B cell responses to respiratory viral infections

By

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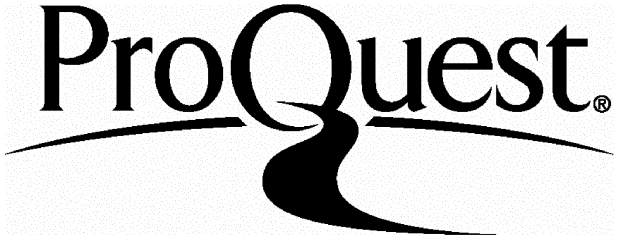
The Edward Jenner Institute for Vaccine Research



This work is dedicated to my parents,

John and Carmel,

for all their hard work that has allowed me to be in the privileged position I am now.

I really appreciate everything you've both done for me, thank you.

Abstract

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract disease during infancy and early childhood. Previous studies have focused mainly on serum antibody responses and have largely neglected the mucosal (local) sites. The nasal-associated lymphoid tissue (NALT) is a mucosal lymphoid tissue located at the entrance to the naso-pharynx and is significant as it is the first site of contact with inhaled antigen suggesting it may play an important role in the immune response to respiratory pathogens. Our aim was to characterise the local humoral response to RSV in the mouse model using cellular and immunological techniques.

Infection of mice with RSV induces an IgA dominated response in the nasal tissues that is extremely short-lived. In addition, very low frequencies of virus-specific antibody forming cells (AFC) were detected in the systemic tissues during the acute phase of infection and only the bone marrow possessed RSV-specific AFC long after infection, but at levels just above background. This is in direct contrast with the well-documented response to influenza virus infection where the AFC responses in the bone marrow are far more robust with frequencies four-fold in excess of those observed with RSV following only a single influenza virus infection. Significantly, the number of RSV-specific AFCs in the bone marrow, but not the D-NALT, can be boosted by subsequent infections with RSV and can attain a level comparable to that seen after a single exposure to influenza virus but only after three consecutive exposures to RSV. In addition, live RSV was still detected within the lungs of mice after secondary and tertiary exposures to RSV demonstrating the potential importance of antibody production at the site of infection.

When compared with influenza virus infection, our observations on the antibody response to RSV during infection highlight some interesting differences. The lack of a robust, boostable, RSV-specific antibody response in the D-NALT may be a key factor in explaining how RSV can re-infect via the nasal route with such apparent ease. This, coupled with the observation that there is only a gradual build up of RSV-specific AFC in the bone marrow over successive infections, may potentially explain why reinfection is so commonplace, requiring multiple infections before even moderate protection is afforded.

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I would like to thank my supervisor Dr. Lisa Hyland for all her valuable advice, guidance and expertise, not to mention patience, without which the last three years would have been much harder. I would also like to thank my co-supervisor, Dr. Torben Lund, for his encouragement and tuition which have been invaluable.

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Finally I would like to thank my wonderful girlfriend Zania who has put up with my many rants and ramblings during the last couple of years and who has, on more than one occasion, helped me retain my sanity and made me feel like I can achieve anything I set my mind to. I appreciate this more than you'll ever know, thank you.

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List of abbreviations

°C	Degrees Celsius
AFC	Antibody forming cell
APC	Antigen presenting cell
BALT	Bronchus-associated lymphoid tissue
BM	Bone marrow
BPL	Beta-propiolactone
BSA	Bovine serum albumin
CD	Cluster of differentiation
CLN	Cervical lymph nodes
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
D-NALT	Diffuse nasal-associated lymphoid tissue
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
FAE	Follicle associate epithelium
FCS	Foetal calf serum
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissue
GC	Germinal centre
GM	Monosialoganglioside
HEV	High endothelial venule
i.n.	Intra-nasal
i.p.	Intra-peritoneal
IEL	Intra-epithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin
IMDM	Iscove's modified Dulbecco's medium
ISCOM	Immunostimulating complex
KO	Knockout
LP	Lamina Propria
LPS	Lipopolysaccharide
MALT	Mucosal-associated lymphoid tissue
MEM	Minimum essential medium
MHC	Major histocompatability complex
MLN	Mediastinal lymph node
NALT	Nasal-associated lymphoid tissue
O_2	Oxygen
O-NALT	Organised nasal-associated lymphoid tissue
PBS	Phosphate buffered saline
PE	Phycoerythrin
PP	Peyers patches
RT	Room temperature
SCID	Severe combined immunodeficiency syndrome
TCR	T cell receptor

TGF	Transforming growth factor
Th	T-helper
TNF	Tumour necrosis factor
v/v	Volume per volume
w/v	Weight per volume

Chapter One: Introduction

The World Health Organisation (WHO) estimates that of the 12.2 million deaths of children under 5 years old worldwide, one third are as a direct result of lower respiratory tract infections (Garenne et al., 1992).

1.1 Epidemiology of Respiratory Syncytial Virus

RSV is the leading cause of severe viral respiratory disease in infants and children (Glezen and Denny, 1973) and accounts for approximately 50% of all pneumonias and up to 90% of all of the reported cases of bronchiolitis in infancy (Chanock and Parrott, 1965; Glezen, 1987). Two thirds of infants become infected with RSV in their first year of life and of these, nearly a third develop lower respiratory tract disease symptoms. More than one in fifty cases develop symptoms severe enough to require hospitalisation and, amongst these children, the mortality rate is high with one in every thousand dying as a result of RSV infection and its ensuing pathology (Holberg et al., 1991; Parrott et al., 1973). By the time infants have reached two years of age, almost all will have been infected with RSV and nearly half will have been infected twice (Henderson et al., 1979) demonstrating how prevalent RSV infection is in the very young.

