to 72 hours. The cough is often associated with sneezing and low-grade fever; and subsequent wheezing. If the LRTI is mild, the symptoms are limited to cough, sneeze and wheeze. Clinical examination reveals moderate tachypnoea, diffuse inspiratory and expiratory crackles and wheeze. The upper respiratory tract symptoms often remain and there is profuse rhinorrhea, intermittent fever and otitis media. The recovery from mild LRTI is within 7 to 12 days (45). If the LRT involvement is more severe the cough and wheeze are more troublesome and there is increasing dyspnoea (46). Examination at this stage reveals increasing tachypnoea, chest hyperinflation with intercostal and subcostal recession as well as a refusal to feed. With more advanced disease the child tires and becomes hypoxic and respiratory failure occurs. Most children who are admitted to hospital will be hypoxic on admission and remain hypoxic for several weeks following

resolution of the illness (45). This persistent hypoxia is thought to be due to ventilation, perfusion mismatch (47).

Following RSV lower respiratory tract infection a significant proportion of children are left with altered respiratory physiology. These children have increased episodes of wheeze, suffer prolonged respiratory symptoms following other respiratory tract infections and are hospitalised more frequently. The recurrent episodes of wheeze have lead to conjecture whether there is an association between RSV LRTI and subsequent asthma, if recurrent episodes of wheeze are used as criteria for making a diagnosis of paediatric asthma. The link between bronchiolitis and subsequent asthma will be discussed later.

Complications of RSV infection, such as apnoea, can manifest themselves at initial presentation. Apnoea occurs in up to 20% of hospitalised children, often before other respiratory signs are present, though the highest risk period is 2-3 days post-admission (48). RSV infection can exacerbate symptoms in children with cystic fibrosis, nephrotic syndrome or congenital heart lesions (49,50).

1.3.2 RSV Infection of Healthy Adults

RSV is a common upper respiratory pathogen in the normal adult population, often leading to upper respiratory tract infection with no major sequelae. The duration of the protective immune response in adults is little better than that seen in children. In one study a cohort of healthy adult volunteers were exposed to recurrent experimental RSV following natural RSV infection. Over the 26-month study period 73% of the subjects had two induced infections, with 47% having three or more infections (51).

RSV is now recognised as a major pathogen in the elderly population, where there is a greater risk of developing lower respiratory tract infection, often as pneumonia (52,53). The risk of developing RSV pneumonia is associated with pre-existing cardiac or respiratory pathology (54). Increased population density, such as old people's homes enhances the risk of outbreaks.

1.4 Immunobiology of Bronchiolitis

Though RSV infection is associated with great morbidity, the mortality rate in the developed world is low leading to a paucity of tissue for study. Most reports on the pathological changes seen in human RSV bronchiolitis are over 30 years old.

RSV has a predilection for ciliated respiratory epithelium. Acute RSV infection is associated with disruption or destruction of the epithelial layer and loss of ciliated epithelium in the bronchioles, between 75 and 300μm in diameter (55). Syncytia form in the infected epithelium due to virally-mediated cell to cell fusion, the characteristic hallmark of RSV infection. In the recovery period there is a regeneration of the ciliated epithelium, but this takes up to 14 days to return to full function, whereas the rest of the respiratory epithelium is replaced within a few days (55). Under the epithelial layer there is submucosal and adventitial oedema, and the damaged epithelium is shed. The epithelial plug formed can act to prevent air entering the distal airway, leading to atelectasis and collapse. If there is partial obstruction, this can lead to distal airway hyperinflation, as seen in bronchiolitis. The summation of these effects leads to the clinical picture of increased lung volume with increased expiratory resistance combined with patches of collapse that is seen in bronchiolitis (56,57).

Associated with the submucosal and adventitial oedema is a peribronchiolar infiltrate of lymphocytes, plasma cells and macrophages (58). These cells will have been recruited locally as well as from the circulation. Studies of children who have died during RSV upper respiratory tract have revealed that the lymphoid infiltrate is an early feature of the disease process, and this lower respiratory tract infiltration is seen in children who clinically have only had upper respiratory tract infection. The changes seen in the airways are localised to the epithelial layers, with no evidence of fibrosis and remodelling taking place that may distort the structure of the bronchial tree. During early childhood the airway is still developing, with formation of the terminal airways and alveolar sac not complete until the age of 5.

1.4.1 Immune Response to RSV by Infants

The antibody titre to a specific pathogen often defines the degree of immunity to re-infection by the organism. In the newborn infant, maternal immunoglobulin G (IgG) antibodies, transferred late in pregnancy play a significant role in reducing the incidence of new infections during this period. The continuing protection of upper respiratory tract of infants occurs when the infant is breast-fed.

The antibody response to most infections involves initial production of immunoglobulin (Ig) M, the levels of this antibody fall as the individual recovers from infection and is replaced by the production of IgG, systemically, and IgA at mucosal surfaces. Elevated IgE levels to the RS virus has been observed in infants with bronchiolitis (59,60).

1.4.2 Maternal Antibody Status

Initially it was thought that maternal antibodies might play a part in the immunopathology of bronchiolitis. In its simple form this hypothesis is unlikely, as maternal antibodies are at their highest during the first 6 weeks after delivery, during which time the incidence of RSV infection is low. If, however, protective antibodies are a minor subset of maternal immunoglobulins reactive to RSV, they would decay first creating a situation where RSV infection occurs in the presence of antibodies capable of inducing immunopathology.

The developing foetus acquires maternal IgG antibodies through the placenta actively; the final levels just prior to delivery are higher than those found in the maternal circulation. The majority of the IgG transfer occurs during the last trimester. This means that prematurity is a risk factor for RSV infection, as the final trimester transfer of protective antibodies will not have taken place. This was shown by De Sierra (61), who demonstrated that infants born under 28 weeks gestational age benefit from the administration of hyperimmune globulin.

The level of protection against RSV bronchiolitis correlates with acquired maternal antibody, but does not seem to be protective against RSV upper respiratory tract infection. Lamprecht (62) showed in a small study of 15 children with pneumonia and 19 with bronchiolitis that, though high maternal neutralising antibody titres did not protect against RSV infection there was an inverse correlation between the maternal antibody titres and severity of pneumonia. Ogilvie demonstrated that in a group of 100 children followed for 12.months, 29 children who developed RSV infection their maternal IgG levels to RSV were significantly lower than in the children who did not develop RSV infection in the first 6 months of life (63). Studies on cord blood neutralising antibody titres have shown similar findings. Glezen was able to show that in 68 children who developed culture-proven RSV infection, cord blood neutralising antibody titres were lower than in a control group of 500 cord bloods taken from children who did not develop infection, but were born at a similar time (64).

The importance of antibodies against RSV has further been highlighted by the use of hyper-immune intravenous gamma globulin, with neutralising activity against RSV infection for prophylaxis and treatment in high-risk groups, such as children with bronchopulmonary dysplasia (BPD) or congenital heart disease. The results of large field trials with hyperimmune globulin show that regular monthly intravenous administration in high-risk groups is of benefit. There is a reduction in the incidence of lower respiratory tract infection, a reduction in hospitalisation rates, days in hospital and also days spent in intensive care (ITU) (65). These findings are very similar to the clinical pattern in infants who have acquired high maternal antibody. It is interesting to note that although there is a reduction in the rate of acute otitis media associated with the administration of hyperimmune globulin, upper respiratory tract infections are not reduced in infants with high antibody levels (66).

The levels of neutralising antibody in the maternal circulation dictates the levels found in the foetal and neonatal circulation. The levels of acquired maternal antibodies will rapidly fall during the first six months following delivery through the normal clearance and degradation mechanisms.

1.4.3 Antibodies Acquired through Breast-Feeding

Infants also acquire antibodies from the mother through breast feeding, and some studies have shown that levels of RSV specific antibodies in breast milk correlates with protection against bronchiolitis (67,68). Normal breast milk contains relatively low levels of IgG and IgM, but high levels of secretory IgA, which coats the nasopharynx of the infant during feeding. One report that has followed the IgA levels in breast milk from newly lactating women has shown that there are high levels of RSV specific IgA in colostrum (75%), this remains high at 6 months post-partum (59%) (69), the maintenance of high levels of antibodies is thought to be due to maternal re-infection during the lactating period.

1.5 Antibody Response to RSV Infection

The immune response against RSV differs between, the response of the mature immune system and the response of the infant's immune system.

1.5.1 Changes in Antibody Response with Maturation of Immune System

The infant immune system continues to mature during the first year of life; this maturation could account for the variability seen in the antibody responses to RSV. The dynamic maturation process of the immune system has implications for both the initial infection and for any subsequent infections. If there were a rapid neutralising antibody response to the initial RSV infection, it would lead to quicker resolution of the initial infection, leave residual high neutralising antibody titres, which will reduce the risk of subsequent re-infection.

Deficient immune responses to RSV infection occur in the first six months of life (70,71), though another study has suggested that the worst antibody responses may be localised to the first two months of life (72). The main characteristic of the immature immune system is the poor response to carbohydrate antigens, leaving good responses to protein with IgG1 and IgG3 subclasses, but poor antibody IgG2 and IgG4 responses to polysaccharides (71,73). As the infant matures the immune response to carbohydrate residues does improve, with a rise in predominantly IgG1 and IgG2 responses. Never the less the protective quality of the response is also inadequate in infants infected between 4 and 8 months of age. The antibody titres against both the F and G surface proteins of RSV are 10 to 12 times lower than those observed in children over the age of 12 months (74).

1.5.2 Antibody Response to RSV Infection

Studies have demonstrated that the level of neutralising antibody response following primary infection is inversely proportional to the risk of further infection. The level of the antibody response against the F protein has been shown to reduce the risk of re-infection

(75) and children with low levels of IgG1 are at a higher risk of developing bronchiolitis (76). The differences between RSV sub-type surface proteins are highlighted by the finding that antibody responses to the F protein are not sub-type specific, while those against the G protein are (77).

In the mucosal antibody response to RSV infection, the major immunoglobulin produced is IgA, there is a rise in 55-65% of RSV infections during the first six months of life (78). This antibody can be found adherent to the surface of infected cells obtained during nasopharyngeal aspiration of children with RSV infection (79). The presence of IgA in aspirates is often associated with a fall in shedding of RSV, suggesting that IgA may play a significant part in this. The other immunoglobulins can be found during nasopharyngeal aspiration of infected children, though IgA is the initial antibody found (80).

The protective immune response in adults is nearly as poor as in an infant population, though infection is rarely associated with lower respiratory tract involvement. In one study adults who had had a proven RSV infection were challenged repeatedly with RSV 2, 4, 8, 14, 20 and 26 months following the initial infection. Each challenge episode would be expected to boost the individual's protective immunity, as well as exposing them to the possibility of re-infection. The risk of developing a RSV infection was 25% at each challenge, though 50% were infected at 2 months, rising to 66% by 8 months. By the end of the study 73% of test subjects had 2 or more infections, while 43% had 3 or more. The development of high titres of antibodies against F and G were associated with some protection, though even with the highest antibody titre the re-infection rate was reduced by only 25% (51).

1.6 Innate immune response following RSV exposure

Recently, other factors that are produced by the airways as part of the innate response to infection have been found to be of importance. The airway epithelium is important in the innate immune response, this has already been demonstrated for cytokine production, but there are other factors that are epithelialy derived and may have a significant role.

Nitric oxide is one of the factors that have been investigated; the other is defensins, epithelial-derived proteins.

1.6.1 Nitric Oxide (NO)

The production of nitric oxide (NO) from human tissues has been difficult to establish, though it has been confirmed recently for both epithelial cells (81) as well as macrophages (82). It is known that NO can exert a regulatory role for other respiratory viruses by reducing cytokine production (83-85).

There has been a recent study that has highlighted the increased production of iNOS (inducible nitric oxide synthase) following RSV infection of epithelial cells (86). Recently there has been a study that has demonstrated an increase in NO associated with experimental RSV infection of healthy volunteers(87). The in-vitro and in-vivo stimulation of iNOS has been shown for other respiratory viruses as well recently(88).

1.6.2 Defensins

Human beta defensins (hBD) are members of a family of highly conserved, cationic peptides that have a broad spectrum of antimicrobial properties against gram-negative and gram-positive bacteria, fungi and enveloped viruses(89). Members of the defensin family are found in many species of both the plant and animal kingdom, including insect, fish and mammals. Alpha defensins are produced by either neutrophils or by Paneth cells in the

intestine. A wide range of epithelial derived tissues can produce beta defensins. Defensins are sensitive to alterations in the salt concentration in airway lining fluid, and have a reduced effect in airways of cystic fibrosis patients (90). Most research that has been conducted on the defensins has focused on the role of defensins and bacterial infection, with pathogens associated with cystic fibrosis airway colonization having been studied(91). There have been no previous studies that have investigated the role of this family of peptides in RSV disease.

1.7 Cell-Mediated Immunity

1.7.1 Maturation of Cell-Mediated Immunity

Initial RSV infections occur in infants during a period when the immune system is maturing, this has been demonstrated for the humoral arm of the system. During period of maturation, exposure to antigens, environmental stimuli and respiratory pathogens may alter the final immune phenotype of an individual. The significance of early infections driving a Th1-type phenotype has been established from several population studies (92,93) (94). Paradoxically RSV infection is associated with the opposite effect, early infection leading to bronchiolitis is associated with atopy in later life.

The neonatal immune system is still maturing as RSV infection strikes. A recent study focused on the immune maturation of T cell cytokine responses, and demonstrated that there are important changes in the first two years of life (95). At birth both atopic and non-atopic populations have a Th2 skew to their responses to common allergens, in the non-atopic population this is progressively suppressed during the following twelve months, while the atopic population continues to have a Th2 response, with low IFNγ production. Thus the Th2 responses that are seen in children with RSV bronchiolitis may be the natural response of either atopic or non-atopic population at this particular age, but the infection may skew the phenotype, such that Th2 responses are maintained in the child. The responses seen in this population may be a marker of the response seen in children with a Th2 predominance, rather than the cause of an increase in the risk developing asthma later in life, following bronchiolitis; this will be discussed later.

1.7.2 Cell-Mediated Immune Response to RSV

The cell-mediated, or T cell, responses to RSV infection in infancy are more difficult to define and characterise, when compared to the antibody response. The majority of the findings discussed are derived from murine models of RSV infection. Most murine models of RSV infection do not study primary RSV infection, instead they focus on the immune

response to re-challenge with RSV, following priming with either RSV or RSV surface proteins.

It has long been believed that the cell-mediated immune (CMI) response to RSV is critical in terms of both viral clearance and the immunopathology seen with infection. Studies that have focused on the antibody status and severity of children with RSV infection have not been able to demonstrate any relationship. The situation is different for the CMI response to RSV infection.

1.7.3 RSV Infection in immuno-compromised Individuals of Different Ages

Though exposure to RSV leads has a high infection rate and also spreads to the lower respiratory tract infection in a large proportion of children, the rate of hospital admission is only 2% and the mortality rate is only 0.3% of the hospitalised population. The mortality rate in children with pre-existing cardiac or pulmonary diseases is much higher (96). Initial estimates of the mortality rate in children with either congenital heart disease or bronchopulmonary dysplasia was thought to be as high as 30%, especially in children with cyanotic congenital heart disease. More recent estimates now place the mortality rate in this population at 9% (97). The high mortality rate is due to the decompensation of the underlying disease secondary to the large ventilation, perfusion mismatch induced by RSV infection. The virus can also lead to prolonged respiratory morbidity in the paediatric cystic fibrosis population (98).

The immuno-compromised adult population has a significantly higher risk of RSV infection being associated with pneumonia and increased mortality (99). Almost any compromise of cell mediated immunity is associated with increased mortality. Reported outbreaks of RSV infection in bone marrow transplant units have been associated with mortality rates of greater than 50% (100,101).

Though the role of protective CMI has been established by studies on immuno-compromised patients, the role of non-protective CMI in the immunopathology of RSV has not been established.

1.7.4 Lymphocyte Proliferative Responses to RSV

Infants with RSV bronchiolitis do show evidence of lymphocyte transformation activity (LTF), a marker of CMI (102-104). The LTF responses are seen more often in infants under the age of six months, compared to children above that age, 78% vs. 46% (102), which lead to the hypothesis that the immunopathology observed during bronchiolitis might be due to an exuberant CMI response to RSV.

The observation that prior treatment with formalin-inactivated RSV (FI-RSV) leads to an increased in disease severity, morbidity and mortality following infection with live RSV led to the cessation of field trials of FI-RSV vaccine (105). Post-mortem studies on two of the children that died following administration of this vaccine showed that they both had eosinophilic pulmonary infiltrates (105). Subsequent CMI studies on this group of children showed that they also had an increased LTF compared to control children (106).

There have been in-vitro and in-vivo studies into T cell cytokine responses to RSV infection. The in-vivo studies have focused on trying to find markers of Th2 activation in peripheral blood, either an increase in soluble IL-2 receptor (sCD25), or interleukin (IL) - 4 or IL-5 (107). Though there were higher levels of sCD25 in children with acute infection, compared to both controls and convalescence, there was no correlation with disease severity; IL-4 was below the limits of detection in the peripheral circulation (107). FACS analysis of peripheral blood from children with RSV bronchiolitis shows that there is a fall in CD8+ cells in the peripheral circulation, which returns to normal during convalescence (108), suggesting pulmonary recruitment of CD8+ cells during the acute infection.

The initial and memory T cell responses may be different, as shown by Anderson and Roman. In children with acute RSV, stimulated peripheral blood mononuclear cells (PBMC) produced an increase in IL-4/IFNγ ratio, though absolute levels of both cytokines fell, the relative fall of IFNγ was greater (109). Anderson et al. looked at the in-vitro production of cytokines from stimulated PBMC from adults and children, and showed that there was a predominant Th1 skew to the cytokine production (110). The majority of adults produced IFNγ and IL-2, with less than 30% producing IL-5 and none producing IL-4.

1.7.5 Murine Studies with RSV

Murine studies focused on primary RSV infection have demonstrated the dose-response relationship between viral load and murine morbidity and cytokine production(111). Primary RSV infection is associated with brisk IFNγ production in bronchoalveolar lavage (BAL) fluid and draining mediastinal lymph nodes (112). The main cellular sources for IFNγ were CD8+ and CD4+ T cells.

The majority of murine infection experiments have used a re-challenge model, where the mouse is primed initially with RSV surface proteins or FI-RSV, and then challenged with live RSV and the response measured. Studies conducted in this way have focused on the T cell responses, and shown that priming mice with a F protein vaccinia recombinant (vvF) leads to a Th1-type response, while using a G protein (vvG) recombinant, or FI-RSV leads to a Th2-type response (113-116). T cell sub-set adoptive transfer experiments from mice primed with vvG have shown that transfer into naïve mice of either CD4+ or CD8+ can lead to an increase in pulmonary pathology. The transfer of CD4+ cells, the main sources of IL-4 and IL-5 lead to an increase in pulmonary eosinophilia similar to that seen in the mice primed with vvG (117,118).

Alterations in the cytokine milieu following priming have been shown to alter the immune response to subsequent RSV challenge. The reduction in Th2-type cytokines after priming

will reduce subsequent pulmonary pathology. This was seen when IL-4 alone (115), or IL-4 plus IL-10 were depleted (116), and this led to a reduction in pathology. Further modulation of the Th1 response, either by the addition of IL-12 at initial priming does lead to a fall in the production of the Th2 cytokines IL-4 and IL-5. Though there is a fall in these cytokines, a reduction in pulmonary eosinophilia and an increase in IFNγ production, the clinical end-point is similar, a pulmonary lymphocytic infiltrate replacing the eosinophilic one (119,120). T cell sub-set depletion experiments have shown that CD4+ cells are the main effector cells, producing IFNγ and reducing viral load (121).

Overexpression and knockout mice have been used to study the importance of this cytokine in the clearing of RSV in primary infection experiments. Absent IL-4 is associated with rapid viral clearance, whereas overexpression is associated delayed viral clearance and low CTL response, but there is normal viral clearance by both groups on rechallenge (122).

Reduction of the Th1 response in mouse strains that do not develop an eosinophilic response to RSV rechallenge does lead to pulmonary eosinophilic infiltrates. This reduction in the Th1 response was carried out by disrupting IFNγ production, or by reducing CD8+ T cells, the major source of IFNγ (123,124).

In summary, murine studies show the importance of T cell sub-sets in the immune response against RSV and how important the Th1 and Th2 cytokines are in skewing subsequent responses to RSV. The murine studies also give some insight into the responses that were seen in children, who were vaccinated with FI-RSV, similar pathology is seen when mice were primed with FI-RSV and then challenged with RSV.

1.8 The Similarity between Immune Responses to Bronchiolitis and Asthma

The results from studies in humans and mice to RSV infection demonstrates how viral infection can skew the immune response from a predominantly Th1-type response, to a Th2-type response. A Th2-type response is the predominant response seen in atopic individuals, with significant IL-4 and IL-5 production, IgE production and involvement of eosinophils. Many of these features are seen in the immune response to bronchiolitis.

1.8.1 Evidence of Mast Cell and Eosinophil Involvement in RSV Infection

There is ample evidence that the development of an IgE response to RSV is associated with mast cell degranulation and liberation of various mediators. The action of these mediators is to cause the recruitment and activation of eosinophils and lymphocytes. Several studies have investigated the release of mast cell components. Welliver showed that children who developed a wheezing illness associated with RSV infection had higher titres of RSV-IgE and also higher levels of histamine in nasal secretions were isolated in this group (60). In-vitro testing of mononuclear cells from children with bronchiolitis have shown an increased capacity to release histamine (125,126).

Other mast cell products, including arachidonic acid-derived mediators, such as leukotrienes are elevated during the first few days of RSV infection in those children who develop bronchiolitis. Elevated levels of leukotriene C_4 (LTC_4) may persist for up to 28 days post-infection, this may be one of the effector agents involved in the prolonged wheeze associated with RSV infection (127). However, other important mast cell degranulation components, such as tryptase, have not been detected in children with RSV bronchiolitis (128).

Eosinophilic cationic protein (ECP) has been used a marker of eosinophil activity being one of the commonest mediators released on eosinophil activation. Several studies have

compared different groups of children with RSV infection, from children who had URTI only, to those who developed a non-wheezy LRTI (pneumonia) to those who developed a wheezy LRTI (bronchiolitis). A common finding has been elevated ECP in the group of children who develop a wheezy LRTI (129,130). Interestingly when RSV bronchiolitis is compared to non-RSV bronchiolitis, elevated ECP levels are only observed in children with RSV bronchiolitis (131), raising the possibility that there may be different mechanisms involved in the aetiology of wheeze associated with bronchiolitis.

1.8.2 Immunoglobulin E Responses

The production of immunoglobulin E antibodies (IgE) is associated with both parasitic diseases, particularly helminthic infections and atopy. The majority of IgE production occurs in local mucosal associated lymphoid tissue, rather than in the major lymphoid areas, such as the spleen. Though IgE is considered a mucosal associated immunoglobulin and is secreted, IgE differs from IgA in that the secreted form is identical to the form in the circulation, i.e. translocation to the mucosal surface does not involve a secretory component.

Individuals with atopy have higher IgE levels in the circulation than a normal individual, though the serum levels of IgE often do not reflect the severity of the atopic disease. A large proportion of IgE is surface bound to mast cells and basophils through a tight association with the high affinity IgE receptor (FcεRI). The Th2-type cytokines interleukin (IL)-4 and IL-5 drive the class switch to IgE and for continued IgE production.

The binding of antigen by mast cell surface-bound IgE leads to cross-linking of the IgE, which in turn leads to mast cell degranulation. Mast cell degranulation causes a classical type-I hypersensitivity reaction, the release of pre-formed and newly synthesised mast cell products. These products can lead to the recruitment and activation of eosinophils and lymphocytes.

In RSV bronchiolitis there is often an IgE response; although the development of IgE responses, and the associated Th2-type cytokines are rare in other respiratory viral infections. The initial observation that RSV infection can lead to an IgE response was made by Polmar (132). Welliver confirmed this observation in further studies (59). The IgE response can be observed both in an increase in IgE in respiratory secretions and serum IgE levels, though the serum levels are often lower than the IgE levels found at the mucosal surface (133). Initially the IgE produced in response to a RSV infection is bound to infected epithelial cells, then as the infection proceeds IgE is found free in mucosal secretions.

Although the production of IgE antibodies associated with RSV infection is firmly established, in more recent studies the nature of this IgE response been clarified. The majority of the IgE produced is polyclonal and non-specific, but in a significant proportion of children there is a RSV specific IgE response (RSV-IgE). Studies comparing the children who develop RSV-IgE with those who develop non-specific IgE responses, reveals specific responses in children at higher risk of developing recurrent wheezing episodes in the 48 months following initial RSV infection (70% vs. 20%) (134).

Other studies have investigated the significance of polyclonal IgE responses to RSV infection. A combination of human and animal studies reveal possible new pathways for subsequent events. Forster (135) showed that in the first 24 months following RSV infection there is an increase in sensitisation to various common aeroallergens. Studies by Noma (136) and Shenghan (137) show similar findings. Several murine RSV infection models, using BALB/c mice exposed to ovalbumin demonstrate the increased risk of sensitisation following RSV infection (98,138).

Thus there is evidence that children who develop IgE responses to RSV have an increased risk of mast cell degranulation following binding and cross-linking of surface bound IgE. In these children RSV re-infection results in virus binding to RSV-IgE leading to mast cell degranulation. The second mechanism is seen in children who develop polyclonal IgE

responses; IgE recognition of common aeroallergens induces a persistent inflammatory cascade.

1.9 Association Between Bronchiolitis and Subsequent Asthma

For many years there has been controversy as to whether RSV bronchiolitis can trigger the development of asthma in later life (139-142). Both diseases show similar pathology, with small airway inflammation and wheeze. The question as to whether bronchiolitis predisposes to subsequent asthma or whether bronchiolitis develops in children with an atopic predisposition who are likely to develop asthma remains controversial. Several studies have followed children who have suffered RSV bronchiolitis monitoring pulmonary function and the development of atopy. The main difficulty in collating these studies is the variability in diagnostic criteria used, both in the diagnosis of bronchiolitis and the confirmed of establishment of atopy.

This review will only refer to prospective studies of children following bronchiolitis, thus establishing an accurate diagnosis of bronchiolitis. The diagnosis of atopy or asthma, has varied from confirmation of airway reactivity, serum IgE levels, skin prick tests (SPTs) to aeroallergens or IgE RAST measurements for common aeroallergens, to a simple clinical diagnosis based on three episodes of wheezing illness.

Some studies have only a limited follow up of children for no more than three years following the initial episode of bronchiolitis, while other studies have followed children for up to ten years after the episode.

The majority of studies have been able to demonstrate some functional airway abnormality following infantile bronchiolitis, though one study failed to demonstrate any changes (143). Most of the other studies provide a consistent message. Studies of children in the years immediately following an episode of bronchiolitis suggest that in the first year after the infection there is an increased risk of developing sensitisation to common

aeroallergens (135,144,145). Studies focused on functional aspects of the airways demonstrated abnormalities in the first year after bronchiolitis, but these settled with time. Such a three and a half year study (146,147) found that at the two year follow up there was a high risk of lower respiratory tract symptoms (82%), though this fell in the subsequent year (69%). Other parameters of airway abnormality also fell, including LRTI lasting more than two weeks (36% to 31%); percentage of subjects with lower respiratory tract symptoms for more than 100 days per annum (33% to 17%) and hospital readmission with wheezing illness (13% to 7%). A similar study conducted by Korppi et al. (148,149) also concluded that the percentage of children with recurrent wheeze following bronchiolitis fell over time. When subjects were assessed in the first year after infection, 76% of children had wheeze, this fell the following year to 58% (148), but at the end of the study 4.5 to 6 years after the initial infection 25% of children still had wheezy episodes (149). In this final group of children with persistent wheeze there was a higher incidence of atopic dermatitis and atopic rhinitis.

Sigurs et al. (150) were able to show in a group of 147 children followed up for three years that an episode of bronchiolitis was the biggest risk factor for the development of asthma. The diagnosis of asthma was a physician diagnosis, defined as three episodes of recurrent wheeze. However previously highlighted studies have demonstrated that bronchiolitis increases the risk of wheezing episodes following infection, and this could confound the clinical diagnosis of asthma. The only study that confirms an increase in the prevalence of atopy/asthma was a long-term study by Wennergren et al. (151), though the asthma severity was graded mild to moderate.

One study has observed the oxygen saturation following bronchiolitis and been able to show abnormalities in the oxygen saturation of children admitted to hospital, which were still present on discharge from hospital (45). Long-term follow-up of these children revealed abnormalities in the oxygen saturation for the first three to four years following infection (152). In 21% of the children there were abnormalities present eight years after the initial infection, though there was a comment on the reversible nature of this abnormality no measures of bronchial hyperreactivity were included in the study. These

findings of persistent hypoxia after the initial event have not been reproduced by other studies.

One study that followed children for up to seven years after an episode of bronchiolitis and showed an increase in bronchial hyperreactivity, though no increase in atopy(153). Other long-term studies (eight to ten year follow-up) into the children who have had bronchiolitis have not been able to demonstrate any abnormalities in static lung function, bronchial hyperreactivity or episodes of recurrent wheeze (154,155). Long-term longitudinal studies, where the study population was assessed at several different time points after infection reveal why this apparent contradiction is present. A study conducted on children followed up for ten years that children who had bronchiolitis were more likely to suffer with wheezing episodes (42% vs. 21%). There was an increase in bronchial hyperresponsiveness in this group (6.2% vs. 4.5%), but there was no increase in atopy, as measured by SPT or serum IgE. The largest study conducted was the Tucson study, which followed a group of children for thirteen years after birth and annually assessed several different parameters (156-158). This study has demonstrate that though there was an increased risk of infrequent and frequent wheeze following RSV LRTI at the age of 6, which was maintained at the age of 11, this was lost by the age of 13 (158).

In conclusion there is little substantial evidence from population-based studies that RSV bronchiolitis predisposes to asthma in later life. However there is strong evidence that RSV bronchiolitis can lead to a transient increase in airway reactivity, which returns to normal within a few years. The prevalence of asthma increases after airway problems associated with bronchiolitis fall and continue to rise (158) suggesting that there are two different populations. Objective measures of atopic status, such as IgE, SPT or IgE-RAST do not increase long-term following RSV bronchiolitis, though there is evidence that RSV infection may cause an increase in sensitisation to aeroallergens in the first year after infection (135). Thus the conclusion, from a review of the available literature, does not support the hypothesis that RSV bronchiolitis is associated with an increased risk of atopic asthma, though there may be a transient rise in non-atopic asthma.

1.10 Evidence for different cell line infection

1.10.1 Evidence of epithelial infection

There have been several studies into the pathogenesis of RSV infection of epithelial cells. Evidence from animal studies demonstrate that bovine RSV infection leads to distinct airway changes, with loss of ciliary activity and viral replication, as ascertained by plaque assay (159). More recent studies have focused on the changes seen in the cell ultrastructure following RSV infection of polarised epithelial cells. It has shown that RSV matures and buds off from the apical epithelial surface in polarised epithelial cell lines (160). There are no previous studies that have tried to ascertain the viral load in infected cell lines by using real time quantitative PCR (TAQMAN). In the past viral load has been estimated by plaque assay, but this does not measure incomplete non-infectious virus (approx 10%) which may play an important part in the immunobiology of the disease (25).

Commercial reagents are used in microbiology laboratories to confirm RSV infection from nasal and nasopharyngeal aspirates. The reagents use fluorescent tagged monoclonal antibodies against RSV surface proteins (Dako, UK). This approach has been used in past studies to confirm infection of epithelial cells.

1.10.2 Evidence of macrophage infection

Early studies of RSV infection of macrophages suggested that RSV was unable to replicate in macrophages. However a series of reports in the late 1980's demonstrated that RSV could replicate in macrophages, though there was limited replication in monocytes (161). Reports from Panuska and colleagues suggested that replicative infection in alveolar macrophages could continue for several days (162), but the release of cytokines by infected macrophages could alter the susceptibility of surrounding macrophages to infection (163). Work by Midulla et al. (164) has shown that different populations of

monocytes and macrophages have different susceptibility to RSV infection, with monocytes being more susceptible than alveolar macrophages. The number of cells infected was increased by activation of the cells by lipopolysaccharide (LPS). Macrophages derived from cord blood are more susceptible to RSV infection compared to adult monocyte-derived macrophages (MDM) (164).

Monocyte-derived macrophages (MDM) have been used as an alternative to the use of alveolar macrophages and monocytic cell lines, such as U937. The work presented in this thesis uses the techniques outlined by Andreesen et al. (165), and this technique has been employed in RSV research before by Midulla et al. (164) and also Villani (166), Adair (167) and Tsutsumi (168).

1.11 Cytokines produced following RSV exposure

The cytokines are a group of small to medium sized proteins and glycoproteins that mediate a vast range of processes on most cells. In the scope of this thesis the cytokine response is important in the inflammatory process, though they do have other roles in the neuroendocrine system as well as in the nervous system.

There is a subgroup of cytokines known also as chemokines, an abbreviation of **chemo**tactic cyto**kine**, as the name suggests these small proteins are potent leukocyte chemoattractants. Members of this group are further sub-divided based on a structural motif in the molecule where 4 conserved cysteine residues form disulphide bridges. The groups are then named dependant on the amino acids between the bridges, thus there are CXC, CC, C and also CXXXC (or CX3C) chemokines.

1.11.1 Interleukin 1(IL-1)

Interleukin 1 (IL-1) is the term for two polypeptide mediators, interleukin 1 alpha (IL-1α) and interleukin 1 beta (IL-1β). The whole of the IL-1 system includes the agonists IL-1α and IL-1β; two high affinity IL-1 receptors IL-1RA and IL-1RB; a receptor antagonist IL-1Ra and also a specific activation system IL-1 converting enzyme (ICE)/caspase 1.

IL-1 can be produced by a wide variety of immune and non-immune effector cells, such as lymphocytes, monocytes and epithelial cells in response to infection and inflammation. The transcription of IL-1 is up regulated by several cytokines including IL-1 and IL-2. IL-1 production is down regulated by IL-4, IL-10 and IL-13 (169-171). Glucocorticoids are good inhibitors of IL-1, both at transcriptional and post-transcriptional levels. Prostaglandins do not alter the transcription of IL-1, though they can block release at the post-transcriptional level (172). The protein production of IL-1 requires the initial protein product of IL-1 synthesis to be cleaved from pre-IL-1 to the active form by caspase 1.

Thus though IL-1 mRNA production increases in response to the stimuli of infection and inflammation the active gene product is controlled by other factors and is closely linked to cellular apoptosis in response to infection.

The action of IL-1 is similar to the cytokines, IL-6 and TNF-α. IL-1 can act on T lymphocytes as a co-stimulant of proliferation and also activates them and stimulate T cells into releasing further cytokines, e.g. IL-1 targets the IL-2 promoter. There is stimulation of cytokine production in monocytes by IL-1. In neutrophils the main effect is to reduce apoptosis and promote neutrophil survival. At a whole organism level there is the promotion of the acute phase response, IL-1 is a key component in this, along with IL-6.

1.11.2 Previous study of Interleukin 1 response to RSV challenge

IL-1α production from RSV-challenged epithelial cells has been reported by several groups (173-176). Tsutsumi and colleagues (86) have shown IL-1β production from RSV-infected epithelial cells. Investigators such as Salkind, have compared the IL-1 response of RSV with other viruses and suggest that there is relative suppression of the IL-1 response compared to the IL-1 response demonstrated when other viruses infect epithelial cells(177).

Some studies of macrophage RSV infection have only described IL-1 production and not specified whether it was IL-1α or IL-1β (178,179). Other studies have studied IL-1β from adult macrophages(168) or from neonatal macrophages (180), only Soukup and colleagues have studied the production of both IL-1α and IL-1β in macrophages infected with RSV and shown elevation of both cytokines(181).

There have been few studies that have investigated the production of IL-1 in a clinical context, Becker et al. (182) did investigate local nasal cytokine mRNA production following RSV infection was not able to detect IL-1 mRNA from the nasal mucosa.

1.11.3 Interleukin 4 (IL-4)

Interleukin 4 (IL-4) is produced by the Th2 type subset of T lymphocytes, as well as mast cells and basophils. IL-4 causes activation of B cells (183), causes B cell proliferation (184), differentiation and Ig chain switching (185). IL-4 causes increased MHC class II expression on monocytes and macrophages, causes differentiation of monocytes to macrophages (186), but inhibits cytokine production from macrophages.

1.11.4 Previous studies of Interleukin 4 response to RSV challenge

The vast majority of studies that have investigated IL-4 responses following RSV infection have been in the murine model of RSV infection. Roman et al (109) demonstrated that there was an alteration in IL-4/IFN-γ ratio of stimulated PBMC from children who had RSV infection, suggesting either an increase in IL-4 or a fall in IFN-γ. This has also been shown by Bendelja et al. (187). The latter study demonstrated that CD8 cells produced IL-4 more often than the CD4 T cell population.

One study has tried to establish whether IL-4 is present in the aspirates of children with RSV infection, the results from this study were not able to demonstrate any detectable IL-4 production (107).

1.11.5 Interleukin 6 (IL-6)

Interleukin-6 (IL-6), with IL-1 is a major constituent of the acute phase response. IL-6 has a wide range of functions on immune cells; it can induce responses in B cells, T helper cells, T cytotoxic cells and NK cells (188,189). In B cells IL-6 acts as a late stage differentiation factor inducing activated B cells to form plasma cells. IL-6 acts on T cells to promote proliferation and maturation (190). IL-6 has an effect on many haemopoietic cells, especially multilineage progenitors. The actions of IL-6 can lead to the inhibition of

IL-1 and tumour necrosis factor alpha (TNF-α) (191,192), both of these two cytokines are strong inducers of IL-6 production.
IL-6 can be produced by a wide variety of cellular sources, though the most significant source is cells from the monocyte/macrophage lineage. Epithelial and endothelial cells can produce IL-6 in response to IL-1 and TNF-α. T cells, B cells and mast cells have demonstrated the ability to produce IL-6. The production of IL-6 from macrophages can be reduced by IL-4, IL-10 and glucocorticoids (193-195).

IL-6 transcription is regulated by certain response elements found in the promoter region of the IL-6 gene; these include NFIL-6, NF-κB, cAMP response element binding protein (CREB) and the glucocorticoid receptor (GR). It is also known that the tumour suppression gene p53 inhibits IL-6 promoter-driven expression.

1.11.6 Previous study of Interleukin 6 response to RSV challenge

Other investigators have previously studied the production of IL-6 in response to RSV challenge. Investigations on epithelial cells have been conducted on a wide variety of epithelial cell lines. These studies have shown that there is a dose dependant increase in IL-6 production following RSV infection of A549 cells (196) and that there are differences in the timing of peak cytokine production depending on the cell line. Noah and colleagues found the peak increase in IL-6 production at 96 hours post-infection for the BEAS-2B cell line (197), while Tristram demonstrated peak IL-6 production 24 hours post-infection when a primary epithelial cell line was used (198).

The results from previous studies on macrophage production of IL-6 following infection show differing patterns, depending on the macrophage source. The majority of previous studies have used alveolar macrophages to investigate the interaction between macrophages and RSV (178,181,199), one study has used milk macrophages (200) and others have used cord blood macrophages (168,201).

There is evidence that neonatal or cord blood macrophages are more resistant to infection and produce lower levels of IL-6 following infection (168,201). The source of the macrophages demonstrate differing results, from studies performed on cord blood derived macrophages reveal prompt production of IL-6 peaking within 24 hours of challenge, while alveolar macrophages do not reach peak production until 24-48 hours post-infection. The results of stimulation with inactivated RSV in cord blood cells provoke negligible IL-6 production; the studies with alveolar macrophages do not report the results of using UV-inactivated RSV. The results from murine alveolar macrophages challenged with UV-inactivated RSV reveal significant production of IL-6 leading investigators to conclude that IL-6 production by macrophages does not rely on viral replication to occur (202).

Clinical studies that have investigated the airway and serum production of IL-6 in children with RSV infection have shown that though Becker et al. (182) were not able to detect IL-6 mRNA with children with RSV infection other researchers have been able to. Matsuda et al. (203) were able to demonstrate elevated IL-6 protein levels in all the children they studied with RSV infection, as were Sheeran et al. (204). Hornsleth et al. (205) demonstrated elevated IL-6 protein levels in the serum with an increase in the IL-6/TNF-α ratio from nasal aspirates, with the elevation correlating to disease severity in terms of length of hospital stay, respiratory support and infiltrates on chest x-ray.

1.11.7 Interleukin 8 (IL-8)

Interleukin 8 (IL-8) is a member of the chemokine family, the chemokines (**chemo**tactic cyto**kines**) are an important group of recently described mediators; they are pivotal in recruiting inflammatory effector cells to sites of infection and inflammation. The chemokines are divided into several sub-groups depending on their internal structure and the intervening amino acids between disulphide bonds. The groups are CXC, which includes IL-8; CC, which includes RANTES; C, which includes lymphotactin, and CXXXC (or CX_3C), which includes Fractalkine.

Interleukin 8 (IL-8) is a representative member of the CXC chemokine family. IL-8 transcription can be increased by the addition of IL-1, TNF-α, reactive oxygen and nitrogen intermediates. The IL-8 gene contains many regulatory elements, such as NF-κB, NF IL-6, AP-1, AP-2 and AP-3, as well as the glucocorticoid response element.
There are a wide variety of cellular sources for IL-8 including: monocytes/macrophages, endothelial/ epithelial cells (206), lymphocytes (207), neutrophils (208), eosinophils (209), fibroblasts (210) and smooth muscle cells (211). The production of IL-8 can be stimulated by the inflammatory cytokines, IL-1α or β, TNF-α and also lipopolysaccharide (LPS), hypoxia (212), adherence (213). The presence of the glucocorticoid response element (GRE) means that IL-8 expression can be down regulated by dexamethasone (214).

The actions of IL-8 include neutrophil activation, as judged by degranulation, shape change and chemotaxis (215). There is evidence of stimulation of the respiratory burst and superoxide production (216). IL-8 also leads to the alteration in intergrins on the cell surface, these bind strongly to their ligands, the intercellular adhesion molecules (ICAM) and promote margination of neutrophils (217). Il-8 exerts its activity through two high affinity receptors, CXCR1 (IL-8RA) and CXCR2 (IL-8RB) (218,219). IL-8 is the sole ligand for CXCR1, while CXCR2 is a promiscuous receptor that binds other CXC cytokines, such as GRO-α/β/γ. There is low affinity binding to the Duffy blood group antigen (DARC).

1.11.8 Previous studies of Interleukin 8 response to RSV challenge

Past investigation of the production and regulation of IL-8 following RSV infection of epithelial cells have shown that RSV challenge of A549 cells does lead to an increase in IL-8 production within 24 hours of challenge (197) and that IL-8 production is both time and infecting viral dose dependant (196). Most cell lines when stimulated with RSV provide an increase in IL-8 concentration, from A549 (196), through BEAS-2B (197) to MRC5, WI38 and Hep-2 cell lines (220). Recently there have been investigations that

have shown that viral replication is not necessary for the production of IL-8 (221). IL-8 production is stimulated by IL-1α, one of the pro-inflammatory cytokines, which can act in an autocrine fashion on epithelial cells. Current studies are investigating the regulation of IL-8 activation and the role of transcription factors in regulating this.

Though macrophages are know to be a major source of IL-8, there have been relatively few studies that have investigated the release of this chemokine in response to RSV challenge. The two main studies have demonstrated conflicting results; Becker et al. (199) demonstrated an increase in IL-8 production from alveolar macrophages challenged with RSV. The release of IL-8 occurred within the first 24 hours and similar results were observed if UV-inactivated were used for the challenge. Panuska et al. demonstrated a fall in IL-8 production by alveolar macrophages in response to RSV infection, while there was an increase in other cytokines/chemokines (180).

Other CXC chemokines, such as GRO-α have been studied in primary epithelial cell culture, where there is high constitutive expression of GRO-α and no increase in GRO-α expression following RSV infection (222).

In-vivo studies have investigated the production of IL-8 following RSV infection. Becker et al. (182) were able to demonstrate a high constitutive IL-8 mRNA expression from nasal epithelium, there was an increase following infection. Saito et al (222) was also able demonstrate high constitutive IL-8 mRNA expression, but were not able to demonstrate an increase with RSV infection in tissues derived from the upper airway. Sheeran et al. (204) were able to demonstrate increased IL-8 protein levels in both nasal wash samples, and also from tracheal aspirates of intubated children, and the IL-8 levels from the nasal washes were inversely correlated to disease severity. Harrison et al. (220) were also able to demonstrate elevated IL-8 protein levels from the lower airways of children intubated with RSV infection. A recent study by Noah et al. (223) was able to demonstrate an increase in IL-8 protein following RSV infection from the nasal mucosa, but was unable to demonstrate an increase in IL-8 mRNA. Thus past in-vivo studies have not demonstrated

an increase in IL-8 mRNA following RSV infection, but have demonstrated high IL-8 protein levels in both the upper and lower airways following infection, this could suggest either release of pre-formed IL-8 or that IL-8 is produced by cells that are recruited into the airway. There are no studies that have investigated the IL-8 response in blood to RSV infection.

1.11.9 Interleukin 10 (IL-10)

Interleukin 10 (IL-10) is produced by a wide variety of cells, such as monocytes following stimulation by LPS (224); B-lymphocytes stimulated by *Staph aureus* (225) and activated T cells as well (226). Initially IL-10 was classified as a Th2 type cytokine, though this holds for murine immunology, in humans it is produced by Th0, Th1 and Th2 cells (226,227). TNF-α can stimulate IL-10 production (228); while IFN-γ, IL-4, IL-13 and IL-10 all inhibit the production of IL-10 by LPS stimulated monocytes (229-231).