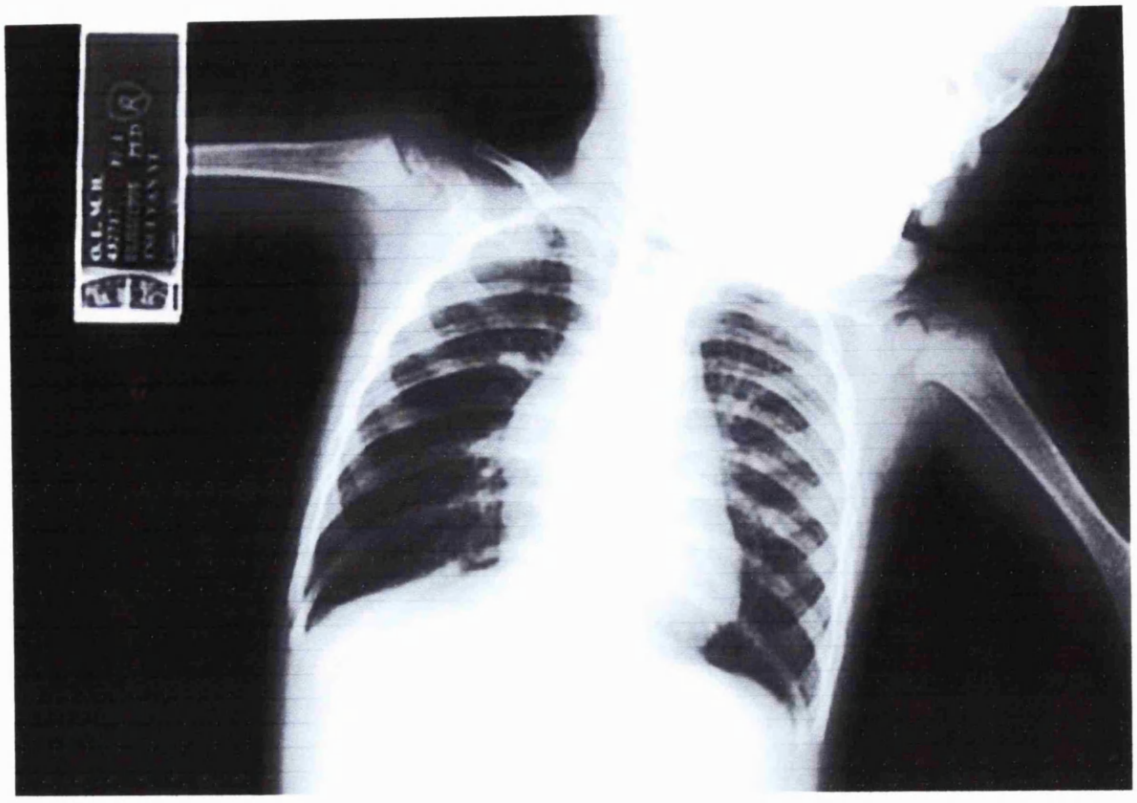


Figure 1.1: A chest X-ray of a child with acute bronchiolitis due to respiratory syncytial virus infection.

Taken from http://www.info.gov.hk/dh/diseases/CD/RSV.htm (Hong Kong Department of Health, Disease Prevention and Control Division).

The less serious socio-economic consequences of RSV infection are clear. In the United States alone RSV infection is responsible for the hospitalisation of an estimated 50,000-80,000 infants each year (Shay et al., 1999) at a projected cost of $365-585 million per annum (data correct as of 2001) (Stang et al., 2001). Classical epidemiological studies in Washington have demonstrated that RSV infections spread rapidly, possibly due to a prolonged period of shedding combined with high titres of virus in the nasal secretions. It was also noted that half of all infants will contract RSV during the first epidemic encountered in their community (Hall et al., 1976; Parrott et al., 1973). This finding was mirrored in the United Kingdom. During a six year study in Newcastle hospitals five consecutive RSV epidemics were monitored. They showed that one in every fifty live

births were admitted to hospital with RSV infection, which was a much higher incidence than was the case with influenza virus where the ratio was between one in one-hundred to one in five-hundred (Martin et al., 1978).

Although these data pertain to the developed countries, it is not exclusive to them. Studies on acute respiratory tract infections in children under 5 years of age from developing countries (Argentina, Colombia, Guatemala, Kenya, Nigeria, Pakistan, Papua New Guinea, the Philippines, Thailand and Uruguay) demonstrate that RSV is also the most frequent cause of lower respiratory tract infections in these countries, accounting for 70% of all patient cases (Selwyn, 1990).

However, in addition to the very young, adults with underlying chronic conditions or immunosuppresion are susceptible to RSV induced pathology (Hall et al., 1986; Harrington et al., 1992). Nosocomial infections are a prime example of how RSV can be potentially lethal; in the environment of a bone marrow transplant ward RSV presents a serious threat to life (Khushalani et al., 2001) and, once pneumonia develops, the chance of patients dying significantly increases (Harrington et al., 1992).

It is not just the very young and immunocompromised that are susceptible to the effects of RSV. As medical treatments improve and life expectancy increases, the expanding elderly population is also at risk from this virus. In fact it is currently the leading cause of respiratory disease within this community (Falsey et al., 1995) and, as such, presents a far more serious risk to life than influenza virus. This was confirmed during a study performed on hospitalised elderly patients. Here, adults aged 65 years or older, presenting acute cardiopulmonary or influenza like symptoms, were assessed. Of 1580 patients, 10% were diagnosed with RSV and 11% with influenza A virus. The death

rates however, were less similar with 10% of the RSV patients dying as opposed to only 6% of the influenza A virus patients (Falsey et al., 1995). Indeed, for many years infection with influenza virus has overshadowed infection with RSV so that the effect of RSV on the population has been obscured (Nicholson, 1996). In fact, from 1975 to 1990 it is estimated, from statistical modelling, that the impact of RSV infection was much greater than that of influenza (Nicholson, 1996). To further emphasise these results it has been shown that excess morbidity and mortality in persons aged 65 or over peaks when RSV activity is highest in the community as judged by viral isolates recovered from children (Fleming and Cross, 1993). The significance of the effect of RSV on the elderly and immunocompromised cannot be undervalued as it is estimated that the economic burden of RSV infection in the USA during 1999 for older adults was $150-$160 million for pneumonia alone and, if chronic conditions are included, could be far higher (Han et al., 1999).

1.1.1 Incidence of RSV infections

RSV exists as distinct serotypes, in humans the major serogroups are A and B and comprise 6 major genotypes of virus that can be isolated throughout the world (Cane and Pringle, 1995). Although both serotypes are infectious, type A appears to be slightly more virulent and tends to be isolated more frequently (Walsh et al., 1997). As with influenza A virus, RSV epidemics occur yearly and are most prevalent during the cold seasons, through winter to spring (**Figure 1.2**), with outbreaks being abrupt in onset and lasting anything up to five months (Hall and Douglas, 1981; Kim et al., 1973a). RSV is able to re-infect individuals very efficiently and it is presumed that the alternation of the viral subgroups during seasons may contribute to this process.

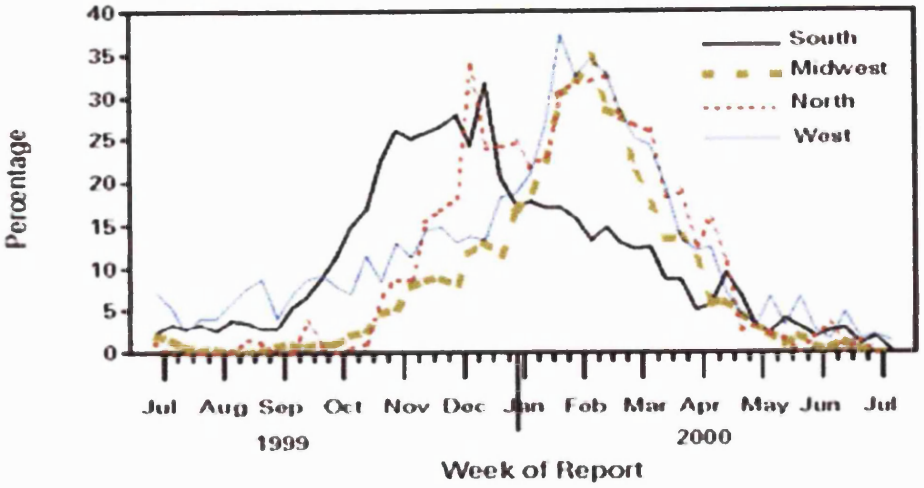


Figure 1.2: Percentage of specimens testing positive for respiratory syncytial virus by region and week of report. United States, July 1999 – July 2000.

Graph taken from:http://www.medscape.com/content/2000/00/41/42/414284/art-mmwr4948.03.fig1.gif (Medscape.com).

1.2 The *Paramyxoviridae*

RSV is an enveloped, non-segmented, negative-sense, single-stranded RNA virus which belongs to the family *Paramyxoviridae* and lies within the order *Mononegavirales*. The *Paramyxoviridae* can be further subdivided into two subfamilies: the *Paramyxovirinae* which comprises Sendai, measles, mumps and Newcastle disease virus; and the *Pneumovirinae* of which RSV is a member.

RSV follows a similar pattern of attachment, penetration, uncoating, replication, assembly and release to other viruses. What unites the *Paramyxoviridae* is that the virions enter the target cell by surface fusion and replicate solely within the cellular cytoplasm. Protection against cellular enzymes is afforded by an RNase resistant helical nucleocapsid which surrounds the viral genome and, once replication and assembly are

complete, progeny virions acquire their lipid envelope during the budding process at the plasma membrane (Fields et al., 2001).

RSV exists as irregular spherical particles of approximately 150-300nm (**Figure 1.3**) that represent the nucleocapsid within a lipid envelope. This envelope is a lipid bilayer derived from the host plasma membrane and contains three virally encoded transmembrane surface glycoproteins (G, F and SH) which are arranged into virion 'spikes' of approximately 11-20nm. Evidence that the infectious particles contain only a single functional copy of the RSV genome comes from the fact that infectivity is lost with single hit kinetics when the particles are exposed to UV light (Dickens et al., 1984). Within the nucleocapsid, which is a symmetrical helix, there are four nucleocapsid proteins, the large polymerase subunit L, the phosphoprotein P, the anti-termination factor M2-1 and the major nucleocapsid protein N. In total eleven genes and their encoded proteins (**Figure 1.4**) have been identified and account for all of the significant open reading frames (ORF's) within the RSV genome and will be briefly discussed now.

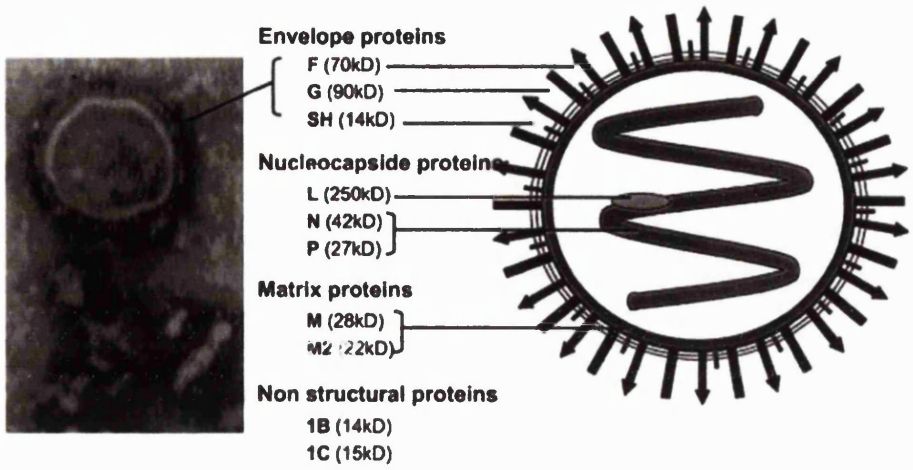


Figure 1.3: Negative contrast electron micrograph (A) and schematic diagram (B) of RSV virion showing the different RSV proteins. Taken from Brandenburg *et al.* (Brandenburg et al., 2001).

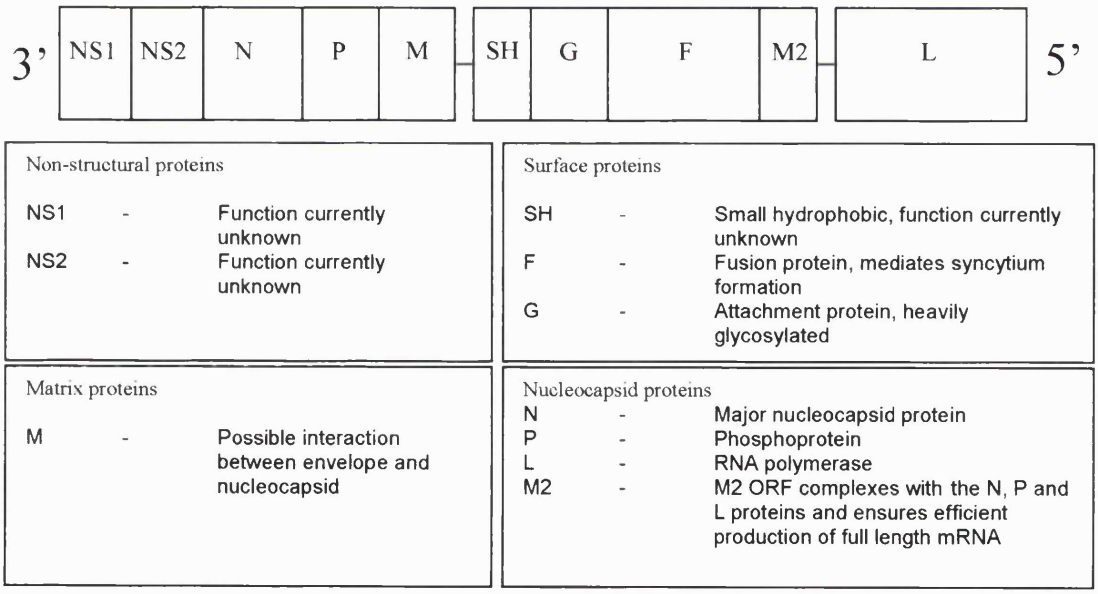


Figure 1.4: Schematic representation of the RSV genome and the functions of the individual proteins (where known).

1.2.1 The fusion (F) protein

The fusion or F protein was initially identified through the observation that selected F-specific monoclonal antibodies were able to inhibit cell-to-cell fusion in tissue culture (Walsh and Hruska, 1983). This ability to fuse cells together leads to the formation of syncytia – a notable form of cytopathic effect – and may serve the virus well as an alternative route of spread via direct contact (**Figure 1.5**). However, it is perceived that the main purpose of this protein is to facilitate viral penetration of the cell via fusion between the virion envelope and the host cell plasma membrane, thereby allowing the nucleocapsid entry into the cytoplasm. In functional experiments RSV F protein has been successfully expressed in Vesicular Stomatitis Virus (VSV) resulting in syncytium formation in cell culture and, more conclusively, affording VSV the ability to enter cells by surface fusion (Kahn et al., 1999; Olmsted et al., 1986).

The F protein is a highly conserved protein amongst the *Paramyxoviridae* family (Collins et al., 2001) and is synthesised as a 67 kDa precursor (F_0) which undergoes proteolytic cleavage to produce two disulfide-linked polypeptides named F_1 (from the C terminus) and F_2 (from the N terminus) (Collins and Mottet, 1991). The part of the F protein that enters the cell membrane is situated at the N terminus of the F_1 polypeptide (hydrophobic fusion peptide), whilst the transmembrane segment is located close to the C terminus. Adjacent to these two regions are two heptad repeat sequences, HR-N and HR-C, that form stable 'trimer-of-hairpins-like' structures that undergo a conformational change to enable the viral and cell membranes to be apposed prior to viral entry (Lopez et al., 1998) (Hacking and Hull, 2002).