The action of IL-10 can act on monocytes and macrophages to reduce their adhesion (232), reduce production of pro-inflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-8 and IL-12. It does increase the production of IL-1RA from monocytes (230,233-236). There is a reduction in antigen presentation from monocytes, following IL-10 administration; this action is due to a reduction in co-stimulatory molecules (230,232,237,238), reduction of IL-12 production and reduction in MHC class II expression. The T cell production of IL-2 and TNF-α is inhibited by IL10. IL-10 synergises with IL-2 to induce B cell proliferation and differentiation (239).

1.11.10 Previous studies of Interleukin 10 response to RSV challenge

There have been few studies of the IL-10 response of mononuclear cells in response to RSV challenge. Panuska et al (180) were able to demonstrate an increase in IL-10 mRNA following RSV and LPS co-stimulation of alveolar macrophages. Stimulation of PBMC

with the G protein of RSV has been shown to increase IL-10 production at low levels and inhibit it at higher levels (240).

Elevated levels of IL-10 have been found in the nasal washes and tracheal aspirates of children with RSV infection, and are correlated with WBC in nasal washes (204). In-vitro studies of PBMC taken from children with RSV bronchiolitis have shown that increased IL-10 production in the convalescent phase is associated with prolonged wheeze (241).

1.11.11 Interleukin 12 (IL-12)

Interleukin 12 is an unusual cytokine as it is composed of two sub-units (p35 and p40); both are needed for active IL-12. The genes are found on chromosome 3 and 5 respectively (242). Disulphide bonds link the two subunits. Neither sub-unit by themselves has significant activity compared to the heterodimer, the p40 sub-unit may stabilise the p35 sub-unit in a confirmation that allows binding to the IL-12 receptor (243). The p35 sub-unit has homology with IL-6 and G-CSF (244), while the p40 sub-unit shares homology with the IL-6 receptor (245). Monocytes, macrophages as well as B cells produce IL-12 (246). Its production is increased in response to LPS and killed *Mycobacterium tuberculosis*. Its actions are to activate NK cells (247), up-regulate expression of adhesion molecules on cytolytic cells (248). It also stimulates proliferation of stimulated T cells (249). There is an enhancement of the production of IFN-γ from lymphocytes (247,250). It is also important in the generation of Th1 type responses (251), IL-12 also reduces the IL-4 stimulated production of IgE from B cells (252).

1.11.12 Previous studies of Interleukin 12 response to RSV challenge

There are no studies that have investigated the IL-12 response from RSV challenged monocytes or that have investigated the IL-12 response in-vivo following RSV bronchiolitis.

1.11.13 Interleukin 18 (IL-18)

IL-18 is a recently described Th1-type cytokine; it was originally designated IFN-γ releasing factor (IGRF) as its actions are to stimulate the production of IFN-γ (253).

IL-18 can be considered a Th1 cytokine (along with IL-2, IL-12 and IL-15), but has activities beyond this action alone. IL-18 is released in a pro form, proIL-18, which is cleaved by caspase-1 (ICE), thus there are similarities to IL-1β (254). It acts through its receptor IL-18R that is comprised of IL-18Rα (which is a member of IL-1 receptor family, previously designated IL-1Rrp or IL-1 receptor-related protein) and IL-18Rβ (also designated AcPL which is related to IL-1R acessory protein). The signal transduction pathway is similar IL-1's with IRAK and NIK activation and NF-κB signaling (255). IL-18 production is constitutive from cultured peripheral blood mononuclear cells, but can be induced by various cytokines, including: IL-1, TNF-α, IL-6 and IL-10. IL-18 can activate T cells to synthesize IL-2, GM-CSF and TNF-α, while suppressing IL-10 production (254). There are reports that IL-18 can lead to broad-spectrum chemokine release and this could promote eosinophil recruitment (256).

1.11.14 Previous studies of Interleukin 18 response to RSV challenge

There are no previous reports of IL-18 being studied in the context of RSV infection.

1.11.15 Tumour necrosis factor alpha (TNF-α)

Tumour necrosis factor alpha (TNF-α) is an inflammatory cytokine that is produced mainly by macrophages and monocytes (257). The transcriptional regulation of TNF-α is through NF-κB, but other factors are important. The AU rich 3' untranslated sequence of TNF-α mRNA is important in regulation of TNF-α production as it renders the mRNA

less stable; the AU sequences are recognition sites for the binding of ribonucleases. TNF-α has several effects, it induces monocyte differentiation (258), and mitogenic for fibroblasts as well as B and T lymphocytes (259,260). TNF-α activates monocytes and macrophages and can lead to increased production of IL-1, IL-6 and IL-8. TNF-α induces neutrophil adherence to endothelial cells, activates phagocytosis and enhances the oxidative burst (261-263). There is evidence that TNF-a also has antiviral activity against VSV and HSV-2 (264), it can also lyse cells that are virally infected.

1.11.16 Previous studies of TNF-α response to RSV challenge

There has been conflicting evidence for TNF-α production by epithelial cells infected by RSV, Fielder et al. (221) was unable to detect TNF-α mRNA or protein following RSV challenge, though Patel and others have demonstrated an increase in TNF-α mRNA by PCR (86,173,175,176).

TNF-α is one of the major cytokines produced by macrophages and monocytes in response to RSV challenge. It is hypothesised that TNF-α has anti-viral effects and can restrict viral replication in cells. There is evidence that this is the case with RSV infection, Midulla (164), Cirino (163) and Franke (265) all have produced evidence that TNF-α can restrict RSV replication in macrophages and epithelial cells, though there is no evidence of complete protection against infection. Midulla (164) studied infection of monocytes by RSV and demonstrated that the addition of LPS lead to the production of TNF-α and a reduction in the percentage of monocytes staining positive for infection by RSV. Cirino and colleagues (163) showed that RSV-infected alveolar macrophages produced a variety of cytokines, though prior treatment of alveolar macrophages did not affect viral infection or replication, prior treatment with TNF-α lead to a reduction in viral replication by 30-60%. Franke and colleagues (265) studied the effect of RSV infection in murine alveolar macrophages, they demonstrated that resting macrophages became more heavily infected when compared to activated macrophages that produced high levels of TNF-α.

The differential production of TNF-α was similar to the differential observed with IL-6, and has been demonstrated in milk macrophages (200) and neonatal monocyte-derived macrophages (168,201). Macrophages from older donors produce greater amounts of TNF-α and do so for longer after RSV infection; cord blood macrophages produce the majority of their TNF-α within the first 24hours.

Becker was able to show that replicating RSV was required for sustained production of TNF-α, though UV-inactivated RSV did stimulate TNF-α production (199). The production of TNF-α was the greatest out of all the cytokines produced in the study by Becker (199).

Studies of clinical RSV infection have been able to demonstrate that infection with RSV does lead to the infection of alveolar macrophages(266), Matsuda showed that there was an increase in TNF-α production from nasal aspirates in the acute phase of RSV infection, and that this rapidly declined during convalescence (203). There are no reports in the literature that correlate TNF-α levels with disease severity.

1.11.17 Interferon alpha (IFN-α)

IFN-α is a collection of peptides with a high degree of structural homology. One of the major mononuclear cell responses to viral infection is the production of IFN-α. IFN-α together with IFN-β are classed as a type-1 interferons (because of their acid stability) (267). The major stimulus for IFN-α production is double stranded RNA (dsRNA) – as found during viral replication (268), its production leads to an increase in 2-5 adenylate synthetase and dsRNA-dependant protein kinase (269). IFN-α also synergises with TNF-α to activate inducible nitric oxide synthetase (iNOS); it can also cause CD8 T cell expansion and activation. IFN-α has an inhibitory effect on IL-12 production (270).

There is evidence that the production of IFN-α has an anti-viral effects and it may protect other airway cells from infection.

1.11.18 Previous studies of Interferon alpha (IFN-α) response to RSV challenge

The role of IFN-α in RSV infection has been controversial for many years, though more recent studies indicate that it may be important. Previous clinical reports have not been able to demonstrate any relationship between IFN-α production and severity of RSV infection (271), or level of F protein shedding (272). There is little correlation between the age of infection and IFN-α production, though there is significantly lower production during the first three months of life (273).

Clinical intervention trials with IFN-α in children with RSV infection have not been able to demonstrate a reduction in signs and symptoms (274) or viral shedding (275). In-vitro studies have shown variable results, but the degree of maturation of monocyte-derived macrophages (MDM) is an important component in the level of IFN-α production. A comparison between RSV and parainfluenza virus 3 (PIV3) infection of MDM, demonstrated that RSV infection leads to higher IFN-α production than PIV3 (161), this contradicts the findings of another study, that RSV infection leads to lower IFN production when compared to other viral infections (271).

Results from previous studies have demonstrated that macrophages have no constitutive production of IFN-α, but post-RSV challenge there is an increase (161,180). The production of IFN-α by macrophages in the lung may be a significant factor in the subsequent immune response.

1.11.19 Interferon gamma (IFN-γ)

Interferon gamma (IFN-γ) is a Th1-type cytokine, that also has anti-viral properties (276). IFN-γ has several other actions, including enhancing MHC class II expression (277), activating macrophages (this is antagonised by IFN-α), and regulation of IgG production (278). The only sources of IFN-γ are NK cells and T cells. When T cells are stimulated they either produce IFN-γ in an antigen specific manner, or in response to antibodies or mitogens.

1.11.20 Previous studies of Interferon gamma response to RSV challenge

Though IFN-γ production in PBMC following RSV challenge has been investigated in the past, there are few reports of the production of IFN-γ in monocytes or macrophages following RSV challenge. From initial experiments by Midulla et al. (164) there was evidence that IFN-γ reduced macrophage infection by RSV. In a study of milk macrophages Sone was not able to demonstrate any increase in IFN-γ production following RSV challenge (200). A study by van Schaik et al. that investigated the responses in children with virus-induced wheeze, demonstrated an increase in IFN-γ in children with wheeze. In this study there was no characterisation of the causative virus (279).

1.11.21 RANTES (Regulated upon Activation Normal T cell Expressed and Secreted)

The CC sub-group of chemokines includes Regulated upon normal T cell expressed and secreted (RANTES). RANTES is produced by a wide variety of cells. Several cell lines, not just T lymphocytes (280), as suggested by its name, can produce RANTES; these include fibroblasts (281), endothelial/epithelial cells and monocytes (282). These cells

produce RANTES in response to different stimuli, T lymphocytes produce it early in their development; fibroblasts produce it in response to IL-1 and TNF-α; while endothelial/epithelial cells produce it in response to stimulation by TNF-α and IFN-γ.

The activities of RANTES include: T lymphocyte, monocyte and NK cell chemotaxis (283); alteration of $CD4^+$ lymphocyte and monocyte cell surface β-intergrin expression, leading to increased adhesion to endothelial surfaces. RANTES also leads to T lymphocyte activation and transendothelial migration via upregulation of the secretion of matrix metalloproteinases (284). Recently RANTES has also been shown to have an effect on eosinophil and basophil chemotaxis and activation. There are several chemokine receptors that have RANTES as one of their ligands, these are CCR1, CCR3 and CCR5, and none of these receptors have RANTES as their sole ligand.

1.11.22 Previous studies of RANTES response to RSV challenge

There are few previous studies of RSV infection of either epithelial cells cell lines that have studied the effect upon RANTES production. Studies of epithelial infection with RSV have shown that infection of primary epithelial cells (198,222,285), and cell lines (285-287) lead to the production of RANTES. Becker et al. was able demonstrate a time and dose dependant release of RANTES from epithelial cells.

There have been no studies that have investigated the RANTES production from macrophages that have been challenged with RSV.

Previous studies have demonstrated that there is an increase in RANTES production from nasal epithelium in culture that is infected with RSV (222), also the nasal mucosa does produce RANTES mRNA after infection (223). Studies of lower airway infection have shown that RANTES protein can be detected from intubated children (204,220), though lower airway levels of RANTES are inversely correlated to disease severity.

1.11.23 Monocyte chemoattractant protein-1 (MCP-1)

Monocyte chemoattractant protein –1 (MCP-1) belongs to a sub-group of the CC chemokines that are involved in monocyte/macrophage chemotaxis; to date there are four members of this family have been described in humans. MCP-1 is produce by a wide variety of cells including: fibroblasts, monocytes/macrophages, epithelial cells and smooth muscle cells (288). The MCP-1 promoter region contains binding sites for AP-1 as well as NF-κB (289). It is known that cells stimulated with cytokines, such as IL-1β, TNF-α and IFN-γ can all lead to an increase in MCP-1 production, as can stimulation with bacteria, measles virus, LPS and PHA. Steroids can inhibit the production of MCP-1. MCP-1 binds to CCR1, CCR2 and DARC.

1.11.24 Previous studies of MCP-1 response to RSV challenge

There have been three studies investigating the production of MCP-1 from epithelial cells. Two studies have not been able to demonstrate any significant increase in production of MCP-1 over control cells following RSV infection (222,285). Both studies used primary epithelial cell cultures and the BEAS-2B cell line and both studies demonstrated high constitutive production of MCP-1 with no further increase in MCP-1 production following RSV infection. Only one study has been able show an increase in MCP-1 production following RSV infection (290). There have been no studies that have investigated the production of MCP-1 from macrophages following RSV infection.

The only clinical study that has investigated MCP-1 production following RSV infection was a study conducted by Noah et al., where healthy adult volunteers were infected with RSV and nasal lavage fluid was analyzed. There was an increase in both MCP-1 mRNA and protein production associated with the duration of viral shedding only (223).

1.11.25 Macrophage Inflammatory Protein (MIP-1α and MIP-1β)

The proteins macrophage inflammatory proteins 1α/β (MIP-1α/β) are members of the CC chemokine family and are produced by a variety of cells, including macrophages /monocytes, lymphocytes, mast cells and epithelial cells (291). The main targets for these chemokines are lymphocytes and haemopoietic precursor cells (292-295). The promoter site for MIP-1α contains binding sites for C/EBP and NF-κB; the MIP-1β promoter contains CRE binding sites. Production of MIP-1α is up regulated by IL-1, LPS, TNF-α and IFN-γ.

1.11.26 Previous studies of MIP-1α response to RSV challenge

There has been one prior study that has demonstrated MIP-1α production from epithelial cell lines infected with RSV (220). MIP-1β production following RSV infection of both primary epithelial and established cell lines has been studied by Becker et al., they were not able to demonstrate any significant increase in production following RSV infection (285).

There have been no previous studies that have investigated the production of either MIP-1α or MIP-1β from macrophages in response to RSV challenge.

Clinical studies have been able to show that upper respiratory tract infections are associated with the production of MIP-1α (296), Sheeran was able to demonstrate and increase in MIP-1α in the nasal wash and tracheal aspirates of children with RSV infection (204). Recently Garofalo was able to establish that there was an inverse correlation between MIP-1α in nasopharyngeal secretions and oxygen saturations in children with severe bronchiolitis (297). There are no clinical studies in the literature that have studied the MIP-1β response during RSV infection.

1.11.27 Eotaxin

The CC chemokine eotaxin was also studied; this chemokine has homology with MCP-1 and MIP-1α. Studies have demonstrated its production from epithelial cells and alveolar macrophages. It has specific chemoattractant properties and activation properties for eosinophils.

1.11.28 Previous studies of Eotaxin response to RSV challenge

There are no prior studies that have focused on the eotaxin production by A549 pneumocytes or other respiratory epithelial cell lines in response to RSV infection. The eotaxin response to influenza virus has been studied and this shows that there is constitutive production of eotaxin mRNA and that this is up regulated by influenza virus infection. There are no clinical studies that have investigated the production of eotaxin in response to RSV infection. The pathology of RSV infection does suggest that eotaxin may play a role, with evidence of significant aggregation of eosinophils to the respiratory bronchioles.

1.12 Study Hypothesis

The investigation hypothesis is that respiratory syncytial virus infection stimulates the early production of cytokines that can stimulate and skew the immune response of incoming lymphocytes towards a Th2 type phenotype, where the response is similar that seen in acute asthma.

The hypothesis to be tested by the clinical study, with regards to the response of macrophage/monocytes to RSV infection was that the IL-10/IL-12 response would follow the IFN-γ/IL-4 response that is observed and would occur at the later time point. The response would follow the lymphocyte response and lead to a predominant Th-2 type response.

1.13 Research Objectives

The research objectives for this project include:-

i) Establishing a model for RSV epithelial infection.

ii) Establishing methodology for culture of monocytes and development into a macrophage phenotype

iii) Demonstrating epithelial and macrophage infection by RSV

iv) Establishing techniques for measuring cytokine mRNA and protein production.

v) Establish a technique to quantify viral load during RSV infection.

vi) Investigate the epithelial innate responses to RSV infection.

vii) Collect a cohort of children with atopic parents and follow them through their first RSV season.

viii) Sample the nasal secretions of the children a very early stage of upper respiratory tract symptoms and again later on.

ix) Correlate the in-vivo response to RSV infection with the in-vitro response

Chapter 2 - Methods

2.1 Introduction

The work presented in this section was performed in the Southampton University's departments of Medicine and Microbiology. The in-vivo work presented in this thesis was performed in conjunction with Dr Julian Legg, Department of Paediatrics, Southampton University. The study was designed in conjunction with Dr Legg; Dr Legg performed the sample collection, the analysis was performed by both of us and used techniques optimised by myself.

The work described took place between August 1996 and July 1999.

2.2 Cell culture Techniques

2.2.1 A549 cells

The A549 cell line is a cell line that has been established from Type II alveolar pneumocytes. This cell line has the characteristic features of Type II cells, with the ability to produce surfactant and is extensively used as a cell line that models cells of the alveolar space. The cells divide rapidly and achieve confluence quickly with little cell over growth occurring. This cell line has been established as the most common cell line used in investigating the effects of RSV infection in airway epithelial cells.

Culture

The cells were obtained at passage number 80-85 from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK) and all the experiments took place between passage 90 and 95.

The cell line was propagated in $75cm^2$ tissue flasks (Nunc, Paisley, UK), with 20 ml of medium in each flask. The cells were passaged every 5 to 7 days depending on the degree of confluence achieved, and original seeding density.

Culture Medium

All the cell lines used in this series of studies were propagated in Minimal Essential Medium (MEM) (Life Technologies, Paisley, UK) supplemented with Glutamax-I. The medium had additional gentamicin (250μg/ml), amphotericin B (1.25μg/ml), sodium carbonate (24mM), HEPES buffer (20mM) (all supplements from Sigma-Aldrich, Poole, UK).

When the medium was used to propagate the cells, 10% foetal calf serum (FCS) (Life Technologies, Paisley, UK) was added to the culture medium. If the medium was being used for experimental purposes the FCS concentration added to the culture medium was reduced to 1%.

Passage of cells

The cells were initially washed twice with phosphate buffered saline (PBS) to remove any contaminating foetal calf serum and dead cells, before the addition of 5ml of trypsin/EDTA mixture (2.5g/l and 0.2 g/l respectively) (Sigma-Aldrich, Poole, UK) to the cell monolayer. The monolayer was incubated at room temperature for 1 minute, and then the majority of the trypsin/EDTA mixture was decanted off leaving enough remaining fluid to cover the monolayer. There was a further 2 to 3 minute incubation, until the cells were observed to be detaching from the tissue culture flask. The cells are then detached from the tissue culture tray by gentle agitation. Once the cells were detached from the flask 10ml of culture medium was added to the flask to inactivate the trypsin/EDTA. The mixture was thoroughly mixed by pipetting the mixture to ensure that there was even dispersal of the cells.

To continue propagation of the cell line 2ml of the final mixture was added to a new 75 cm^2 tissue culture flask containing 20ml of culture medium, mixing of the solutions was achieved by thorough pipetting. The flask was maintained in an incubator at 37°C with an atmosphere containing 5% CO_2. The culture medium was changed three days after splitting.

2.2.2 16HBE cell line

The 16HBE cell line is an incompletely SV-40 transformed bronchial epithelial cell line. This was obtained from ECACC (Porton Down, UK). It is used to represent the bronchial epithelium, its culture requirements are similar to those for the A549 cell line, but 5ml, instead of 2ml, of the final mixture after inactivation of the trypsin/EDTA by tissue culture medium was added to a 75cm^2 tissue culture flask with 17ml of tissue culture medium.

The cell line was propagated in 75cm^2 tissue flasks (Nunc, Paisley, UK), with 20 ml of medium in each flask. The cells were passaged every 7 days. For propagating the cell line, the monolayer had to be incubated with 5ml trypsin (2.5g/l) / EDTA (0.2 g/l) mix for approximately 5-10 minutes at 37°C to ensure complete detachment of the cells. Once the cells are detached and 10ml of culture medium has been added, 5ml of the resultant mixture has to be added to 20ml of fresh culture medium to ensure good propagation of the cell line. Using the concentrations outlined above the cell monolayer required passaging every 7 days. This cell line also requires a medium change after 3 days of splitting to remove dead and non-adherent cells, as these reduced the growth of the adherent cells.

2.2.3 Hep2 cell line

The culture conditions for the Hep2 cell line were exactly the same requirements as for the A549 cell line. This cell line was also obtained from ECACC (Porton Down, UK), this cell line was only used to grow RSV stocks for the subsequent experimentation.

2.2.4 Experimenting With A549/Hep2 Cell Lines

These two cell lines required monolayers to be grown to confluence. The cells were transferred to tissue culture trays for experimentation. Cells were stripped from the 75cm^2 tissue culture flasks by the method employed for passaging the cell lines. The cells were

re-suspended in MEM with 10% FCS and diluted to a concentration of 1 x 10^5 cells/ml. The cells were counted using a Neubauher chamber and trypan blue to count viable cells. If cells were to be experimented on in 24 well trays, then 1ml of the mix was added to each well and 2ml if 12 well trays were used. The cells were cultured overnight at 37°C and 5% CO_2 prior to experimentation. Prior to experimentation the cells were washed twice with PBS, before the growth medium was replaced with experimental medium; the experimental media was 500µl of MEM with 1% FCS per well. The cells achieve approximately 90% confluence following overnight culture. The cell line is allowed to equilibrate with this new medium for a minimum of 60 minutes prior to any experimentation.

Cell counting

Cell counts were performed using a Neubauer counting chamber (Wolf Scientific supplies, York, UK). The cell suspension is thoroughly mixed prior to an aliquot being removed; this was diluted 1 in 10 with 8% solution of trypan blue dye. Approximately 500µl of the resultant mix was taken and placed on a counting slide and counted using an inverted phase microscope (Leica instruments, Leica (UK) Ltd,. Milton Keynes, UK). An average of the two centre fields was taken, to establish the concentration of the cells in solution.

2.2.5 16HBE Cell line – Experimental

The 16HBE cell line had a slower growth rate than the other cell lines used, thus the interval between seeding of the wells in tissue culture trays to achieving 90% confluence was 72 hours. The seeding densities used were the same as for the other cell lines, 1 x 10^5 cells for a 1ml well and 2 x 10^5 cells for a 2ml well. The cells were cultured at 37°C and 5% CO_2. The same conditions were employed for experimentation, with 500ml of MEM with 1% FCS used for experimentation, following the well being washed twice with PBS after removal of the culture medium.

2.3 Generation of RSV Stores

The original stock of RSV-A2 that was used for the experiments was obtained from Professor Openshaw's laboratory, St. Mary's Hospital, London. The identity of the virus stock was confirmed by sequencing the F and G genes (see section 2.3a for sequencing protocol) and comparing them to the known sequence of RSV A2 strain and also restriction enzyme analysis of the F and G genes.

Primers for the F and G genes were constructed on the basis of known sequences for these genes obtained from the EMBL database. The primers were designed so that they covered the conserved regions of the F and G protein genes.

2.3.1 Generation of RSV Stock

To inoculate a new batch of Hep-2 cells, the cells were grown in bulk, until six $75cm^2$ tissue culture trays were confluent. The growth medium was discarded and the cells washed once to remove any dead cells, following this 2ml of culture medium, warmed to 37°C, was added to each flask. An aliquot of frozen RSV stock with 1×10^6 virus/ml was rapidly thawed and warmed to 37°C, before being added to the tissue culture flask. The tissue culture flask was kept at 37°C and gently agitated for one hour under normal atmospheric conditions, to ensure good coverage of the monolayer by the virus stock. After this, a further 6 ml of medium was added to the flask and it was incubated at 37°C in 5% CO_2 for several days, once there was evidence of cytopathic effect affecting 50% of the cells, the cells were harvested.

To harvest the cells, a cell scraper was used to strip the cells off the surface of the tissue culture flask. Once the cells and culture medium was harvested the samples from all the culture flasks were mixed. The samples were spun at 500g for 5 minutes to remove the majority of the cellular debris that was present. The samples were aliquoted into 1 ml samples, so that there would not be any further freeze thaw cycles, which would reduce

the virus viability. For subsequent experimentation single aliquots of RSV stock would be thawed for use, none were re-frozen after the initial thawing. The aliquots were stored at –135°C until used. This was used as the stock virus for all the epithelial and macrophage experiments performed.

2.3.2 Sequencing of RSV genome

The theory of the sequencing reaction is similar to the polymerase chain reaction. The sequencing reaction contains a heat stable DNA polymerase enzyme, the deoxy-nucleotides for the 4 bases, with a certain proportion of terminator nucleotides and one primer. As the reaction proceeds there will be multiple versions of the copy of the template made, the length will vary depending on the termination of the extension due to the terminator nucleotides. The reaction mix is then separated using a polyacrilamide gel, sequence can be worked out by the varying lengths of oligonucleotides.

A sample of the RSV stock solution was taken and diluted 1000 fold. A 9.5µl sample of this solution was then used as the template for the sequencing reaction. A commercial sequencing reagent was utilised for the sequencing reaction. Thermo Sequenase (Amersham, Little Chalfont, UK) reaction mixture 9.5µl, diluted RSV template 9.5µl and primer 1µl (3.2 pmol).

The sequencing reaction was performed in 200 µl reaction vessels, and performed in thermal cycler (Tetrad PTC-225, MJ Technologies, Waltham USA). The reaction was processed as per manufactures instructions, 25 cycles of 96°C 15seconds, 48°C 10seconds annealing time and 60°C 4minutes extension time.

The reaction mixture was purified by precipitating the reaction mixture by the addition of 53µl 60mM sodium acetate in 95% ethanol. The mixture was cooled to 4°C for 10 minutes, following which it was centrifuged at 10000 g at 4°C for 30 minutes. The supernatant was removed and the pellet was washed with 75% ethanol, followed by a 5-

minute spin at 10000g. The pellet was vacuum dried and stored at –20°C until it was reconstituted for sequencing.

The samples were then run on an ABI 3700 sequence analyser (Foster City, USA). The separation occurred using a acrylamide 6% gel.

Gel Formation

A 6% sequencing gel was made by adding the following constituents in order; 32ml Sequagel (Flowgen, UK) to 13% v/v 10x TBE concentrate (recipe given below), 6% v/v UHQ, 5% w/v ammonium persulphate (APS) (Sigma Aldrich, Poole, UK) and 0.05% v/v Temed (Kodak, Rochester, US).

The recipe for 1 litre of 10 times concentrate of TBE was:
TRIS 1M, Boric Acid 1M and EDTA 1M. The chemicals were dissolved initially in 500ml of distilled water, and then this was made up to 1 litre.

Once the sequencing gel chemicals had been mixed, with the Temed added last, the resultant mixture was poured between two firmly clamped sequencing plates separated by a pair of spacers, and any air bubbles were removed with a bubble catcher (Promega UK, Southampton, UK). The gel was left to set for 2 hours at room temperature prior to use. Once set, the plates were positioned in the sequencer and a comb was used to form either 24 or 36 wells for the samples to be run. Running buffer (1x TBE) was added to the anode and cathode wells. Air was carefully expelled from these wells, using a syringe (Becton Dickinson, UK) with a curved needle attached, prior to the loading of the samples.

The samples that had been stored at –20°C were re-dissolved with loading buffer prior to their loading. 400µl of formamide (Sigma Aldrich, Poole, UK) was de-ionised for 30 minutes then this was mixed with 80µl EDTA (50mM, pH 8.0) and 10% v/w dextran blue was added to the mix. 5µl aliquots of the mixture were used to dissolve the samples to be

sequenced. The mixed sample was heated to 90°C for 2 minutes prior to being loaded onto the gel. The gel was run for 8 hours in total at 30 watts.

The sequence was then confirmed by studying the output of the analysis. The study sequences were then compared to the F and G protein gene sequences present on the EMBL database to confirm the strain of RSV used for the experiments.

2.3.3 PCR primer design

The initial primer sequences were decided by reviewing the conserved areas of the RVS genome within the F and G protein. The final PCR product was designed to be approximately 100 nucleotides in length for each of the assays. This length was chosen as primers were also used for the real-time PCR process as well, and there was a requirement for those primers to be approximately 100 nucleotides in length (see section 2.6.3). The primers were optimised by using the GeneRunner software package (Hastings Software Inc., Hastings, New York, USA). The primers were optimised so that each had a similar CG percentage and theoretical annealing temperature, each primer was assessed to ensure there were a minimum number of secondary structural problems. These problems included hairpins, palindromes and internal loops and also risk of primer dimmers, where the 3' end of each primer anneals to another 3' end. By use of the software the above problems were reduced to a minimum or eliminated completely.

The PCR primers were synthesised commercially (Oswel, Southampton, UK) using phosphoramidite chemistry (298) and the resultant primer was obtained in desiccated form, this was reconstituted using sterile UHQ water to the required concentration prior to use any sequencing or PCR reactions.

2.4 Standard PCR Technique

The polymerase chain reaction (PCR) was used to identify the presence of cDNA that corresponded to mRNA of certain messenger sequences of various cytokines and their

receptors (299). The standard reaction mixture for conventional RT-PCR of samples: each reaction used 5 µl of template cDNA, following dilution of the original cDNA sample this was 1/40 of the total cDNA formed. The reaction volume used was 25 µl, this comprised of the *Taq* DNA polymerase buffer (2.5 µl), 25mM magnesium chloride solution variable amounts, 4µl of 1.25 mM of each dNTP (Promega, UK), 0.75 Units *Taq* DNA polymerase (Promega, Southampton, UK) and UHQ water to make reaction volume up to 25µl.

The reaction conditions for each set of primers was optimised for magnesium concentration, annealing temperature and extension time dependent on the length of the PCR product, though the base reaction conditions were similar for all the reactions. The base conditions are listed here, and the magnesium concentration and overall reaction conditions are listed in table 2.2.

Reaction conditions:
Denaturing 94°C for 15 seconds
Annealing x°C for 20 seconds
Extension 72°C for 30 seconds
(where x is the annealing temperature and varies for each PCR reaction, this can be found in table 2.1)

The number of cycles was between 30-40 for most products, to identify their presence in template cDNA.

Table 2.1

Table of thermal cycler conditions for various programs used.

Thermal Cycler Conditions:	
A	94°C(15s); 50°C(20s); 72°C(30s)
B	94°C(15s)-ramp @ 1.5°C/s-50°C(20s); 72°C(30s)
C	94°C(15s)-ramp @ 1.5°C/s-50°C(20s)-ramp @ 1°C/s-72°C(30s)
D	94°C(15s)-50°C(20s)-60°C(30s)-72°C(30s)
E	94°C(15s); 55°C(20s); 72°C(30s)
F	94°C(15s)-57°C(20s)-60°C(30s)-72°C (30s)
G	94°C(15s)-ramp @ 1.5°C/s-55°C(20s)-ramp @ 1°C/s-72°C(30s)
H	94°C(15s); 63°C(20s); 72°C(30s)
I	94°C(15s); 60°C(20s); 72°C(30s)

If a PCR reaction was positive, the reaction was repeated several times, with differing cycle conditions i.e. 25, 30, 35 and 40 cycles. The product for each cycle condition was run on an agarose gel and then the product was quantified. The semi-quantitative analysis of samples is described in section 2.5. The commonest cycle number was 35 cycles.
All reactions were carried out using a Peltier effect thermal cycler (Tetrad PTC-225, MJ Technologies, Waltham, USA) using 200 µl thin-walled reaction tubes (Robbins Scientific, UK). This thermal cycler employed heated lids to prevent sample evaporation.

2.4.1 Optimisation of PCR process

There are several parameters that can alter the efficacy of the PCR process. These factors include: magnesium concentration, primer concentration, annealing temperature and extension time.

For each of the PCR's the above parameters were altered to find optimal conditions and used for subsequent reactions. Initially the theoretical annealing temperature, as calculated from the composition of the oligonucleotide primers and confirmed by the software used to help manufacture the oligonucleotides (GeneRunner, Hastings Software, USA). The

extension time was set at 30 seconds as all the PCR products were less than 700 base pairs and the calculated extension rate would have covered this length (Promega, Southampton, UK). The initial primer concentration was set at 1µl of 1µM of each primer. A series of reactions were set up to have set concentrations of all other reactant except the magnesium concentration, this was varied through from 0.5µM through to 3.5µM and this series of reactions were conducted at annealing temperatures –10, -5 and +5°C around the annealing temperature. Once the reaction was complete the reaction products were run on an agarose gel (see section 2.4b), the gel was stained and visualised. The efficacy of each reaction was assessed by the amount of product in each reaction vessel. The ideal annealing temperature and magnesium concentration was ascertained by this method.

2.4.2 Product identification

The identification and confirmation of any PCR products was by two methods, initially 10 µl of the contents of the reaction vessel were mixed with 2µl of loading dye and run on an 2% agarose gel, against a standard ladder (MBI Fermentas, Newcastle, UK). A voltage of 10V/cm was applied across the gel for one hour.

The gel was stained in 300ml of 1:10000 dilution of SYBR-Green dye solution (Molecular Probes, Leiden, Netherlands), the gel was visualised by either viewing on ultra-violet light-box and taking a Polaroid photograph, or scanning the image using a blue laser and the Storm 850 imager (Molecular Dynamics, Sunnyvale, USA). The size of the PCR product could be estimated using the 100 base pair (bp) DNA ladder that was run along side the products. The base pair ladder could also be used to quantify the amount of PCR product as each band of the ladder was of a set concentration.

2.4.3 Sequencing of PCR products

For each new set of PCR primers that were used (irrespective of whether the primer sequences came from a previously published sequence or had been designed by myself),

the initial PCR product that was confirmed to be of approximately the correct length was sequenced to confirm the validity of the reaction.

The primers that were designed by myself are indicated on table 2.3, the rest of the primers have the original reference cited by them. The gene sequences held on the EMBL database were used as the templates to design primers and to confirm sequencing products with.

The primers that were designed by myself were done by designed so that the primers would amplify sequences that spanned introns; this guaranteed that only the mRNA would be detected, because if the DNA sequence was amplified the PCR product would be of a different size. The primer sequences were analysed for theoretical annealing temperatures (see section 2.4b), to ensure that was equal for both primers.

Once the PCR reaction was complete, 5µl of the product from each reaction vessel that had not been run on an agarose gel was used as the template for the sequencing reaction. A commercial set of reagents was used for the sequencing reaction. Thermo Sequenase (Amersham, Little Chalfont, UK) reaction mixture 9.5µl, diluted template (previous product from reaction vessel) 9.5µl (5µl and 4.5µl UHQ) and primer 1µl (3.2 pmol). Each primer required a separate sequencing reaction. If there was difficulty with the sequencing reaction, the PCR product was purified and then the sequencing reaction was repeated.

The purification was by running the PCR product on an agarose gel, visualizing it and cutting it out of the gel. The band was cut out of the gel, this was placed in a reaction tube and heated up to 65°C, liquefying the gel and allowing the addition of glass milk binding complex, this is a commercial reagent (Bio101, New York, USA), which binds the PCR product, allowing the removal of the gel. The PCR product is then eluted off the bonding complex and dissolved in water and then desiccated until needed for the sequencing reaction.

The sequencing reaction was carried out in 200 µl reaction vessels, and performed on the Tetrad thermal cycler. The reaction products were processed as per manufacturers instructions, 25 cycles of 96°C 15seconds, 48°C 10seconds and 60°C 4minutes.

The samples were then run on an ABI 3700 sequence analyser (Foster City, USA). The separation occurred using a 6% acrylamide gel.

2.4.4 Sequencing Gel formation

The sequencing gel is made of the following constituents, these were added in the order described below and mixed thoroughly, the final constituent ammonium persulphate (APS) catalysing the setting of the gel. The resultant mixture was poured between horizontal two sequencing plates, separated by thin plastic spacers down the long axis of the plates. Eight bulldog clips held the plates firmly together. Once the gel had been poured any bubbles had to be removed, as any bubbles distort the smooth running of the gel. The air bubbles were removed using a bubble catcher (Promega, UK).

A 6% sequencing gel was made of the following constituents:
32ml Sequagel (Flowgen, UK)
13% v/v 10x TBE concentrate
6% v/v UHQ
0.05% v/v Temed (Kodak, Rochester, US)
5% w/v APS (Sigma Aldrich, Poole, UK)

The gel was left to set for 2 hours at room temperature prior to use. Once set the gel was positioned vertically in the sequencer. A comb was inserted into the top of the gel to form either 24 or 36 wells for the samples. Any air was expelled from the wells by using TBE via a 5ml syringe with a bent 21gauge hypodermic needle (Sterilin, UK). Running buffer (1x TBE) was added to the anode and cathode wells. The anode chamber took 180ml of running buffer, and the cathode required approximately 800ml of running buffer.

The recipe for 1 litre of 10 times concentrate of TBE (10x TBE) was:

TRIS 1M

Boric Acid 1M

EDTA 1M

The chemicals were dissolved initially in 500ml of distilled water, and then this was made up to 1 litre.

2.4.5 Running of samples

The desiccated samples had been stored at –20°C, these samples were re-dissolved with loading buffer prior to their loading. The loading buffer was made of 400µl of formamide (Sigma Aldrich, Poole, UK) that was de-ionised for 30 minutes prior to mixing with 80µl EDTA (50mM, pH 8.0), 10% v/w dextran blue was added to the mix. From the stock loading solution, 5µl aliquots were used to dissolve the samples to be sequenced. The mixed sample was heated to 90°C for 2 minutes prior to being loaded onto the gel. Once the gel had been loaded it was run for 8 hours with a voltage of 300v applied across the electrodes, 30watts and using analysis filter A. The analysis software was able to capture the sequence of each sample that was run. The data was analysed using proprietary software (Applied Biosystems, Foster City, USA) on an apple Macintosh Computer (Palo Alto, USA). The sequences of the PCR products were compared to EMBL database sequences to confirm the sequence of the cytokine genes that had been amplified.

Table 2.2

A table of the primer sequences used for conventional RT-PCR

Primer	Sense/Anti-sense Sequence	PCR product size	Primer site on mRNA	Accession Number	Cycler Conditions
β-actin	TGACGGGGTCACCC	661	509-1170	J00074	A
	ACACTGTGCCCATCTA				
IL-1α*	GTCTCTGAATCAGAAATCCTTCTATC	420	120-540	NM000575	B
	CATGTCAAATTTCACTGCTTCATCC				
IL-1β*	AAACAGATGAAGTGCTCCTTCCAGG	388	174-562	NM000576	C
	TGGAGAACACCACTTGTTGCTCCA				
IL-2*	GAATGGAATTAATAATTACAAGAATCCC	222	191-413	NM000586	A
	TGTTTCAGATCCCTTTAGTTCCAG				
IL-4*	AACACAACTGAGAAGGAAACCTTC	276	247-523	M13982	D
	GCTCGAACACTTTGAATATTTCTC				
IL-5*	TGTATGCCATCCCCACAGAA	100	605-705	J03478	C
	CAGAGTCTCATTGGCTATCAGCA				
IL-6*	ATGAACTCCTTCTCCACAAGCGC	628	1747-2375	AF372214	A
	GAAGAGCCCTCAGGCTGGACTG				
IL-8*	ATGACTTCCAAGCTGGCCGTGGCT	289	3748-4037	AF385628	B
	TCTCAGCCCTCTTCAAAAACTTCTC				
IL-10*	CTTGTCTGAGATGATCCAG	310	276-586	XM_001409	E
	CTCATGGCTTTGTAGATGCC				
IL-11	ACTGCTGCTGCTGAAGACTCGGCTGTGA	320	709-1029	M57765	F
	ATGGGGAAGAGCCAGGGCAGAAGTCTGT				
IL-12p40*	ATGTCGTAGAATTGGATTGGTATCCG	358	105-463	NM_002187	A
	GTACTGATTGTCGTCAGCCACCAGC				
IL-13	CGGTCATTGCTCTCACTTGCCTT	352	1824-2176	AF377331	A
	TTACCCCTCCCTAACCCTCCTT				

Primer	Sense/Anti-sense Sequence	PCR product size	Primer site on mRNA	Accession Number	Cycler Conditions
IL-15	ATGAGAATTTCGAAACCACATTTG	410	317-727	NM000585	C
	CCATTAGAAGACAAACTGTTCTTTGC				
IL-16	AGCGAGCACGGAGCTTCCCC	410	958-1368	NM004513	G
	CATGGATGCCGTCTAATTGCTTT				
IL-18	ATGGCTGCTGAACCAGTAGAAGACA	571	178-749	XM_006289	E
	ACTTTTTGTATCCTTGATGTTATCAGGAG				
TGF-β	GCCCTGGACACCAACTATTGCT	161	727-888	NM_015927	C
	AGGCTCCAAATGTAGGGGCAGG				
IFN-γ*	ATGAAATATACAAGTTATATCTTGGCT	501	109-610	NM_000619	C
	GATGCTCTTCGACCTCGAAACAGCAT				
TNF-α*	CGGGACGTGGAGCTGGCCGAGGAG	355		XM_012478	H
	CACCAGCTGGTTATCTCTCAGCTC				
GM-CSF*	ACACTGCTGAGATGAATGAAACAGTAG	286	148-434	E01817	C
	TGGACTGGCTCCCAGCAGTCAAAGGGGATG				
RANTES	ATGAAGGTCTCCGCGGCACGCCTCGCTGTC	252	1-252	AF266753	B
	CTAGCTCATCTCCAAAGAGTTGAT				
MCP-1*	TCTGTGCCTGCTGCTCATAGC	510	64-435	X14768	B
	GGGTAGAACTGTGGTTCAAGAGG				
MIP-1α	CAGGTCTCCACTGCTGCC	252	87-339	XM_008450	A
	CACTCAGCTCCAGG				
GRO-α**	ACTCAAGAATGGGCGGAAAG	333	814-1147	U03018	I
	TGGCATGTTGCAGGCTCCT				

* Jung et al.

** Yang et al.

2.5 Semi-Quantitative analysis of PCR products

Two alternative methods of visualising PCR products were considered. The first method and the method that was subsequently used was to run PCR product on an agarose gel with a voltage of 10 volts/cm of gel length, then subsequent staining of the gel in a 1:10000 solution of SYBRGreen (Molecular Probes, Leiden, Netherlands). The second method considered, but discarded as this altered the electrophoretic mobility of the PCR products in an unpredictable way.

The movement of a PCR product is based on overall charge and molecular weight of the product, the two methods should have produced different results, but the results should have been comparable. Initial experiments performed to evaluate methods were unable to produce consistent results for the second method, with identical products run in adjacent lanes having different eletrophoretic movement. The only hypothesis to account for this is that there was a variable amount of intercalation of SYBRGreen during the initial mixing, leading to differing molecular weights and hence variable migration. The variable migration meant that the products could not be solely identified by weight (when compared to a standard PCR ladder), and that sequencing of each PCR product would be required, which would have been impractical on a large scale.

The final result was visualised by either using ultraviolet (UV) light and taking a Polaroid photograph of the gel, or by using a commercial image analyser. The Storm 850 image analyser (Molecular Dynamics, Sunnyvale, USA) is able to analyse gels that have been stained with SYBRGreen and quantify the intensity of the PCR product and thus the relative amount of PCR product. A standard DNA ladder (MBI Fermentas, Newcastle, UK) of known concentration of DNA per rung was run every 20-25 samples, the intensity of the 300bp rung was used as the standard and all each sample was corrected for the intensity of the standard, allowing individual rows to be compared with each other.

The results of all the cytokine PCR's that were run were all compared to the "house keeping" gene β-actin, as standard amounts of starting template (1μl of 100ng/ml) had been used. The DNA concentration was calculated at the start of the reverse transcriptase procedure and quantification was by the absorbance at 260nm. All the samples had been handled under the same condition and had undergone a similar number of freeze-thaw cycles, had been aliquoted at the same time and frozen at –80°C until processed. The use of 208 well gels made it was possible to run all the PCR products for a single cytokine under investigation on a single gel. The processing of all the samples and internal controls for each cytokine simultaneously reduced observational errors in processing the samples.

2.6 TAQMAN Quantitative Polymerase Chain Reaction

The TAQMAN system (Applied Biosystems - ABI, Foster City, USA) was used to provide quantitative measures of mRNA copy numbers of target genes. The apparatus required for the process was the ABI Prism 7700 sequence detector, attached to an Apple Macintosh G3 computer (Macintosh, Palo Alto, USA) running Sequence Detection Software (SDS – ABI, Foster City, USA).

The reaction process is similar to that for a normal PCR, though with the use of AmpliTaq Gold™ (ABI, Foster City, USA) the reaction mix has to be heated to 95°C to activate the enzyme. This ensures a "hot-start" reaction and increased specificity, as this method eliminates incomplete strands being synthesised as the temperature increases from ambient temperature. The enzyme used has significant 5' exonuclease activity. An internal probe, which has been double labelled, is also used. The primers and probes are designed so the annealing temperature is 60°C, this temperature is also the ideal temperature for extension to occur. The enzyme will extend as far as the probe, before its exonuclease activity becomes apparent. The enzyme cleaves the probe and proceeds with the extension process.