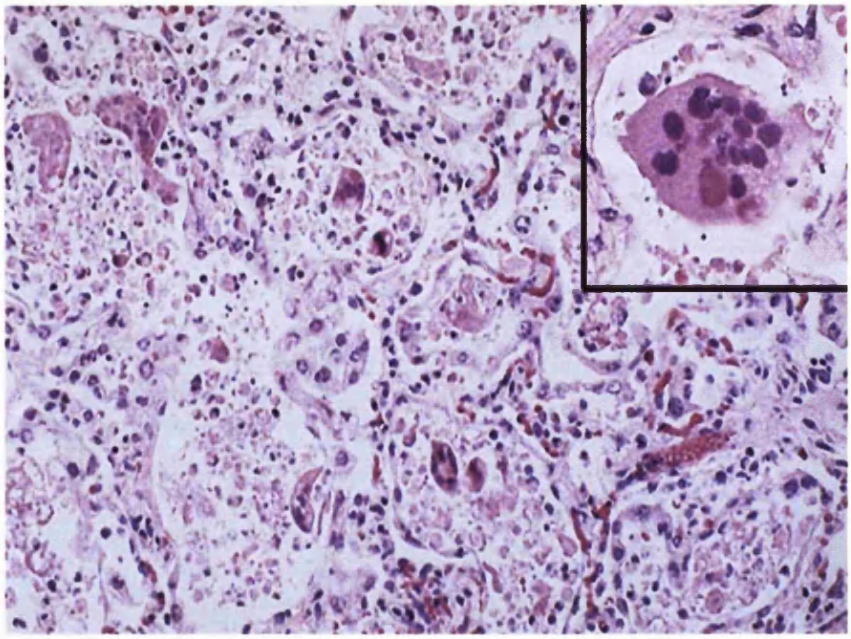


Figure 1.5: Histology of respiratory syncytial virus (RSV) in a child. Note the giant cells which are part of the viral cytopathic effect. The inset demonstrates a typical giant cell with a round, pink intracytoplasmic inclusion.

RSV has an absolute requirement for the F protein (Teng and Collins, 1998) as fusion of the viral envelope or infected cell membranes with uninfected cell membranes is an essential step in the virus life cycle. This process requires the F protein to be intact and is enhanced by the G protein.

1.2.2 The attachment (G) protein

The G glycoprotein was originally identified as the RSV attachment protein through experiments in which the binding of radio-labelled RSV particles to cultured cells was blocked by specific anti-G, but not anti-F antibodies (Levine et al., 1987; Walsh and Hruska, 1983).

The G protein is a glycosylated type II transmembrane protein of 289-299 amino acids depending on the viral strain. It consists of an N terminal cytoplasmic domain, a hydrophobic anchor region and an ectodomain. Structurally, G is a highly glycosylated transmembrane protein (Collins and Mottet, 1992; Wathen et al., 1991) that appears similar in composition to members of the mucin family - host glycoproteins that form a protective barrier on the respiratory, gastro-intestinal and reproductive tracts (Collins et al., 2001). This may suggest that the host immune system does not recognise these glycoproteins as 'foreign' and may therefore lead to some form of immune evasion by the virus, though it must be noted that this is only speculation and has not been investigated. However, it may be feasible to suggest that the attachment protein may play a role in the modulation of the immune response through the release of secreted G from infected cells. Twenty-four hours post-infection, secreted G protein comprises 80% of all G protein released from cells (Collins et al., 2001) and as such may serve as a decoy to trap virus neutralising antibodies, thereby influencing the immune system (Cane, 2001). Recombinant RSV in which the secreted and membrane bound G protein have both been deleted are still able to infect some cell types as efficiently as wild-type virus. This mutant form of the virus has a much lower efficiency of infection in the airways of both humans and mice (Karron et al., 1997; Teng et al., 2001) indicating that the G protein is not essential for cell attachment but acts as an accessory protein which increases the efficiency of the infection process (Teng and Collins, 1998).

1.2.3 The small hydrophobic (SH) Protein

The small hydrophobic (SH) protein is a small integral membrane protein (14kDa) that is attached to the viral envelope by a hydrophobic signal anchor sequence at the C-terminus and is orientated extracellularly (Collins and Mottet, 1993). To date its

function remains unknown with no apparent effect on the efficiency of formation or passage of RSV virus-like particles (Teng and Collins, 1998). When SH is expressed in unison with G and F, there does appear to be some cumulative effect upon the efficiency of syncytium formation, suggesting that SH may enhance the function or functions of one or both of the glycoproteins (Heminway et al., 1994) however the main purpose of SH remains unknown.

1.2.4 The nucleocapsid (N), polymerase (L) and phospho-(P) proteins

The N, P and L viral proteins constitute the viral replicase and are necessary and sufficient for RNA replication, however there is little data available on their individual functions (Collins et al., 1996; Grosfeld et al., 1995; Yu et al., 1995). The N protein (42 kDa) is essential for transcriptional activity and can be co-purified with nucleocapsids where it is tightly bound to RNA protecting it from RNase degradation (Garcia et al., 1994). The large polymerase subunit, L (250 kDa) is an RNA polymerase that contains six discrete segments with highly conserved polymerase motifs (Stec et al., 1991). The phosphoprotein, P (27 kDa) appears to function as a chaperonin for soluble N protein (Fields et al., 2001) and is essential for transcriptional activity (Hacking and Hull, 2002).

1.2.5 The matrix (M) protein

The matrix protein (28 kDa) is a non-glycosylated inner virion protein that plays an important role in the formation of virus-like particles as demonstrated by the inability of M protein deficient particles to passage (Teng and Collins, 1998). In addition it has also been proposed that the M protein renders the nucleocapsid transcriptionally inactive before packaging and mediates the association of the nucleocapsid with the nascent

envelope although neither of these functions has been definitively proven (Collins et al., 1996).

1.2.6 The M2 protein (M2-1 & M2-2)

The M2 gene contains two overlapping open-reading frames (ORF's), ORF 1 and ORF 2 which encode two separate proteins, M2-1 and M2-2 which are 22 kDa and 11 kDa respectively (Collins et al., 1990). M2-1 co-localises with N and P (Fearns et al., 1997; Garcia et al., 1993) and is a transcription anti-termination factor essential for viral viability (Collins et al., 1999a; Collins et al., 1996; Fearns and Collins, 1999; Hardy and Wertz, 1998) ensuring efficient production of full length mRNA (Collins et al., 1996; Hardy and Wertz, 1998). M2-2 has been suggested to function as a negative regulatory factor (Collins et al., 1996; Collins et al., 1990; Hardy and Wertz, 1998) but it is not an essential viral component as the expression of the M2-2 gene can be ablated in recombinant virus with no major detrimental effects (Bermingham and Collins, 1999; Jin et al., 2000a). However, RNA replication in the ΔM2-2 virus is reduced in comparison to wild-type, suggesting that M2-2 does have a role in the regulation of RNA synthesis.

1.2.7 The non-structural proteins, NS1 and NS2

NS1 and NS2 are only detected in trace amounts in preparations of purified virions and have therefore been designated as non-structural. NS1 co-precipitates with the M protein (Evans et al., 1996) and is strongly inhibitory to RNA transcription and replication (Atreya et al., 1998). Although NS2 also inhibits RNA transcription and replication, relatively high levels of expressed protein are required and, in opposition to

NS1, NS2 co-localises with the N but not M protein (Weber et al., 1995). Individual deletion of NS1 and NS2 from recombinant RSV attenuated virus growth *in vivo* and *in vitro* and the simultaneous deletion of both proteins had an additive effect (Buchholz et al., 1999; Teng and Collins, 1999; Teng et al., 2000; Whitehead et al., 1999) confirming that NS1 and NS2 serve a role, either singly or in tandem, in RNA synthesis.

1.3 The structure of the human respiratory tract and lung

The respiratory tract can be separated into two distinct portions; the upper respiratory tract and the lower respiratory tract. The upper respiratory tract contains the pharynx and the nose whilst the lower respiratory tract comprises the larynx, trachea, bronchi and lungs.

1.3.1 The upper respiratory tract

The first anatomical structure encountered by an airborne viral particle is the nose (**Figure 1.6**). This has an internal and external portion and the external cavity is divided centrally by the nasal septum. When air first enters the nostrils it passes through the vestibule which is lined by coarse hairs that filter out large dust particles. Within the internal portion of the nose, the nasal cavity, there are three shelves (formed by projections of the superior, middle and inferior conchae) that subdivide each side of the nasal cavity into a series of groove-like passageways known as the superior, middle and inferior meatuses all of which are lined with mucous membranes. This membrane is comprised of pseudostratified ciliated columnar epithelium which denotes that all the luminal cells secrete mucous or bear cilia upon their surface. The purpose of the mucous is two-fold, firstly it moistens the air in preparation for gas exchange later on

and secondly, it traps foreign particles. These are then moved toward the pharynx via the muco-ciliatory escalator so that they can be eliminated from the respiratory tract by expectoration or swallowing. After the nose, the next structure encountered is the pharynx which is a funnel-shaped tube approximately thirteen centimetres in length. The pharynx follows the conchae, starting at the internal nares and extending down to the cricoid cartilage. It is located posterior to both the nasal and oral cavities and its wall is comprised of skeletal muscle lined with mucous membrane. The pharynx serves as a passageway for food and air and is notable immunologically as it houses the tonsils, which are multiple aggregations of lymphatic nodules. The superior portion of the pharynx, the nasopharynx, lies posterior to the nasal cavity and extends to the plane of the soft palette with the posterior wall containing the pharyngeal tonsil. The nasopharynx receives air from the nasal cavity and also packages of particle laden mucous from the surrounding structures. As with the nasal cavity the nasopharynx contains pseudostratified ciliated columnar epithelium and the associated cilia move the mucous down towards the inferior part of the pharynx. The intermediate portion of the pharynx, the oropharynx, lies posterior to the oral cavity and, due to its opening to the mouth, functions as both a respiratory and digestive passageway. Because of this dual nature the oropharynx is lined with non-keratinised stratified squamous epithelia as a defence against the continual abrasion by food particles. Additionally two further pairs of tonsils, the palatine and lingual, are found within the oropharynx.

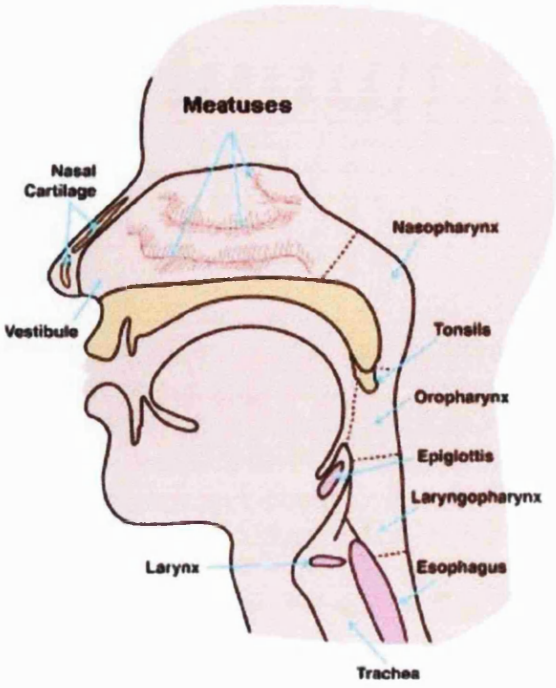


Figure 1.6: The upper respiratory tract.

Image taken from http://www.radiation-scott.org/deposition/respiratory.htm - Bobby R. Scott, Ph.D., Lovelace Respiratory Research Institute.

1.3.2 The lower respiratory tract

The next structure encountered after the pharynx (**Figure 1.7**) is the larynx and is the origination of the lower respiratory tract. This is a short passageway connecting the laryngopharynx with the trachea and, as with the majority of the tissues of the upper respiratory tract, is lined with pseudostratified ciliated columnar epithelium. The role of the mucous here is as a fail-safe for any particles that get through the upper respiratory tract defences and, whereas the cilia in the upper respiratory tract move mucous and trapped particles downwards, the cilia in the lower respiratory tract move them upwards towards the pharynx. The trachea or windpipe continues the respiratory passageway and is a tubular structure about twelve centimetres in length and two and a half centimetres in diameter that is supported by sixteen to twenty C-shaped rings of cartilage. It is

located anterior to the oesophagus and extends from the larynx to the superior border of the fifth thoracic vertebra where it divides into right and left primary bronchi. At this point there is an internal ridge, formed by the posterior and inferior projections of the last tracheal cartilage, called the carina, and here the mucous membrane of this structure is one of the most sensitive areas of the larynx and trachea for triggering a cough reflex, emphasising how important the removal of foreign particles is at this point. The bronchi after the carina are then designated primary bronchi with the left branch leading to the left lung and the right branch leading to the right lung. The right primary bronchus is more vertical, shorter and wider than the left and as a result an aspirated object is more likely to enter the right primary bronchus than the left. Again the bronchi are lined with pseudostratified ciliated columnar epithelium and, on entering the lungs, divide to form smaller secondary (lobar) bronchi, one for each lobe of the lung (three lobes of the right lung, two lobes of the left lung). These secondary bronchi then continue to branch forming smaller tertiary (segmented) bronchi that further divide into bronchioles. As this branching becomes more extensive several changes may be observed; firstly, the epithelium changes gradually from pseudostratified ciliated columnar epithelium in the bronchi to non-ciliated simple cuboidal epithelial in the terminal bronchioles which is more adapted to the functions of absorption and secretion. Secondly, in regions where non-ciliated cuboidal epithelium is present inhaled particles are now removed by macrophages in lieu of the muco-ciliatory escalator.