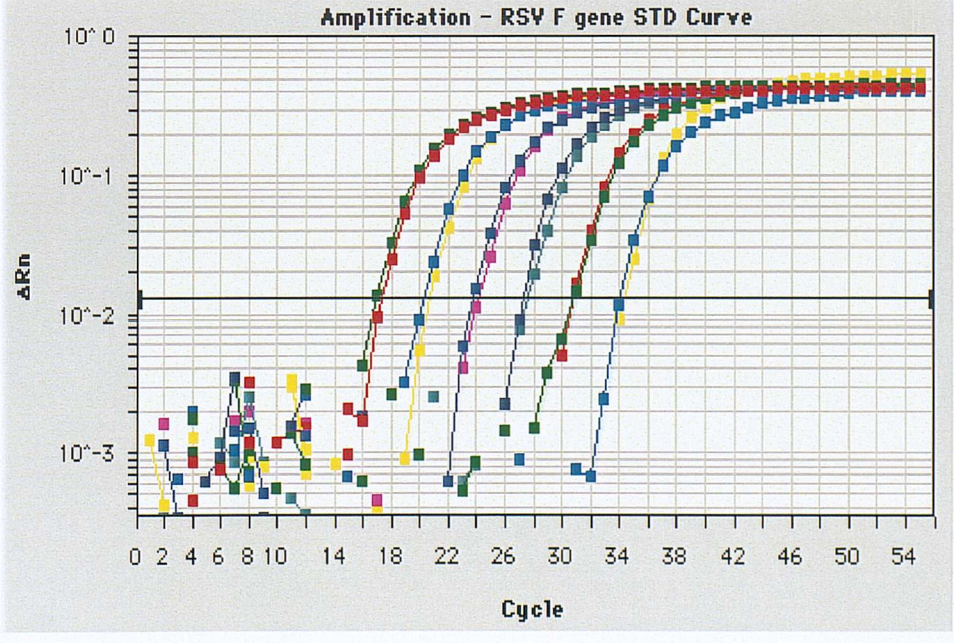
2.6.1 Theoretical Background to Taqman

The internal probe that is used in the Taqman process is double-labelled. It has both FAM and TAMRA dyes at either end. The TAMRA dye acts as a quencher to the FAM, ensuring that there is no net fluorescence from the intact probe. The cleavage of the probe by the Taq DNA polymerase liberates the FAM dye from the quenching effect of the TAMRA dye. Thus in one cycle, one probe and set of primers will attach to one strand of target DNA, the probe will be cleaved by the DNA polymerase and liberate the FAM and TAMRA from the probe. One DNA strand can only bind one probe, which in turn can only liberate one FAM on cleavage, thus as the cycles progress the increase in fluorescence is a direct indication of the increase in the amount of DNA present in the reaction vessel (see figure 2.1). The heat-cycling process can lead to changes in the overall fluorescence of the reaction, to account for this there is a passive reference dye (ROX) also present in the reaction buffer.

The change in the fluorescence in each single reaction vessel can be monitored, and the changes expressed relative to the passive reference dye. This is expressed as the ΔR_n, and for each reaction vessel the ΔR_n can be plotted against the number cycles that has occurred (see Figure 2.2).

Figure 2.2

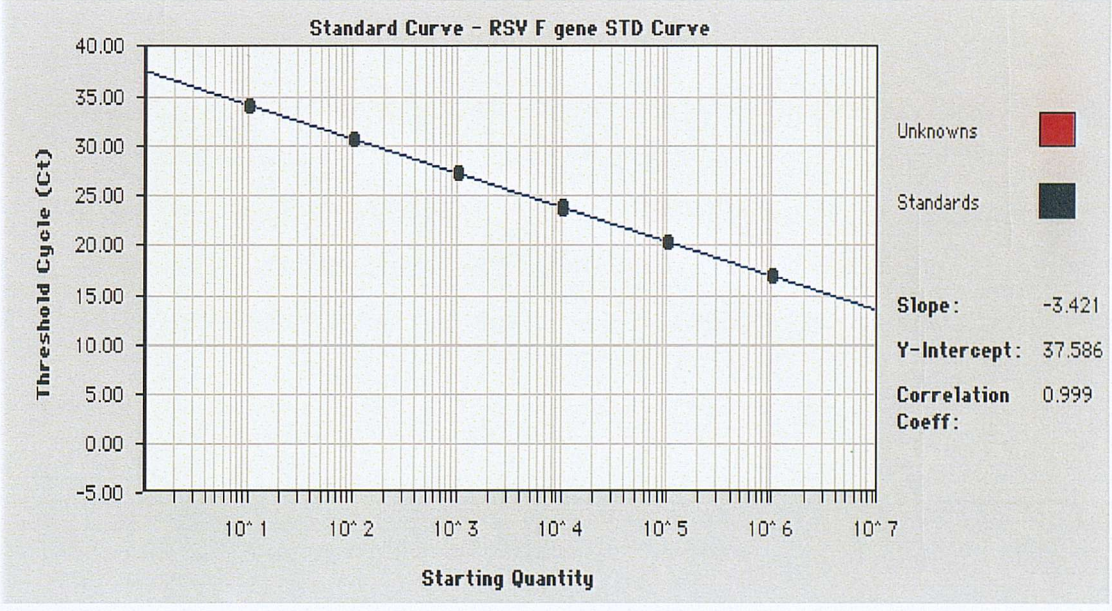
Output from amplification plots for RSV standards taken from TAQMAN™ output.



A reaction is considered to be positive if the ΔR_n crosses a certain threshold, designated the threshold value. The threshold value is arbitrary, but is taken as 10 times the standard deviation from the average baseline between cycle number 3 and 15 (In Figure 2.2 the threshold value is designated by the horizontal line that bisects 10^{-1} and 10^{-2} on the ΔR_n axis). The cycle at which the plot of the ΔR_n against cycle number crosses the threshold value is designated the threshold cycle (C_T). If known quantified standards are also run in reaction vessels alongside unknown samples, then a graph of starting DNA quantity can be plotted against threshold cycle (C_T), Figure 2.3. The unknown samples can have their starting DNA quantity extrapolated from the standard curve constructed, assuming that there is no inhibition or alteration in the reaction kinetics between the standards and the unknowns.

Figure 2.3

A Standard Curve Constructed from the threshold cycle values for various standards.



2.6.2 Reagent and Reaction Conditions

The reaction tubes used for this process and lids were obtained from Applied Biosystems Ltd, and were either obtained as single reaction vessels or strips of eight reaction vessels. The reagents used for each 25 µl reaction were:

cDNA	5µl
TAQMAN buffer A	2.5µl
$MgCl_2$	1µM
Probe	500nM
Primers	300nM
dATP/dCTP/dGTP	200nM
dUTP	400nM
AmpErase UNG	0.01u/µl
AmpliTaq Gold	0.05u/µl
UHQ water	6.125µl

The reaction conditions used for all the reactions were as follows: an initial hold at 50°C for 2 minutes to activate the AmpErase- uracil-N-glycosylase (UNG), followed by 10 minutes at 95°C to inactivate the enzyme and activate the AmpliTaq Gold ™. The actual PCR process was 50 cycles each as a 2 stage process, 95°C for 15 seconds, followed by combined annealing and extension at 60°C for 60 seconds. This was possible as all the primers had been constructed so that their annealing temperature was 60°C.

The use of AmpErase-UNG in the reaction eliminated the risk of contaminating samples. This recombinant enzyme acts on single and double-stranded dUTP containing DNA, releasing uracil from the complex. The enzyme's actions are specific, with no cross-reaction with RNA or dT-containing DNA. The inclusion of this enzyme and dUTP during the PCR process eliminated contamination of an experiment by the products of a previous experiment, as all final PCR products by this method will contain UTP.

2.6.3 Construction of DNA Standards for Taqman

By using a separate method to quantify the DNA standards used to construct the standard curve, absolute quantitation of DNA could take place, rather than relative quantitation.

Initially PCR primers were constructed that spanned the area of interest; these were constructed using the methods outlined previously. The primers for these products were constructed so that at each end restriction enzyme sites were incorporated. Once the area had been amplified using the standard PCR method for 50 cycles the sample was purified by using the Geneclean methodology (Bio 101). The PCR product was then cleaved using the restriction enzymes EcoHR1 and BamH1 (Promega, Southampton, UK). The reaction conditions were 0.5µl of each enzyme with 2µl of universal buffer A (Promega, UK) and 18µl of sample incubated for 1 hour at 20°C. a similar process was under taken for the plasmid that the PCR products were going to be ligated into, this was the pCI plasmid (Promega, Southampton, UK). Heating the sample to 42°C for 5 minutes inactivated the restriction enzymes. The samples were then ligated together by the addition of DNA T4 ligase (Promega, Southampton, UK), ligase buffer and 15mg/ml ddT and 9mg/ml ATP (Promega, UK), the process is termed "sticky end ligation" as the use of the restriction enzymes guarantees that the PCR product is ligated in only a particular orientation into the vector; this is a more efficient process than "blunt-end ligation".

Once the plasmid with the PCR product had been manufactured it was used to transform E. coli, and then could be grown up in large amounts. The plasmid was inserted into E.coli, the process of transformation by the following method.

2.6.4 Calcium Chloride Method of Transformation

A sample of stock E.coli grown up overnight in LB medium at 37°C. A sample of this was taken the following day and inoculated into 100ml fresh LB medium [the LB is made up of 10g tryptone (Sigma, UK), 5g yeast extract (Sigma, UK), 5 g sodium chloride (Sigma, UK), 1ml of 1N sodium hydroxide adjusted to pH 7.5 in 1l of sterile water] that was

agitated gently at 50Hz at 37°C.After 2 hours of further growth the cells were taken as four 20ml aliquots and spun 3000rpm for 10 minutes at 4°C, the supernatant was decanted off and 20ml of ice cold 0.1M $CaCl_2$ added, this solution was stored for 20 min at 4°C. After this the solution was re-spun at 3000rpm and the supernatant was removed and the cells were re-suspended in 4ml of 0.1M ice-cold sterile $CaCl_2$ solution, this mixture was left for one hour before the ligation reaction mixture was added. The ligation reaction mixture was added to 300µl of the competent cell solution, gently mixed and left on ice for one hour. Then the solution was placed in a water bath at 42°C for 2 minutes and then back into ice. 1 ml of fresh LB was added to the transformation mixture and incubated at 37°C for 30 minutes. The solution was mixed and then 50µl samples were plated onto LB agar plates (made with LB as indicated at start of section with the addition of 20g agar (Sigma, UK) for each 1 litre of broth, 50ml of LB agar was poured into petri dishes (Sterilin, UK)). The solution was plated out and grown up overnight. The following day each individual colony was plated onto LB agar plates with ampicillin (as previously with 10 ml stock solution of sterile filtered ampicillin 5mg/ml (Sigma, UK) to each litre of LB). The plates were subjected to a further overnight incubation and any colonies that grew up were ones that had been transformed by the plasmid. These are then grown up in bulk in LB solution to provide large amounts of the plasmid.

2.6.5 Purification of Plasmid

This preparation was performed in the University department of Molecular Microbiology, with the assistance of Dr Liu. Large volumes of the transformed E.coli were grown up in LB, the plasmid was extracted using a commercial preparation method (Qiagen maxi prep, Qiagen, USA). 20ml of overnight growth of cells were used per maxi-column used. Spinning the solution at 3000g for 10 minutes pelleted the cells, the supernatant was removed and the cell pellet was resuspended in 11ml of buffer B1 per maxi column. 300µl lysozyme solution and 500µl of proteinase K solution are added and the mixture is incubated at 37°C for 30 minutes. This reaction is terminated by the addition of 4ml of buffer B2 and this is incubated at 50°C for 30 minutes this lyses the cells completely. The

lysate is vortexed and then applied to the maxi column. The columns are washed with two 15 ml aliquots of QC buffer; once washed the plasmid DNA is eluted off by washing the column with 15ml QF buffer. The DNA can be precipitated by the addition of 10.5ml of isopropanol, then the mix is spun at 5000g for 10 minutes, the supernatant removed and the sample washed with 70% ethanol, re-spun and then vacuum dried and resuspended in UHQ water.

Buffer constituents –
B1 - 50 mM EDTA, 50 mM Tris/HCl, 0.5% Tween 20, 0.5% Triton X-100
B2 - 3 M GuHCl, 20% Tween 20
QC - 1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0
QF - 1.25 M NaCl, 50 mM Tris/HCl, 15 % ethanol, pH 8.5
(All constituents Sigma Aldrich, Poole, UK)

The plasmid, with the PCR standard spliced in can be reconstituted and a series of dilutions made. The theoretical mass of this plasmid can be calculated and using the A260 (absorbance at 260 nm) the amount of plasmid in the sample calculated. Thus the true number of DNA copies is known and this solution diluted to form a series of standards for use in the construction of the standard curve described earlier in section 2.6. This series of standard dilutions were aliquoted into small volumes and these were stored at –80°C.

An alternative method for construction of the standard curve was to amplify the initial PCR product several times and then perform serial dilutions on this, after initial sequencing of the PCR product to confirm correct amplification.

Table 2.3.

Table of TAQMAN primers and probes used

Primer	Sense/Anti-sense Sequence	Position on mRNA	Probe Sequence	Accession Number
eotaxin	AGGAGAATCACCAGTGGCAAA	1650-2117	1786-1818	U46572
	TCCTGCACCCACTTCTTCTTG			
hBD-1	CCTTCTGCTGTTTACTCTCTGCTTACT	82-188	118-148	U50931
	CCACTGCTGACGCAATTGTAAT			
hBD-2	CCCAGCCATCAGCCATGA	10-171	106-140	Z71389
	TTTGTTTATACCTTCTAGGGCAAAAGACT			
RSV-F	TGCAGTCACATTTTGTTTTGCTT	61-161	88-125	M11486
	TTCTCAGAGCACTAAGATAGCCTTTG			
RSV-G	TCAAATGGAAACCTTCCACTCAA	795-895	836-867	M11486
	TGGGTGGAGATGAAGGTTGTG			
GAPDH	GGGAAGGTGAAGGTCGGAGT	64-293	172-209	M33197
	TGGAAGATGGTGATGGGATTTC			

2.7 Growth of Monocyte derived macrophages

Several different methods of experimenting on macrophages have been described; these include using alveolar macrophages obtained by bronchoalveolar lavage; monocytes from cord blood; macrophages from milk; or as in this study using blood monocytes, which take on a macrophage morphology during culture conditions. For the series of experiments a steady supply of safe, screened blood was required, this was obtained from the South West Blood transfusion service (Southampton, UK). The samples used were buffy coat preparations taken for use in plasmaphoresis, any surplus samples were available for use. The blood was screened for a variety of blood borne pathogens prior to use (HIV 1+ 2, Hepatitis B + C).

2.7.1 Blood Preparation

Once the sample was obtained from the Blood Transfusion service the sample was processed to reduce the number of red cells in the sample and remove the majority of platelets; by removing the red cells it increased the probability that the monocytes were to come in contact with the plastic base of the well and adhere to it. The platelets were removed, as they were a significant source of cytokines. A sample was taken at the beginning and at the end of the processing to confirm the removal of red cells and platelets. The sample was analysed using the flow cytometer (Becton Dickinson, USA), Haematology laboratory Southampton General Hospital. The cell concentrations at the beginning and at the end of the processing (Table 2.4).

Table 2.4

Representative table of Cellular composition of buffy coat pre and post-purification, the percentage of cells left after purification n = 10

Cell type	Concentration before purification (cells/L)	Concentration after purification (cells/L)	Percentage of original cells left after purification
Red blood cells	1.77×10^{12}	0.005×10^{12}	1.1
Total white blood cells	12.7×10^{9}	11.8×10^{9}	37
Neutrophils	2.16×10^{9}	1.48×10^{9}	27
Lymphocytes	9.67×10^{9}	9.24×10^{9}	38
Monocytes	0.738×10^{9}	0.968×10^{9}	52
Eosinophils	0.078×10^{9}	0.016×10^{9}	8
Basophils	0.086×10^{9}	0.111×10^{9}	52
Platelets	781 x 109	15.1×10^{9}	0.8

The table above demonstrates that though there is a loss of all cell types during the purification process, the process employed was successful at removing the majority of the red blood cells and platelets.

The procedure involved using a 50ml conical bottomed test tube (Alpha laboratories, Southampton, UK) for this procedure. 20ml of Histopaque 1077 (Sigma-Aldrich, Poole, UK) was placed at the bottom of the test-tube, 25ml of buffy coat was carefully overlaid on this, ensuring the interface between the two solutions was not disrupted, and this was repeated until four separate test tubes, using 100ml of the buffy coat, had been prepared this way. The tubes were centrifuged at 700g for 30 minutes (IEC- Centra 4, Basingstoke, UK), with a slow start and no braking, to ensure the interface remained intact. The red

cells were separated from the mononuclear cells on the basis of density. The mononuclear cells settled at the interface between the serum and the Histopaque in the lower layer. The mononuclear cells were aspirated from the interface using a broad tip 1ml pasteur pipette (Alpha laboratories, Southampton, UK). The mononuclear cell suspension aspirated from 2 tubes (approximately 7ml each tube) and placed into a 25ml plastic universal container (Alpha laboratories, Southampton, UK), this was diluted to 20ml with mixture of PBS, 20mM glucose and 10mM EDTA (ehtylenediamine tetracetic acid) (to be referred to as diluent for the rest of this paragraph). The mononuclear cells were washed of contaminating Histopaque by centrifuging the mixture at 800g for 10 minutes; this cell pellet was then re-suspended in 10ml of diluent. The remaining platelets and red blood cells were removed by layering the resultant mixture on to 30ml of FCS (in 50ml conical bottomed test-tube) and spinning it at 300g for 15 minutes, once finished the supernatant was decanted off and the cell pellet was re-suspended in 10ml of diluent and the procedure was repeated.

Finally, the cell pellet was re-suspended in Dulbeco's Minimal Essential Medium (DMEM) (Life Technologies, Paisley, UK), with 20mM HEPES buffer (Sigma-Aldrich, UK) and antibiotics (penicillin 50μ/ml and streptomycin 50μg/ml), (Sigma Aldrich, UK), to give a final concentration of 0.5×10^6 monocytes/ml. A 1 ml sample of the final suspension was analysed on a coulter counter. The analysis of cell type was based on predefined characteristics of cell size and lobularity (see table 2.4).

One ml aliquots of this mononuclear cell suspension were placed into individual wells of 12-well tissue culture trays (Costar, Cambridge, USA). The trays were incubated at 37°C, with 5% CO_2 for one hour for monocyte-plastic adherence to occur. After one hour the supernatant was aspirated and replaced by 1ml/well of the macrophage growth medium.

The macrophage growth medium consisted of macrophage serum-free medium (SFM) (Life Technologies, UK), HEPES buffer 20mM (Sigma-Aldrich, UK), penicillin and streptomycin (50u/ml and 50μg/ml respectively) (Sigma-Aldrich, UK) and recombinant granulocyte-macrophage colony stimulating factor (GM-CSF) 1ng/ml (Preprotech,

London, UK). The monocytes were cultured under standard conditions (37°C and 5% CO_2) throughout the maturation process. Following overnight incubation the medium was aspirated and the cells were washed twice with warmed (to 37°C), sterile PBS. Fresh macrophage growth medium was added to the wells and during five-day culture the medium was changed every 48 hours using the same method of washing off dead cells and debris prior to replacement of the medium.

Within 4 days the cells had taken on a macrophage-like morphology, the phenotype of the cells was confirmed by non-specific esterase test and also by staining for surface markers. The cells were ready for experimentation with by 120 hours after initial processing.

Prior to experimentation the cells were washed twice with warmed, sterile PBS and the culture medium was replaced by macrophage culture medium (serum free medium (SFM)) without GM-CSF or antibiotics. The cells were allowed to equilibrate with this medium for one hour prior to any experimentation.

For the experimentation, RSV diluted (in SFM) stock solution was added to each of the tissue culture tray wells. The solution was gently agitated onto the cells for one hour on a rocker rocking at a rate of 40Hz. At the end of the incubation period the medium was washed off and replaced by 2ml of fresh SFM and the subsequent incubations were undertaken at 37°C with 5% CO_2. As specific time points in the experiment arrived one tray would be removed (a separate tray was used for each time point), the supernatant was aspirated, transferred to an eppendorf test tube and spun down at 15000g for 2 minutes to remove cellular debris, prior to transfer to a separate test tube and freezing at –80°C until required for experimentation. The remaining cells had Trizol agent added to the well, the cell/Trizol mixture was mixed and transferred to a new test tube and frozen at –80°C until required for reverse transcription.

2.8 Confirmation of macrophage morphology

The confirmation of the development of macrophages from monocytes was by staining for macrophage specific surface markers. There were several markers that were used, this included the use of non-specific esterase (NSE), which is not found on monocytes. The other markers that were studied included CD16, CD 32, CD46, CD64 and macrophage mannose receptor (MMR).

Instead of growing monocytes in the 6 well trays, 4 well chamber slides (Nunc, UK), the culture conditions were exactly the same as for the tissue culture plates as outlined in section 2.7. Once the culture had been completed, the cells were washed twice with PBS and then fixed with ice-cold methanol for 10 minutes. Once the cells were fixed and the methanol evaporated off, the first antibody – i.e. mouse anti-human receptor antibody at 1:50 dilution of stock solution (all antibodies from Dako, UK). This incubation was undertaken at 37°C for 15 minutes in a humidified box. After this incubation the chambers were washed with two rinses of PBS before the second antibody was added. The second antibody added was a fluorescent rat anti-mouse antibody at a 1:20 dilution of the stock solution. This incubation was at 37°C in the dark for 15 minutes. After the second antibody incubation the chambers were washed a further two times with PBS. The gasket was removed to remove the chambers from the slide and then mounting solution was added and a whole slide coverslip was added and the slide was viewed using a fluorescent microscope (Leica, Germany).

The slides were viewed and the cells staining with the above surface markers confirmed that the cells were macrophages.

2.9 Confirmation of infection

The confirmation of replicative infection in the cell cultures used was by two separate methods. The initial confirmation was by the use of commercial diagnostic reagents,

which relied on the surface staining of RSV surface proteins (both F and G proteins) on the surface of infected cells (Dako, Ely, UK).

2.9.1 Immunofluorescence

Cells were cultured in chamber slides (Nunc, UK) and then infected with RSV at 1 moi, control slides with Hep2 cell supernatant were used. Chamber slides were fixed at 24 and 48 hours following RSV infection and these were viewed under the fluorescent microscope to confirm infection. The methodology was similar to that used to confirm macrophage morphology.

25µl of stain, a commercial preparation by Dako (Ely, UK) normally for the clinical diagnosis of early RSV infection in children, was added to each chamber slide, which was then enclosed in a humidified box and incubated at 37°C for 15 minutes. The slide was washed in PBS and allowed to air-dry before mounting a coverslip and viewing the slide using fluorescent microscope (Leica, Germany). This procedure was repeated, both with the A549 cell line and the monocyte derived macrophage cells. In each case negative controls of uninfected A549 cells or macrophages were employed. The manufacturer provided the positive control.

2.9.2 Polymerase Chain Reaction (PCR)

Initial experiments performed on epithelial cell lines and macrophages were assayed for RSV F protein. The results are presented in chapters 3 and 4. Each time course experiment had RNA extracted and cDNA formed from it was assayed for evidence of RSV F protein gene expression. The levels of F gene expression were quantified and the absolute levels of RSV gene copy numbers for each experiment were established, to confirm infection and replication of the virus. The quantification of F gene expression was made using the TAQMAN quantitative PCR technique. The methods for this technique were outlined earlier in this chapter.

The methods highlighted in section 2.6c were employed to construct F protein standards for use in the TAQMAN PCR method.

2.10 Reverse transcription and storage of samples

2.10.1 Initial Sample Storage

Each experimental sample was taken and placed on ice at the time of sampling. Following this the supernatant was aspirated off and spun at 15000g for 2 min to remove any cellular debris and stored separately from the cell monolayers. The tissue culture plate was placed in storage for between 2-3 weeks by adding 0.5ml of *RNAlater* solution (Ambion, Austin, USA) to each well, so that the cell monolayer was covered. The sample was then placed at –20°C, until further processing was performed. Initial experiments had been performed to confirm that there was no sample degradation over this period using this methodology, compared to lysing the cells immediately with Trizol and then storing this.

2.10.2 RNA extraction from stored samples

The extraction method employed was a commercial modification of the phenol/ chloroform method described by Chomczynski (300). The samples that had been stored at –20°C with RNAlater were warmed to room temperature; and the RNAlater was aspirated off the cells. Then 0.5ml of Trizol reagent (Life Technologies, UK) was added to each well of the tissue culture plate and left for 5 minutes, to ensure complete cell lysis. The solution was then transferred to a 1.5ml plastic test tube (Eppendorf, UK) and 100µl of chloroform (BDH, Poole, UK) added and the mixture was vortexed for 15 seconds and left to stand for 5 minutes. The samples were then centrifuged at 10,000g for 15 minutes at 4°C, to separate the aqueous and non-aqueous layers. 150 µl of the upper aqueous layer, containing the RNA was carefully taken off and transferred to a fresh test tube containing 300 µl 100% isopropanol (BDH, UK) at –20°C. The solutions were mixed by inverting the test tube and placed at –20°C overnight, to allow the RNA to precipitate.

After the overnight precipitation of the RNA, the samples were spun at 10,000g for 10 minutes at 4°C to pellet the precipitated RNA. The supernatant was aspirated, leaving the RNA pellet, which was washed by adding 500 µl of 75% ethanol, the test tube was vortexed and then spun for 5 minutes at 10,000g at 4°C. The supernatant was aspirated and the pellet was vacuum-dried in a desiccator for 2 minutes. The RNA pellet was re-suspended in 18µl UHQ water by pipetting, and then placed in a water bath at 55°C for 10 minutes to dissolve the RNA pellet. The sample was then treated with 1µl DNase / 2µl DNase buffer (Life Technologies, UK) to remove any contaminating DNA, the sample was incubated for 15 minutes at room temperature, followed by 10 minutes at 65°C to inactivate the DNase.

Random hexamers (Promega, Southampton, UK) were annealed to the RNA sequences by heating a mixture of 18 µl of the sample solution with 2µl of mixture of random hexamers (Promega, Southampton, UK). The mixture was heated to 70°C for 10 minutes, snap cooled on ice for 5 minutes. A 20 µl mixture containing 1µl (50units) RNA-dependant DNA polymerase (without the RNase H activity) (Superscript - Life Technologies, UK), 4µl of 10x buffer, 4 µl bovine serum albumin, 5 µl of 0.1M dithiothretol (DTT) (Life Technologies, UK), 2.5 µl (10mM) dNTPs (Promega, Southampton, UK) and 2.5 µl RNasin (RNase inhibitor) (Promega, Southampton, UK) was added and incubated for one hour at 37°C, at the end of the incubation the Superscript was inactivated by heating the sample to 95°C for 5 minutes.

Each sample was diluted five-fold by the addition of 160 µl of UHQ to the final resultant mixture, in any subsequent PCRs 5 µl of sample was used as the template. The samples were stored at –70°C, until they were required for analysis, if multiple analysises were required on a series of samples once the sample was thawed it would be aliquoted in 5µl measures, so that the main sample did not under go repeated freeze-thaw processes.

2.11 Sample Collection for Community-Based RSV Study

2.11.1 Subjects

The study was approved by the Joint Ethics Committee of Southampton University Hospitals. Ninety-one babies were recruited antenatally on the grounds of having at least one atopic, asthmatic parent (established by a questionnaire concerning symptoms, diagnosis by a specialist and treatment for asthma plus at least one positive skin-prick test to a common aero-allergen). Eighty-eight infants were subsequently monitored through their first winter - 1st November 1997 to 31st March 1998. This study period was used to coincide with the peak rate of infantile RSV infections as determined from Southampton General Hospital Public Health Laboratory virological data.

2.11.2 Study Protocol

Whenever a baby developed respiratory symptoms, parents were asked to telephone the study team within 24 hours. A researcher then visited the family at home where a respiratory questionnaire was completed and clinical examination performed. A nasal lavage specimen was collected. If the nasal lavage specimen tested positive for RSV by enzyme immunoassay (EIA), a further home visit was made 5 to 7 days after the initial onset of respiratory symptoms. At this visit, a further questionnaire and examination were performed, another nasal lavage carried out and a 5-10 ml specimen of peripheral venous blood collected. A clinical diagnosis was made according to predetermined diagnostic criteria:-

a) Acute upper respiratory tract infection (acute URTI) - was defined as new-onset rhinorrhea with or without fever or cough but without bronchiolitis.

b) Acute bronchiolitis (AB) was diagnosed if the infant had all 3 of tachypnoea (>60 breaths per minute), subcostal recession at rest and inspiratory crackles on auscultation

2.11.3 Nasal Lavage- Collection and Cytokine Analysis

The infant was held in the supine position and a soft latex/rubber 8 FG (Vygon, Cirencester, UK) catheter introduced into one nostril with the tip positioned in the nasopharynx. 2.5 ml of phosphate buffered saline (PBS) was then instilled into the nostril with simultaneous suction through the catheter. The catheter was attached to a standard mucus extractor connected to a portable suction device and was gradually withdrawn through the nasal cavity as the PBS was infused slowly over 8-10 seconds. This procedure was then repeated in the other nostril. After collection, the nasal lavage fluid was immediately placed on ice and returned to the laboratory for analysis within 3 hours. The total unprocessed specimen was measured, vortexed vigorously and an aliquot tested for RSV by EIA (RSV Testpack, Abbott Diagnostics, Abingdon, UK). A further 500µl aliquot was mixed with 2 mls of virus transport medium for storage at -70°C. The remaining specimen was filtered through a 100µm cell strainer to remove mucus and cell debris and centrifuged at 400g for 10 minutes. The supernatants were separated, put into aliquots and stored at -70°C until analysis.

2.12 Immunoassay based on Wallac System

The protein assays for several of the cytokines were based on immunoassays for the protein. These were performed using paired antibodies against the cytokine and using recombinant cytokines to act as the standard. The various buffer ingredients used in the Wallac Delfia (Turku, Finland) based assay system are shown in the tables below.

Coating Buffer (pH 9.6)

Sodium Hydrogen Carbonate (BDH)	35mM
Sodium Carbonate (BDH)	15mM
Sodium Azide (BDH)	0.05% (w/v)

Blocking Buffer - diluted in phosphate buffered saline

Bovine Serum Albumin (Sigma)	1% (w/v)
Sucrose (BDH)	5% (w/v)

Assay Buffer and Diluent (pH 7.8)

Sodium Chloride (BDH)	150mM
Tris HCl (BDH)	25mM
Bovine Serum Albumin (BSA)	0.25% (w/v)
Bovine Gamma Globulin (NBS Biologicals)	0.023% (w/v)
Diethylene Triamine Penta acetic acid (DTPA) (Sigma)	10μmol
Sodium Azide (BDH)	0.025% (w/v)
Polyoxyethylene ether W-1 (Sigma)	0.1% (v/v)

Wash Buffer 40x concentrate (pH 8)

Sodium Chloride (BDH)	4M
Tris HCl (BDH)	1M
Tween 20	0.02% (v/v)

(BDH and Sigma both Poole, UK)

2.12.1 Assay Protocol

All incubations took place at 37°C and 100μl of reagent was added to the well, unless otherwise stated. The concentrations of antibodies used are given in table 2.4. Each wash was performed using 400μl of wash buffer, with blotting dry after the last wash.

The assay plates, 96 well FluoroNunc Maxisorp plates (Nunc, Paisley, UK), were coated with capture antibody diluted in carbonate coating buffer. The plates were sealed and incubated at room temperature overnight. The wells were aspirated prior to the addition of the blocking buffer the wells. A volume of 250μl of blocking buffer was added to each of

the wells, after an hour's incubation the wells were washed five times prior to the addition of samples and standards.

The experimental supernatants were diluted with the assay/diluent buffer, as were the recombinant cytokine standards. A standard curve was generated by performing a 2.5 fold dilution series of the standards. The negative controls or blanks were diluent buffer only. The standards and samples were incubated for two hours; this was followed by five washes prior to the addition of the biotinylated detector antibodies, followed by a further two-hour incubation. This incubation ended in five washes of the plates followed by the addition of Europium (Eu)-labelled streptavidin (100ng/ml) and a one-hour incubation. The plate was washed a further five times and Delfia enhancement solution was added to the wells. The plate was shaken on a Titertek shaker for 10 minutes and the fluorescence was measured on a Wallac 1234 Delfia fluorometer (Wallac, Turku, Finland). The Delfia enhancement solution releases the Europium into colloidal solution and this fluoresces under certain conditions. This is a linear process and thus has advantages over other enzyme-based systems, which are non-linear.

2.13 Nitric Oxide Assay

A commercial set of reagents were used to perform the nitric oxide assay, based on the on the analysis of nitrite concentrations (Calbiochem, Nottingham, UK). The assay is a quantitative colorimetric based assay. The assay could not work if a nitrate or nitrite containing tissue culture media was used. Nitric oxide in solution rapidly reacts with water to from nitrate ions; these are measured by this assay. The assay enzymatically converts nitrate to nitrite and the change is associated with a colorimetric change, which can be picked up by spectometer.

2.13.1 Assay protocol

Serial dilutions of sodium nitrate were used to form the standard curve for the assay, ranging from 2M through to 0.5 mM. 80ml of sample or standard were added to each well, 80ml Griess reagent (Calbiochem, UK) was added to each well, the microtitre plate was sealed and left fro 20 minutes, then was placed in a microtitre reader (Anthos Labtec, USA), with a measurement filter of 570nm and reference filter of 690nm. The values for each individual well could be calculated from the standard curve that was constructed.

2.14 Second Messenger Inhibitors to investigate signalling

To further investigate the mechanism behind alterations in cytokine production of macrophages following RSV challenge the responses to a variety of inhibitors were investigated.

Wortmanin is a cell permeable covalent inhibitor of phosphatidyl 3-kinases (PI 3-kinase). Gliotoxin acts as a NF-κB inhibitor, though at higher concentrations induces apoptotic cell death. Stausporine is a broad-spectrum protein kinase inhibitor, though is slightly more selective for protein kinase C compared to protein kinase A. Herbamycin A is a tyrosine kinase inhibitor, all of the above mechanisms are recognised secondary messengers that have a role in a variety of cell signalling processes. The final agent used was methyl αD

manopyranoside (αMM), this is a mannose analogue, this was used to investigate whether the macrophage mannose receptor was important in RSV binding and stimulation of cytokine release.

The cell culture conditions employed were similar to the normal culture conditions, though in the one hour equilibration step used prior to experimentation the agents were added.

2.15 Statistical Methods Employed in analysis

Different statistical analyses were performed for the different type of experiments. For the in-vitro experiments, the results were initially analysed by non-parametric tests (Mann-Whitney-U), as there was no evidence of the results forming a normal distribution. If the result of the analysis demonstrated statistical significance the analysis was repeated with a parametric test (paired t test). All the analysis was performed using SPSS version 10.01 (SPSS Inc., USA). If multiple analyses were being performed on the same sample, then bonferroni correction was used to compensate for the multiple analyses being performed.

For the analysis of the in-vivo data, statistical advice was obtained from the Univesity statistical department. As the distributions of the nasal lavage and PBMC cytokine values and the derived cytokine ratios were skewed to the right, the data was logarithmically transformed to obtain approximate normality before analysis. Cytokine responses change over the first year of life, to account for the age differences between the groups, the transformed cytokine levels and ratios were compared between the bronchiolitis and upper respiratory tract only groups by using analysis of covariance (ANCOVA), in which age was the covariate. The magnitude of difference between groups for this data is summarised using the geometric mean ratio (gmr) together with a 95% confidence interval. The comparison of viral load was made using Mann-Whitney-U, for the other continuous variables unpaired student's *t* test was used. The analyses were performed using SPSS 9.0 (SPSS Inc., USA).

Chapter 3 - Cellular responses to RSV Challenge

The relative contributions of different lower airway cell types were investigated by the use of single cell type cultures and monitoring the response of these cells to RSV challenge. For lung epithelial cells the type II pneumocyte cell line A549 was used, this has been well established in the field of RSV research. Other cells were considered and initially analysed, including Hep 2 cells and the incompletely SV40 transformed human epithelial cell line 16HBE, but were not ultimately used for analysis.

In the methods section (section 2.12) the rationale for using monocytes that were cultured to take on the macrophage phenotype were used for this series of experiments.

3.1 RSV Replication in Challenged Cells

The initial series of investigations in both A549 cells and the monocyte-derived macrophages (MDM) was to determine if they sustained RSV replication. Two separate methods of analysis were employed to confirm this. The first method employed was by analysing the production of viral RNA by the quantitative TAQMAN RT-PCR method. The second method was to use commercially available fluorescently tagged antibodies against RSV and detect the surface expression of RSV proteins on the cells to be studied.

Following RSV challenge of epithelial cells there was an increase inn viral RNA production. There was a dose dependant relationship between the initial RSV challenge dosage and the subsequent viral load, see figure 3.1. There is an apparent maximum production from the cells, such that a starting number of 10^6 cells will generate 10^7 RSV copies. Differing starting doses, expressed as multiplicity of infection (moi) did have an effect on the number of virions produced, see figure 3.2, but ultimately a ceiling is reached of about 10 virions per cell.

Figure 3.1

F gene copy number expression against time following RSV challenge in A549 pneumocytes

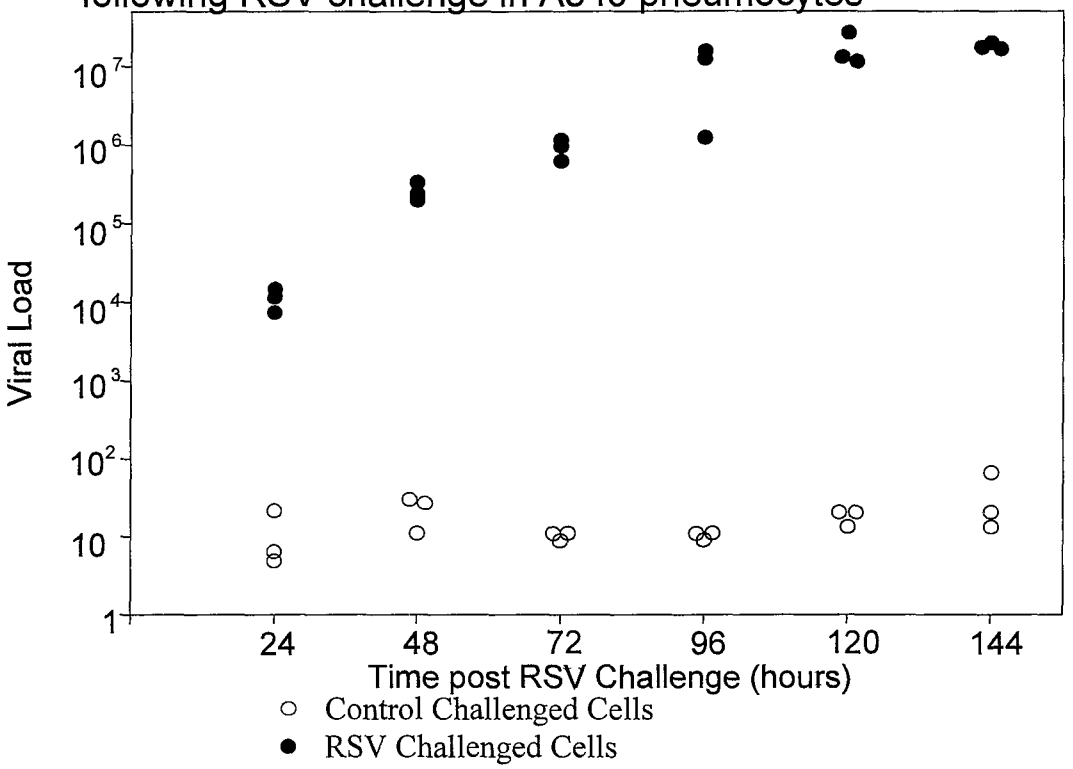
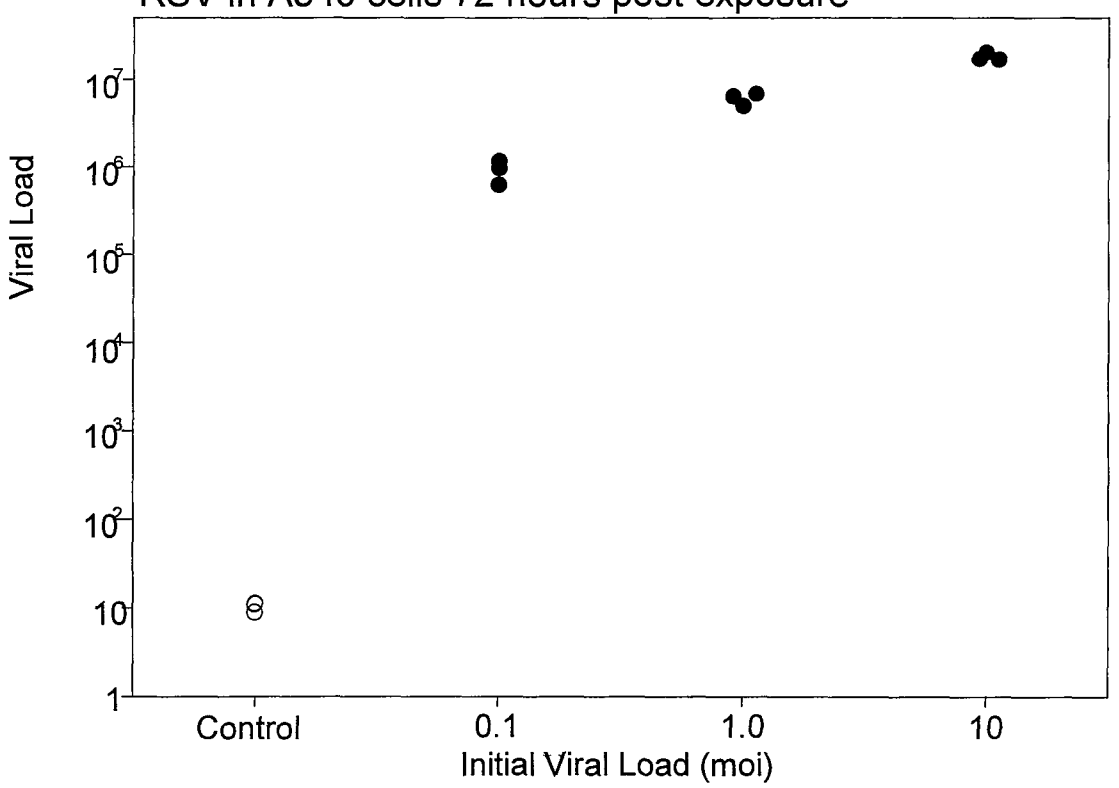


Figure 3.2

F gene copy number expression against challenge dose of RSV in A549 cells 72 hours post exposure



The staining of infected A549 monolayers with fluorescently tagged antibodies against RSV is demonstrated in figure 3.3. This experiment provides further confirmation that RSV replication does take place in A549 cells and there is surface expression of the RSV surface proteins. This experiment does provide alternative confirmation of the quantitative PCR results.

The result from RSV challenge of macrophage culture demonstrates that there was limited RSV replication in macrophages; see figure 3.4 and figure 3.5 for the demonstration of increased viral surface protein. The results shown in figure 3.4 are expressed as F gene copy numbers per GAPDH gene, these results are presented in a slightly different form from the results for epithelial cells because there were variable numbers macrophages in each well and variable survival following RSV challenge. The variation in adherence accounted for the majority of the difference in cell numbers between wells.

Figure 3.4

F gene/GAPDH gene expression for Macrophages challenged with RSV

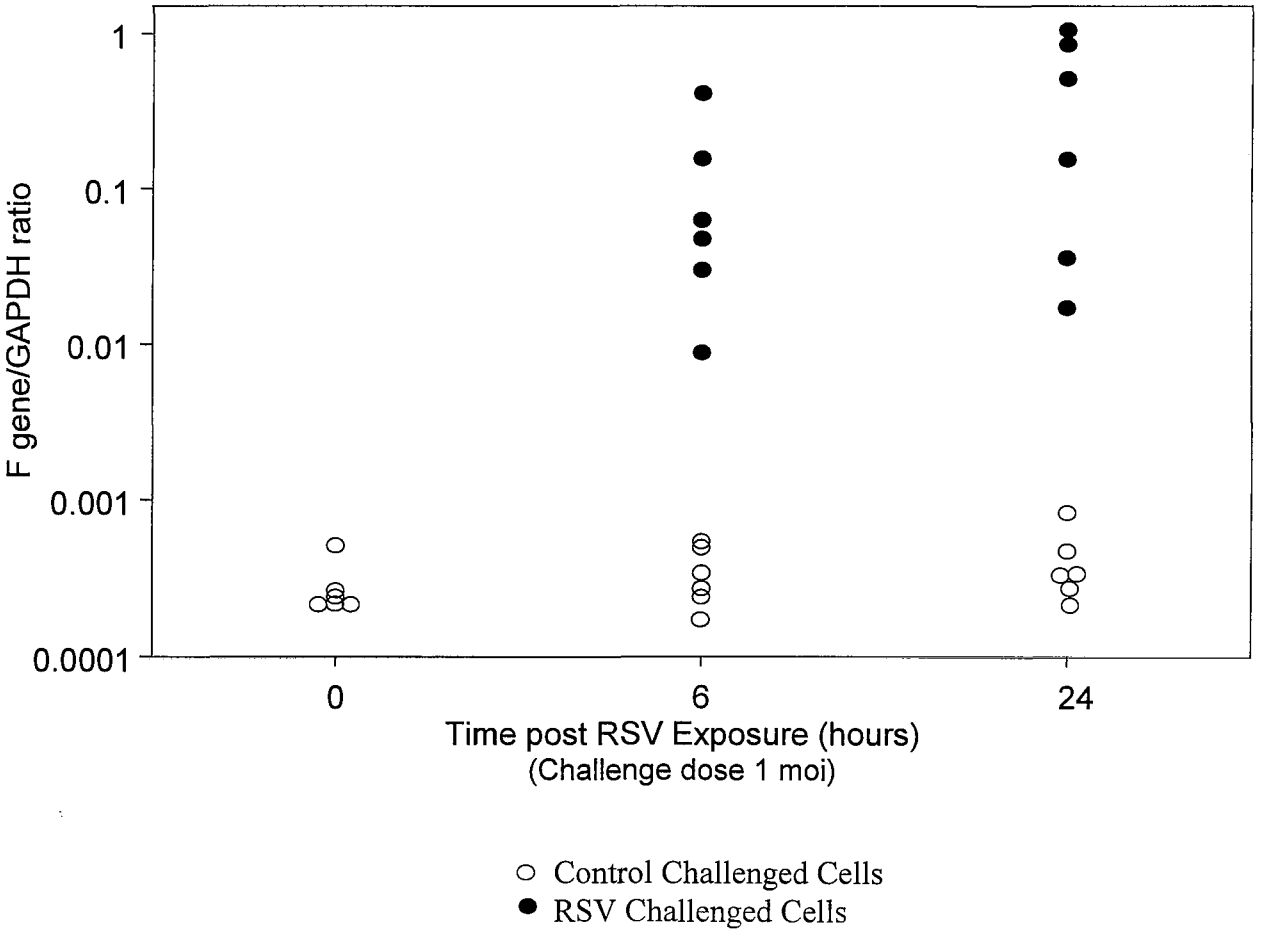
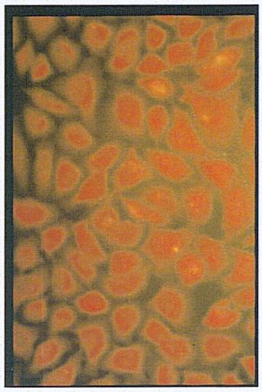


Figure 3.3

A549 type II pneumocytes 72 hours after RSV challenge.

a) Control A549 cells
b) RSV Challenged A549 cells

a)



b)

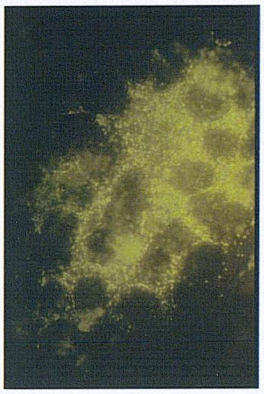


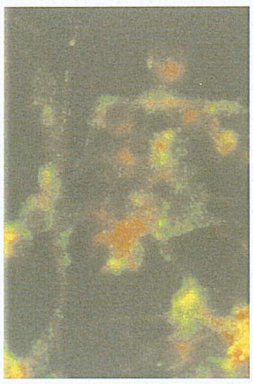
Figure 3.5

Monocyte derived macrophages 48 hours after RSV challenge.

i) Control challenged macrophages
ii) RSV Challenged macrophages

i)

ii)



In all slides there is a red counter stain to highlight the cells, with green fluorescent stain for RSV surface proteins.