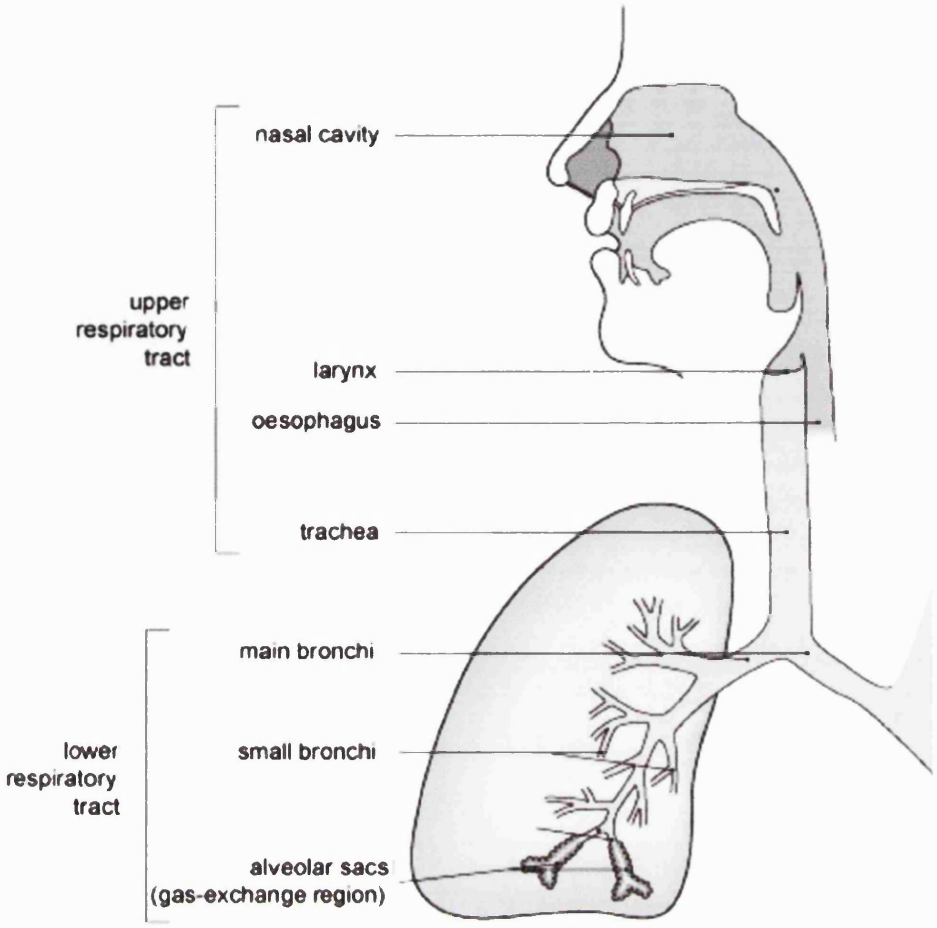


Figure 1.7: The upper and lower respiratory tracts.

Image taken from http://www.bupa.co.uk/wellness/asp/factsheets/lung_cancer.asp.

The final structures of the respiratory tract are the lungs and these extend from the diaphragm to just slightly superior to the clavicles and lie against the ribs anteriorly and posteriorly. Within this structure the secondary bronchioles have divided into tertiary bronchioles with ten per lung, each individually supplying a lung section known as a bronchiopulmonary segment. Within each bronchiopulmonary segment are smaller compartments called lobules and these lobules are wrapped in elastic connective tissue and contain an arteriole, a venule, a lymphatic vessel and a branch from a terminal bronchiole. These terminal bronchioles further subdivide into microscopic respiratory

bronchioles which, in turn, subdivide into alveolar ducts. The alveolar ducts are surrounded by numerous alveoli and alveolar sacs and this is the point at which gaseous exchange occurs. Immunologically, these areas are associated with alveolar macrophages which are avidly phagocytic and influence the activities of both dendritic cell (DC's) and T cells in the lungs. Alveolar macrophages pick up antigen and can leave the alveoli either via the muco-ciliatory escalator or through the alveolar epithelium (Thepen et al., 1994) and will be discussed in more detail later on.

1.3.3 Immune mechanisms of the respiratory tract

With the exception of perhaps the skin the lung is the organ that is subjected to the most exposure to microbial pathogens (**Figure 1.8**) and, as such, any immune response must be highly effective and efficient. However, there is a fine balance between an appropriate and an inappropriate immune response and this can be particularly evident within the respiratory tract, especially when observed in the context of infection with RSV.

As previously described the structure of the respiratory tract is quite extensive and complex in its architecture and this in itself may present many problems especially if inflammatory processes are initiated. To defend itself the respiratory tract has developed a multi-layered defence system that starts with mechanical barriers and then progresses to more complex mechanisms as they are required.

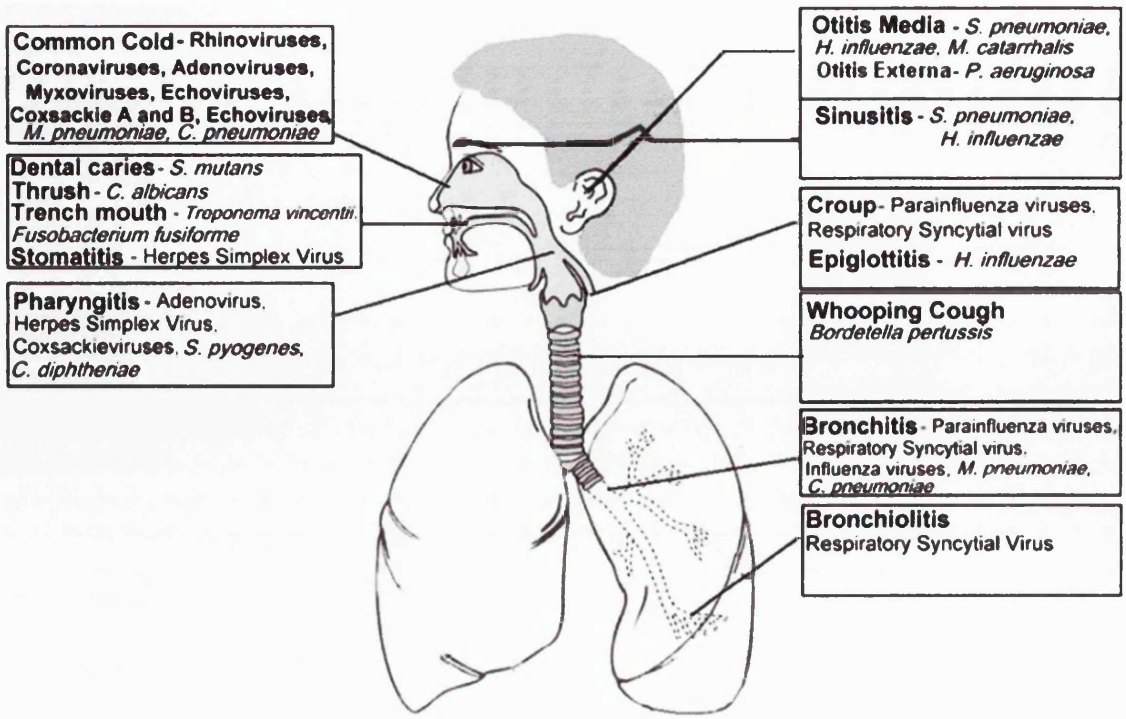


Figure 1.8: Microbiological diseases of the respiratory tract. Image taken from http://www.kcom.edu/faculty/chamberlain/Website/lectures/lectureintrourt.htm. Neal R. Chamberlain, Ph.D.