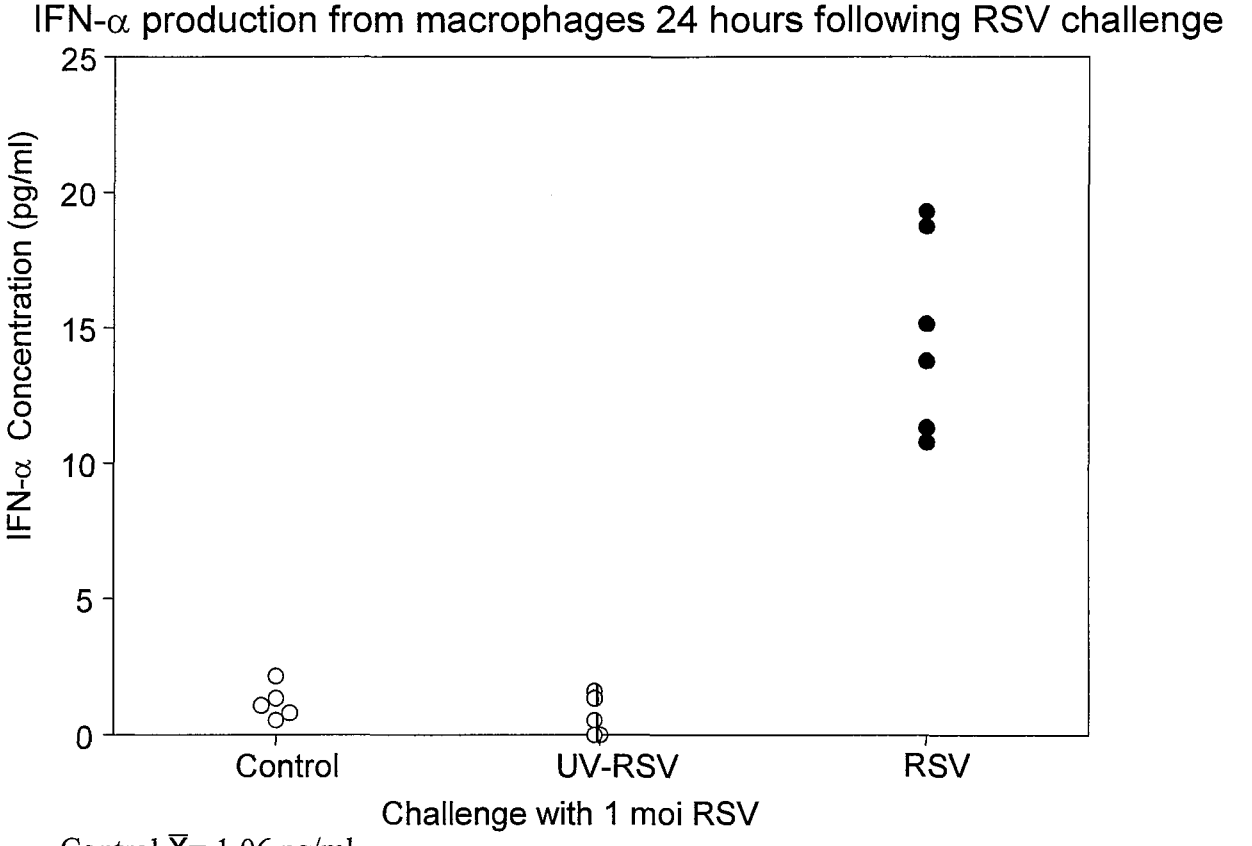
The results shown above confirm that A549 cells are infected by RSV and that the subsequent virus production proportional to the initial infecting viral dose. RSV challenge does show that there is replicative RVS infection of macrophages, but this is not as permissive as replication in A549 cells. Results for both different cells was confirmed by fluorescent staining of the cells.

3.2 Anti-viral Product Release

3.2.1 Interferon Alpha (IFN-α)

There is evidence that the production of IFN-α has an anti-viral effects and it may protect other airway cells from infection, see section 1.20.9. The role of IFN-α in RSV infection has been controversial for many years, though more recent studies indicate that it may be important. My initial experiments were not able to elicit any IFN-α production from A549 pneumocytes in response to RSV challenge, but there was evidence of an IFN-α response by macrophages to RSV challenge, see figure 3.6.

Figure 3.6



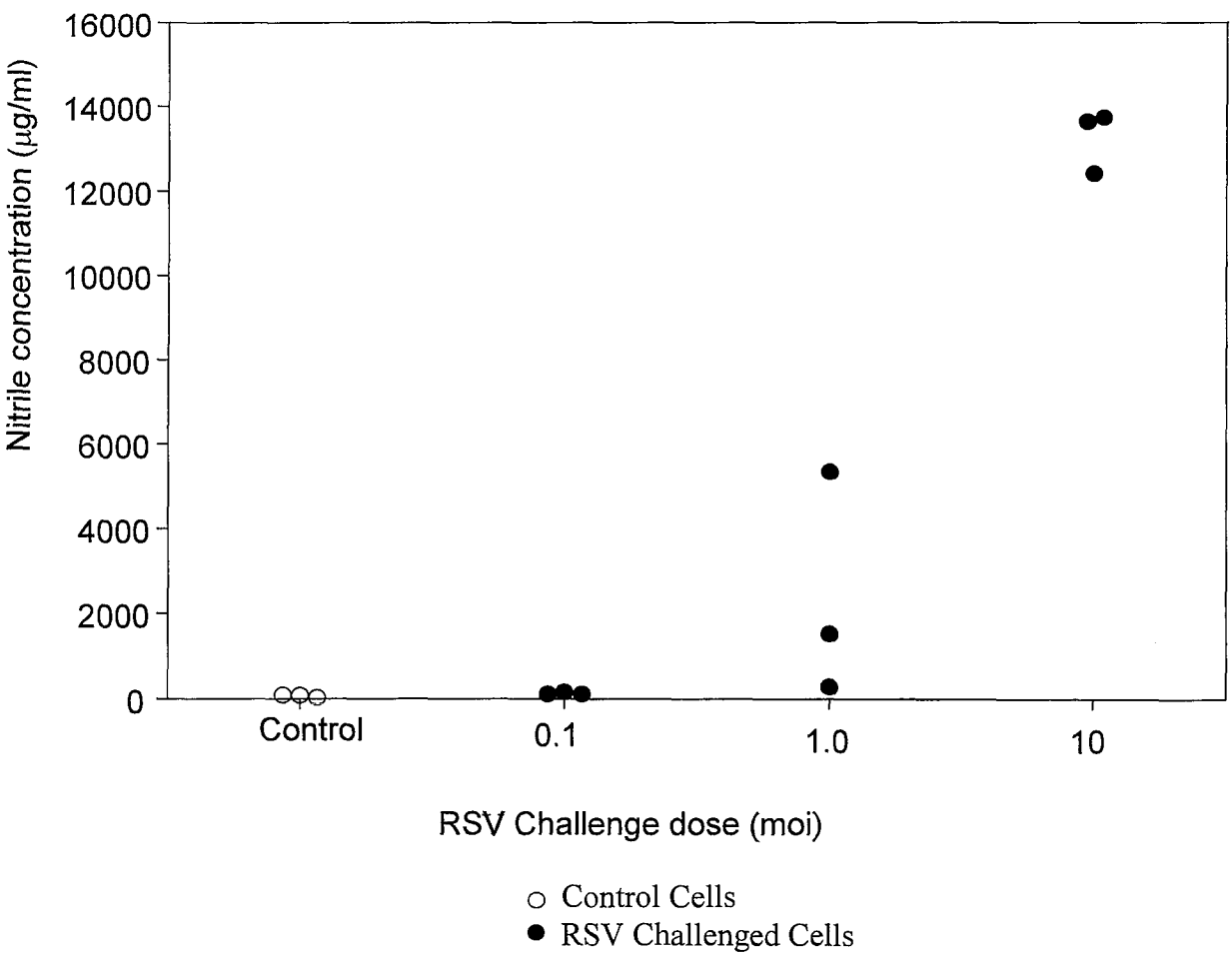
Control $\bar{X}$= 1.06 pg/ml
UV-inactivated RSV $\bar{X}$= 0.58 pg/ml,
RSV Challenged $\bar{X}$= 14.83 pg/ml, produced the largest response
There was no significant difference between control and UV-RSV challenge, but there was a significant difference between both of these and RSV challenge $p<0.01$

No PCR had been optimised to detect IFN-α mRNA, hence no data is provided to confirm or refute if there is an increase in IFN-α mRNA.

3.2.2 Nitric Oxide (NO)

The role of human macrophages in the production of nitric oxide has been controversial, for many years there was no direct evidence that they did produce NO, though this has changed recently, see section 1.6.1. As it was not possible to assay NO directly a nitrate assay was used as a surrogate marker. Despite attempts to establish a PCR to detect an increase in inducible nitric oxide synthase (iNOS), this was not successful, thus there is no confirmation of an increase in iNOS associated with an increase in NO, as measured by nitrate measurements.

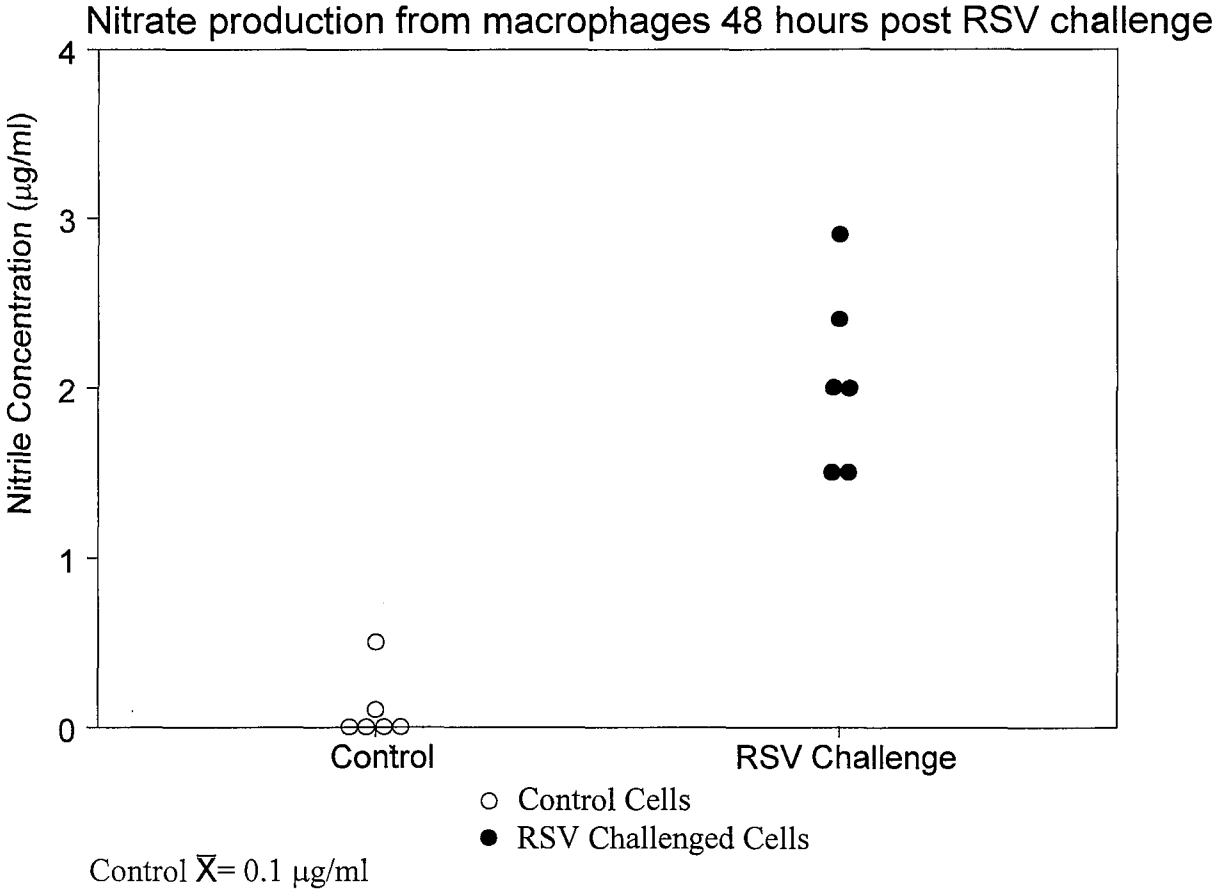
Figure 3.7
Nitrate Concentration in A549 cells 144 hours post-RSV challenge



The culture media used for the macrophages had to be changed to a nitrate-free media (DMEM) 24 hours prior to RSV challenge, the cytokine/chemokine response of the macrophages in the DMEM media was confirmed and demonstrated to be similar to macrophages challenged in serum-free media, as used for all the other experiments performed. The surrogate marker of NO production, nitrate production was assayed. The results demonstrate that there was limited NO production from A549 cells and that this occurred after prolonged incubation with RSV. There was evidence of a relationship between initial viral infecting dose and subsequent NO production.

RSV challenge of macrophages does lead to the production of NO, as measured by an increase in nitrate concentration. The nitric oxide synthase inhibitor, mercaptoethylguanidine (MEG) was employed to investigate the role of NO in RSV killing. MEG is a cell permeable inhibitor of iNOS and has potent anti-inflammatory activity. The addition of MEG did no alter the viral load in the macrophage culture, though there was significantly lower production of nitrate from the macrophages. Macrophages had lower nitrate production than A549 pneumocytes.

Figure 3.8



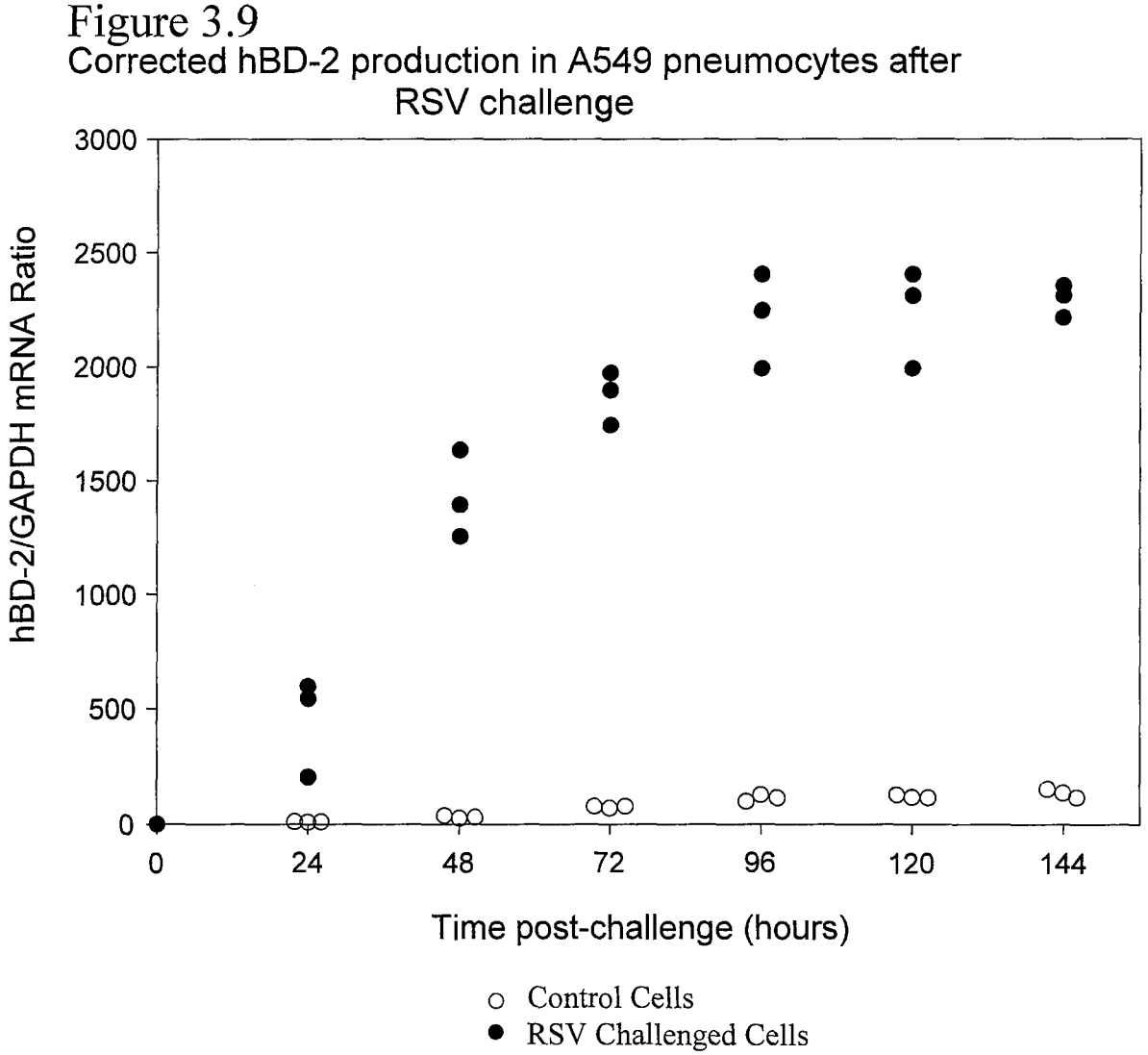
Control $\bar{X}$= 0.1 µg/ml

RSV Challenged $\bar{X}$= 2.06 µg/ml, produced a significantly greater response $p<0.01$

3.2.3 Defensins

Human beta defensins (hBD) are members of a family of highly conserved, cationic peptides that have a broad spectrum of antimicrobial properties against gram-negative and gram-positive bacteria, fungi and enveloped viruses, see section 1.6.2. There have been no previous studies that have investigated the role of this family of peptides in RSV disease. There are no paired antibody sets available for the development of an ELISA against the human beta defensins, thus only studies of beta-defensin mRNA production following RSV challenge were performed. Initial experiments were performed that demonstrated that there was constitutive, non-inducible production of hBD-1. The results from the study of hBD-2 mRNA production demonstrate that there is an ongoing increase in hBD-2 mRNA over the duration of the experiment.

Figure 3.9



3.3 Cytokine Production by Epithelial cells and Macrophages

There are a wide variety of cytokines that can be produced by both epithelial cells and macrophages. The cytokines can be divided into several groups, including: cytokines that are pro-inflammatory; chemokines and cytokines that can regulate the incoming T cell response. The results will be presented for each cytokine produced by both macrophage and epithelial cells.

Pro-inflammatory Cytokines

The group of pro-inflammatory cytokines includes IL-1, IL-6 and TNF-α.

3.3.1 Interleukin-1 (IL-1)

Interleukin-1 alpha (IL-1α) mRNA or protein production by epithelial cells was not detected after RSV challenge (data in appendix).

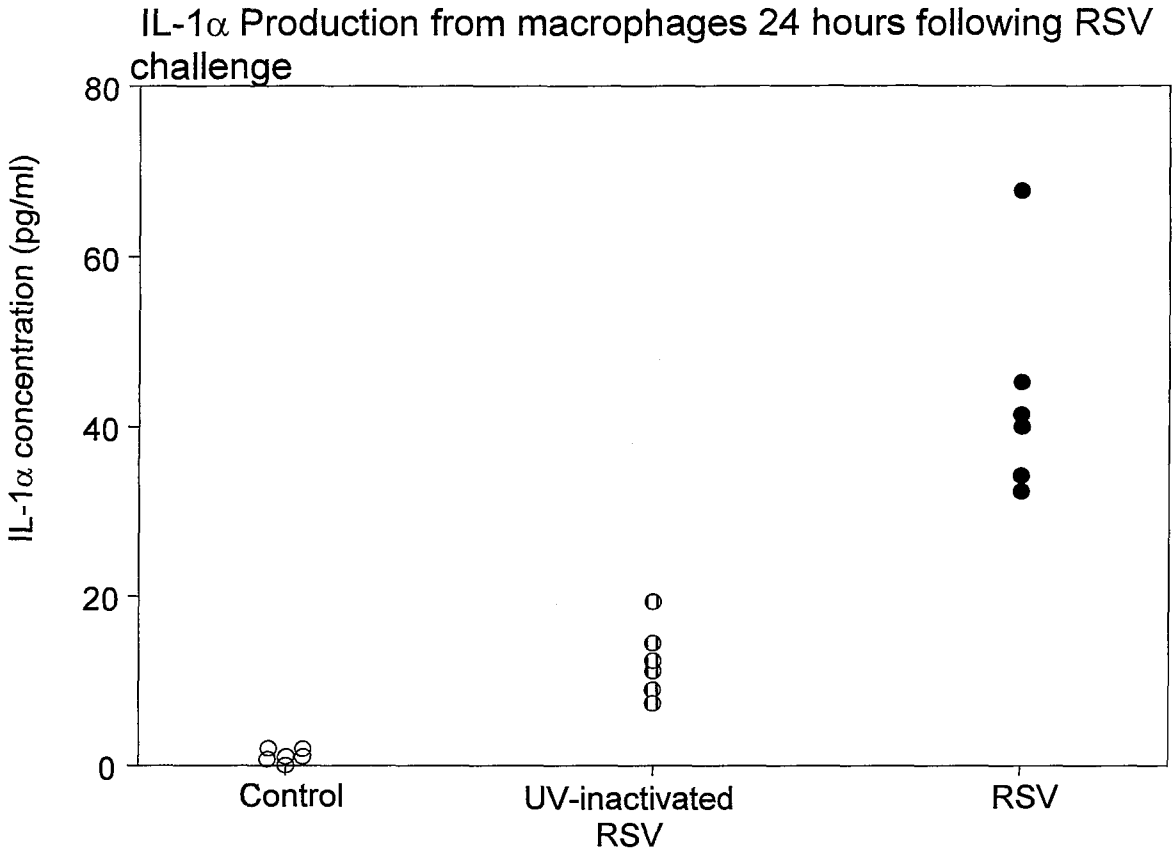
Interleukin-1 beta (IL-1β) mRNA or protein were not produced by epithelial cells either following RSV challenge (data in appendix).

Figure 3.10

The electrophoretic gel for IL-1β PCR, lanes are: ladder, control epithelial (uninfected cells), RSV challenged epithelial cells 24 hours after challenge and positive control.

Interleukin-1 alpha (IL-1α) production by macrophages was demonstrated by ELISA, in contrast to the absence of production by RSV-challenged epithelial cells, there was evidence of an increase in IL-1α protein.

Figure 3.11



Control $\bar{X}$ =1.07 pg/ml
UV-RSV $\bar{X}$=12.25 pg/ml
RSV $\bar{X}$=43.68 pg/ml was the greatest response.
The control response was significantly lower than the response to UV-RSV ($p<0.01$), both of these were significantly lower than the response to RSV ($p<0.01$).

Interleukin-1 beta (IL-1β) mRNA production was increased following RSV challenge, see figure 3.8. The production of IL-1β protein from macrophages challenged with either UV-inactivated RSV or RSV was significantly elevated compared to control cells, see figure 3.10.

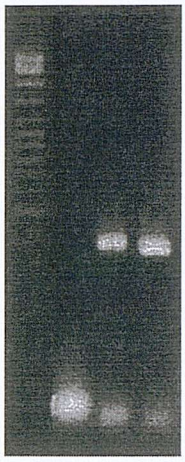
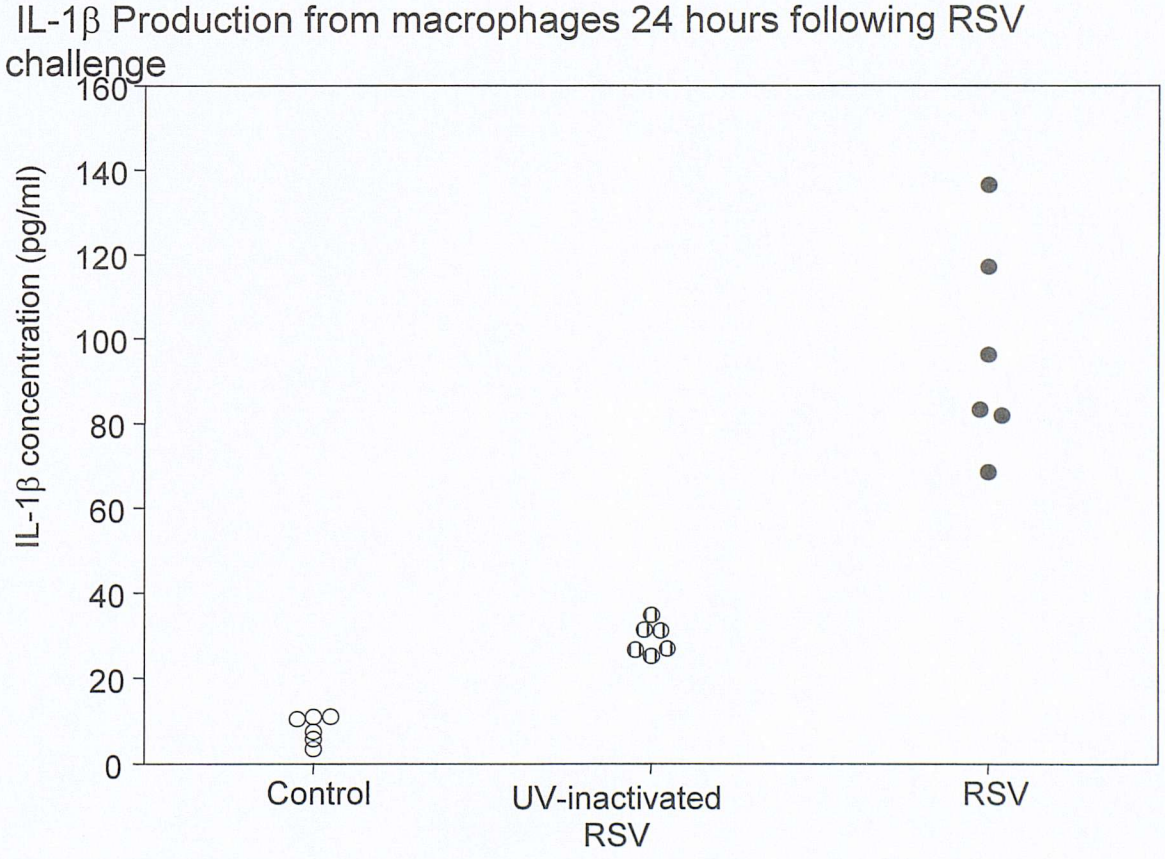


Figure 3.12

The electrophoretic gel for IL-1β PCR, lanes are ladder, control (uninfected cells), RSV challenged macrophages and positive control,, 24 hours after challenge.

Figure 3.13



Control = 8.1 pg/ml
UV-inactivated RSV = 29.31 pg/ml
RSV Challenged = 97.2 pg/ml
The control response was significantly lower than the UV-RSV response ($p<0.01$), both of these were significantly lower than the RSV response ($p<0.01$).

3.3.2 Interleukin 6 (IL-6)

Interleukin-6 is another pro-inflammatory cytokine. There was an increase in IL-6 mRNA production within 24 hours of challenge of A549 cells, see figure 3.14. There is evidence of an increase over time of IL-6 protein production over time, with a significant increase in protein compared to control at 24 hours, see section 3.15.

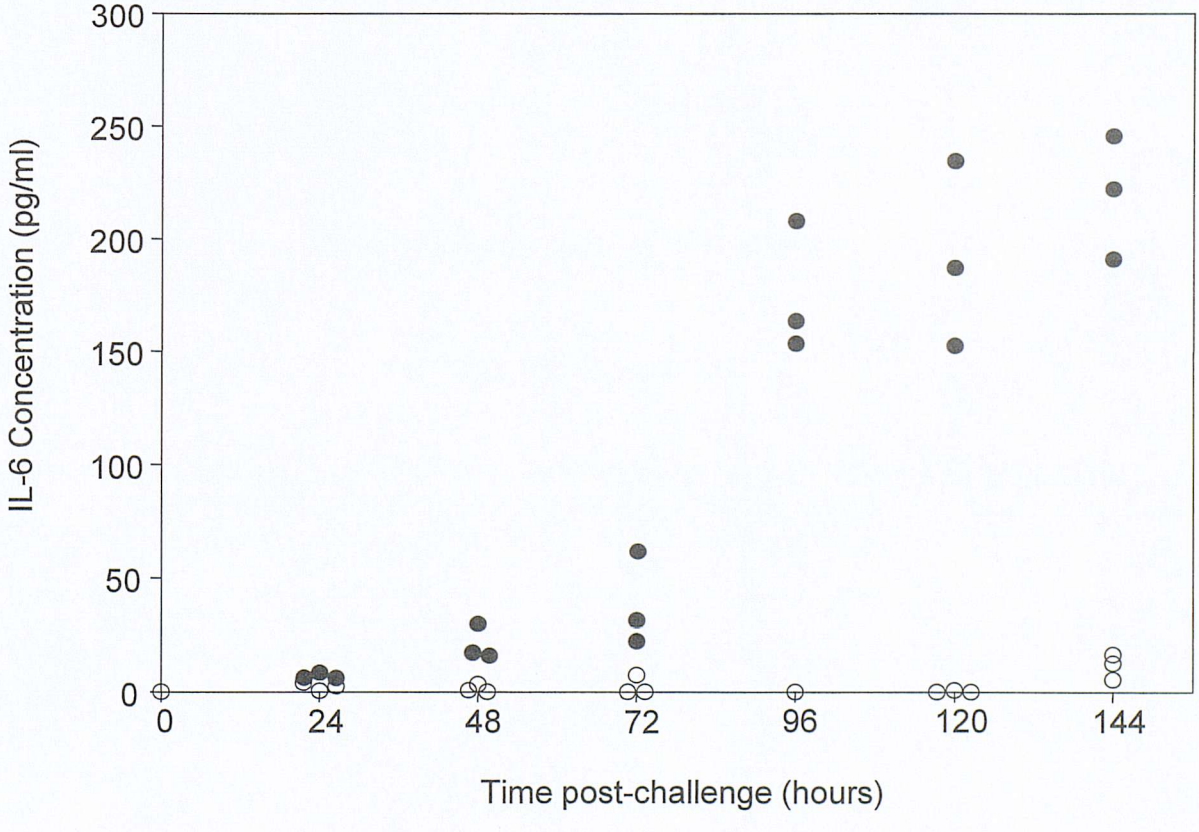
Figure 3.14

Electrophoretic gel of mRNA production from epithelial cells 24 hours after RSV challenge

Lane order: ladder, control cells, RSV challenged epithelial cells

Figure 3.15

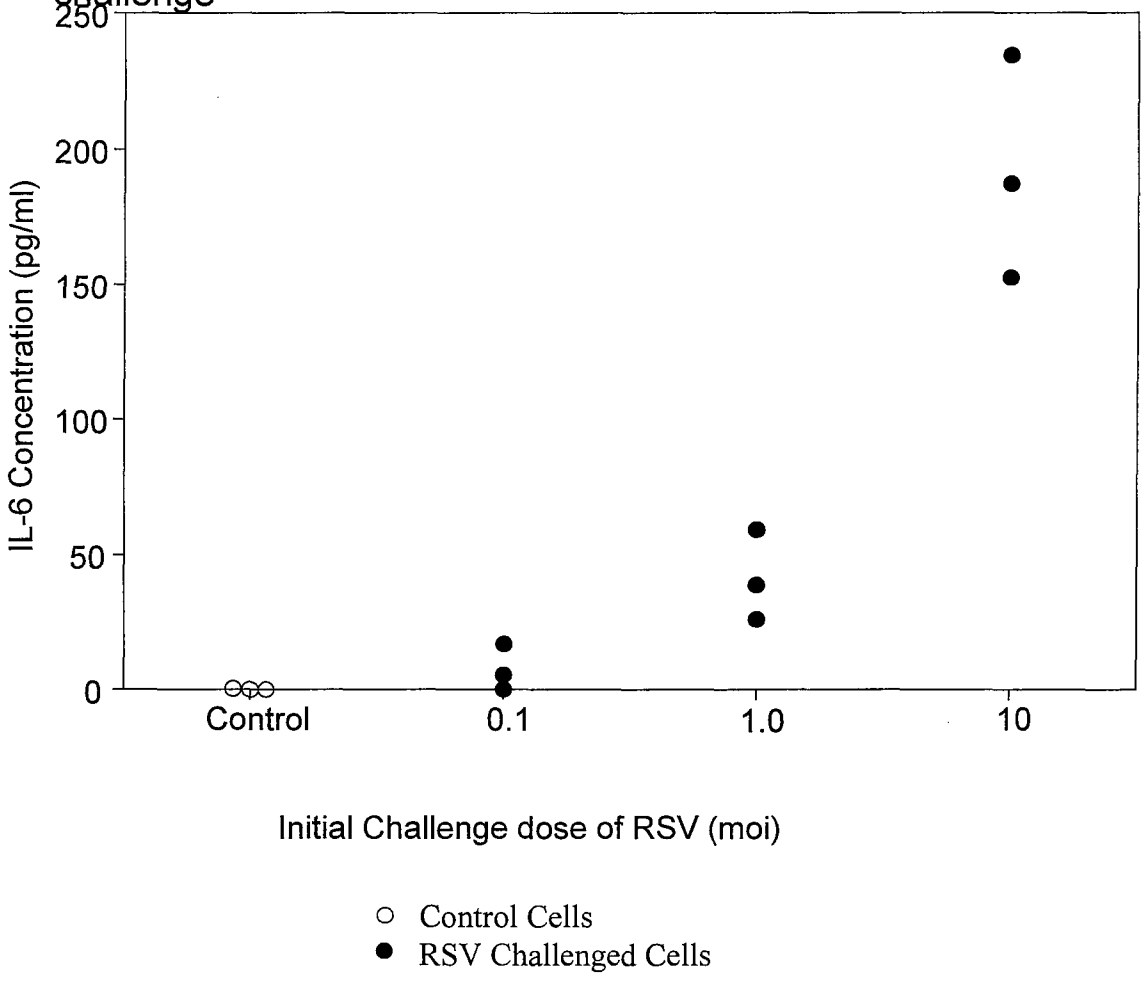
IL-6 Production by A549 pneumocytes following RSV challenge



There is evidence of a initial viral infecting dose dependant increase in IL-6 production, this was most apparent at 72 hours post RSV challenge.

Figure 3.16

IL-6 Production by A549 pneumocytes 72 hours post RSV challenge

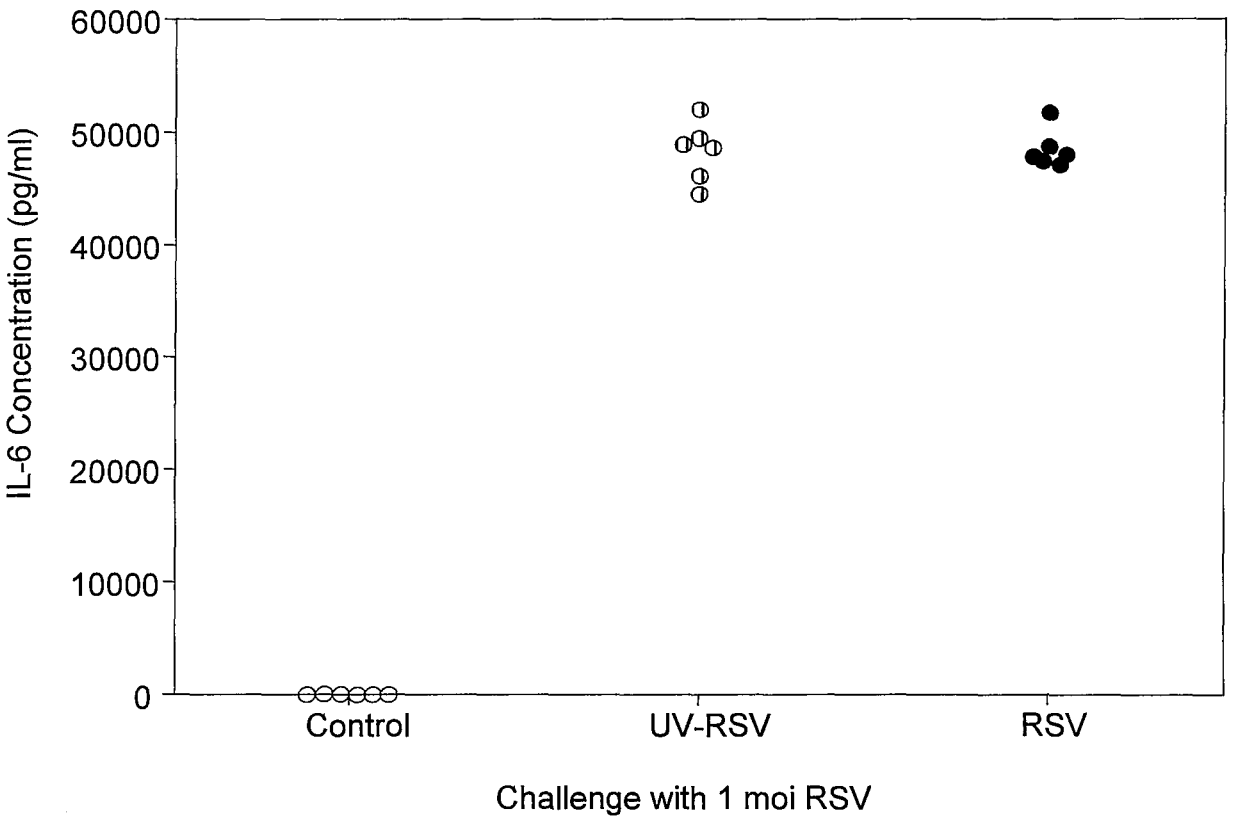


There was a significant difference between the IL-6 concentration of control, 0.1 moi and 1.0 and 10 moi challenged A549 pneumocytes.
There was a significant difference between 1.0 and 10 moi.

There was an increase in IL-6 mRNA production from both UV-inactivated and RSV challenged macrophages; this can be seen in figure 3.18. The amount of IL-6 produced from macrophages was significantly greater than that seen from epithelial cells following RSV challenge. There was a significant increase in IL-6 following UV-RSV challenge, confirming the PCR findings.

Figure 3.17

IL-6 Production by macrophages 24 hours following RSV challenge



Control = 18 pg/ml
UV-inactivated RSV = 4827 pg/ml
RSV Challenged = 4850 pg/ml
The control production was significantly lower than either UV-inactivated, or RSV challenge ($p<0.01$), there was no significant difference between UV-RSV and RSV challenge concentrations

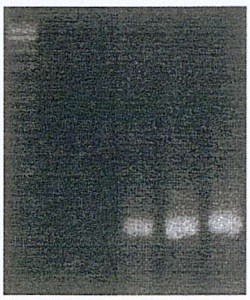


Figure 3.18

The electrophoretic gel for IL-6 mRNA production 24 hours following RSV challenge of macrophages.

Lanes order is: ladder, control (uninfected cells), UV-inactivated RSV challenged macrophages, RSV challenged macrophages and positive control.

3.3.3 Tumour Necrosis Factor alpha (TNF-α)

TNF-α is an inflammatory cytokine that is produced mainly by macrophages and monocytes (see section 1.11.14); this is reflected in the negligible production of TNF-α observed when epithelial cells were challenged with RSV. There is no evidence of an increase in TNF-α protein production over the time course of the experiment, suggesting that maximal production had occurred within the first 24 hours and there was no significant production after this (see figure 3.20). There was no correlation between the initial virus infecting dose to subsequent TNF-α protein production. This is the first report of TNF-α production following A549 challenge by RSV.

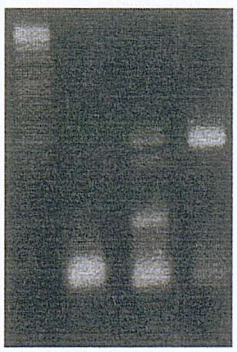


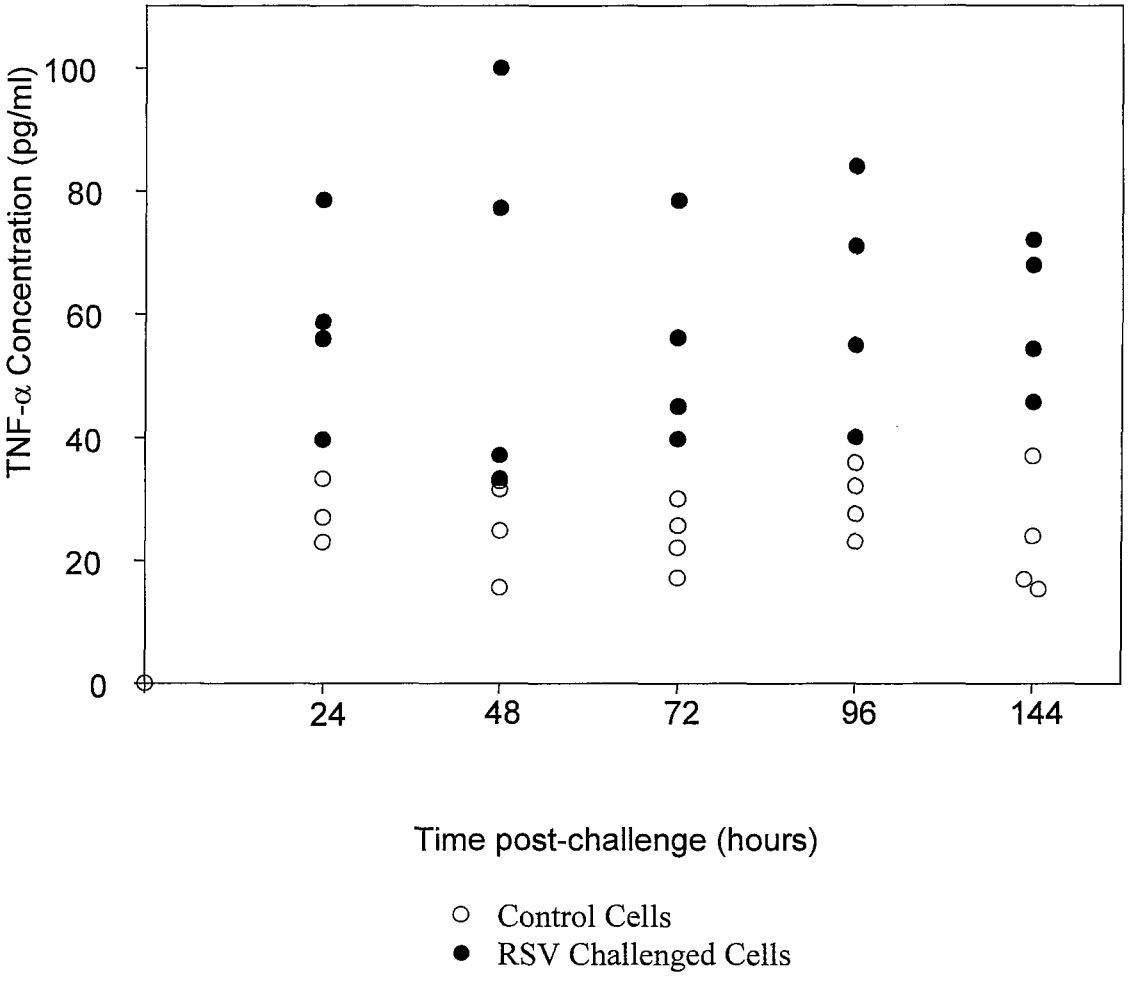
Figure 3.19

TNF-α mRNA production 24 hours after RSV challenge

Lane order: ladder, control (uninfected), RSV-challenged, positive control.

Figure 3.20

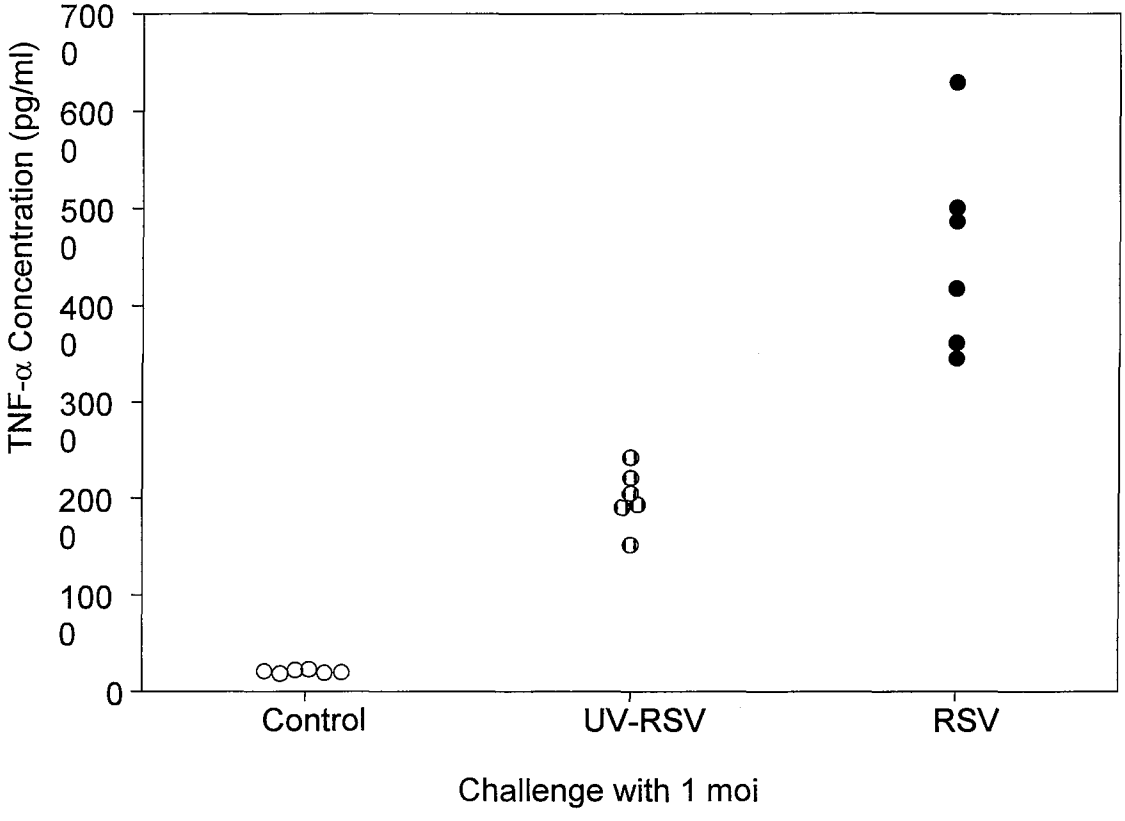
TNF- α Production in A549 pneumocytes following RSV Challenge



The results from my experiments on the TNF-α production from macrophages following RSV challenge reveal TNF-α production to be one of the largest amounts of cytokine produced following RSV infection. Maximal production of TNF-α occurs within the first 24 hours following RSV challenge and, though UV-inactivated RSV does lead to a significant increase in TNF-α production this was significantly lower than the TNF-α production by live replicating RSV, see figure 3.21. The results for TNF-α are in keeping with the previously published reports on TNF-α production following RSV infection of macrophages.

Figure 3.21

TNF-α Production in Macrophages 24 hours post RSV challenge



Control = 203 pg/ml
UV-inactivated RSV = 2001 pg/ml,
RSV Challenged = 4560 pg/ml
Control production of TNF-α was significantly lower than UV-RSV or RSV challenge ($p<0.01$), UV-RSV was also significantly lower than RSV challenge ($p<0.01$)

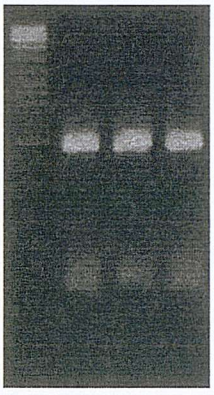


Figure 3.22
TNF mRNA production 24 hours following RSV challenge
Lane order: ladder, UV-inactivated RSV, RSV challenge, and positive control

Chemokines

Chemokines (chemotactic cytokines) are an important group of recently described mediators; they are pivotal in recruiting inflammatory effector cells to sites of infection and inflammation. The differential recruitment of immune effector cells to the site of local infection may dictate the subsequent localised immune response to infection and the subsequent systemic effect. The effects of the various chemokines investigated are outlined in section 1.11.21-26.

3.3.4 C-X-C Chemokines (Interleukin 8, IL-8)

The CXC chemokines recruit neutrophils to the site of inflammation; this group includes IL-8, growth related oncogene (GRO)-α/β/γ and ENA-78. The results from in-vitro epithelial experiments demonstrated an increase in IL-8 mRNA following RSV challenge of epithelial cells (See figure 3.23). There was significant, time-dependant production of IL-8 protein from RSV challenged epithelial cells (Figure 3.24). There was also evidence that there was a correlation between the initial infecting RSV dose and subsequent IL-8 protein production (See figure 3.25).

Figure 3.23

IL-8 mRNA production 24 hours following RSV challenge of A549 cells

Lane order: ladder, uninfected A549 cells, RSV challenged A549, positive control

Figure 3.24
IL-8 Production from A549 pneumocytes following RSV Challenge

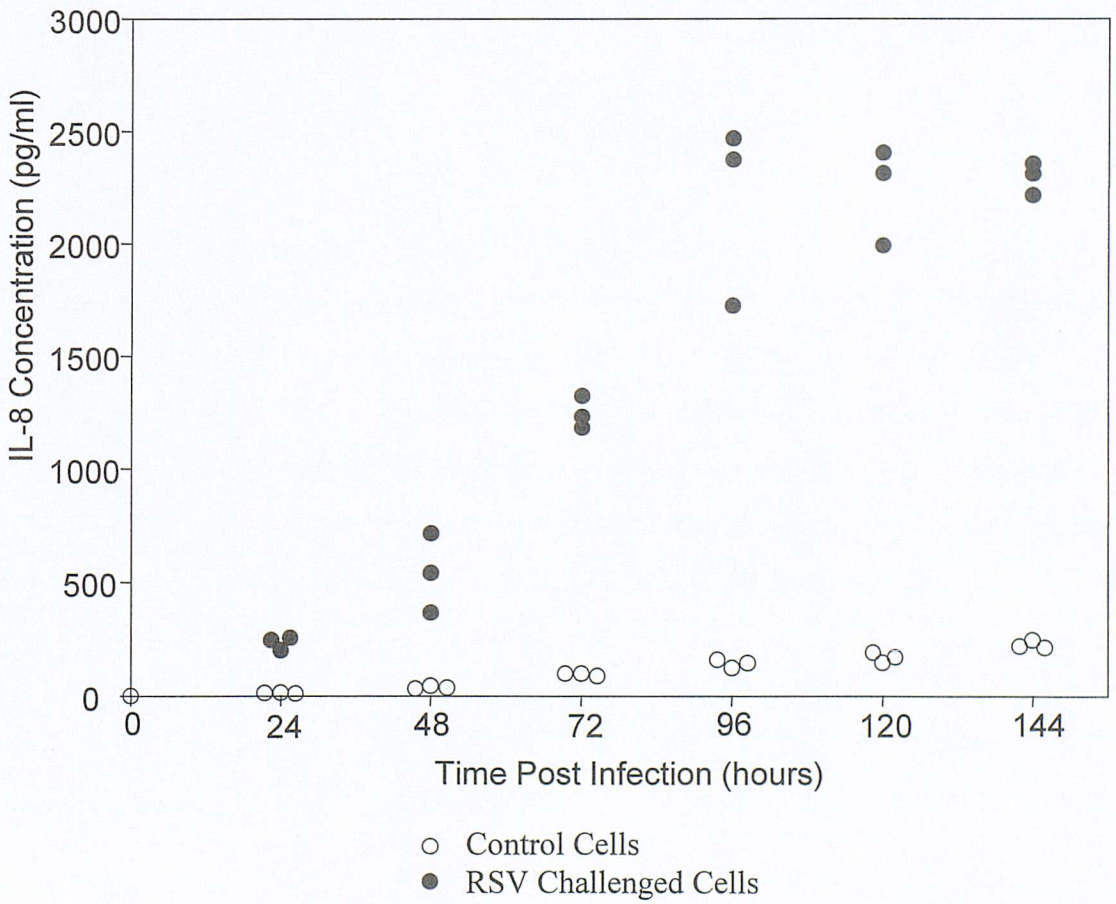
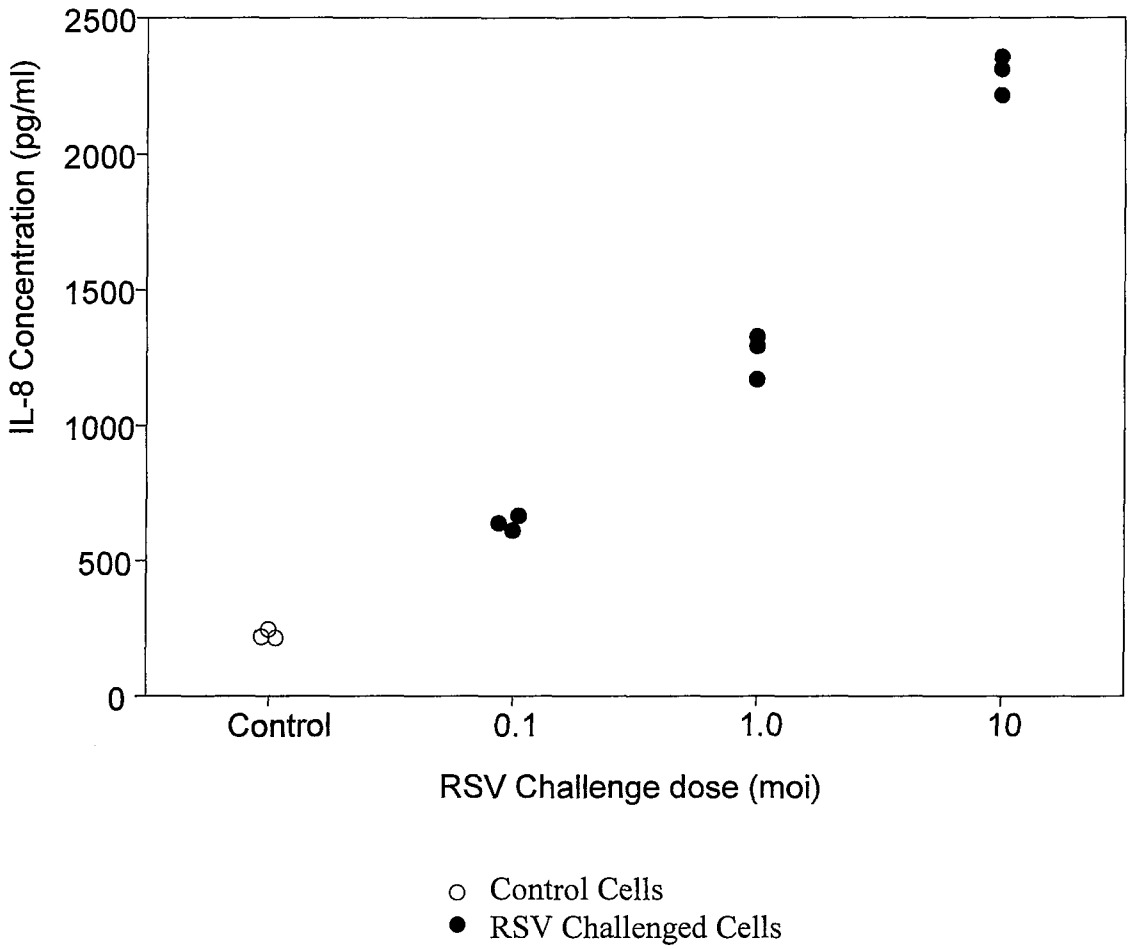


Figure 3.25

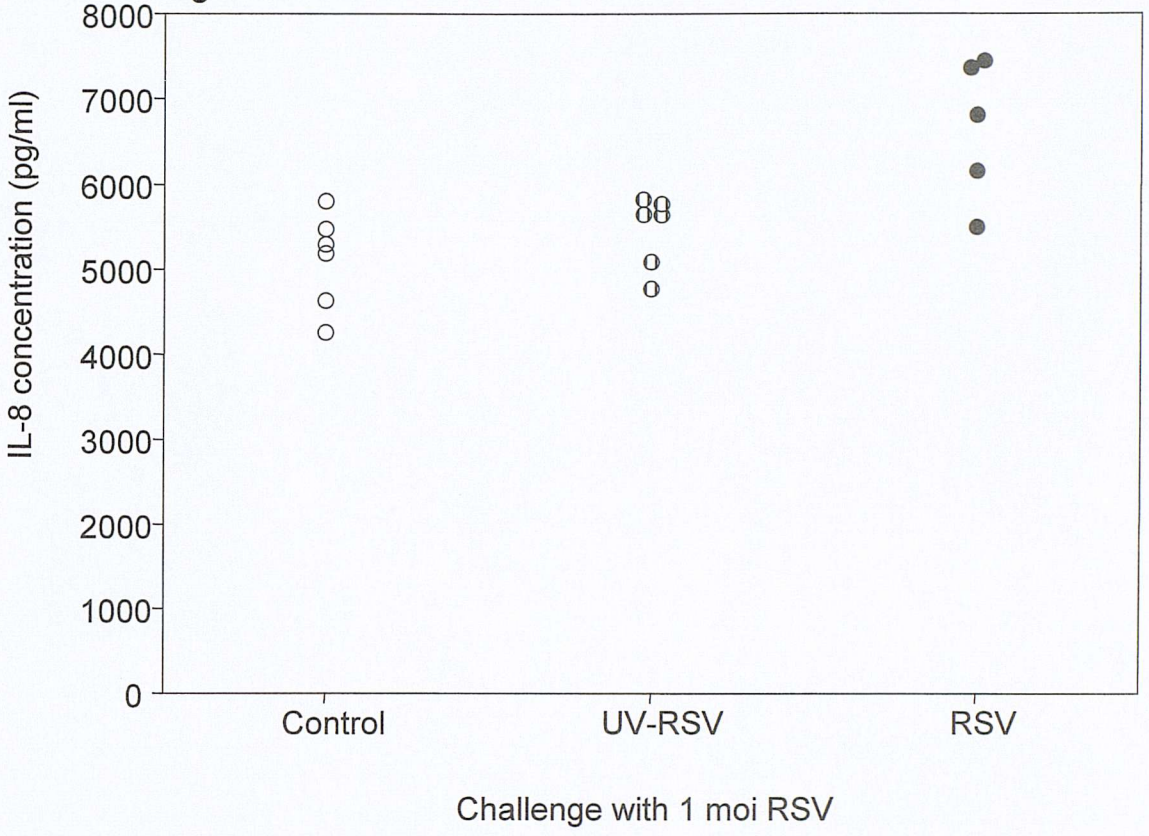
IL-8 production in A549 pneumocytes 120 hours post RSV challenge



The culture conditions employed for the culture of monocyte-derived macrophages does lead to constitutive production of IL-8 protein (see Figure 3.26). The results of analysis of IL-8 protein reveal that there is a significant difference between control and RSV challenged MDM until 48 hours post challenge. The results from the study of IL-8 mRNA showed an increase in IL-8 mRNA production by both un-challenged and challenged cells (see Figure 3.27). These results do suggests that the culture conditions employed for the growth and differentiation of the macrophages does activate them as well.

Figure 3.26

IL-8 Production from Macrophages 6 hours Following RSV Challenge



Control = 5104 pg/ml
UV-inactivated RSV = 5452 pg/ml
RSV Challenged = 6592 pg/ml
There was no significant difference between control cells and UV-RSV, though there was a significant difference between these two and RSV challenged cells ($p<0.05$)

Figure 3.27

The IL-8 mRNA production from macrophage derived monocytes 24hours after challenge

Lane order: ladder, unchallenged cells, challenged cells, positive control

3.3.5 Growth related Oncogene alpha (GRO-α)

GRO-α is another CXC chemokine that has been described as a potent neutrophil chemoattractant. Epithelial tissues are the main source and were looked for using PCR only to look for any increase in GRO-α mRNA. No ELISA was available to investigate any increase in GRO-α protein.

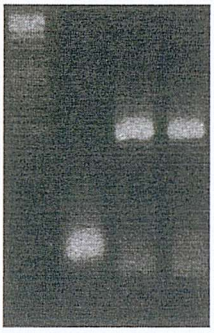


Figure 3.28

GRO-α mRNA production 24 hours following RSV challenge of A549 cells.
Lane order: ladder, unchallenged cells, challenged cells, positive control.

There is evidence of an increase in GRO-α mRNA following A549 infection with RSV, this is not seen in the uninfected cells. And can be seen at 24 hours post infection.

CC Chemokines

The CC Chemokines main action is to promote lymphocyte chemoattraction to sites of inflammation and infection. The CC chemokine family is extensive, so I focused on three different chemokines from this family and studied their production in epithelial and macrophages in-vitro.

3.3.6 RANTES

Regulated upon Activation Normal T cell Expressed and Secreted (RANTES) is produced by a wide variety of cells (see section 1.11.20). There was evidence of an increase in RANTES mRNA following RSV infection of A549 cells (see figure 3.29). The results for RANTES protein production also demonstrate an increase in RANTES following RSV

infection of A549 cells (see figure 3.30). Though a rise in RANTES was seen by 24 it did not reach statistical significance until 72 hours post-challenge. There is an increase in RANTES over time and there is a correlation between initial RSV infecting dose and subsequent RANTES production (see figure 3.31).

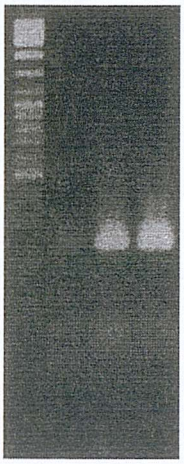


Figure 3.29

RANTES mRNA production from A549 cells 24 hours following RSV challenge

Lane order: ladder, control cells, RSV challenged cells, positive control

Figure 3.30

RANTES Production from A549 pneumocytes following RSV Challenge

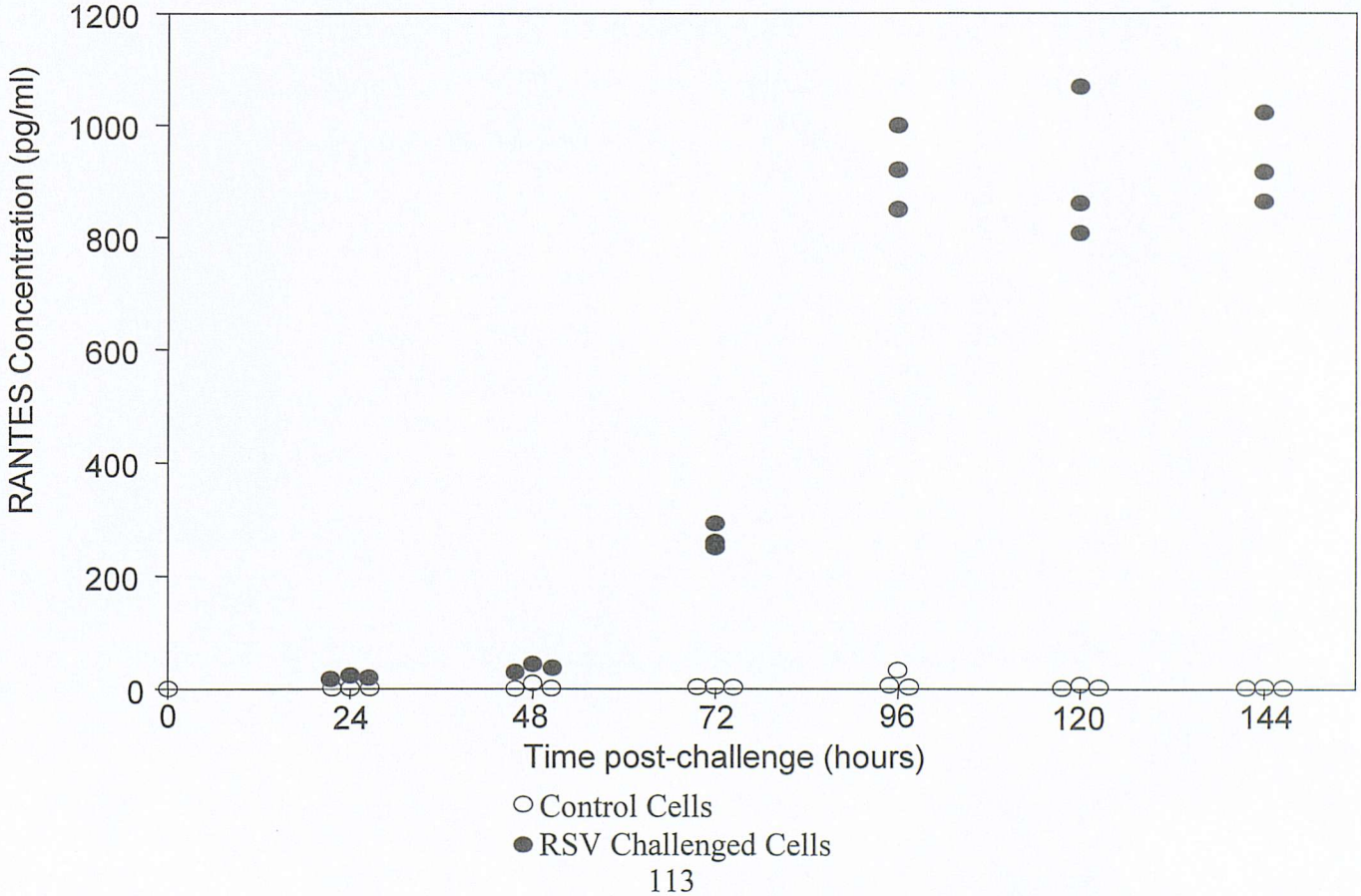
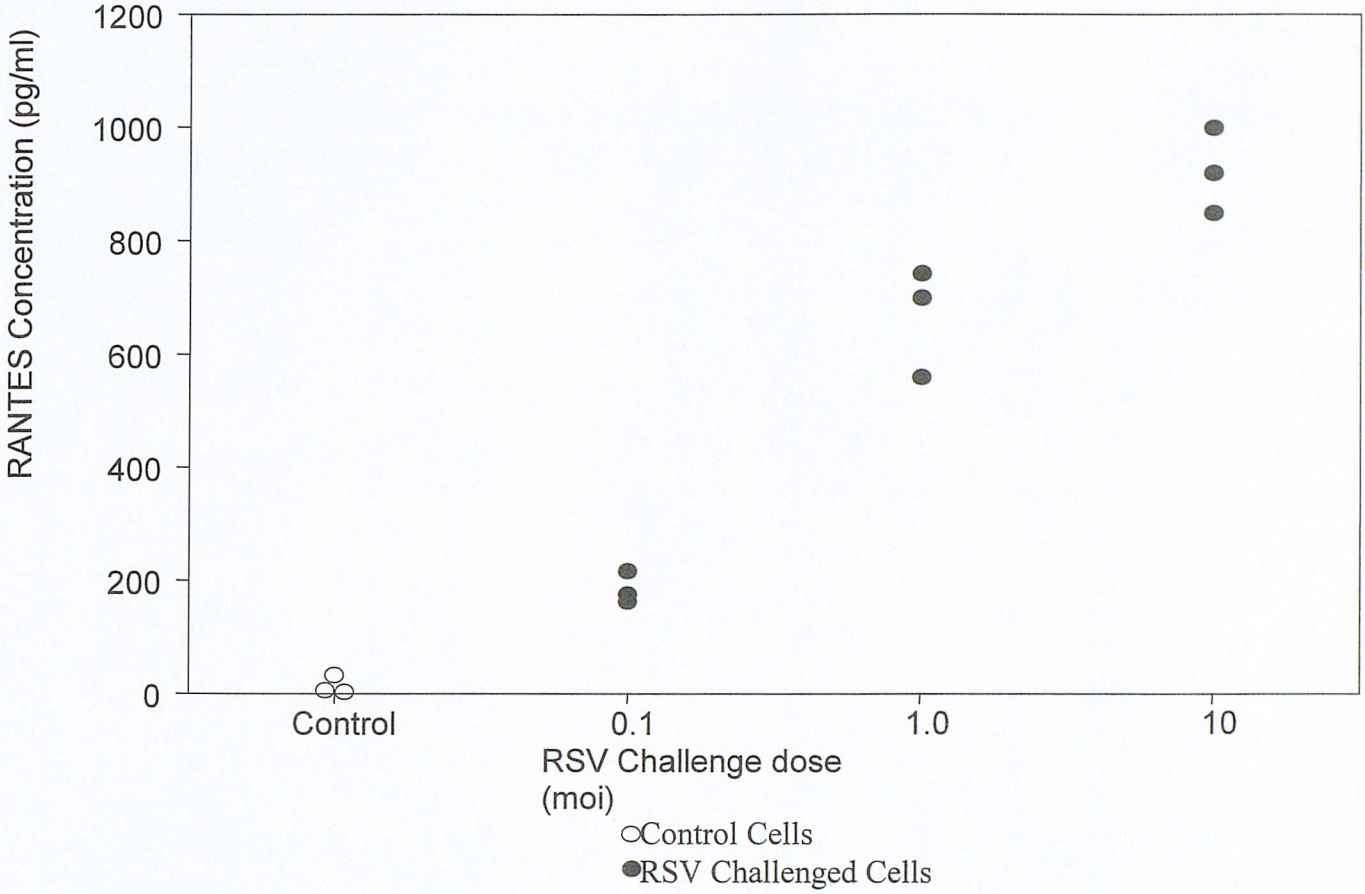


Figure 3.31
RANTES production in A549 pneumocytes 96 hours following RSV challenge



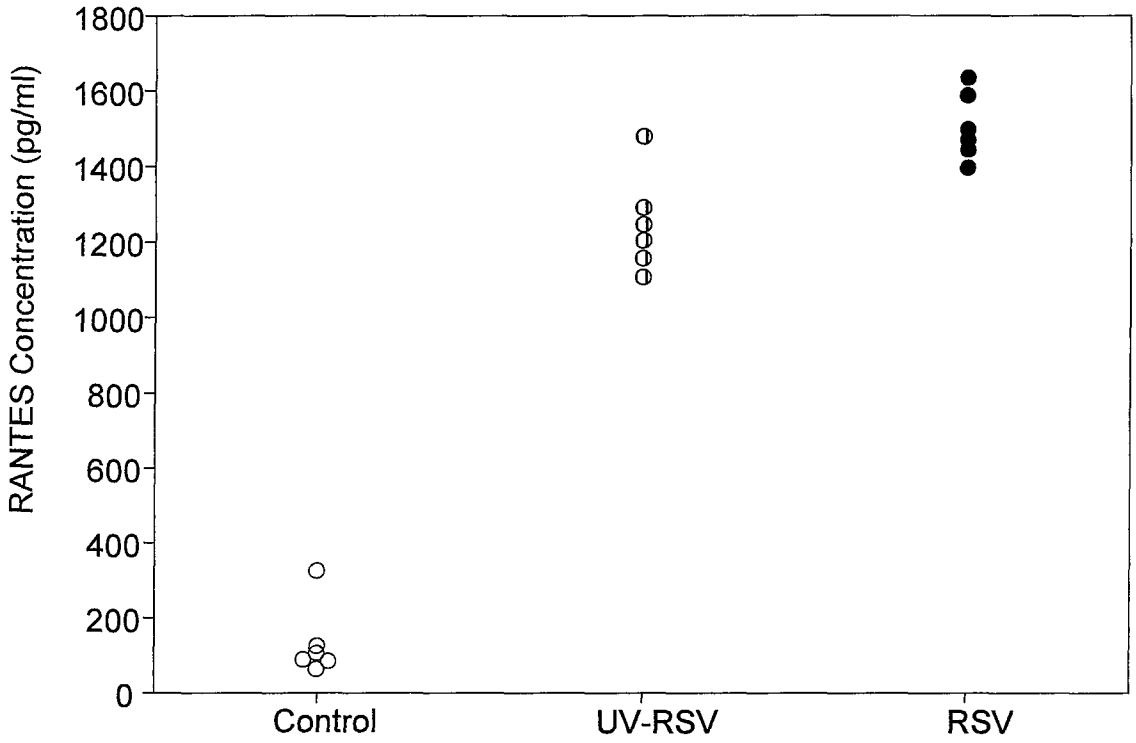
The experiments that investigated the production of RANTES from macrophages following RSV challenge do demonstrate an increase in RANTES production, for both mRNA and RANTES protein. There was an increase in RANTES production from macrophages challenged with RSV as well as macrophages challenged with UV-inactivated RSV (see figures 3.32 and 3.33).

Figure 3.32

RANTES mRNA production from macrophages 24 hours post-stimulation with RSV

Figure 3.33

RANTES production from macrophages 24 hours following RSV challenge



Challenge with 1 moi RSV

Statistical analysis of RANTES production from MDM following RSV challenge demonstrates that:

Control = 145 pg/ml

UV-inactivated RSV = 1247 pg/ml

RSV Challenged = 1504 pg/ml

Control cells RANTES protein production was significantly lower than UV-RSV and RSV challenged cells ($p<0.01$), there was no significant difference between UV-RSV and live RSV RANTES production.

3.3.7 Monocyte Chemotactic Protein 1 (MCP-1)

MCP-1, like RANTES belongs to the CC sub-group of chemokines that are involved in monocyte/macrophage chemotaxis (see section 1.11.22); to date there are four members of the MCP family have been described in humans.

The results of experiments that investigated the production of MCP protein following RSV challenge show that there is constitutive MCP production from A549 cells in prolonged culture (see figure 3.35). Following RSV challenge there is an increase in MCP mRNA at 24 hours post challenge, but no evidence of an increase in MCP mRNA production from control cells (see figure 3.34). Variation in the initial infecting dose of RSV added to the epithelial cells does lead to differential MCP production, though this not seen after 72 hours due to the constitutive MCP production from the epithelial cells (see figure 3.36).

Figure 3.34

MCP mRNA production from A549 cells 24 hours post RSV challenge

Figure 3.35

MCP-1 Production from A549 pneumocytes following RSV Challenge

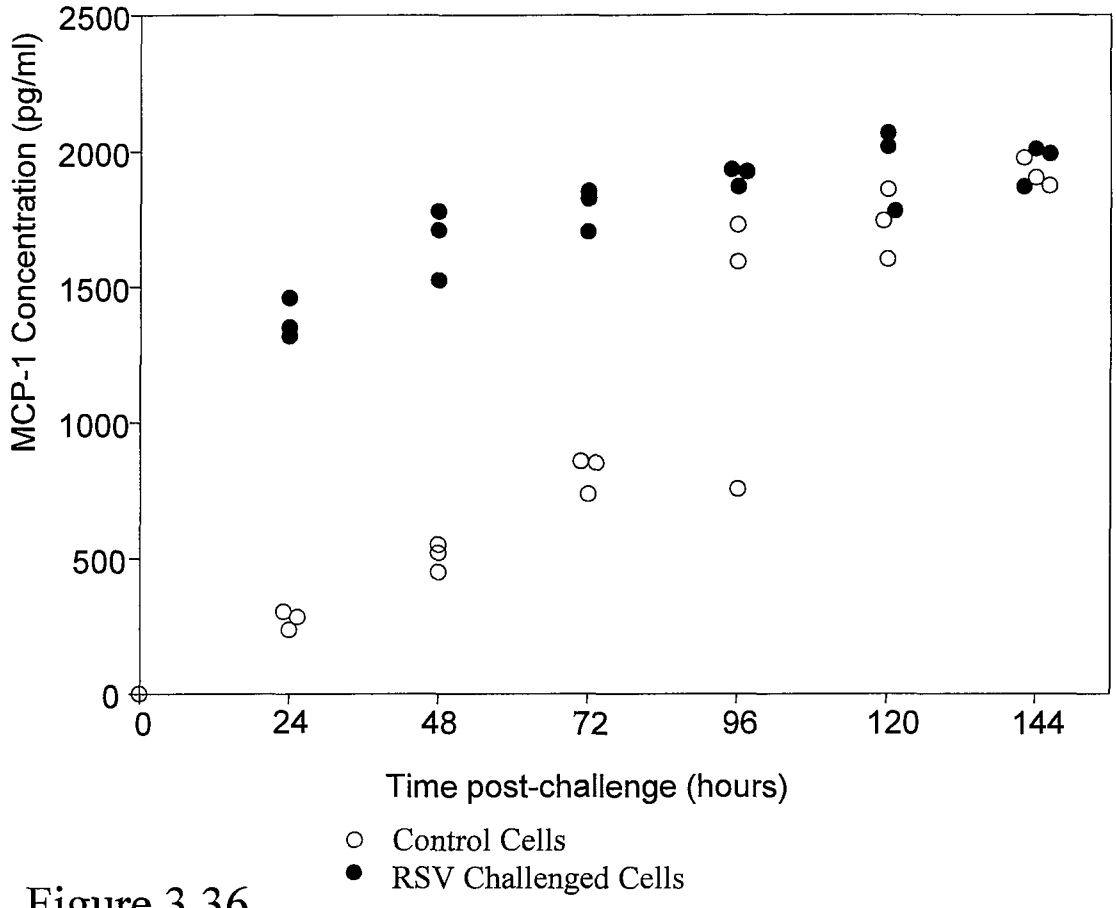
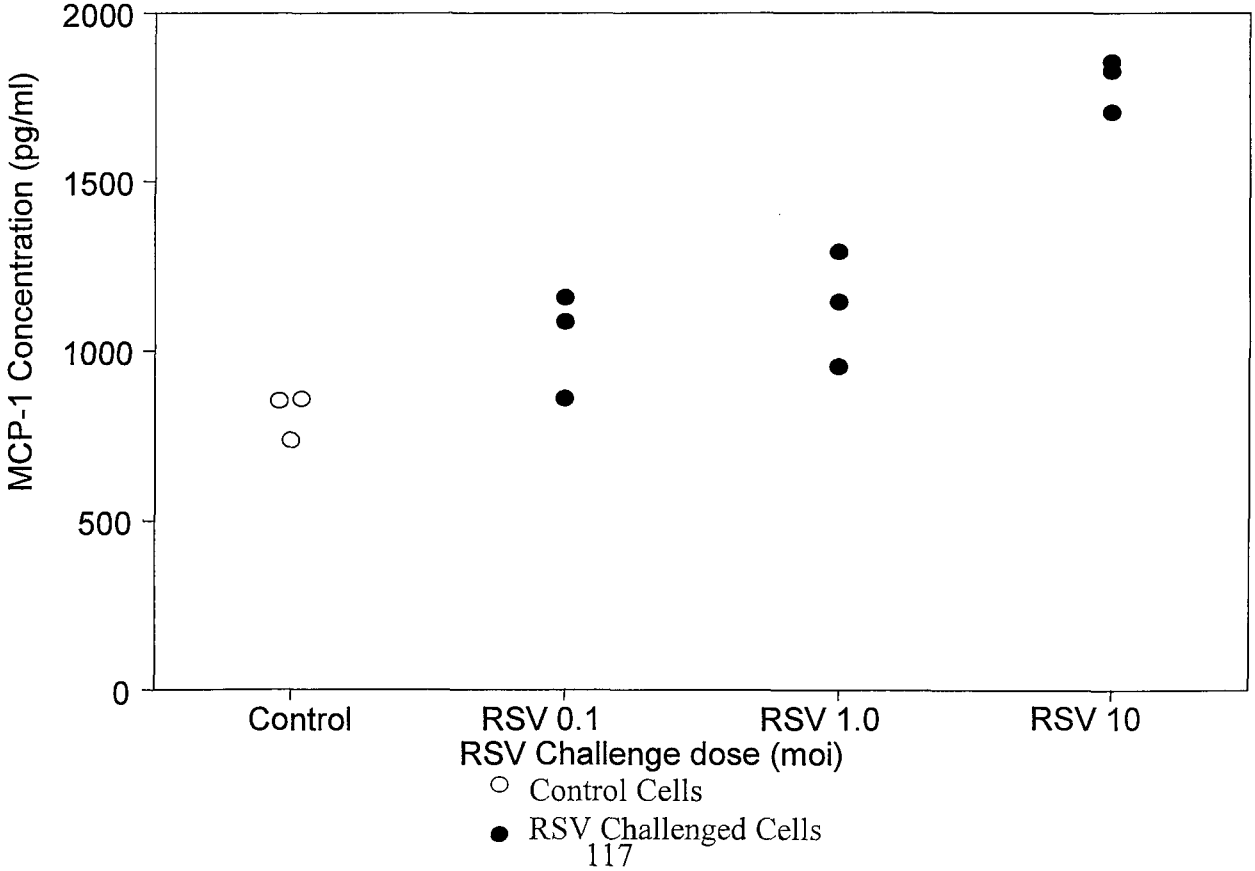


Figure 3.36

MCP-1 production in A549 pneumocytes 72 hours post RSV challenge



There is constitutive production of MCP mRNA and protein from macrophages.

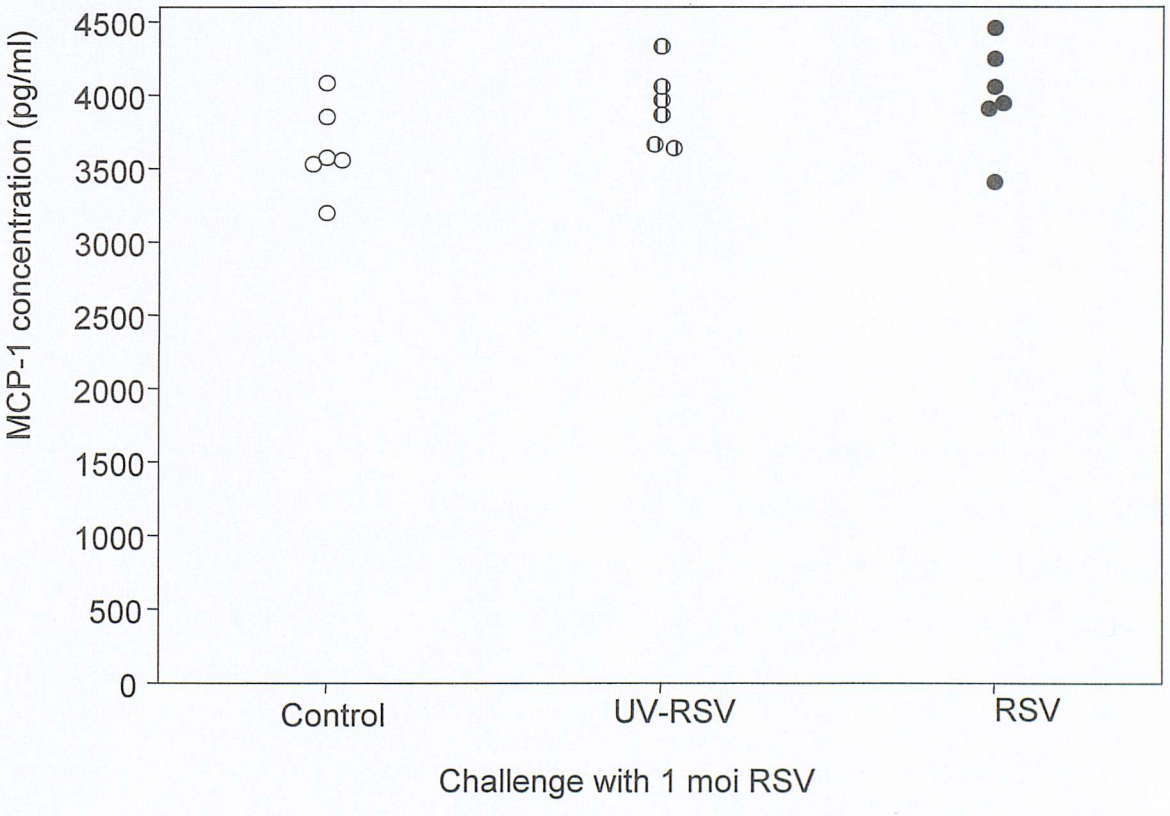
Figure 3.37

MCP mRNA production from macrophages challenged with RSV.

Lane order: ladder, control, RSV challenged, positive control.

Figure 3.38

MCP-1 production from macrophages 24 hours following RSV challenge



Control $\bar{X}$= 3628 pg/ml
UV-inactivated RSV $\bar{X}$= 3917 pg/ml
RSV Challenged $\bar{X}$= 3999 pg/ml
There was no significant difference in MCP-1 production from control cells, UV-RSV or live RSV.

3.3.8/9 Macrophage Inflammatory Protein (MIP-1α and MIP-1β)

The proteins MIP-1α/β are produced by a variety of cells, including macrophages and monocytes, see section 1.11.24. The results from the experiments that investigated the production of MIP-1α mRNA and protein from A549 cells demonstrate limited MIP-1α production. The 48-hour post-RSV challenge demonstrated the best response to RSV challenge (see figure 3.40). I was not able to demonstrate any significant MIP-1β production following RSV challenge.

Figure 3.39

MIP-1α mRNA production from A549 cells 24 hours following RSV challenge
Lane order: ladder, control, RSV challenge, and positive control.

The macrophage response to RSV challenge is difficult to establish, as under the culture conditions employed for macrophage propagation there is a high level of constitutional MIP-1α production (see figure 3.41). It was not possible to obtain useful PCR results to establish whether there is an increase in MIP-1α mRNA production as well, the smearing seen with the investigation of MIP-1α mRNA production from epithelial cells was worse.

Figure 3.40

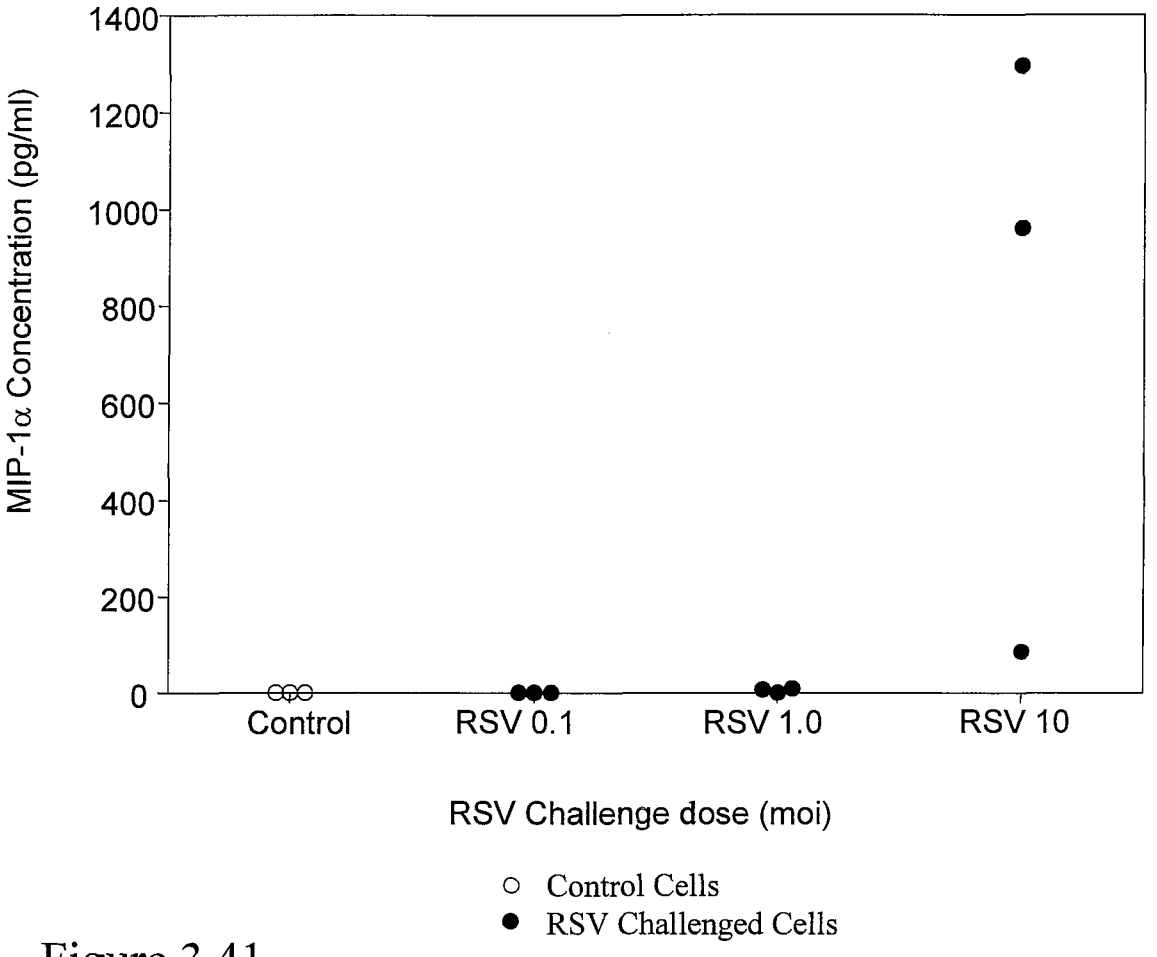
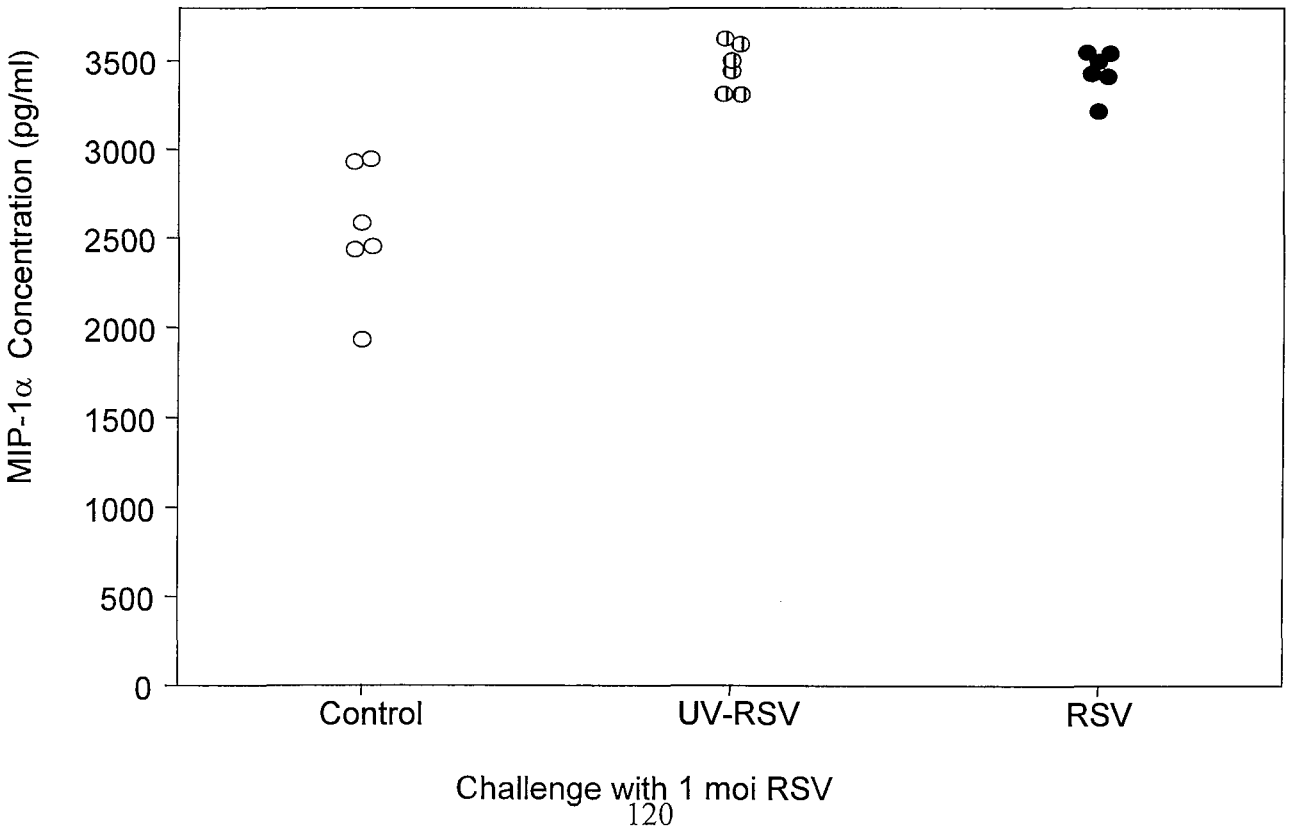


Figure 3.41



The results for MIP-1α protein production from macrophages demonstrate the following.