The first defences a pathogen encounters are mechanical in nature and exist as a form of barrier protection. Here, mucins, of the muco-ciliatory blanket which line the surface of the airways, act by trapping micro-organisms and clearing them via ciliary movement, finally expelling them by expectoration or swallowing when they reach the pharynx. The next level of defence primarily involves a range of soluble mediators both constitutive and inducible that are produced by the cells of the respiratory tract. One of these key defences is airway surface fluid (ASF), a medium that contains several proteins with anti-microbicidal activities. Of particular importance with regard to anti-RSV infection are the collectins. The collectins are lectins that can bind carbohydrates located on the surface of many viruses and bacteria (Crouch et al., 2000; Madsen et al., 2000) in order to act as opsonins. One of the key collectins is SP-A, a pulmonary

surfactant expressed abundantly by epithelial cells that consists of a layer of phospholipids associated with several surfactant proteins (Korfhagen and Whitsett, 1997). Specifically, bronchio-alveolar lavage (BAL) fluid from RSV infected infants has been shown to contain decreased levels of SP-A (Kerr and Paton, 1999). *In vitro*, SP-A has been shown to neutralise RSV by binding to the F protein (Ghildyal et al., 1999) indicating that it may play a role in the defence of the lungs from RSV. This is a theory reinforced by the fact that SP-A -/- mice have higher viral titres and numbers of inflammatory BAL cells compared to SP-A +/+ controls (LeVine et al., 1999).

1.3.4 Neutrophils

Neutrophils represent 93% of the total cell population in the upper airway and up to 76% of the total cell population in the lower airway during infant RSV bronchiolitis (Everard et al., 1994). Neutrophil inflammation is dependent on IL-8 produced by macrophages and respiratory epithelial cells during RSV infection (Wang and Forsyth, 2000). IL-8 expression is rapidly elevated soon after RSV exposure, demonstrating that the inflammatory response is initiated even before RSV infection has become established (Fiedler et al., 1995). *In vitro* studies have shown that, once recruited, neutrophils rapidly adhere to RSV infected cells through epithelial ICAM-1 dependent processes. They then become active and augment tissue damage through the detachment of epithelial cells infected with the virus (Wang and Forsyth, 2000; Wang et al., 1998). Once at the site of infection, the cytotoxic effect of neutrophils is maximised by their retention at that site. The longer the infection persists, the greater the neutrophil recruitment into the airways, thus infants with delayed adaptive responses who are slow to clear virus are potentially subjected to increased neutrophil recruitment and

degranulation and the associated consequences of epithelial cell damage that follows (McNamara and Smyth, 2002).

1.3.5 Alveolar macrophages

Macrophages are formed when monocytes migrate into tissue. Alveolar macrophages, found within the alveolar ducts, are the predominant mononuclear cells in the airspaces of the lung, usually exceeding lymphocytes by a ratio of 10:1. Along with respiratory epithelial cells alveolar macrophages are the first cells to encounter RSV in the airways (Kimpen, 2001).

Alveolar macrophages themselves are susceptible to RSV infection although the infection is abortive after a few rounds of replication (Becker et al., 1991). *In vitro* studies with RSV-infected alveolar macrophages show that they respond to infection by secreting cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α) (Becker et al., 1991; Panuska et al., 1995). These inflammatory cytokines increase vascular permeability and the activation and recruitment of lymphocytes, neutrophils, NK cells and, potentially, eosinophils to the site of infection (Olszewska-Pazdrak et al., 1998). Additionally, studies both *in vivo* and *in vitro* demonstrate that alveolar macrophages co-express RSV specific proteins and HLA-DR and therefore may function as effective APC's potentially regulating the local immune response or lung injury due to this virus (Midulla et al., 1993). With regard to the infant or neonatal immune system it has been noted that the inflammatory and immunoregulatory cytokines such as IL-6 and TNF-α, are produced by neonatal, as well as previously primed, adult macrophages, however neonatal macrophages appear to produce these cytokines less efficiently than adults (Matsuda et al., 1996). Additionally, decreased apoptosis of RSV infected monocytes

was observed in cord and adult samples but the effect was more pronounced in the neonatal cells (Krilov et al., 2000) suggesting that RSV may be equipped with some mechanism to prevent apoptosis which may be a cause of the more severe disease seen in infants.

1.3.6 Eosinophils

Eosinophils are bone marrow derived granulocytic leukocytes that are specialised to release toxic mediators in IgE-mediated immune responses. As such they are usually only associated with the innate response to helminthic parasites (Janeway, 2001) with only very small numbers of these cells present in the circulation; the majority of eosinophils being resident in tissues, especially in the respiratory, gut and urogenital sub-epithelium.

Several groups have demonstrated that, during the course of an RSV infection, eosinophils are recruited to and degranulate within the airway mucosa (Domachowske and Rosenberg, 1999; Garofalo et al., 1992; Sigurs et al., 1994). This evidence for this has been reinforced by the post-mortem findings in children who were immunised with an ill-fated experimental RSV vaccine in the 1960's (Kim et al., 1969). In addition, significantly higher concentrations of eosinophilic cationic protein (ECP) have been shown to be present in the nasopharyngeal secretions of children with bronchiolitis compared to children whose symptoms were restricted to the upper respiratory tract (Garofalo et al., 1992). RSV infected respiratory epithelial cells secrete a number of cytokines, chemokines and growth factors; in particular, epithelial derived RANTES provides a strong chemotactic stimulus for eosinophils *in vitro* (Becker and Soukup, 1999; Olszewska-Pazdrak et al., 1998; Sigurs et al., 1994). In addition, eosinophils are

susceptible to infection by RSV (Kimpen et al., 1996) and can induce the production of oxygen radicals to prime eosinophils for enhanced superoxide generation and leukotriene C4 release as well as inducing transcriptional events that lead to the production of RANTES and MIP-1α (Kimpen et al., 1992; Olszewska-Pazdrak et al., 1998). Thus, by providing an appropriate range of inflammatory mediators, RSV infection of epithelial cells can activate eosinophils and uninfected epithelial cells, further triggering degranulation. This could suggest that the RSV induced inflammatory process initiated by epithelial infection but is then amplified via the secretion of chemokines that recruit eosinophils.

Eosinophils can mediate the destruction of RSV virions *in vitro* (Domachowske et al., 1998) and since Weller *et al.*, have suggested that human eosinophils may be capable of processing and presenting virions to T cells (Weller et al., 1993), it may be possible that the interaction of eosinophils with RSV may contribute to the immune pathology of inflammatory damage that is observed within the airway mucosa (Garofalo and Haeberle, 2000).

1.3.7 NK cells

Natural killer (NK) cells are large lymphoid cells with prominent intracellular granules that make up a small fraction of peripheral blood lymphoid cells. These cells bear no known antigen specific receptors but are able to recognise and kill a limited range of abnormal cells. They are important effectors in the innate immune response mediating significant levels of cytotoxicity that are activated whenever viral interferon (α/β) is induced (Garofalo and Haeberle, 2000) however it is not clear what the role of NK cells is within the context of RSV infection. What may be significant is that infection of the

airway by RSV is a potent stimulus for IFN-β production and this is known to stimulate autocrine upregulation of MHC I by these cells (Garofalo et al., 1996). Studies in mice have shown that the majority of lymphocytes recovered from the bronchio-alveolar lavage (BAL) fluid during the first days of primary RSV infection have the phenotypic characteristics of NK cells and are responsible for much of the IFN-γ produced at this time (Hussell and Openshaw, 1998). NK cells are activated by IFN-β, IL-15, MCP-1,2,3, IL-12, MIP-1α, RANTES, TNF-α and MIP-1β (Biron et al., 1999) which are found in the RSV infected lung (Becker and Soukup, 1999; Kimpen, 2001; Sorkness et al., 2000) and IL-12 and TNF-α in particular, synergistically upregulate IFN-γ production by NK cells (Janeway, 2001).