Control = 2546 pg/ml

UV-inactivated RSV = 3463 pg/ml,

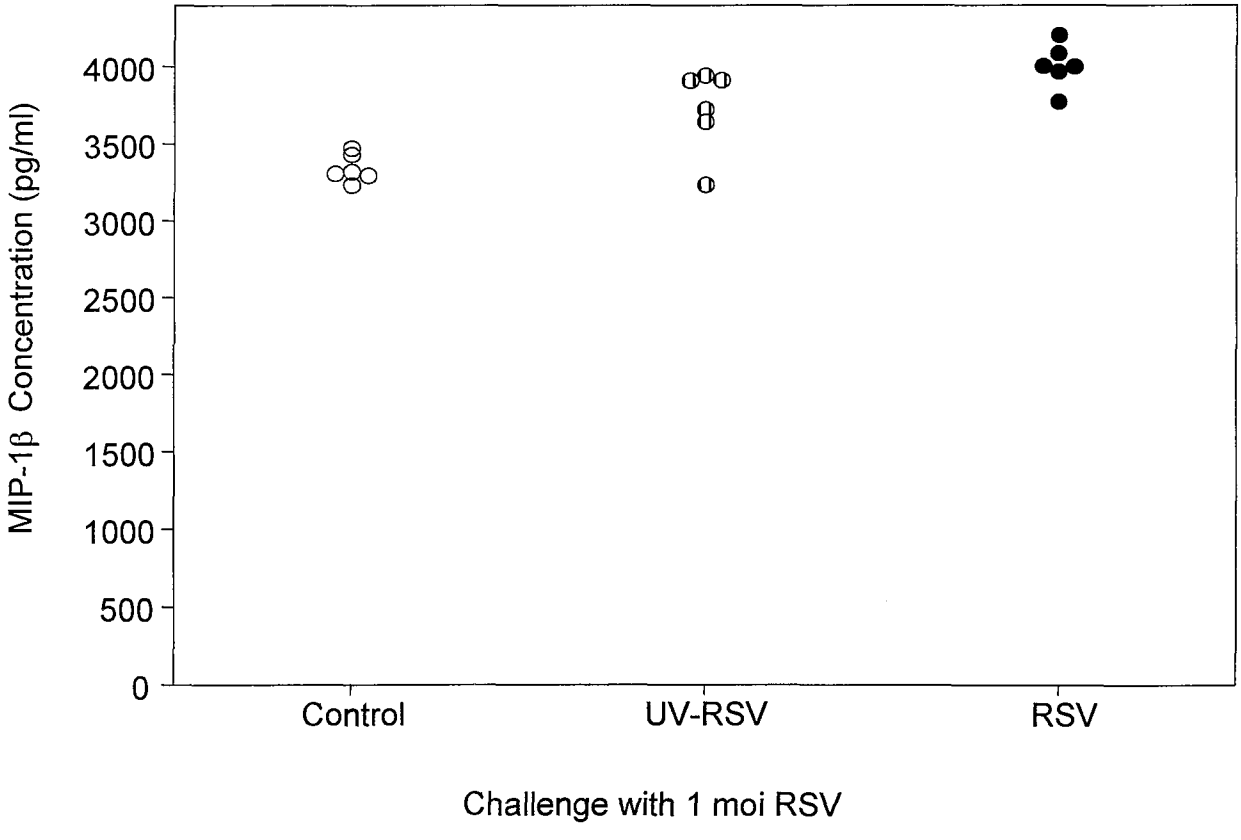
RSV Challenged = 3438 pg/ml

The production of MIP-1α from control cells was significantly lower than following UV-RSV or live RSV challenge ($p<0.05$), there was no significant difference between UV-RSV and live RSV.

The results for MIP-1β are similar to those for MIP-1α from macrophages following RSV challenge; I was unable to perform analysis for MIP-1β production from epithelial cells, due to technical reasons. The results for MIP-1β demonstrate that under the culture conditions employed there was constitutive production of MIP-1β from the control macrophages.

Figure 3.42

MIP-1β production from macrophages 6 hours following RSV challenge



The statistical analysis for the production of MIP-1β from macrophages challenged with RSV are:

Control = 3339 pg/ml

UV-inactivated RSV = 3724 pg/ml

RSV Challenged = 4002 pg/ml

The production of MIP-1β from control cells was significantly lower than following UV-RSV or live RSV challenge ($p<0.05$), there was no significant difference between UV-RSV and live RSV.

3.3.10 Eotaxin

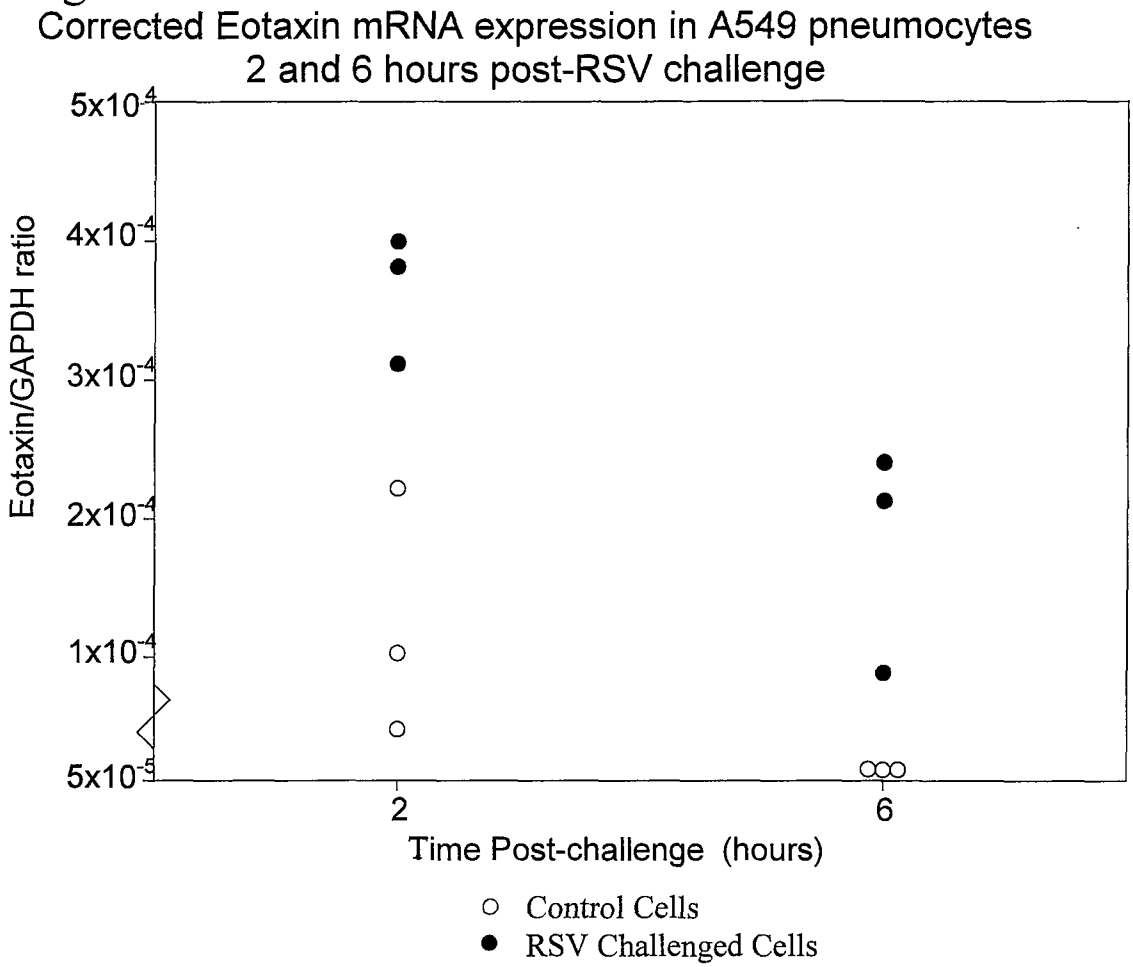
The CC chemokine eotaxin was also studied; it has specific chemoattractant properties and activation properties for eosinophils, see section 1.11.26. There was no evidence of eotaxin mRNA production by PCR from macrophages following RSV challenge, and this was not studied further. Eotaxin ELISA did not detect any protein production following RSV challenge of A549 pneumocytes, though when TAQMAN PCR was performed, there was evidence of eotaxin mRNA production (see figure 3.44), the PCR reaction products were run on an electrophoretic gel and the results are shown in figure 3.43. The eotaxin protein production following RSV challenge may have been below the threshold for the assay employed; the supernatant was assayed at the standard time points (every 24 hours from 24 to 144 hours).

Figure 3.43

Eotaxin production from A549 cells 4 hours following RSV challenge

Lane order: ladder, control, RSV challenged cells, positive control.

Figure 3.44



Control at 2 hours = 1.2×10^{-4}
RSV challenge at 2 hours = 3.6×10^{-4}
Control at 6 hours = 2.5×10^{-5}
RSV challenge at 6 hours = 1.8×10^{-4}
The corrected eotaxin mRNA ratio from RSV challenged pneumocytes was significantly higher than from control pneumocytes at both time points, $p<0.05$ at 2 hours post challenge and $p<0.01$ 6 hours post-RSV challenge.

3.4 Macrophage Production of Cytokines Associated With Lymphocyte Responses

The group of cytokines that are discussed in this section have the potential to be pivotal in skewing any ongoing immune response from airway-based cells. They could alter the milieu of the local environment that incoming naïve lymphocytes are exposed to, and thus may skew the subsequent cytokine response associated with these cells towards either a Th1 or Th2-type response.

There is little literature that supports the production of Th2-type cytokines by epithelial cells and this was initially investigated this by performing PCR for IL-12 IFN-γ and IL-10 on the A549 cell line samples and failed to elicit any evidence of mRNA production. The rest of this section will focus on the production of these critical cytokines by macrophages.

3.4.1 Interleukin-10 (IL-10)

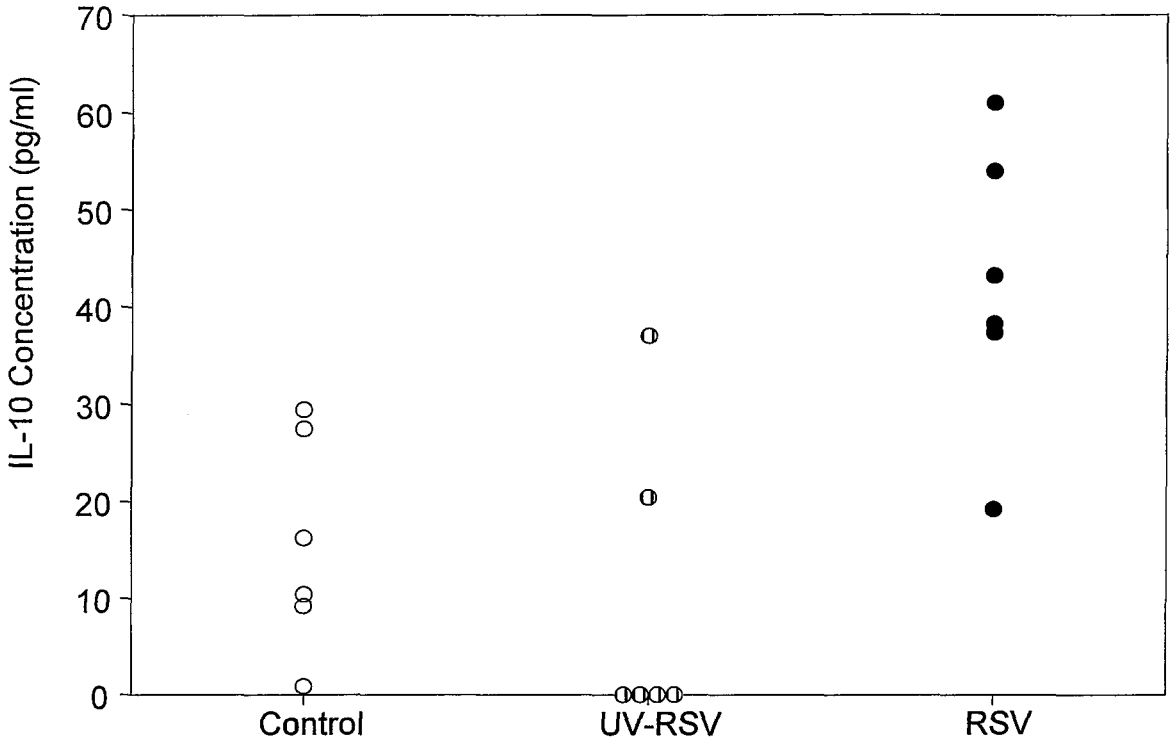
Though this cytokine was initially thought of as a Th2-type cytokine, more recent studies suggest that it has a role in regulating the inflammatory immune response, see section 1.20.5. There is an increase in IL-10 protein production following RSV challenge of MDM, the results from analysis of IL-10 mRNA production reveal that there is an increase in IL-10 mRNA following RSV challenge, but the increase is small see figure 3.41.

Figure 3.45

IL-10 mRNA production 24 hours post-RSV challenge of macrophages
Lane order: ladder, control, RVS challenge, positive control

Figure 3.46

IL-10 production from macrophages 24 hours following RSV challenge



Challenge with 1 moi RSV

○ Control Cells
● RSV Challenged Cells

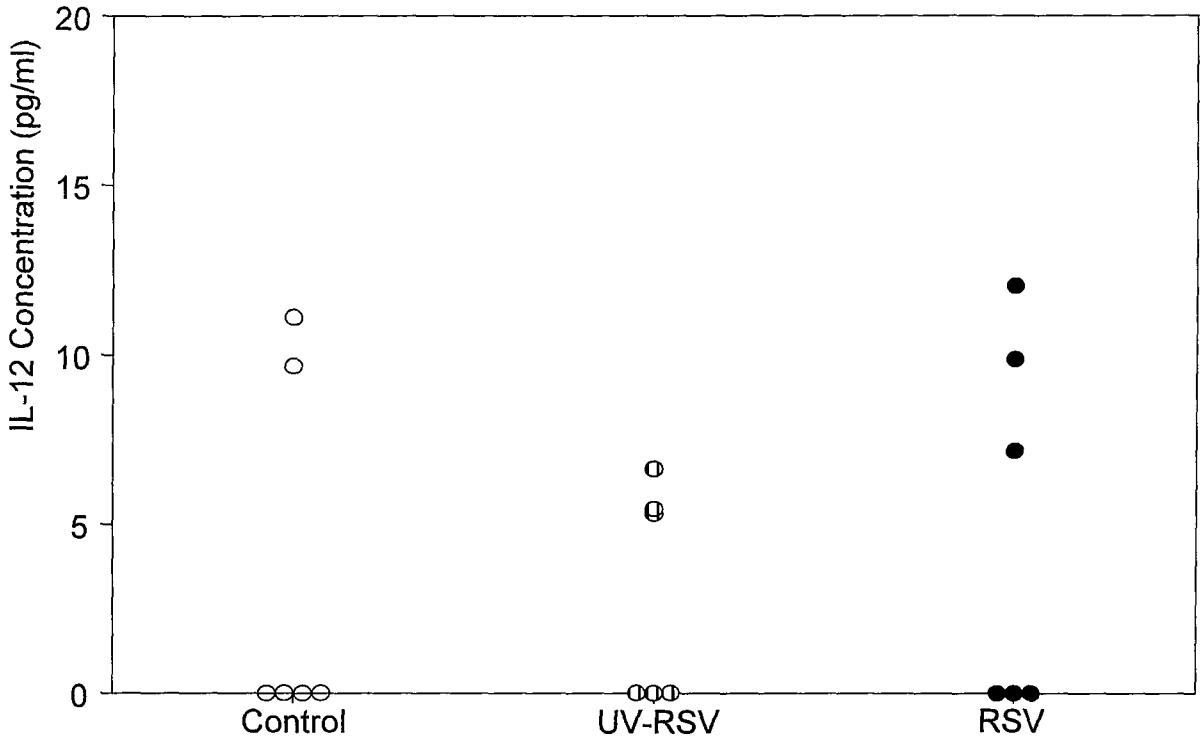
Control = 15.59 pg/ml
UV-inactivated RSV = 12.38 pg/ml
RSV Challenged = 42.17 pg/ml
There was no significant difference between control and UV-RSV challenge, but RSV challenge was significantly greater than either of these responses ($p<0.01$)

3.4.2 Interleukin 12 (IL-12)

Macrophages and monocytes are the main sources of IL-12; it is produced by macrophages in response to several stimuli, such as lipopolysaccharide (LPS), see section 1.20.6.

Figure 3.47

IL-12 production from macrophages 6 hours following RSV challenge



Challenge with 1 moi RSV

○ Control Cells
● RSV Challenged Cells

Control $\bar{X}$= 5.23 pg/ml
UV-inactivated RSV $\bar{X}$= 2.90 pg/ml
RSV Challenged $\bar{X}$= 4.85 pg/ml
There was no significant difference between any of the results

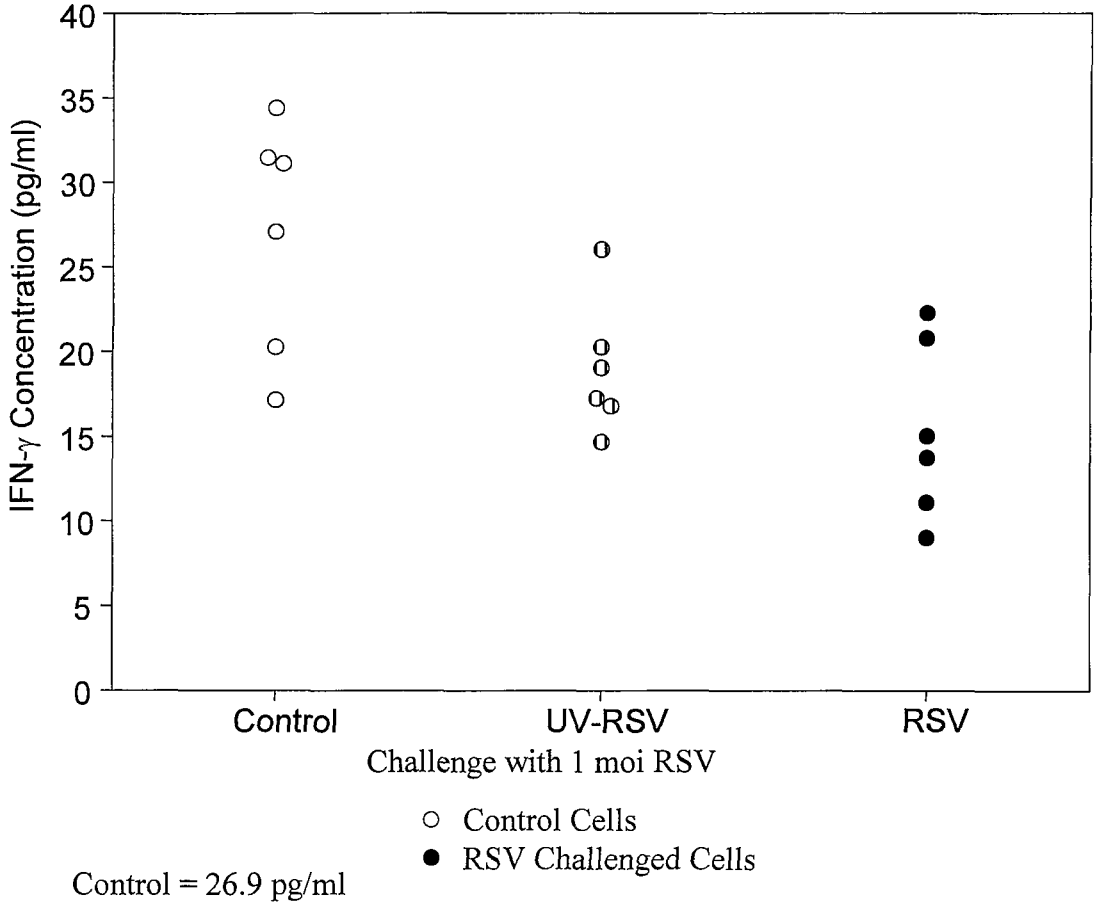
The results of IL-12 mRNA production reveal that there is no increase in IL-12 mRNA following RSV challenge. There was no increase in IL-12 protein production following RSV challenge; similar results were obtained at any of the time points studied.

3.4.3 Interferon –gamma (IFN-γ)

IFN-γ is both a Th1-type cytokine, but also has anti-viral properties; the cellular sources and actions of IFN-γ are discussed in section 1.20.10. The results for IFN-γ production following RSV challenge is different to that found with other cytokines, there is evidence that that there is a fall in IFN-γ production in cells that are challenged with RSV. The investigation of IFN-γ mRNA production did not reveal any IFN-γ mRNA production. The results are unusual and is a novel finding in this system.

Figure 3.48

IFN-γ production from macrophages 24 hours following RSV challenge



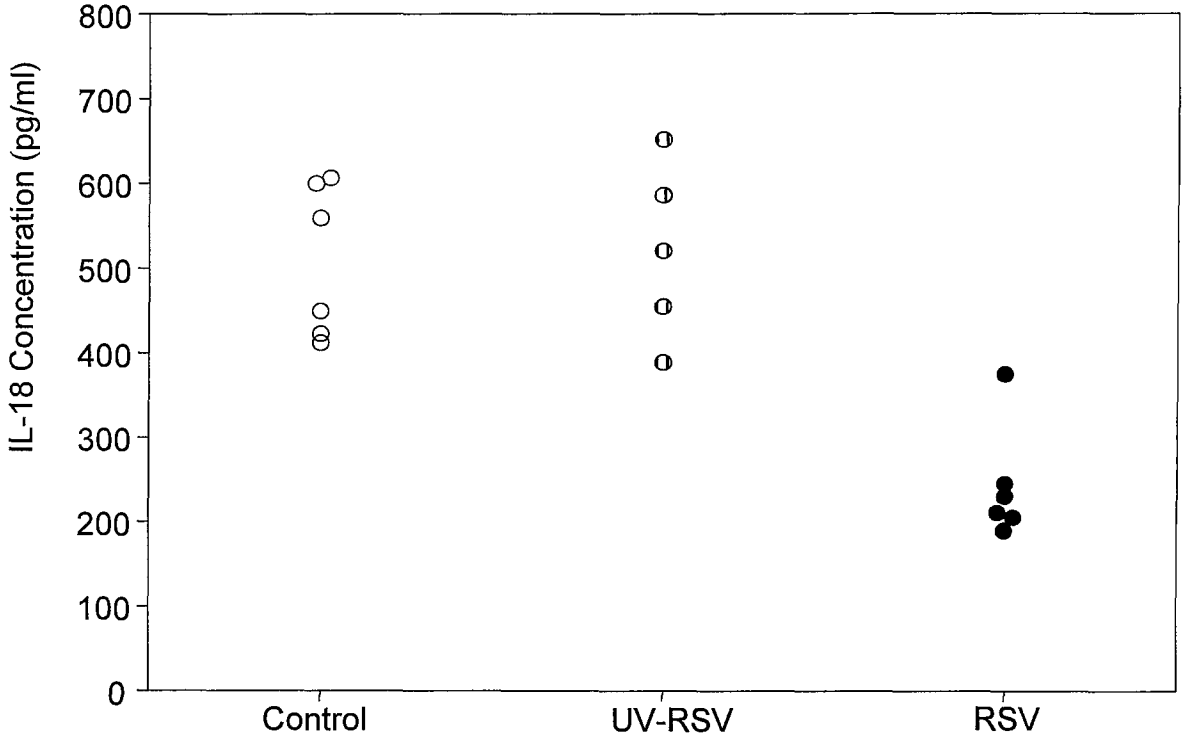
Control = 26.9 pg/ml
UV-inactivated RSV = 19.0 pg/ml
RSV Challenged = 16.01 pg/ml
There was a significant reduction in IFN-γ production by both UV-RSV and RSV challenged macrophages compared to control macrophages ($p<0.05$), but no significant difference between IFN-γ production in UV-RSV and RSV challenged macrophages.

3.4.4 Interleukin – 18 (IL-18)

IL-18 is a recently described Th1-type cytokine; it was originally designated IFN-γ releasing factor (IGRF) as its actions are to stimulate the production of IFN-γ, for a complete outline of IL-18 sources and actions see section 1.20.7. There are no previous reports of IL-18 being studied in the context of RSV infection.

Figure 3.49

IL-18 production from macrophages 24 hours following RSV challenge



Challenge with 1 moi RSV

○ Control Cells
● RSV Challenged Cells

Control = 508 pg/ml
UV-inactivated RSV = 536 pg/ml
RSV Challenged = 253 pg/ml
There was no significant difference between control and UV-inactivated RSV challenged macrophages, though there was a significant difference between these two responses and RSV challenged macrophages ($p<0.05$).

The results for IL-18 show a similar trend as those for IFN-γ, there was suppression of IL-18/IFN-γ with RSV challenge. The study of IL-18 mRNA production from macrophages reveals that there is constitutive IL-18 mRNA production and that the mRNA production mirrors the protein production, with a slight fall following RSV challenge, see figure 3.46.

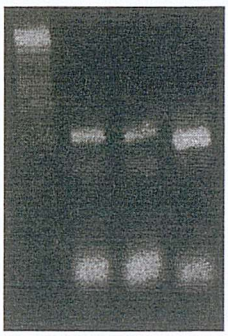


Figure 3.50

IL-18 mRNA production 24 hours post-RSV challenge of macrophages.
Lane order: ladder, control, RSV challenged, positive control.

3.5 The Alteration of Macrophage Cytokine Response

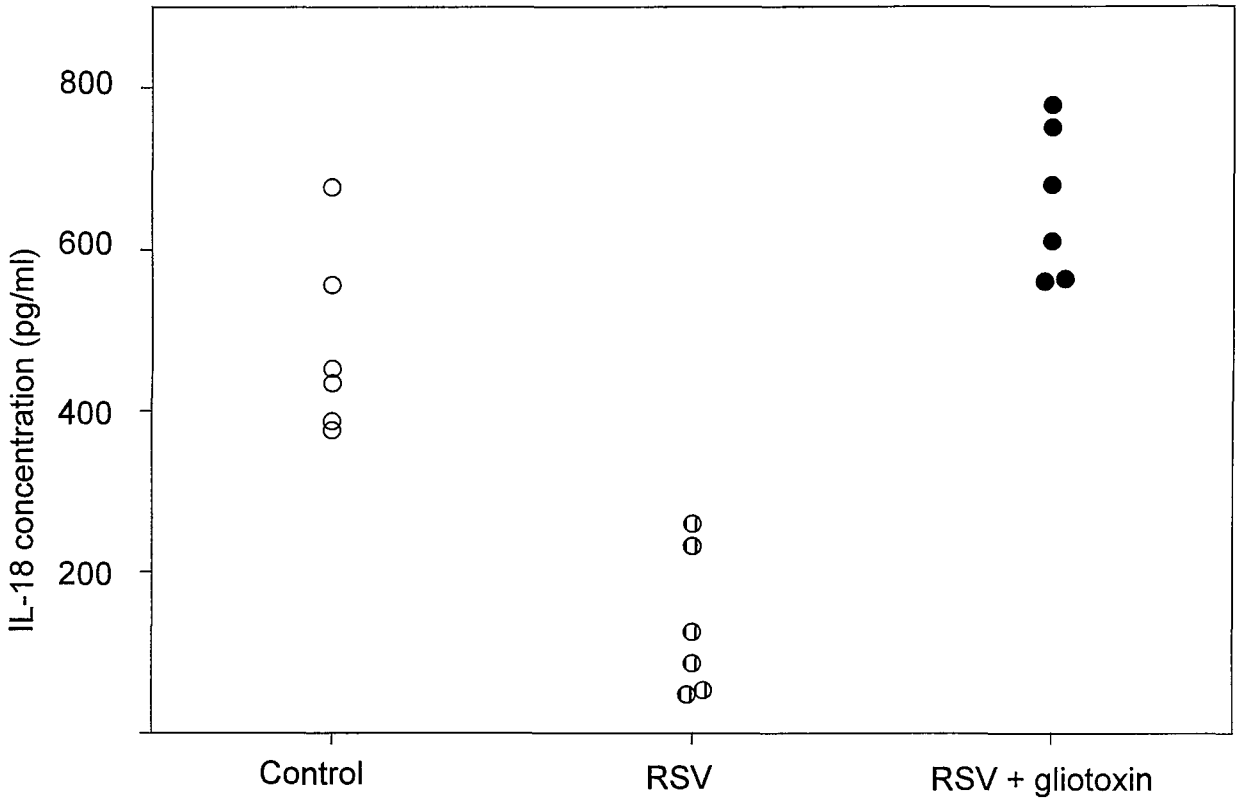
Methyl αD manopyranoside (αMM) was added to the medium 1 hour prior to the addition of RSV, to investigate whether RSV entry and replication in macrophages relied on entry through the mannose receptor. There was no alteration in any cytokine or chemokine response when αMM was added to the experiment. This data indicates that RSV does not use the mannose receptor for macrophage entry.

Stausporine and wortmanin did not alter the production of any of the cytokines/chemokines produced following RSV challenge, indicating that protein kinase C and the PI-3 kinase complex do not play a part in the induction of cytokine responses following RSV challenge in macrophages.

The inhibitors herbamycin and gliotoxin both altered cytokine production following RSV challenge in macrophages. These two inhibitors only altered the IL-18 response following RSV challenge, see figure 3.51 and 3.52.

Figure 3.51

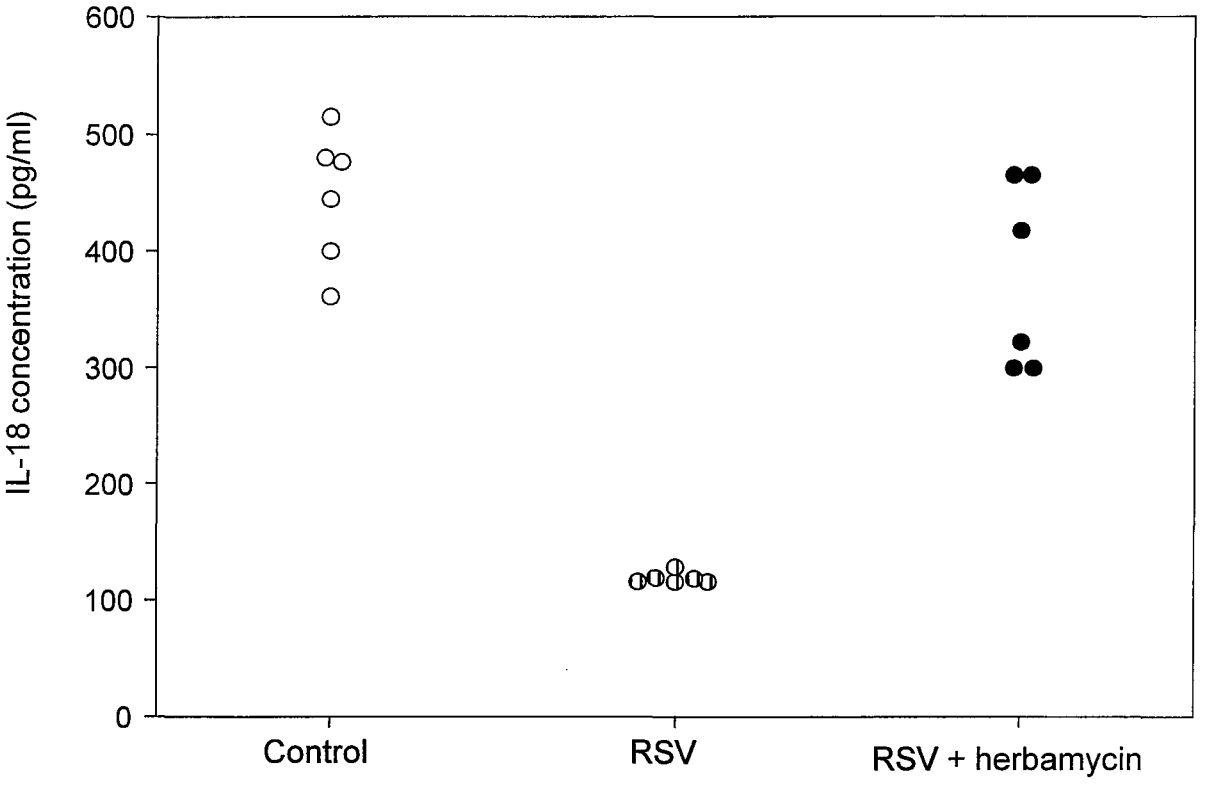
IL-18 Production 24 hours following RSV challenge and the addition of gliotoxin



The results for the response to both herbamycin and gliotoxin are similar; they reverse the reduction in IL-18 protein production from macrophages that was seen with the addition of RSV.

Figure 3.52

IL-18 Production 24 hours following RSV challenge and the addition of herbamvcin



Chapter 4 - Community-based Study of cytokine responses during the early stages of RSV Infection

4.1 Study Outline

91 subjects were initially recruited for this study; they each had at least one atopic asthmatic parent. 88 subjects were followed through the whole RSV season. 17 subjects had no evidence of respiratory tract infection during the study period and were not studied any further. Of the remaining 71 subjects that suffered one or more respiratory tract infection during the study period, 43 had no evidence of RSV infection by EIA on the initial nasal aspirate performed during infection. 19 children developed RSV infection localised to the upper respiratory tract, while 9 children developed RSV-related RSV bronchiolitis during the study period.

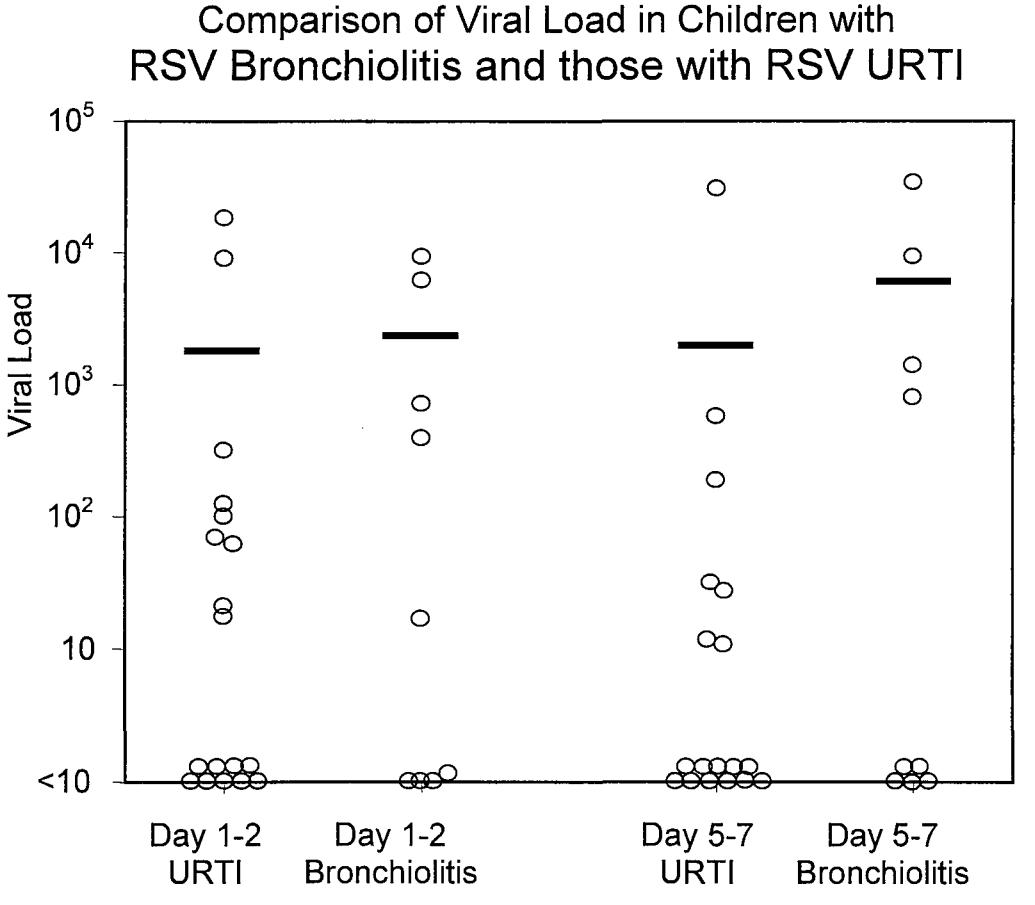
The subject characteristics of each group are outlined in table 4.1.

	Whole group (n=28)	Acute Bronchiolitis (n=9)	Upper respiratory tract infection (n=19)	p value (bronchiolitis vs URTI)
Male Female	15 (54%) 13 (46%)	3 (33%) 6 (66%)	12 (63%) 7 (37%)	0.228
Mean age at RSV infection (days)	195 (116)	147 (84)	219 (125)	0.132
One atopic parent Both parents	16 12	5 4	11 8	1.00
Breast feed>3 months	15 (54%)	5 (56%)	10 (53%)	0.604
Mean gestational age (weeks)	39.54 (1.0)	39.15 (1.2)	39.37 (0.9)	0.324
Mean birth weight (kg)	3.52 (0.6)	3.55 (0.77)	3.5 (0.52)	0.872

4.2 Viral Load

The initial analysis performed on the nasal aspirate samples taken from these children was to establish the viral load in each specimen. The TAQMAN quantitative PCR system was used to quantify the viral load. The results from this analysis showed that there was a marked variability in the viral load (10 – 34000) from the samples (see figure 4.1). There was no significant difference between the loads in children with RSV URTI (1472±1049 at first time point, and 1637±1590 at second) and children with RSV bronchiolitis (1889±1139 at the first and 5089±3769 at the second) at either time point, even though there was a trend for an increase in the mean viral load of samples from children with bronchiolitis between the first and second analysis, while this trend was not present in the children with URTI only.

Figure 4.1

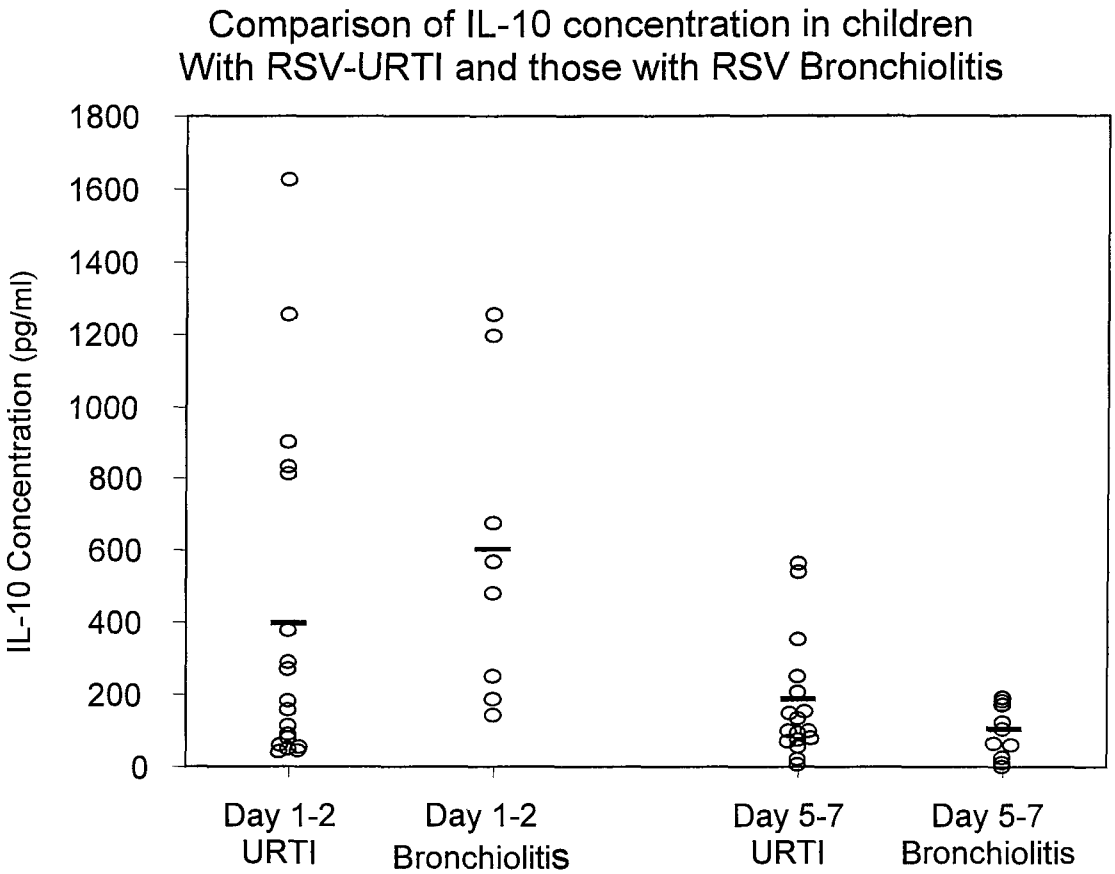


4.3 Cytokine Response

Analyses were performed for selected cytokines produced predominantly by lymphocytes (interleukin 4 (IL-4) and interferon gamma (IFN-γ)) and immune response produced by cells of the macrophage/monocyte lineage (IL-10 and IL-12). The hypothesis being tested was that children that developed RSV bronchiolitis produced a larger Th2 cytokine response, when compared to the children that developed a URTI only, and the Th2 response led to the wheezing illness, the higher incidence of post-bronchiolitis wheeze. If lymphocytes are driving any change in the cytokine balance, then any changes should not be observed until lymphocytes have been recruited to the site of infection, therefore the later day 5-7 samples should show a difference in the cytokine ratios, while the earlier sampling point should have similar ratios.

4.3.1 Macrophage associated cytokines (IL-10 and IL-12)

Figure 4.2

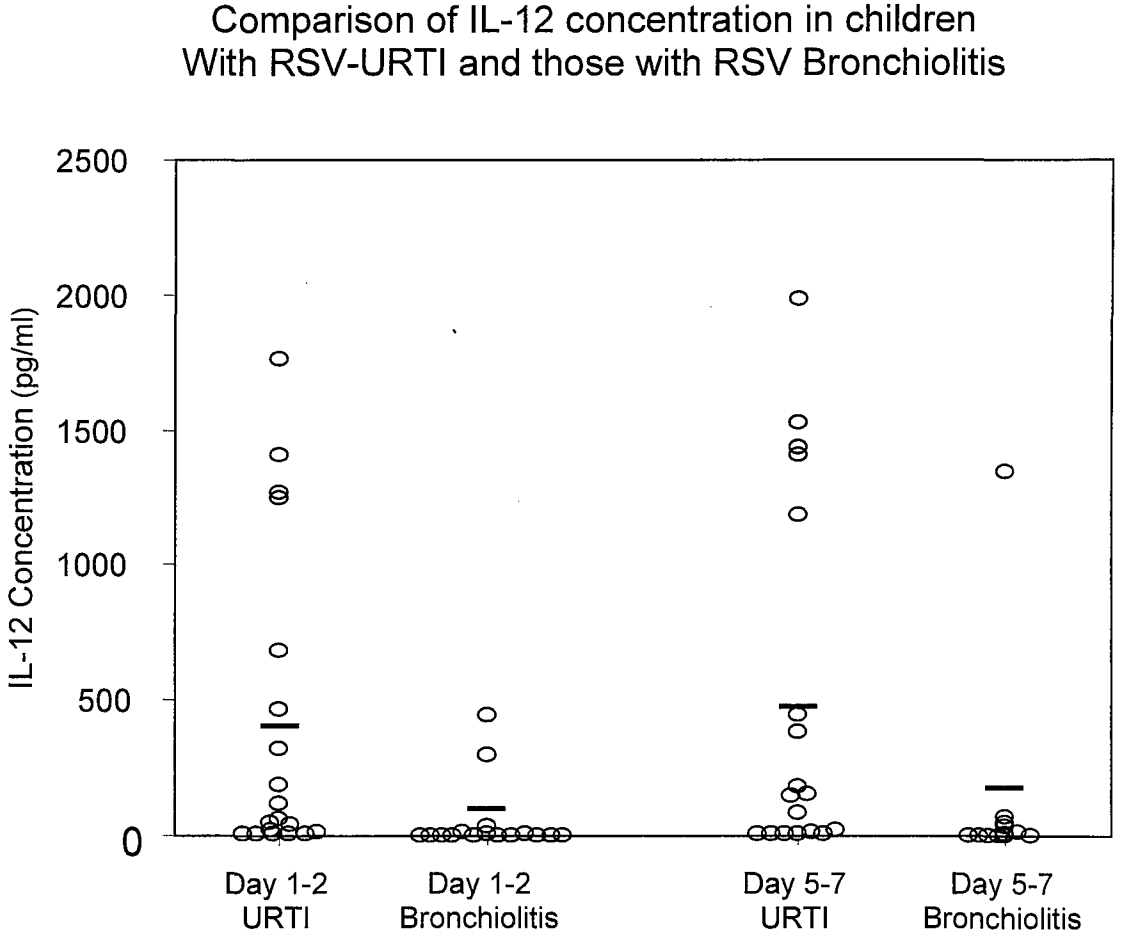


There was no significant difference between the URTI and bronchiolitis samples at either time-point. There was no significant difference between the concentration of IL-10 obtained in the children with URTI between the first and second time points (394pg/ml ± 107 vs. 188pg/ml ±42). There was a significant difference between the concentrations of IL-10 obtained between the two points for children with viral bronchiolitis, 602pg/ml ± 135 vs. 103pg/ml ± 23 ($p<0.01$).

The macrophage-derived cytokines, IL-10 and IL-12 show different production patterns. The Th2 cytokine IL-10 production by both groups of children was higher in the first

sample (day 1-2) than in the second sample (day 5-7) (see Figure 4.2). Interestingly the rate of fall was greater in the children, who develop bronchiolitis, and these children had higher IL-10 concentrations in the first sample period. This finding is surprising as it goes against the hypothesis proposed. The implication is that the skewing in cytokine response may occur during the very early stages of RSV infection and the macrophage/monocyte lineage may play a significant role in this response. If the response is seen in the early stages of infection, it also suggests that host factors are important in the subsequent response.

Figure 4.3

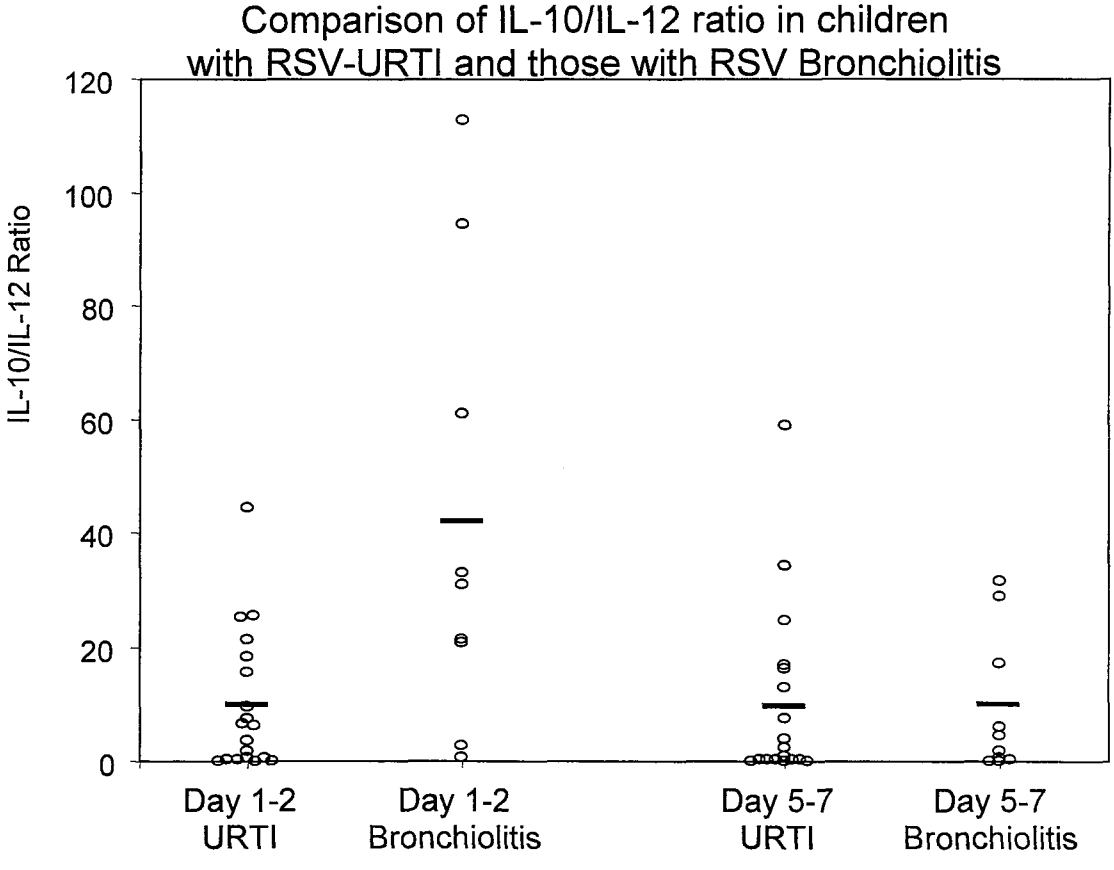


There is no significant difference between the IL-12 concentrations in either group at either of the two time points (Day 1-2 URTI 404±132 vs. Bronchiolitis 94±54, Day 5-7

URTI 473±152 vs., Bronchiolitis 171±147), also there is no significant difference between the concentration of IL-12 at the first time point in either group, nor at the second time point. The data does show that fewer children who developed RSV bronchiolitis mounted an IL-12 response, compared to the children who developed RSV URTI.

The results for IL-12 production show an opposite trend when compared to the results for IL-10 production for children with viral bronchiolitis and URTI. The mean IL-12 concentration from children with viral bronchiolitis was lower than that from children with URTI. There was a slight increase in IL-12 concentration over time, for both groups of children. The majority of children with RSV bronchiolitis had no IL-12 response at either time point, with only one child having significant IL-12 production at the later time point.

Figure 4.4



When the results are viewed as a ratio of IL-10 to IL-12, there are significant differences between the ratios at day 1-2 of the infection, with the IL-10/IL-12 ratio being greater in children with viral bronchiolitis at the first time point, 42 ± 13 vs. 10 ± 2.8 ($p<0.001$). There was no significant time related change in the IL-10/IL-12 ratio in children who developed upper respiratory tract infection only, but there was a significant fall in the IL-10/IL-12 ratio for those children who developed viral bronchiolitis, 42 ± 13 vs. 10 ± 4 ($p<0.05$).

4.3.2 Lymphocyte associated cytokines (IL-4 and IFN-γ)

The data for the Th2 cytokine IL-4 shows a similar response to the IL-10 response seen. The initial IL-4 concentrations (URTI 0.89 ± 0.13 vs. Bronchiolitis 1.54 ± 0.49) are greater than the concentrations at the second time point, and the children with bronchiolitis have higher concentrations of IL-4 than those children that only developed RSV upper respiratory tract infection. Though there was a greater concentration of IL-4 at the initial time point this did not reach statistical significance, but by day 5-7 the differences did became significant, URTI 1.17 ± 0.17 vs. bronchiolitis 0.63 ± 0.11 ($p<0.05$).

Figure 4.5

Comparison of IL-4 concentration in children
With RSV-URTI and those with RSV Bronchiolitis

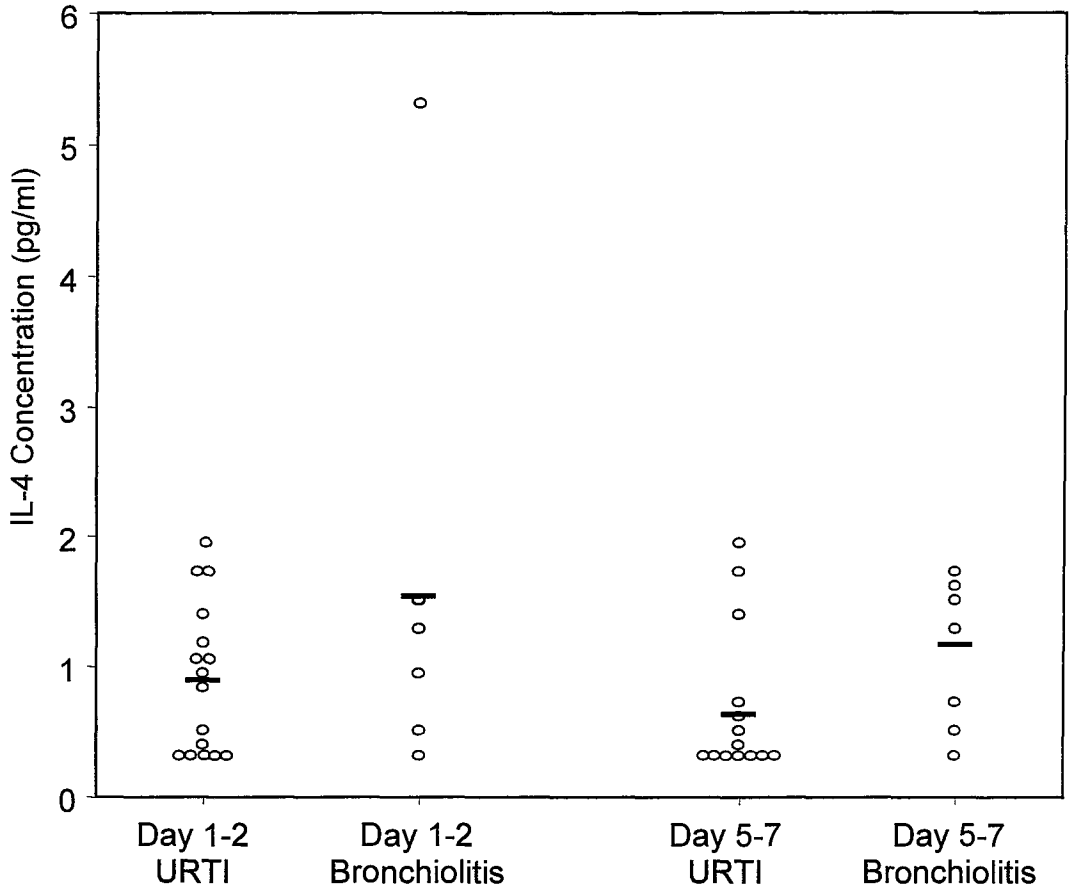
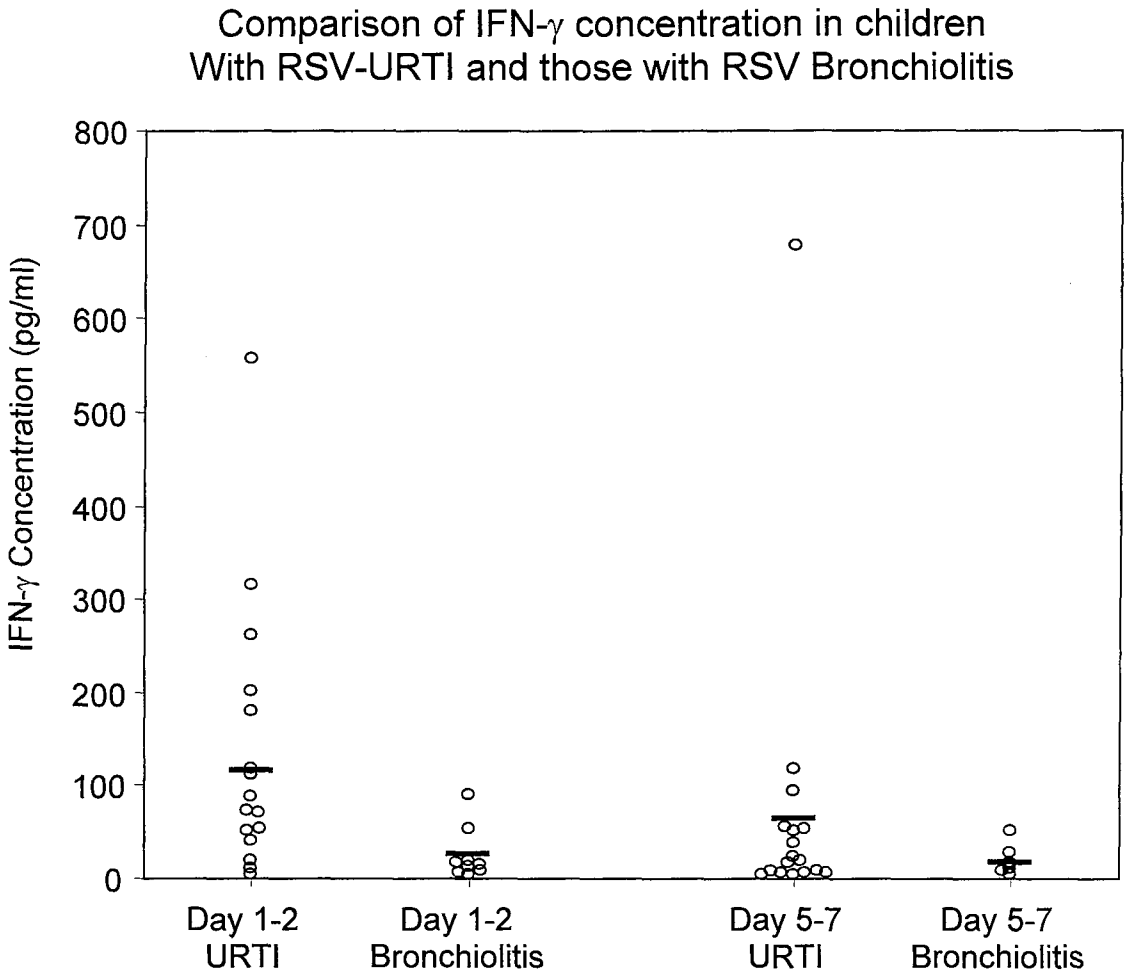


Figure 4.6

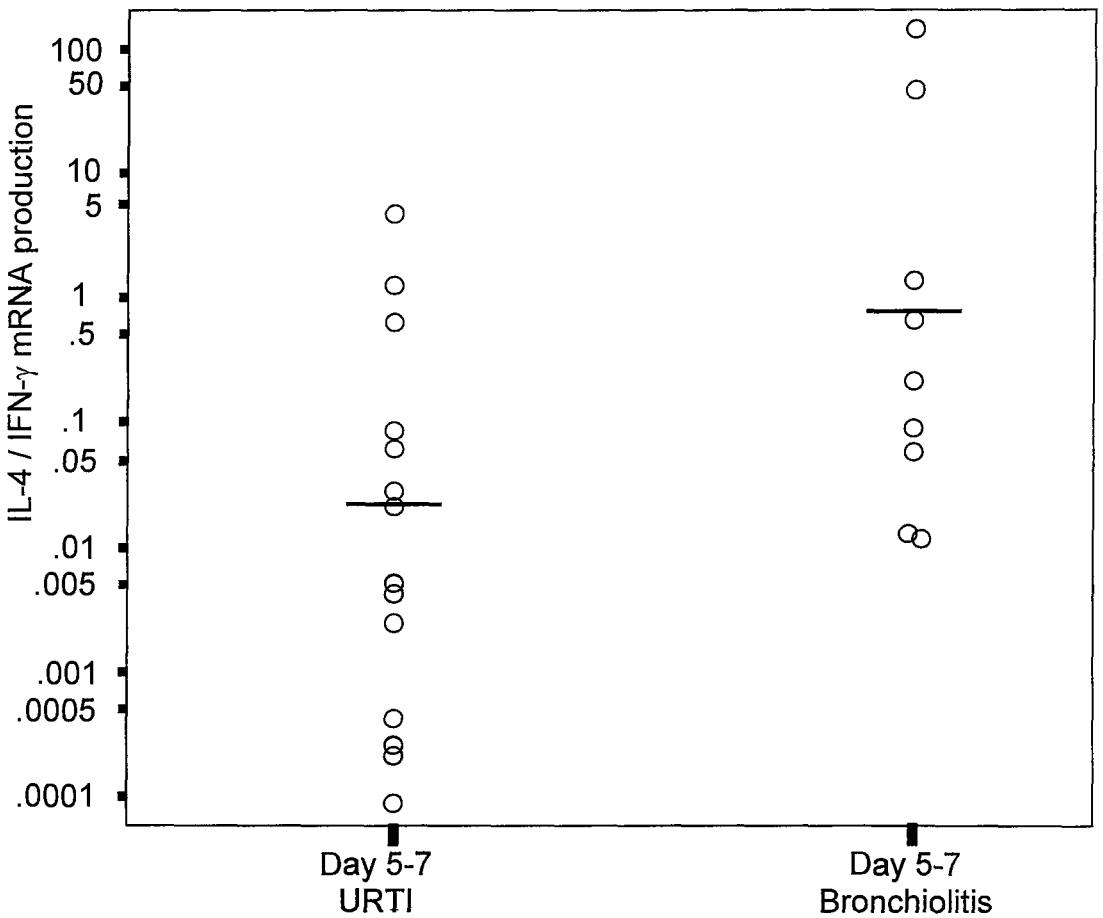


Interestingly children who develop viral bronchiolitis produced significantly less IFN-γ at the first time point compared to children with infection confined to the upper respiratory tract 11.6 ± 3.2 vs. 2.5 ± 0.9 ($p<0.05$). In both groups the IFN-γ concentrations had fallen by day 5-7, and there was no statistical difference between the two groups at this time point (URTI 6.35 ± 3.5 and bronchiolitis 1.6 ± 0.5, see figure 4.6). The initial, higher IFN-γ production by children with URTI may protect them from developing more extensive RSV infection.

For the final time point the children had blood taken and the peripheral blood mononuclear cells (PBMCs) were extracted and stimulated with PHA and the IL-4, IFN-γ, IL-18 and GAPDH mRNA responses were assayed. This was performed to ascertain whether there was a correlation between airway cytokine responses and peripheral blood responses.

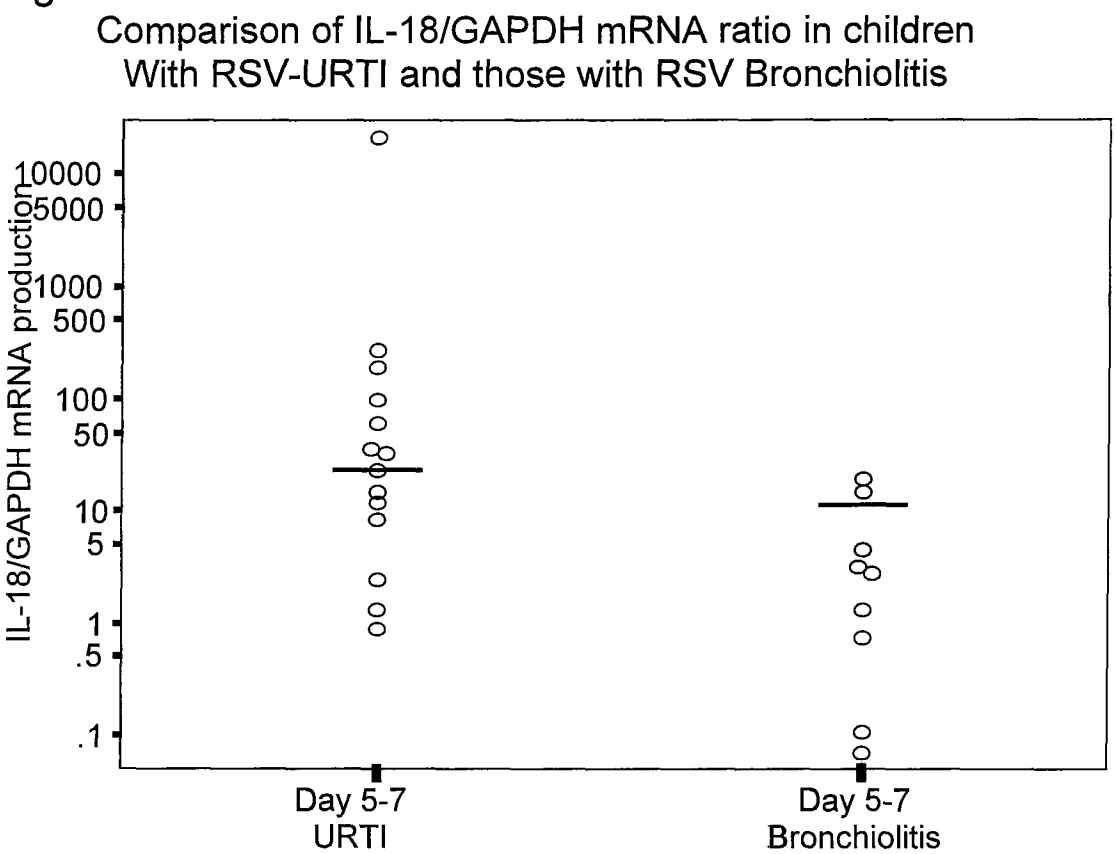
Figure 4.7

Comparison of IL-4/IFN-γ mRNA ratio in children With RSV-URTI and those with RSV Bronchiolitis



The results from the cytokine mRNA ratios demonstrate that there is a significantly higher IL-4/IFN-γ ratio in those children who went onto develop RSV bronchiolitis, compared to those who only had RSV URTI (p=0.01).

Figure 4.8



These results demonstrate that there is a significant reduction in the corrected IL-18 mRNA produced by LPS stimulated PBMC from children with RSV bronchiolitis, when compared to children with RSV URTI (p<0.05).

Chapter 5 – Discussion

5.1 In-vitro Studies of RSV Infection

5.1.1 Evidence of infection

The work presented in this study used the human type II pneumocyte cell line A549 so that comparisons with previous studies could be made. The results will be discussed in order that the results were presented.

Epithelial experimentation

The method employed to demonstrate infection and viral replication was originally established over thirty years ago (301). The use of commercial diagnostic reagents that are normally used to confirm the diagnosis of RSV infection by staining of nasopharyngeal secretions eliminated the risk of cross reactivity and false positive results.

There have been few studies that have tried to quantify the viral load during epithelial cell infection. The method that was employed, using the quantitative PCR technique is novel and its use has not been described in the literature before. The established method to quantify viral load is the plaque assay (159,302,303), this relies on the observation of the cytopathic effect of RSV on epithelial cell monolayers. The process involves using serial dilutions of virus and observing the number of cytopathic events to quantify the viral load, assuming one cytopathic event is due to a single virus.

The results confirm there is evidence of viral replication in epithelial cells (see figure 3.3b) and an increase in viral load was observed over the time course of the experiment (see figure 3.1). There was also a correlation between the initial infecting viral dose and subsequent viral load (see figure 3.2).

The technique employed measured viral load, but also any viral mRNA produced during infection of the cell. This could mean that the figures obtained are actually an overestimate

of the viral load. Alternative methods that could have been employed to more accurately measure the viral load include: trying to measure RSV gene copies only, this would involve performing PCRs employing primers that crossed two adjacent genes, such as the F and G gene; or comparing the viral load as estimated by assessing both F and G copy numbers. The latter technique relies on the fall in the copy numbers for each gene the further 5' it is (19), thus if the viral loads calculated by using PCRs for either F or G protein were the same, then either TAQMAN PCR could be used to quantitate the viral load. The initial technique described, using PCR primers that spanned the 5' end of the G gene and the 3' end of the F gene would only amplify up genomic RSV. The results for all three methods were comparable, validating the results for the method discussed.

This study is novel in that this is the first study that has used the TAQMAN PCR method to try to quantify the viral load of infected cells. This method could be used in the future to accurately analyse the effect of inhibitors of viral adherence, viral replication or attenuators of virulence that could alter viral load. This method provides more accurate results than using the more traditional plaque assay method and would also be quicker than the plaque assay method. The advantages of this method are: speed of analysis and the elimination of the requirement for extensive cell culture facilities. The disadvantages of this method are: the need for presence of significant molecular biology apparatus and significant cloning facilities to establish standard gene quantities, though these could be obtained commercially. This methodology would lend itself to quantitation of a large number of samples and could be used for large scale processing and analysis. The process was used for the quantitation of viral load for the community study of RSV infection; this is discussed later.

Macrophage experimentation

The technical considerations of using both the direct immunofluorescence and TAQMAN PCR are the same for investigation of macrophage infection. The results obtained are similar to those obtained in previous studies, as discussed in chapter 1 (section 1.10.2).

There is evidence for the susceptibility of macrophages to infection by RSV, but macrophage responses to RSV infection are not homogeneous. Panuska has demonstrated that RSV can infect macrophages, while Midulla has demonstrated that macrophage populations cannot be considered to be similar and have differential susceptibility to infection by RSV.

The ideal macrophage population to use for this series of experiments would have been the alveolar macrophages, to emulate the natural local host response to RSV infection in the airways. There where technical restrictions that made this model unfeasible, the number of replicates and time points that were required would not have been possible, as the quantity of cells required for the experiments to be performed would have been unobtainable from performing bronchoalveolar lavages on individuals.

The use of monocytes in culture, that take on a macrophage phenotype has been employed by other researchers in previous studies (see section 1.10.2), and has been shown to be a valid model for macrophage infection response.

The results demonstrate evidence of infection of the monocyte derived macrophages, the viral replication does seem to be limited, figure 3.4 indicates that there was little increase in the RSV load between the 6 and 24 hours after RSV infection. The surface staining with fluorescently labelled antibodies indicates that there was production and expression on the surface of host cells of RSV surface proteins (see figure 3.5).

The experiments presented here confirm that limited RSV replication does occur in peripheral blood-derived macrophages. These experiments confirm the results of previous studies but by using new methodologies there is an indication of the limited viral replication in this type of macrophage.

Anti-viral Product Release

There are certain cytokines, other than TNF-α, that are released from the epithelium and macrophages that can have the effect of reducing subsequent viral replication, thus protecting other host cells from the effect of the infecting virus; this includes IFN-α, nitric oxide and also β-defensins. Though these mediators do not form a recognised grouping they will be discussed in the following section.

5.1.2 IFN-α

There was no evidence of IFN-α production from epithelial cells, and there are no previous reports of RSV infection of epithelial cells leading to the production of IFN-α. There was significant production of IFN-α from macrophages following RSV infection, the production of this cytokine requires replicating RSV (see figure 3.6). There was relatively low-level production of INF-α and no subsequent experiments were performed to establish the significance of this level of IFN-α production on RSV replication, by studying the viral load in relation to IFN-α production.

An observation that was made early in RSV research was that the relative production of interferon, including IFN-α was low following RSV infection of macrophages, when compared to the production following other viruses, such as influenza virus (see section 1.11.17). The concentration of IFN-α produced in this study was greater than previous studies that have investigated IFN-α production by macrophages, this may be due to the known phenomena of increasing IFN-α production from macrophages the longer their duration of culture, increasing culture from one day to seven has been shown to lead to a greater than 200 fold rise in IFN-α production (161).

5.1.3 Nitric Oxide

There was evidence of nitric oxide production from epithelial cells following RSV infection, there was evidence of a relationship between the initial infecting viral dose and subsequent NO production. The production of NO occurred very late in the time course of the infection experiment (see figure 3.7), this suggests that NO production in this cell system relies on the initial production of mediators from epithelial cells that then go and stimulate induction of iNOS and subsequent production of NO from the epithelial cells. Unfortunately there was no confirmatory evidence of an increase in iNOS mRNA production occurring; if there had been evidence of an increase in iNOS mRNA occurring, the timing of this up-regulation of iNOS mRNA could confirm the proposed hypothesis. Other experiments that could confirm the hypothesis would involve the addition of cytokines that have been shown to be produced following epithelial RSV infection, and observe if there was an increase in iNOS mRNA or NO production.

There has been a recent study that has highlighted the increased production of iNOS (inducible nitric oxide synthase) following RSV infection of epithelial cells (86). Further investigations demonstrated that IL-1β, TNF-α and IFN-γ added to infected cells increase iNOS expression, but the addition of these cytokines only increased NO production in infected cells. This finding suggests that the initial infection of the epithelial cells is the permissive factor that allows regulation of iNOS production by cytokines. The addition of neutralising antibodies to the above cytokines did not reduce the iNOS induction. This study demonstrated a rapid induction of iNOS, within 7 hours of infection of cells, thus it would be unusual for there to be such a delay between mRNA production and subsequent protein production. The assay employed may have been relatively insensitive at detecting nitrate production; hence the surrogate of NO production was only observed at the last time point and with the highest infecting viral dose employed, this is not supported by the results of the macrophage production of NO, where the assay was able to measure down to less than 1μg/ml. Recently Kao was bale to demonstrate that NO production from epithelial cells can be reduced by IL-4 or dexamethasone, but is not increased by IFN-γ alone (304).

There are no previous studies of NO production from macrophages following RSV infection. This study demonstrates that there is significant production of NO from infected macrophages and that the production of NO occurs relatively late in the infection process (see figure 3.7). By the time point studied most cytokines had reached maximal production, and as there was no evidence of earlier NO production this suggests that the mechanism of NO production may be similar to that proposed for NO production from epithelial cells, that it may require the earlier production of pro-inflammatory cytokines to regulate the production of NO from infected cells. The nitrate production from macrophages is small when compared to the levels produced by epithelial cells. These results suggest that NO production may play an important role in the pathophysiology of RSV infection and that the epithelial derived NO is less important. From subsequent experiments there was no evidence that NO had any direct anti-viral activity, as inhibition of NO production by MEG did not alter viral load, though it did reduce nitrate levels.

5.1.4 Human β-defensin (hBD)

The experimental data for hBD-2 is only in the form of mRNA data, thus any interpretation relies on the assumption that the increase in mRNA is translated into an increase in protein production. The results from the experimental infection of A549 cells demonstrate that there is a sustained increase in hBD-2 mRNA throughout the time course of the experiment (see figure 3.9). These results are in direct contrast to the results obtained for the production of eotaxin mRNA (see figure 3.43); the results from that experiment demonstrate a rapid increase in eotaxin mRNA, followed by a rapid fall, within a few hours of the initial infection. If the induction of hBD-2 mRNA relied on similar pathways, a similar pattern of induction would be seen and the whole of the time course would not have demonstrated any increase in hBD-2 mRNA, as it would have fallen back to baseline by the 24hour assay point.

A possible hypothesis that would be consistent with the results obtained would be that hBD-2 mRNA induction is not directly due to RSV infection of the A549 cells, but a

similar mechanism to that suggested for nitric oxide; the initial rapid cytokine production from cells may stimulate cells into inducing hBD-2 mRNA production. The rapid and sustained production of pro-inflammatory cytokines may be the trigger for the sustained production of hBD-2 mRNA.

This is the first study to investigate the induction of defensin mRNA following respiratory viral infection. The importance of defensins in airway host defence has been demonstrated by observation of the loss of defensin action due to high salt content in cystic fibrosis patient airways(305). The results for NO production, hBD-2 mRNA production and IFN-α production demonstrate that respiratory epithelium can play a significant role in restricting RSV infection and potentially restricting secondary bacterial infection in infants with RSV bronchiolitis.

Though this series of experiments help to demonstrate the importance of the innate immune system in the pathogenesis of RSV infection in the lower airways, there are other studies that have recently investigated the role of other areas of the innate immune system. There have been recent investigations into the alteration of surfactant production following infection (306) and also the role of parts of the innate immune system in the pathogenesis of infection (307). Clinical studies have begun to define the role of pulmonary surfactant in the pathogenesis of RSV infection (306). Animal studies of RSV infection have identified abnormalities in surfactant function occur following experimental infection (308). There has been a recent study that has indicated that pulmonary surfactant protein A (SP-A) binds to the G surface protein of RSV, and this is via binding of the carbohydrate recognition binding domain and the carbohydrate moieties on the RSV G protein. There is a correlation between SP-A levels and the level of epithelial cell infection, as the cells take up the bound RSV (309). The results obtained during this period of study were not able to confirm this, see section 3.5. Experiments were conducted with the addition of αMM to try to block cellular surface receptors and reduce RSV binding to the macrophage cell surface, no alteration in viral load was demonstrated. In the experiments performed by Hickling the addition of mamman was able to reduce binding between SP-A and RSV (309).

5.1.5 Interleukin –1

Previous studies of RSV infection of epithelial cells have been able to demonstrate an increase in IL-1α following RSV infection. Previous studies have investigated the role of IL-1α as a stimulator of further cytokine production; hence most of the evidence has been indirect. The production of IL-1α has been suggested by the inhibition of secondary cytokine production by neutralising antibodies against IL-1α (see section 1.11.1). The results obtained by this series of experiments were not able to demonstrate an increase in either IL-1α mRNA or IL-1α protein. The previously described experiments may not have been able to detect IL-1α protein, hence the use of an indirect measure of IL-1α protein production, by using neutralising antibodies and monitoring a secondary response. Thus the IL-1α protein production may have been below the level of detection of the immunoassay employed. The lack of detection of an increase in IL-1α mRNA may have been due to the use of a relatively late time point for the mRNA assay, this is discussed later, as there are several cases where this seems to have occurred. The evidence from previous studies suggests that IL-1α is one of the first cytokines to be produced at the site of infection and inflammation. The sampling for IL-1α mRNA at 24hours following infection may have been too late to detect any increase in IL-1α mRNA.

No increase in either IL-1β mRNA or protein production was detected; there have been no previous studies that have investigated the production of IL-1β from epithelial cells. There has been one previous study that has investigated the production of IL-1β mRNA from A549 cells and demonstrated an increase following RSV infection. The late sampling of the cells for IL-1β mRNA may have lead to the negative result, as the previous study showed maximal production at 7 hours following infection (86). The immunoassay used may not have been sensitive enough to detect any increase in IL-1β protein.

In contrast to the results for IL-1 production from epithelial cells, there is evidence of both IL-1α and IL-1β mRNA and protein production from peripheral blood derived macrophages (se figure 3.11 to 3.13). These results indicate that there is early production

of IL-1 from macrophages that have been infected with RSV. The increase in IL-1α and IL-1β protein production following inactivated RSV challenge indicates that a small proportion of the protein production is triggered by RSV binding to cell surface receptors and does not require replicating RSV for the protein production (see figure 3.11 and figure 3.13).

The majority of previous studies have only described IL-1 production, but not specified whether it is IL-1α or IL-1β, though a more recent study have been able to demonstrate the production of both following RSV infection (see section 1.11.1).

These results indicate that RSV can stimulate the production of IL-1 from macrophages, and that this may play a role in the initial inflammatory response.

5.1.6 Interleukin - 6

The results for IL-6 production from A549 cells demonstrate that there is an increase in both IL-6 mRNA and IL-6 protein production following RSV infection. A non-significant increase in IL-6 protein production was seen within 24 hours of infection, this was significant by 48 hours of infection and there was a further dramatic increase demonstrated 72 and 96 hours after infection (see figure 3.14 and figure 3.15). There was evidence of a relationship between the initial infecting dose of RSV and subsequent IL-6 protein production (see figure 3.16).

These results indicate that there is induction of IL-6 mRNA within 24 hours of RSV infection of A549 cells, and that this leads to an increase in IL-6 protein production, the initial increase can be demonstrated within 48 hours of infection, the production of IL-6 continues to rise following infection, with a significant increase in the IL-6 protein production 96 hours after infection. The IL-6 cytokine response is proportional to the initial infecting viral load, thus a graduated response is seen to infection. Previous studies

have demonstrated that there is variability in the cytokine production of different cells lines, with maximal production seen in some 96 hours following infection (197), while in others it is seen within 24 hours (198). Previous studies have not explored the relationship between infecting dose and cytokine response.

The results from the IL-6 protein response to RSV challenge indicate that infection of macrophages is not required for the production of IL-6 from these cells. UV-inactivated RSV mount a similar response to live replicating RSV. This indicates that the IL-6 protein production from macrophages demonstrated following RSV infection is due to the virus docking onto a cell surface receptor and this action probably leads to IL-6 production (see figure 3.17). Similar findings are seen for the production of IL-6 mRNA following RSV challenge, with an increase in IL-6 mRNA seen with UV-inactivated RSV challenge. The IL-6 response from macrophages is quantitatively greater from macrophages than from epithelial cells (see figure 3.15 and 3.17).

Previous studies have been able to demonstrate that macrophages from different sources have different IL-6 responses to RSV infection; this may reflect their relative susceptibility to RSV infection. Macrophages from different cellular sources also have peak IL-6 production at differing times post infection, with cord blood macrophages reaching maximal production within 24 hours and alveolar macrophages taking 48 hours (see section 1.11.5). There have been no previous studies of inactivated RSV on macrophage production of IL-6, except in the mouse model, the results were similar to those observed (202) in this study, that replicating RSV is not required to mount an IL-6 cytokine response to RSV.

5.1.7 Tumour Necrosis Factor -alpha

The results of epithelial cell infection with RSV and subsequent TNF-α production demonstrate that there is low-level induction of TNF-α mRNA production seen 24 hours after epithelial infection (see figure 3.14). The results for TNF-α protein production

indicate that there is an increase in TNF-α protein following RSV challenge of A549 cells, but this occurs soon after infection and has reached a maximal level by 24 hours following infection (see figure 3.20), and there is no subsequent rise in TNF-α production. The TNF-α mRNA results may reflect a similar situation to that seen with IL-1, that the sampling for cytokine mRNA 2 hours after the viral challenge may be too late to observe the majority of the mRNA induction, and that the results obtained are false negative results. The results obtained from protein sampling in these experiments supports this hypothesis. There was no evidence of a relationship between initial infecting viral dose and subsequent TNF-α protein production. This result may reflect the peak TNF-α protein production may have already been reached at an earlier time point and that TNF-α levels reflect the maximal production from the epithelial cells. These results of this study are not in keeping with previous studies on TNF-α production, though these results have been conflicting, with variable reports on the detection of TNF-α mRNA, an increase in TNF-a production has been previously reported by Arnold et al. in A549 cells (see section 1.11.16).

The results for macrophage production of TNF-α mRNA following RSV challenge demonstrate that there is an increase in TNF-α mRNA following both UV-inactivated RSV and live RSV (see figure 3.21). This result for protein production is similar to the result obtained for IL-6, in that some of the production of this pro-inflammatory cytokine can occur with the binding of RSV to a cell surface receptor and does not require infection and replication of RSV before there is induction of the cytokine. The level of TNF-α protein production by challenge with UV-RSV is lower than the level observed with infection of the macrophages by live replicating RSV, suggesting that there may be two components to the TNF-α production, a third of the total TNF-α production being due to RSV cell surface binding, while the rest is due to infection of the macrophage.

The epithelial pro-inflammatory response to RSV infection reveals little IL-1 production, but significant production of IL-6 and TNF-α following infection. The IL-6 response increases over several days following infection, though an increase in mRNA production

seen within 24 hours, little to no increase in TNF-α mRNA was seen and there was no increase in TNF-α protein over time. This data suggests that though some of the pro-inflammatory cytokines may have rapid induction and return to baseline, others do not.

The pro-inflammatory cytokine response observed in macrophages is brisker and quantitatively greater than the epithelial response. There is a significant increase in cytokine protein production. The cytokine production from macrophages may be a two-stage process for both IL-1 and TNF-α, with inactivated RSV leading to an increase in cytokine production, suggesting that viral binding to the cell initiates some cytokine production, though the majority is produced on cell infection. The data for IL-6 indicates that macrophage cytokine production is only reliant on a virus cell interaction stimulating cytokine production. The results for TNF-α production from macrophages, demonstrating low level TNF-α production with inactivated RSV have been reported before. The results for IL-1 and IL-6 are previously unreported.

The greater cytokine response observed from macrophages suggested that this group of cells provide the major early stimulus to initiate the inflammatory response at the site of RSV infection. Some of the response does not require viral infection of cells, though cellular infection may be required for the maximal inflammatory response. Thus there is an extremely rapid pro-inflammatory cytokine response, without the need for cellular infection to occur, stimulating immune activation even before the virus has started to infect the initial cell.

5.1.8 Chemokine production - Interleukin-8

The RSV infection of epithelial cells leads to the induction of IL-8 mRNA production and subsequent IL-8 protein production (see figure 3.23). The cell line studied does have a low level of constitutive IL-8 protein production, though there is a significant increase in IL-8 production following RSV infection that is significantly greater than the constitutive production. Over the time course of the experiment there is a steady increase in IL-8

protein production (see figure 3.24), this begins to plateau 96 hours after infection. There is also a relationship between the initial infecting viral dose and subsequent cytokine production. The steady increase in production over time suggests that there is continuous stimulation of production of IL-8; there are two possible hypotheses. The first is that there is spreading infection of the epithelial cells, and as a different group of cells are infected they produce IL-8. The second hypothesis is that some of the earlier cytokines, which are produced in a rapid manner after infection of the epithelial cells, stimulate the production of IL-8. No experiments were performed to investigate whether viral replication was required for epithelial IL-8 production or not. Recent studies have indicated that the latter hypothesis is the correct one, as IL-1α can stimulate the production of IL-8 (see section 1.11.7).

The results from studying IL-8 mRNA and protein production from macrophages were difficult to interpret. The culture system employed stimulated macrophages to produced IL-8 mRNA and protein (see figure 3.26 and 3.27). There was no significant difference between the IL-8 protein levels from the control macrophages and those exposed to UV-inactivated RSV. There was a statistically significant difference between the macrophages exposed to UV-inactivated RSV and those exposed to live RSV, though this result is probably not clinically significant as there was an extremely high level of background IL-8 protein produced (see figure 3.26). The adherence of macrophages to plastic is known to stimulate the activation of macrophages, and the results observed may be due to the non-specific activation.

There have been few prior studies investigating the IL-8 response of macrophages to RSV infection; this may be due to difficulty in interpreting results if other culture systems employed also stimulate macrophages into high constitutive IL-8 production.

5.1.9 Growth Related Oncogene-alpha

The investigation of GRO-α production is limited, as only mRNA production was investigated. The result for GRO-α mRNA production demonstrates there is an increase in GRO-α mRNA following RSV challenge of A549 cells (see figure 3.28), with no evidence constitutive induction of GRO-α production by the culture conditions. No investigation of GRO-α protein production was performed.

This is the first study to investigate GRO-α mRNA production from A549 cells, the only previous study has investigated primary epithelial cell culture and were only able to demonstrate constitutive production, with no increase following RSV infection (222).

These results indicate that the infected epithelium releases neutrophil chemoattractants and activators; this does not seem to be the case with macrophages. Thus neutrophils are attracted to the site of infection.

5.1.10 RANTES

The results from the experiments investigating RANTES mRNA production from epithelial cells indicate that there was a low background level of constitutive RANTES mRNA production (see figure 3.29). The results from the analysis of RANTES protein production did not demonstrate any constitutive RANTES production (see figure 3.30). After RSV infection there was a low level of RANTES production that rapidly increased after 72 to 96 hours after initial infection. There was a good correlation between initial infecting dose and subsequent cytokine production. The delay in RANTES protein production may be due to the need for the production of another cytokine or mediator, and then this stimulates the production of RANTES protein. Though this hypothesis would correspond with the RANTES protein data, it would not correspond with the mRNA data, where there is evidence of early RANTES mRNA induction, unless the production of

RANTES can be stimulated by both direct cell surface binding or infection as well as by other cytokines acting on the epithelial cells. The experiments presented are the first to demonstrate an increase in RANTES mRNA and protein production following RSV challenge of the epithelial cell line A549.

The results for RANTES production from macrophages are similar to those for the pro-inflammatory cytokine IL-6 (see figure 3.17). Though there was a statistically significant difference between inactivated RSV and live RSV in terms of RANTES protein production, clinically there would be little difference between the two responses. This could be proved if the macrophage response was observed with study of the cell supernatant and investigations using lymphocyte chemotaxis rather than accurate analysis of cytokine levels. This data suggests that the induction of RANTES mRNA and protein production depends on virus-cell surface interaction and not on viral replication.

5.1.11 MCP-1

There is evidence of an increase in MCP-1 mRNA production from A549 cells following RSV challenge (see figure 3.34). The results for MCP-1 protein production reveal that there is constitutive MCP-1 production from the control cells, there was a significant difference between control and RSV challenged cells until 72 hours following challenge, after this there was no significant difference (see figure 3.35). There was a correlation between initial viral infecting dose and subsequent MCP-1 production; this was a valid observation up to 72 hours following RSV challenge, with the continual increase in constitutive production over time this was not valid after this time point (see figure 3.36). The relatively rapid initial increase in MCP-1 protein, followed by a relative plateau, once the constitutive MCP-1 is accounted for suggests that the majority of the protein production occurs within the first 24 hours. Thus the MCP-1 response to RSV infection of epithelial cells is different to the slower, response observed for RANTES. Previous studies investigating have only shown high constitutive MCP-1 production; except for one study

that was able to demonstrate that RSV infection leads to an increase in MCP-1 protein (see section 1.11.24).

The investigation into MCP-1 production from macrophages was not able to produce any difference between the control cells and the infected cells, in this system there must have been activation of the macrophages to produce an increase in MCP-1 mRNA and protein (see figure 3.37 and 3.38), thus no comments can be made on the role of MCP-1 in producing an inflammatory response to RSV infection of macrophages.

5.1.12 MIP-1α and MIP-1β

The results for the MIP-1α mRNA production are difficult to interpret due to technical problems with the PCR for this mRNA. Though there is the suggestion of a band forming in the RSV challenged cells, this is not definite (see figure 3.39). There was an increase in MIP-1α following RSV challenge, though this was only observed when higher infecting doses were employed (see figure 3.40). No correlation could be made between infecting dose of virus and subsequent cytokine response (see figure 3.40).

The analysis of samples for MIP-1α and MIP-1β mRNA at 24 hours following RSV infection was unable to demonstrate any increase in either mRNA. The cytokine protein results for these two cytokines demonstrates similar response to RANTES, where though there is a statistically significant difference between the protein levels for control cells and those infected with RSV there is unlikely to be a clinically significant difference between the responses.

In the study of RSV induced responses in macrophages, the protein levels for both cytokines are increased at 6 hours post challenge, suggesting that the time point used to study for mRNA is too late, as there probably is an early induction of cytokine mRNA

following infection, with levels falling rapidly thereafter. The results demonstrate high constitutive production of the proteins, but there is a statistically significant increase if UV-inactivated RSV or live RSV is used, though clinically this may not be significant as there is a less than 20-25% quantitative difference in the response, between control and challenged cells. These results that the macrophages in the culture system employed do constitutively produce large amounts of both MIP-1α and MIP-1β.

5.1.13 Eotaxin

The data obtained investigating the production of eotaxin from epithelial cells is limited, as there is no protein data. There is evidence that there is an increase in eotaxin mRNA following RSV challenge from epithelial cells (see figure 3.44). The use of the TAQMAN process was useful in this analysis, it demonstrated that eotaxin mRNA production is induced within 2 hours of infection and rapidly returns towards baseline, if the conventional time points had been used, as in previous experiments, there would have been no evidence of an increase in eotaxin mRNA production. The results, if supported by increases in eotaxin protein production indicate that specific eosinophil chemoattractants are also being produced by the epithelium following RSV infection.

The results for the production of CXC and CC chemokines from epithelial cells that have been infected with RSV indicate that infection does lead to the production of neutrophil and lymphocyte and eosinophil chemoattractants to the site of infection. This is coupled to the production of immune cell activator cytokines (IL-1, IL-6 and TNF-α) that are also produced at the site of infection and inflammation.

The analysis of the macrophage response to RSV infection is difficult to elucidate as the culture conditions employed lead to activation of the macrophages, probably due to macrophage adherence to plastic. This activation led to high constitutive cytokine

production for CXC chemokines (IL-8), as well as CC chemokines (MIP-1α, MIP-1β). The epithelial chemokine responses suggest that there is a prolonged production of chemokines from epithelial cells that allows continual recruitment of immune cells to the site of infection, while the activation of these cells may only occur relatively early in the response to infection, as the pro-inflammatory cytokine production by the macrophages is not sustained.

Cytokines associated with lymphocyte responses

5.1.14 Interleukin 10

There was significant up-regulation of IL-10 mRNA and also IL-10 protein production following RSV infection of macrophages, though the magnitude of production was no as large as for some other cytokines. There has been only one previous study that has investigated the production of IL-10 from alveolar macrophages following RSV infection (see section 1.11.10); this study was able to demonstrate a significant increase in IL-10 production following RSV infection. This latter study demonstrated that IL-10 production is relatively late when compared to the other cytokines and that it has an anti-inflammatory effect, reducing IL-1 and IL-8 production. IL-10 may have a role in switching off the cytokine production at the site of infection and regulating the immune response. There has been no evidence of epithelial production of IL-10.

5.1.15 Interleukin 12

There was no evidence of IL-12 protein production from macrophages following RSV infection, these results are in keeping with the previous literature, where IL-12 production from macrophages has not been demonstrated (168).

5.1.16 Interferon gamma

The results for the production of IFN-γ from macrophages are different to the results previously reported. Converse to the results obtained in past studies, was the inability to be able to demonstrate an increase in IFN-γ. mRNA following infection of macrophages. Previous reports have demonstrated an increase in IFN-γ mRNA (168,200), but an inability to detect IFN-γ protein production. Though the results obtained do demonstrate no increase in IFN-γ protein, they do indicate a fall. This is one of the few studies to date that have been ale to demonstrate a fall in cytokine production following RSV infection. The PCR assay that had been established may not have been optimised enough to detect an increase in IFN-γ mRNA and the lower limit for the sensitivity of the protein assays may have made the job of establishing an actual fall in protein difficult. The apparent fall is due to there being constitutive production of IFN-γ by macrophages in the culture system employed, and it is the constitutive production of IFN-γ that is lost.

5.1.17 Interleukin 18

There have been no previous experiments investigating the production of IL-18 from macrophages following viral infection. The results obtained are broadly similar to those obtained for IFN-γ, which would be expected, as IL-18 is IFN-γ inducing factor. The constitutive production of IL-18 demonstrated in the culture system employed is lost with RSV challenge of the macrophages. It is difficult to establish whether there is a reduction in IL-18 mRNA with infection, though it does not seem to have occurred. If the production of IL-18 mRNA had been investigated using the TAQMAN quantitative PCR system, then the effect of viral infection of mRNA production could be observed.

The results for both IL-18 and IFN-γ are difficult to interpret if viral infection only leads to up-regulation of cytokines. There must be feedback loops where there is actually a reduction in cytokine production following infection. This may be due to the induction and protein production of an inhibitor for the cytokine in question, and this may lead on and have a negative feedback, thus there may be stimulation and up-regulation, but the

protein being assayed will fall due to the negative impact of the induced protein, one such system is the SOCS system (suppressors of cytokine signalling). This group of proteins are known to act as regulators of cytokine production.

5.1.18 Investigation of Signal transduction pathway

Recent studies have tried to investigate the signal transduction pathways involved in cytokine up-regulation following RSV infection, one such study is the investigation of IL-8 transduction pathways following RSV infection(310). This group have published further reports, which suggest that protein kinase C, and the MAP (mitogen activated protein) kinases are important in the induction of RSV responses (311). The results from the most recent report suggest that there is a two-step process, with an initial response dependant on RSV binding and a further response that relies on viral replication. This type of investigation could help in establishing the mechanism of RSV – cell attachment and infection and my preliminary data suggested that the MAP kinases were important in the induction of RSV responses.

The results of the experiment that investigated the macrophage IL-18 response to RSV infection (see figures 3.49 and 3.50) demonstrated constitutive IL-18 production, with RSV challenge leading to a reduction in the constitutive IL-18 production. The reduction in IL-18 production was not demonstrated when macrophages were challenged with UV-inactivated RSV. The results for IL-18 production are similar to the results obtained for the investigation of IFN-γ production. There was constitutive production of IFN-γ and following RSV challenge this fell. Only one other research group have been able to demonstrate an association between an increase in viral load and a fall in cytokine production (180). In that report it was a fall in IL-8 that was demonstrated, other research groups have not replicated this. The result obtained is important; it is the only study, to date, that provides evidence that macrophages at the site of primary infection may demonstrate a Th2-type response skew. This cytokine skew may drive any subsequent

immune response towards a Th2-type phenotype. This result was replicated several times in different experiments, underlying its reproducibility.

The last result would need further experimental evidence to investigate the alteration in IL-18 protein; the assay used only measured bioactive IL-18. Further experimentation would need to be performed to establish where the reduction occurred; IL-18 is produced as a precursor (pro-IL-18) and relies on IL-1 converting enzyme (ICE or caspase I) to cleave it and form the bioactive IL-18 protein. IL-18 mRNA studies (see figure 3.50) do not demonstrate any alteration in IL-18 mRNA production following RSV challenge. The use of a quantitative mRNA assay would have been a more accurate method to confirm that there was no reduction in IL-18 mRNA production. Other investigations that are required to investigate this response further include the study of caspase I activity, following RSV infection.

The series of experiments were performed on anonymous blood donor specimens; there was no indication of and no method available to obtain the atopic status of the donor. If this series of experiments could be repeated using blood from identified donors and the groups divided in atopic and non-atopic, more information about the Th-2 type responses following RSV challenge would be obtained. This series of experiments used blood monocytes that had under gone phenotypic changes as a surrogate for alveolar macrophages and this series of experiments should ideally be repeated on alveolar macrophages to confirm described findings. Previous studies have demonstrated the differing responses found between different macrophage populations, and the effect of prolonged culture on cytokine responses from monocyte derived macrophages.

The role of the epithelial cells in driving or sustaining the inflammatory response could be investigated further by studying the alteration in cytokine and chemokine receptors following RSV epithelial infection. The study of chemokine receptor mRNA up-regulation would be a relatively simple undertaking, if primers were designed to be used with the Taqman, then a quantitative study of chemokine receptor mRNA up-regulation following infection could be performed. This study would help to define which receptors are altered

with infection, and also if there is differential receptor expression over time, and whether this correlates with chemokine production. The results of these experiments may highlight targets for clinical intervention in the future.

As with the epithelial experiments, investigation into the alteration in cytokine and chemokine cell surface receptor expression would be important. This experimental information may be more important in the macrophage as this cell type appears to be critical in the linking the innate and adaptive immune system. The later experiments I presented studies the signal transduction pathways involved in RSV infection in macrophages, the only reports of RSV involvement in signal transduction to date are for A549 cells (310,311). The results suggest that MAP kinases are linked in the induction of cytokine responses in both macrophages and epithelial cells. The results describe how there was no evidence that the signal transduction pathway that leads to the production of IL-18 involves protein kinase C or the PI-3 kinase system. There was evidence that the tyrosine kinase pathway was involved, as herbamycin A is a tyrosine kinase inhibitor altered the IL-18 response. There was also evidence that at the concentration used for gliotoxin (nanomolar concentration) NF-κB is important in the regulation of IL-18. These are the first experiments that have investigated the regulation of IL-18 production from macrophages. The results (see figures 3.51 and 3.52) support data that indicates that MAP kinases are important in IL-18 regulation. This is an area that requires further investigation.

5.2 Childhood Community Study

5.2.1 Viral Load

There are several hypotheses relating to the pathogenesis of RSV bronchiolitis. The first hypothesis is that the severity of bronchiolitis is, in part, a function of the degree of development of the infant airway at the time of RSV infection. Thus smaller, younger infants are at greater risk of developing RSV bronchiolitis due to their narrower airways, and these smaller airways develop a higher resistance due to their smaller calibre, which in

turn leads to the need for higher inspiratory pressures to maintain airflow. The higher resistance of these narrower airways leads to the clinical picture of respiratory distress, sub-costal recession, inter-costal recession and increased respiratory rate. The second hypothesis is that in some infants with limited infection of the lower respiratory tract by RSV, an aberrant host immune response induces the development of bronchiolitis. Such a hypothesis suggests that the development of bronchiolitis would be more common in children born to atopic parents. These infants have a predisposition to develop atopy, and the host response to RSV LRTI would be more likely to lead to post-infection wheeze. The evidence for this hypothesis is not in concordance with the population data from the Tucson study (157,158), indicating that the incidence of post-bronchiolitis wheeze falls with age, and at the same time as post-bronchiolitis wheeze falls there is a rise in the incidence of asthma, the populations are different, and there is limited overlap between the two populations. In this study we sought to determine if the severity of infection, as measured by viral load, was a major determinant of the host response as measured by cytokine production.

The results presented in figure 4.1 demonstrate that there is no significant difference in viral load between children who develop upper respiratory tract infection only and those that develop bronchiolitis. There was no significant increase in the viral load over time of children that develop bronchiolitis or those that only had a URTI. This confirms that the severity of infection, as measured by viral load, is not an important determinant of the subsequent cytokine response.

5.2.2 Interleukin 10 response

There was a fall in IL-10 levels in both groups over time, though there was a greater fall in IL-10 concentration in children with bronchiolitis when compared to those that developed URTI only. This result suggests that the skew in immune response to RSV infection between the two groups may occur early in the response (see figure 4.2). As this is seen within the first week of infection, this is probably due to the macrophage cytokine response and is not dependant on the incoming lymphocytes. The macrophage response

will be related to either infecting dose of virus or host response. The results from figure 4.1 indicate that there was no significant difference between the two groups in terms of infecting dose, this suggests that the host response is responsible for the difference in IL-10 levels between the two groups.

5.2.3 Interleukin 12 response

There were no significant difference in the response between the groups at either time point and there was no significant difference in response over time either (see figure 4.3).

Since IL-10 induces a Th2 response and also acts as an anti-inflammatory cytokine, while IL-12 is the key cytokine in initiating a Th1 response and is a potent inducer of IFN-γ, expressing the results for these two cytokines as a ratio of IL-10/IL-12 more accurately reflects the likelihood of RSV infection inducing a predominantly Th2 response. Further, this method corrects for any differential dilution of individual samples that may have occurred in the sampling or processing of the clinical specimens. The results of this can be seen in figure 4.4.

The results presented in figure 4.4 demonstrate that there is a significant difference between the two groups at the earlier time point, with a significant increase in the IL-10/IL-12 ratio for children that developed bronchiolitis. This data suggests that there may be a skew in the immune response occurring very early in the response to infection, and that this is not related to infecting viral load (from the results obtained in figure 4.1). Thus the initial host response is important for the subsequent cytokine response that is produced locally in the airways of infected children. This initial host response seems to skew the immune response away from a Th1 type response towards a Th2 type response, this may lead to the development of the transient wheezy phenotype that is seen in children following viral bronchiolitis.

5.2.4 Interleukin 4 response

The overall IL-4 response in both groups was low. The IL-4 response to RSV infection demonstrates no significant difference between the two groups; there is also no significant difference between the two time points for either group (see figure 4.5). There is no previous evidence that indicates that a significant increase in IL-4 is seen in RSV infection in humans.

5.2.5 Interferon gamma response

There was a lower level of IFN-γ production from children that developed bronchiolitis than those that developed URTI, the initial high levels of IFN-γ fell over time, but there was no significant difference between the initial and later time points or between the two groups subsequently (see figure 4.6). The initial higher levels of IFN-γ may be protective to the URTI group, reducing viral replication and protecting against more extensive disease, though this is not confirmed by the viral load data. The expression of the data as IL-4/IFN-γ ratios does not demonstrate any significant difference. This data was obtained from airway lavage specimens, conflicting data was obtained by Bendelja (312), where the children with more severe infection actually had higher IFN-γ levels, though this data was from stimulated peripheral blood mononuclear cells.

5.2.6 Peripheral blood cytokine mRNA response

The results for the cytokine mRNA responses from peripheral blood mononuclear cells are presented in figure 4.7, they demonstrate that at the final time point analysed there was a correlation between the nasal aspirate cytokine response and the peripheral blood

mononuclear cell mRNA response. There was a significant difference in the IL-4/IFN-γ ratio between the two groups, with the response from children with URTI only being greater than that from children with RSV bronchiolitis.

The IL-4 and IFN-γ responses were expressed as a ratio, similar to the ratios expressed in studies by Roman et al. (109). The IL-18 responses were corrected for cell numbers by expressing as a ratio of GAPDH (house keeping gene).

The results for IL-18/GAPDH ratio demonstrate that the children with URTI only have a greater IL-18/GAPDH ratio that those that develop bronchiolitis (see figure 4.8). This result would be in keeping with the result obtained for IL-4/IFN-γ responses, with the Th1 type cytokine being greater in those children who do not develop bronchiolitis. This data correlates well with previous studies. As IL-18 is IFN-γ inducing factor, and IL-12 is also required for an increase in IFN-γ production, a relative fall in either IL-18 or IL-12 would lead to a reduction in IFN-γ, or an increase in IL-4/IFN-γ ratio, as observed.

There have been very few studies that have investigated the nasal (or blood) cytokine levels from children with RSV infection. Some studies have taken mononuclear cells from healthy individuals and stimulated these with either RSV or RSV surface components and assayed for cytokine production (240). While most studies have focused on obtaining mononuclear cells from children or adults with active infection, and stimulating these cells and assaying the cytokines production (109,110,187,313).

There have been two studies that have investigated the response of PBMC from children with RSV infection to stimulation with PHA or poke weed mitogen (PWM) (109,313). These studies investigated children with RSV bronchiolitis only and compared the responses to healthy controls rather than comparing responses between children with RSV

bronchiolitis and RSV URTI. The results from these two studies demonstrate that RSV bronchiolitis leads to an increase in IL-10 production during convalescence, though not acutely (313). In children with recurrent wheeze there was no correlation between recurrent wheeze and IFN-γ, IL-4 or IL-4/IFN-γ ratio. Roman et al. were able to show alterations in cytokine response in children with RSV infection (109), there was a fall in both IL-4 and IFN-γ concentrations produced from stimulated PBMC, though the greater fall in IFN-γ led to an increase in the IL-4/IFN-γ ratio. Bendelja was able to demonstrate an increase in IL-4 production and a fall in IFN-γ production (187), leading to a similar difference as other studies, between children with RSV and controls. This study was also able to study both CD4 and CD8 cell population cytokine production by flow cytometry and demonstrated that the CD8 T cell population produced IL-4 more often.

The difference between these previous studies and the one performed is the rapidity with which samples were obtained from children with RSV infection and the analysis comparing upper and lower respiratory tract infection with RSV. The ability to sample children within 24 hours of the onset of symptoms enables the cytokine responses at the initiation of the illness to be studied and the division of the population means that these two populations can be compared for any variation in response. The use of viral load measurements by semi-quantitative PCR is also novel, previous studies have used viral load assays based on plaque forming units of virus (204).

The results demonstrate the variability in viral load data for this population, there was no significant difference between isolates at either time point, though there is a trend towards an increase in viral load for children with bronchiolitis, though this did not reach statistical significance. The results show similar trends for the IFN-γ and IL-4 data as demonstrated by the Roman et al. (109), there were falls, over time, for both IFN-γ and IL-4 for both populations, but there was no increase in the IL-4/IFN-γ ratio that was observed in that study. In this study an analysis of stimulated PBMC and measurement of mRNA for IFN-γ and IL-4 was also performed, this mirrored the changes in the nasal aspirates. The results were only expressed as a ratio between IFN-γ and IL-4, therefore the individual changes in

cytokine mRNA were not established, but the overall result is similar to the result obtained by Roman et al..

This study also assayed IL-10 and IL-12 levels, the IL-10 levels fell between the initial sample time and the second sample time for both study populations, while there was an increase in IL-12 in children with URTI only. The results of comparing IL-10 to IL-12 ratios reveals that there was no change in children with URTI only, but there was a significant increase in children with RSV bronchiolitis. In the study by Sheeran et al. (204), where IL-10 concentrations from intubated and non-intubated children were compared, there was a fall in IL-10 concentration with time, similar to the results obtained in this study. Sheeran et al. were able to demonstrate an inverse correlation between severity of RSV infection and IL-10 concentration, this correlation was not found in this study.

The final aspect of this study was the investigation of the corrected IL-18 mRNA response of LPS-stimulated PBMC. For this cytokine the PBMC seemed to follow the trend that had been established in the airway responses for other Th1 and Th2 cytokines, there was a significantly higher level of IL-18 mRNA released from the PBMC of children with URTI only when compared to children with RSV bronchiolitis. This confirms that the skew in cytokine responses is not just a localised airway phenomenon, but is a generalised systemic response.

It is difficult to correlate the results of human studies comparing Th1 and Th2 responses with murine studies, as the murine model of RSV studies the murine response to rechallenge with RSV after initial priming with RSV, formalin-inactivated RSV (FI-RSV) or F/G surface proteins of RSV. The murine results demonstrate that a cytokine skew can be seen, depending on the initial priming agent used; with FI-RSV and G proteins leading to a Th2-type response (314) (315), while F and RSV lead to a Th1-type response (113,316). The relevance of the Th-2 cytokines IL-4 and IL-10 has been demonstrated by depletion experiments in the murine model of RSV infection, with reduction in IL-4 (160) and IL-10 (116) leading to a reduction in the pathology associated with infection. The

augmentation of a Th2-type response by the addition of IL-4 reduces viral clearance (122). The augmentation of the Th1-type response by the addition of IL-12, rather than the depletion of Th2-type cytokines does lead to a reduction in some of the pathological changes, but does not alter survival following RSV challenge (119).

5.2.7 Conclusion

The results from the in-vitro challenge of macrophages with RSV are important, when coupled to the results from the community RSV study, they draw together the in-vitro model and in-vivo results. This in-vivo study is important in demonstrating the very early skew in cytokine profiles that was observed between the two study groups. It also provides a useful method for differentiating between two groups of children infected by RSV. This method of prospectively differentiating between children has not been employed previously. A Th1 profile has been demonstrated in PBMC from children with RSV URTI previously, though the samples were all taken at the convalescent phase for that study.

This study shows that either the change in cytokine profiles occurs within the first 24-48 hours following infection, or that different populations develop URTI or bronchiolitis. The later statement cannot be tested by the methods employed for this study. The results from PBMC for the same groups of children concur with the cytokine responses elicited from the nasal aspirates, proving that there is evidence of a systemic, as well as local airway, skew in the cytokine profiles of children with RSV bronchiolitis, compared to children that develop RSV URTI only.

The limitation of the study is that the population studied was not a normal population. There was a greater likelihood of differences being present between the two groups in this study than in a normal population, as the study population had at least one atopic parent, and were thus more likely to be atopic themselves, increasing the probability that RSV infection will lead to a Th2 response.

This study is being continued until the study population reaches the age of five. During this study the children will have regular IgE and skin prick testing performed to ascertain their atopic status. The final results of this study will be able address the following hypotheses:

i) Does RSV bronchiolitis predispose children to developing an atopic phenotype
ii) Does the maternal antibody level against RSV correlate with the immune response, and does it predispose towards development of bronchiolitis.
iii) When are the differences between those children that develop RSV URTI and RSV bronchiolitis manifest.

The answer to question i) will be able to be answered at the end of the study. During the introductory chapter I have discussed the evidence that supports the statement that RSV infection predisposes to childhood wheezing. The evidence from long term studies shows that childhood wheezing continues for several years after infection, though the prevalence of wheeze in this population falls back to that of the general population by the teenage years. There has been conflicting evidence as to whether RSV bronchiolitis does predispose children to developing atopy, though some recent studies have suggested that it may happen, and there is some experimental evidence to back up this hypothesis.

The second hypothesis has not been discussed in this thesis and forms a part of another study that was performed on this population of children. This study was based on the knowledge that antenatal sensitisation to proteins can occur after 22 weeks gestation (317), it was hypothesised that maternal exposure after this date could lead to protective responses in the infant. Cord blood responses to RSV were observed in a third of children with sensitisation. Stimulation with RSV induced Th1 type responses, with increased IFN-γ and reduced IL-4 production, confirming the protective effects of antenatal sensitisation.

The third hypothesis, stating that there is an early skew in response to RSV infection can be linked in with the data from the in-vitro experimentation on macrophages. The in-vivo data demonstrates that there is a skew in cytokine production towards a Th2 phenotype in

children with RSV bronchiolitis; this is seen with IL-10/IL-12 ratio as well as the IFN-γ/IL-4 ratio. The data from PBMC also demonstrate the reduction in IL-18 mRNA production in children with RSV bronchiolitis. The results from this study are in keeping with the results of previous studies that have demonstrated an increase in Th2 activity, though these studies have investigated cytokine production from activated PBMC from children with RSV and not primary cytokine responses from children with RSV infection.

The importance of early childhood infection in protecting against subsequent atopy has been discussed during the introduction. Though there have been many discussions about the paradoxical nature of RSV bronchiolitis possibly leading to wheezing illness, rather than protecting the child from atopy there is limited evidence to suggest that RSV infection does lead to a permanent skew in the immune response towards atopy. The data from the experiment presented does suggest that RSV infection can lead to skewing of the immune response, but other data does suggest that this may be primed by maternal RSV exposure. The study has not reached a point at which a conclusion can be made as to whether there is a permanent alteration in the immune response. The study population must be taken into account when conclusions are made from this study. Extrapolation of these results to the general population must be done with caution; the study population are more likely to develop atopy compared to the normal population, due to the parental history of atopy.

Subsequent studies in this area could study a cohort of children with non-atopic parents, to confirm that similar results are obtained from a cohort where the prevalence of atopy is likely to be lower. The immune responses of children infected with RSV in the first year of life would be important in designing any future vaccines. The coupling of the maternal antibody status with childhood RSV infections will be important in determining whether maternal vaccination, with a view to childhood protection is possible.

The strength of this study is that it prospectively followed a defined cohort of children for one year; a single investigator to sample the child and categorise the children was used. The ability to perform viral load quantitation was useful and helped to strengthen any

analysis, demonstrating that there is no significant difference in the viral load between the two groups studied.

Despite the significant amount of research that has taken place in trying to understand the immunopathology of RSV bronchiolitis, I feel that the experimental results discussed do help in the understanding of this important childhood disease. The experiments draw together the in-vitro results that demonstrate that RSV infection of macrophages alters their production of IFN-γ and IL-18 and the in-vivo results demonstrating that similar responses are seen in children that develop RSV bronchiolitis and that this response is different from that seen in children that only have URTI.

Chapter 6 – Conclusion

The research hypotheses proposed in section 1.22 - that RSV infection stimulates the early production of cytokines that can stimulate and skew the immune response of incoming lymphocytes towards a Th2 type phenotype, where the response is similar that seen in acute asthma, has been answered.

The results from the in-vitro study of RSV challenge of epithelial and macrophage infection by RSV demonstrate that RSV infection of epithelial cells leads to the production of many different cytokines and chemokines (see figure 3.6 to 3.36). These factors lead to lymphocyte and neutrophil recruitment (see section 1.20) and activation.

The results from in-vitro challenge of macrophages with RSV demonstrate similar results, with the production of numerous cytokines and chemokines (see figure 3.6 to figure 3.34). The in-vitro macrophage data provides novel data on the possible importance of macrophage infection in the pathophysiology of RSV bronchiolitis. This series of experiments demonstrate that there was a skew in the Th1 and Th2 type cytokines produced by macrophages following RSV challenge (see section 3.4.1 to 3.4.4). The cytokine production favours a Th2 type phenotype, with an increase in IL-10, no alteration in IL-12 production and a reduction in IFN-γ and IL-18 production following RSV challenge.

Though this is not the first study to provide data that suggests that RSV infection may lead to a reduction in cytokine response, that is not associated with an increase in cell death, it is the first to demonstrate that this occurs with cytokines that promote a Th1 type response. The results from the experiments with the blood-derived macrophages have demonstrated that they respond conventionally to RSV challenge, with an upregulation in the production of certain cytokines and that this work supports pervious studies in this field; there is evidence of a fall in IFN-g and IL-18 in the system employed.

Though these results, initially, seem to be counter intuitive, recent reports have suggested that there are previously undescribed feedback loops that control cytokine responses. The regulatory mechanisms are termed the suppressors of cytokine signalling (SOCS). This group of proteins were discovered by several groups simultaneously (318-320). This group of proteins are negative regulators of cytokine activation and as such have a similar role to IL-10, TGF-β, decoy and soluble cytokine receptors. Several different cytokines have been shown to be inhibited by the different SOCS proteins. Though several different proteins have been described only the first three have had significant investigation. It has been proposed that SOCS may play a role in the polarisation of T cells towards Th1 or Th2 phenotypes, though there is no evidence for this from experimental studies to date (321). The potential for SOCS proteins to alter T cell polarisation is due to both SOCS 1 and 3 having inhibitory effects on interferon gamma, with SOCS 1 has a more specific inhibitory effect on IFN-γ signalling (322); this is not seen with SOCS 2.

The data presented for IL-18 and IFN-γ response to RSV infection raise the possibility that SOCS proteins may be activated by RSV infection, this in turn would lead to reduction of IL-18 and subsequently IFN-γ and this could be the critical step in switching any immune response towards a Th2 type response.

The data presented in the study of children and PBMC cytokine responses also integrates with the data obtained from the macrophage responses to RSV challenge. The children that develop RSV bronchiolitis have lower corrected IL-18 mRNA responses compared to those children that developed URTI only (see figure 4.8). There is also an increase in the IL-4/IFN-γ mRNA responses of the stimulated PBMC from children with RSV bronchiolitis (see figure 4.7) and this also correlates with these children also having lower IFN-γ concentrations in their nasal lavage specimens (see figure 4.6). This data suggests that following RSV infection there is a fall in IL-18 and IFN-γ at the cellular level, and this also seen in the peripheral blood of children with RSV infection, and at the site of infection in the upper airway, with change being more apparent in those children that develop bronchiolitis.

The signalling pathways for RSV binding and subsequent cytokine production have not been established yet. The results of recent experiments indicate that the MAP kinases have a role in the signal transduction pathways that link RSV binding to cytokine production. For IL-8 there is evidence that signalling via the ERK2 is important, but not through JNK kinase, and that other pathways must be involved as inhibition of MEK1 will only lead to a reduction in IL-8 production by 50% (310). There is much work to be done in this area, as different cytokines have different signal transduction pathways. The final in-vitro experiments presented, investigating the inhibition of IL-18 production, indicate that tyrosine kinases and NF-κB are important in the suppression of IL-18 production following RSV infection of blood-derived macrophages (see figures 3.47 and 3.48).

NF-κB has been implicated in the induction of cytokeratin 17, which is found at the sites of syncytia formation following RSV infection (323).

Some members of the paramyxovirus family are able to disrupt the STAT signalling pathway that induces IFN production, by increasing the degradation of STAT1 and STAT2 (simian virus 5) and thus interfering with both type 1 and type II interferons. This mechanism of disrupting interferon production was also be demonstrated for Sendai virus and also human parainfluenza virus 2 and 3, RSV was not able demonstrate this response, but must have other mechanisms present to be able to continue to replicate in interferon producing cells (324).

Future investigations should investigate the possible role of SOCS induction following RSV infection of macrophages. The signalling mechanisms involved after RSV binding are important and investigation of these, with the use of more specific kinase inhibitors will help to establish the exact signalling pathways involved. The results for IL-8 production (310) and also for IL-6 production following RSV challenge (see figure 3.13) indicate for several cytokines that there are at least two different mechanisms involved in leading to cytokine production, one that involved RSV binding to a cell surface receptor and a second that involves RSV replication. There is evidence that NF-kB is involved following RSV infection (323) and that tyrosine kinases are also involved in signal

transduction, as they alter phosphorylation of tyrosine residues on STAT1 after RSV infection (324). These reports support the experimental data that I have obtained that suggest that both NF-κB and tyrosine kinases are involved following RSV infection of macrophages.

The experimental data presented in chapter 3 was the result of a series of experiments and has demonstrated an increase in both mRNA and also protein for several cytokines and chemokines. These experiments have demonstrated an increase in pro-inflammatory cytokines, as well as chemokines from the CXC and CC families. Recent technological developments, such as oligonucleotides probe-based microarrays are altering the scale of investigating mRNA production. Recent reports have demonstrated the power of this system in establishing the role of NF-κB in the induction of certain genes following RSV infection of epithelial cells (325). This study has demonstrated the ability to investigate a large number of different genes very quickly, and has demonstrated that RSV infection leads to an increase in RANTES, IL-8, GRO-α, tyrosine phosphatase and NF-κB all being up-regulated following RSV infection. Though these results have already been presented in this thesis, it is the ability to screen a large number of genes very quickly and establish links through signal transduction pathways is important, though the cost of this technology and the ability to only monitor mRNA production are important limitations to its use. The use of microarrays leads to the possibility that hypotheses can be generated from the result of large scale screening experiments.

The results from in-vitro infection of epithelial cells and macrophages are important as these results are of greater scope than previously published data and also demonstrate both an increase in mRNA as well as protein levels. For the epithelial data, there are no prior publications that have presented extended time points for RSV infection or shown a correlation between initial infecting viral dose and subsequent cytokine response, this was demonstrated for a large number of cytokines.

Future investigations into the cytokine response to RSV infection should investigate several aspects that have not been investigated yet. These areas include specific investigation into the induction of SOCS following infection and whether this correlates with the reduction in IFN-γ and IL-18 seen in this series of experiments. The dissection of the cytokine and chemokine response to RSV infection would be another important area to investigate further and as Tian has done (325) is to investigate the responses and group them together in terms of gene networks. Many cytokines are produced following RSV infection, the effect of these cytokines has several effects on surrounding cells, and the cells responsiveness to them is not fully understood, by investigating the expression of cytokine and chemokine receptors on epithelial cells, macrophages and lymphocytes (each of which has several ligands) a more comprehensive picture of the systems that are driven by RSV infection would be possible. This investigation could use both quantitative PCR to investigate receptor mRNA induction and either confocal microscopy or cell surface staining and flow cytometry to investigate whether there is autocrine induction of increased receptor expression by the cells that are infected and producing cytokines and chemokines.

The role of the respiratory epithelium in the innate immune response has recently come under renewed interest. The study of sequence homology and the relationship between RSV G surface protein and both the CXC_3 chemokine, Fractalkine (28), and the TNF-α receptor (326) indicate that either RSV may use these surface receptors to bind to cells and facilitate cell entry, or by mimicking them disrupt the immune response. Other recent studies have demonstrated that the innate immune pattern recognition receptors play a significant role in the recognition of RSV and the initiation of immune response (327,328). The evolutionary conserved toll like receptors (TLR) are important in recognising RSV, RSV is recognised by TLR4. These studies have also demonstrated that there a two stages to the response to RSV, the initial response that requires cell attachment and is epithelial cell independent and a second stage that requires the epithelium and requires viral replication (328). The findings of these studies suggest that NF-kB is

important in the response to RSV, a finding that was confirmed by the results of the inhibitor studies using gliotoxin (see figure 3.47).

The role of various components of the innate immune system have been investigated in RSV infection, such as the collectins (in the form of surfactant proteins), to a limited extent. The levels of surfactant proteins from children ventilated with bronchiolitis with have been studied, and alterations have been seen, with reductions in SP-A (329), though no correlation between SP-A level and disease severity. In-vitro studies have been able to demonstrate that SP-A can opsonise RSV and enhance its uptake by PBMC (330). SP-A also binds to RSV and reduces its infectivity (331).

Another arm of the innate immune response to consider is defensin production from the epithelium. Section 3.3.1c demonstrated that there was an increase in hBD-2 production following RSV infection of epithelial cells; this is the first study that has investigated the human epithelial defensin response to RSV infection. The results from the study suggest that the defensin response is not related to direct viral infection, but in response to the cytokine response following RSV infection. There is evidence to support this hypothesis, with evidence that IL-1β can up-regulate hBD2 mRNA expression(90). Recent studies have demonstrated that β-defensins also play a role in the adaptive immune response. HBD binds to the chemokine receptor CCR6 and is chemotactic for immature dendritic cells and memory T cells (332). HBD-2 also acts on mast cells to release histamine and prostaglandin D2 (333), thus the defensins released after RSV infection may have a more significant role to play in the wheezy type phenotype seen after RSV infection.

The results of the experiments performed suggest that RSV infection does lead to defensin production, subsequent other studies indicate that this arm of the innate immune system may bridge to the adaptive immune system as well. Future studies should investigate the possible role of defensins in the histamine release that has been found in some in-vivo studies with RSV infection (334). The in-vitro experimentation could involve the study of defensin protein production associated with RSV infection, then the depletion of defensins and whether this alters the histamine release. The role of defensins in special respiratory

populations, such as individuals with cystic fibrosis would be useful. Cystic fibrosis patients have impaired defensin responses, due to the salt content of their airways; the altered activity of their defensins may alter their histamine response.

The results of the study that investigated NO production following RSV infection indicate that there is an increase in NO following RSV infection of both epithelial cells and macrophages. Studying the NO levels of children with RSV infection would be useful, a repeat study would be able to investigate whether the severity of the infection leads to an increase in NO production in the airways, the use of NO synthase inhibitors would be able to elucidate the significance of NO production during RSV infection. In the studies suggested, the potential effect of disrupting the innate immune response to RSV could be monitored by studying alterations in the viral load.

This thesis has demonstrated several novel techniques to investigate the pathophysiology of RSV infection of the airways. It is the first occasion that quantitative PCR has been used to study RSV load during experimental in-vitro and in-vivo infection. The range of mediators investigated is broader than previous studies, and has demonstrated a broad range of cytokine activation following RSV infection. The in-vitro experimentation involving the investigation of the innate immune system has been the first report investigating the role of antimicrobial peptides in the host response to RSV infection. Further studies should investigate this further by in-vivo sampling during and after RSV infection. The possible link between the innate and the adaptive immune system needs further investigation. The role of RSV infection altering defensin expression and altering chemokine receptor expression, and whether an alteration in these does lead to alterations in lymphocyte chemotaxis. Future studies will be needed to establish the genetics of the host response to RSV, and whether this is due to polymorphisms in the innate immune system. There is evidence for other disease processes that gene polymorphisms in CD14 and TLR4 do alter the host response to infection (335).

The results from the macrophage in-vitro work demonstrate a significant cytokine release following RSV infection, with certain responses, such as the IL-18 and IFN-γ response

being noted for the first time. These results suggest areas of further study, where the role of the macrophage in helping to determine the adaptive immune response to RSV can investigated further. The signal transduction pathways for RSV stimulation of IL-18 production need further evaluation as well; the initial brief studies have highlighted areas that should be studied further.

The study hypothesis has been answered; there is evidence that an early difference is seen between the children that develop RSV bronchiolitis, compared to those that have RSV disease limited to the upper respiratory tract. The results of the in-vitro macrophage response to RSV challenge support the data obtained during the clinical study of RSV infection. There is evidence that the children that develop RSV bronchiolitis do have an immune response that is skewed towards a Th2 type response, with an increase in IL-10, IL-4 and a reduction in IL-18 and IFN-γ. The second hypothesis has also been answered; the respiratory epithelium can mount a significant innate immune response to RSV infection, with an increase in IFN-α, NO and defensins.

Appendix

The data presented in chapter 3 is the result of a single series of experiments on one sample of monocyte-derived macrophages. Several series of experiments were conducted, but as they were from different donors and there was no record of atopic status or RSV immunity the results were not combined. Additional data from RSV challenge macrophage experiments, the time points studied were the same as used for each figure in the main results section. The data is presented with the mean and standard error of the mean beneath.

Pro-inflammatory cytokines

Interleukin – 1α

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	1.2	2.8	9		2.7	0.3	332.1
	2.5	5	11.4		2.3	0.4	687.9
	3.7	5.7	18.4		3.4	11	520.8
	1.9	3.6	3.5		2	14.2	345.5
	0.8	2.5	3.9		2.1	2.7	373.3
	1.1	5.4	6.9		4.4	10.1	635.2
Mean	1.9	4.2	8.9		2.8	6.5	482.5
SEM	0.4	0.6	2.3		0.4	2.5	63.3

Interleukin - 1β

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	62.1	8.5	1650.4		6.2	11	23.9
	63.2	8.8	3253.5		11.8	13.9	14.5
	82.3	276.7	2708.4		13.3	26.7	21.9
	28.6	295.7	2381.9		11.4	19.2	24.4
	24.2	76.2	2415.8		8.9	17.1	17.3
Mean	47.6	140.1	2683.5		10.3	17.6	20.4
SEM	10.1	52.5	292.7		1.2	2.7	1.9

Interleukin-6

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	288.5	396.6	767.4		12.3	2186.2	6769.2
	194.7	232.9	518.9		7.7	2904.4	8032.1
	335.2	362.5	422.9		18.2	3301	5671.8
	272.9	216.3	961.6		48.1	2735.8	10374.7
	370.8	322	627.6		9.2	3037.5	6776.8
	346.1	382.2	526.7				
Mean	301.4	318.8	637.5		19.1	2833.0	7524.9
SEM	26.0	31.6	80.5		7.5	186.2	804.5

TNF - α

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	186	183.1	196.5		160.4	193	8154.8
	169	218	173.6		182.4	145.7	13082.1
	169.3	188.4	192		284.7	705.1	12949.8
	178.2	204.1	180.5		173.2	1201.3	12434.6
	158.2	185.7	224.5		180.8	253.5	11520.6
	180.8	179.9	220.5		188.4	645.8	14601.3
Mean	173.6	193.2	197.9		195.0	524.1	12123.9
SEM	4.1	6.0	8.5		18.4	166.6	893.5

TGF-β

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	91.7	79.5	82		72.4	72.3	73.3
	86.6	78.6	84.7		73.4	74.8	75.5
	86.9	77.4	85.9		83.4	72.8	73.7
	87.7	80.9	81.9		73.2	72	72.8
	84.3	79.6	84.6		72.1	71.3	74
	85.2	78.9	85.4		70.1	74.5	72.8
Mean	87.1	79.2	84.1		74.1	73.0	73.7
SEM	1.1	0.5	0.7		1.9	0.6	0.4

Chemokines

IL-8

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	853.4	1288.2	1913.5		4253.7	5085	6388.2
	730.7	1127.1	2209.4		5184.5	5817.5	6809.7
	1075	1483.1	2476		5293.2	5636.3	6152.9
	1298.8	1653.1	2679.9		5797.1	5641.5	7357.6
	1009.7	1343.4	1869.4		5470.2	5764.7	7437.9
	767.1	893.8	1811.4		4627	4768.1	5407.9
Mean	955.8	1298.1	2159.9		5104.3	5452.2	6592.4
SEM	87.8	108.8	146.0		231.5	173.6	315.5

RANTES

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	62.4	1156.9	1587		63.4	171.1	552.2
	88.5	1479.3	1496.7		62.9	530	558.7
	104.9	1204.2	1443.9		61.8	117.2	393.3
	84.1	1246.5	1394.9		77.7	72.3	576.6
	124	1291.2	1634.7		61.9	224.4	767.6
	324.8	1106.5	1469.7		62.5	121.3	947.9
Mean	131.5	1247.4	1504.5		65.0	206.1	632.7
SEM	39.6	53.4	36.8		2.5	68.2	79.6

MCP-1

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	286.4	282.9	324.3		701.3	508.8	2897.4
	212.2	357.6	340.6		505	467.2	3084.3
	181.1	333.8	353.5		590.4	1077	3170.5
	201	491.7	287.3		395.4	1127.4	3059.3
	113.8	303.5	357.7		675	547.4	3122.9
	152	491.6	516.2		456.4	955	2566.5
Mean	191.08	376.85	363.27		553.92	780.47	2983.48
SEM	23.96	37.77	32.31		49.91	124.50	91.58

MIP-1α

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	1564.9	1821.7	1829		1239.1	2898.7	3386
	1669.1	1920.2	1991.5		1683.7	3038.9	3427.6
	1351.7	1720.9	1759.8		1836.7	2888.2	3369.7
	1811.2	1724.6	2247		2700.1	2493.8	3435.7
	1689.7	1715.7	2162		1795.2	3147.1	3491.1
	1386.8	1582.6	1858.1		1597	2884.8	3269
Mean	1578.9	1747.6	1974.6		1808.6	2891.9	3396.5
SEM	73.7	46.5	79.7		198.4	90.4	30.8

MIP-1β

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	2644.67	4012.28	3914.92		2827.71	4124.45	3790.51
	2999.77	3871.43	4197.66		3192.66	3928.23	4055.48
	3207.54	3774.44	3981.1		3076.51	3709.46	3947.67
	3810.74	3739.46	4005.37		3586.73	3697.23	3635.23
	3110.8	3719.71	4075.97		3196.67	3773.96	3943.87
	2961.99	3503.46	3773.63		2961.38	3567.84	3845.73
Mean	3122.6	3770.1	3991.4		3140.3	3800.2	3869.7
SEM	158.1	69.2	58.7		106.3	80.6	60.0

Cytokines that Skew the Immune Response

IL-10

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	18.2	0	0		0	63.8	161.8
	47	0	5.7		0	9.5	297.3
	0	1.5	17.3		0	0	220.7
	0	0	18.7		0	0	144.2
	82.7	1.8	0		0	0	147.3
	0	5.1	6.5		0	6.2	233.1
Mean	24.7	1.4	8.0		0.0	13.3	200.7
SEM	13.8	0.8	3.3		0.0	10.2	24.7

IL-12

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	35.7	29	82.3		8	41.5	26.7
	43	64.7	55.3		9	5.4	37.9
	1.6	27.3	34.5		6.6	10.5	39.3
	0	55.9	62.6		6.6	12	25
	11.6	65.6	19.8		4.5	8.6	24.5
	14	58.6	27.3		2.5	8.3	14.3
Mean	17.7	50.2	47.0		6.2	14.4	28.0
SEM	7.3	7.1	9.7		1.0	5.5	3.8

IFN-γ

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	31.1	19	15		93.3	96.7	66.8
	20.3	17.3	9		88.4	43.4	38.3
	34.4	16.8	11.1		49.6	31	81.7
	31.5	26	26.9		76.7	32.8	47.8
	17.1	14.7	20.8		75.5	43.9	47.3
	27.1	20.3	13.7		78.1	59.9	54.1
Mean	26.9	19.0	16.1		76.9	51.3	56.0
SEM	2.8	1.6	2.7		6.2	10.0	6.4

IL-18

	Control	UV-RSV	RSV		Control	UV-RSV	RSV
	600	459.8	230		949.4	555	356.6
	422.7	692.3	210.7		853.1	405.6	355.1
	559	618.6	205.2		1001.3	371	373.8
	411.8	389.4	245.5		679.3	533.5	300.9
	606.2	524.1	375.5		838.3	310.9	352.6
	449	763.6	590		764.7	369.9	355.8
Mean	508.1	574.6	309.5		847.7	424.3	349.1
SEM	36.8	58.2	61.7		48.1	40.0	10.1

The data for the response of A549 cells following RSV challenge are presented below.

IL-1α

Time	0	24	48	72	96	120
Control	12.96	18.81	13.86	12.7	22.2	12.29
	20.29	9.74	10.94	15.4	9.45	12.7
	30.00	8.84	12.83	10.5	16.7	12
Mean	21.12	12.465	12.54	12.7	16.1	12.54
SEM	8.88	5.51	1.47	2.46	6.39	0.23
RSV	13.22	11.79	17	23.52	11.64	12.36
	11.01	10.5	25	12.3	9.7	9.27
	13.2	8.01	8.4	9.8	7.56	12.9
Mean	12.5	10.1	16.8	15.24	9.6	11.5
SEM	1.29	1.89	8.2	7.26	2.04	1.97

IL-1β

Time	0	24	48	72	96	120
Control	40.53	12.99	18.48	16.16	29.63	16.38
	27.06	25.08	14.58	14.13	22.35	16.99
	16.92	11.79	17.11	20.57	12.61	16.80
Mean	28.17	16.62	16.72	16.95	21.53	16.72
SEM	11.84	7.35	1.97	3.29	8.53	0.31
RSV	17.78	15.72	22.73	31.36	15.53	16.48
	14.70	14.13	33.40	16.42	12.99	12.36
	17.62	10.78	11.29	13.18	10.08	17.27
Mean	16.7	13.54	22.47	20.32	12.86	15.37
SEM	1.73	2.52	11.05	9.69	2.72	2.63

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