1.4 The adaptive immune response

The adaptive immune response is the response of antigen-specific lymphocytes to antigen, including the development of immunological memory. Adaptive immune responses are generated by the clonal selection of lymphocytes and are distinct from innate and non-adaptive phases of immunity which are not mediated by clonal selection of antigen-specific lymphocytes (Janeway, 2001).

The common lymphoid progenitor gives rise to lymphocytes. There are two major types of lymphocyte: B lymphocytes (B cells) and T lymphocytes (T cells) both of which will be discussed in this chapter.

1.4.1 T cell responses to RSV

The field of T cell research following RSV infection has been fraught with difficulty. Contradictory findings and opposing theories have slowed the development of our knowledge of the mechanisms of the T cell response to natural RSV infection significantly and, at present, there are still many unanswered questions regarding the role of T cells within the disease pathogenesis of RSV.

Whereas antibodies are currently thought to function in a protective role, T cell responses have been demonstrated to be essential for clearance of the virus once infection has become established (Bangham et al., 1985; Bangham and McMichael, 1986). In normal children viral excretion ceases within one to three weeks, whereas children who are immunodeficient in their cellular response may shed for prolonged periods of time (Fishaut et al., 1980). The fact that RSV infection in mice can be aborted by the adoptive transfer of RSV-specific $CD4^+$ and $CD8^+$ T cells further emphasises the role of T cells in the complete resolution of infection. As expected, depletion of $CD4^+$ and $CD8^+$ T cell subsets from these mice prolongs the period of RSV replication but also appears to alleviate the symptoms of RSV illness suggesting that the host immune response, rather than viral cytocidal effect, is the primary determinant of RSV disease in mice (Graham et al., 1991c). From this Graham *et al.* postulated that antibody may be an illness sparing mechanism for protecting mice from RSV and that T-lymphocytes are an important determinant of illness (Graham et al., 1991c).

1.4.2 $CD4^+$ T cells

In contrast to their protective role, passively transferred $CD4^+$ and, to a lesser extent, $CD8^+$ T cells also appear to augment pathology in RSV infected mice (Alwan et al., 1992; Cannon et al., 1988). Studies using the BALB/c model have demonstrated that $CD4^+$ T cells can prove detrimental to the host by mediating immunopathology and exacerbating disease (Alwan and Openshaw, 1993; Alwan et al., 1992; Graham et al., 1991c). Mirroring an ill-fated 1960's RSV vaccine trial in children, vaccination of rodents with formalin-inactivated RSV (FI-RSV) preparations resulted in T cell-mediated enhanced lung pathology upon subsequent challenge with wild-type virus (Connors et al., 1992; Tang and Graham, 1994; Waris et al., 1996). This pathology is characterised by an infiltrate of eosinophils and correlates with a Th2 immune response (Connors et al., 1992; Waris et al., 1996) the effects of which could be abrogated by simultaneous administration of IL-4 and IL-10 specific mAbs but not IFN-γ or IL-2 specific mAbs (Connors et al., 1994). Depletion of $CD4^+$ T cells from these mice completely eliminated pulmonary histopathology whereas depletion of $CD8^+$ T cells had only a modest effect (Connors et al., 1992).

The enhanced pulmonary pathology exhibited by FI-RSV described above can also be stimulated by priming BALB/c mice with a recombinant vaccinia virus expressing RSV G protein (Graham et al., 2000; Openshaw et al., 1992). The development of this pathology requires the presence of either the G and/or SH protein in the challenge virus suggesting that the G and/or SH proteins have important effects on the inflammatory and innate response to RSV infection (Tripp et al., 1999). Similarly, mice primed with vaccinia virus expressing RSV F or M2 proteins develop extensive pulmonary inflammation, characteristic of a memory T cell response, but without lung eosinophilia

following RSV challenge (Openshaw et al., 1992). These results can be explained in part by the fact that the G protein does not generate a detectable MHC class I restricted $CD8^+$ T cell response but does induce a strong memory G protein specific $CD4^+$ T cell response in the lungs that is characterised by a mixture of Th1 and Th2 cytokine secreting cells (Graham et al., 2000; Srikiatkhachorn and Braciale, 1997a; Srikiatkhachorn and Braciale, 1997b; Srikiatkhachorn et al., 1999). This is in contrast to the effects seen after priming with the F protein where MHC class I restricted CTL are generated, as are Th1, but not Th2, cytokine producing $CD4^+$ T cells (Srikiatkhachorn and Braciale, 1997b). Thus, observations have been made that suggest that the lung pathology and pulmonary eosinophilia induced by either FI-RSV or recombinant vaccinia virus G immunisation may result from the selective priming of virus specific $CD4^+$ T cells and as such implicates Th2 production as an important factor in the disease process.

Interestingly approximately half of the $CD4^+$ T cells infiltrating the lungs of mice primed with the RSV G protein utilise a single Vβ gene (Vβ14) with remarkably limited CDR3 diversity. Deletion of these Vβ14 bearing $CD4^+$ T cells *in vivo* abolishes the Th2-like pulmonary pathology (Varga et al., 2001) suggesting that a novel subset of $CD4^+$ T cells may be crucial in the development of pathology during human RSV infection. Furthermore the balance of $CD4^+$ Th1 and Th2 subsets may be key in determining whether pathology occurs, with Th1 $CD4^+$ T cell responses generally favouring enhanced viral clearance and Th2 $CD4^+$ T cell responses prolonging virus replication and, in some cases, leading to enhanced immunopathology (Alwan et al., 1994; Fischer et al., 1997; Graham et al., 1991c; Graham et al., 1994; Maloy et al., 2000; Tang and Graham, 1997).

As immunisation with RSV G protein or FI-RSV results in similar disease profiles following RSV infection it has been proposed that G epitopes are responsible for vaccine enhanced disease, a theory that is reinforced by the identification of a G protein epitope associated with eosinophil induction (Sparer et al., 1998). However, the epitope in question is a component of wild-type RSV that is known to induce Th1 responses (Graham et al., 2000) and this would seem to contradict all the data so far that points towards the association of the G protein with Th2-biased responses. Other studies however have shown that it is possible to elicit both Th1 and Th2 responses from this same epitope on the G protein which implies that MHC-ligand complexes derived from RSV G protein do not direct the differentiation of $CD4^+$ T-lymphocytes towards a particular Th1 or Th2 phenotype (Srikiatkhachorn et al., 1999). Work by Varga *et al.* (2000) has recently demonstrated that the G protein specific memory $CD4^+$ T cell response in the BALB/c mouse is in fact directed against a single $I\text{-}E^d$ restricted immunodominant epitope and that this single epitope elicits a memory response yielding both Th1 and Th2 effector $CD4^+$ T cells (Varga et al., 2000).

Although most of the studies described above suggest that sensitisation to RSV G protein induces a predominantly Th2 response, recent evidence shows that the G specific memory $CD4^+$ T cell response in G sensitised mice challenged with RSV is mainly comprised of IFN-γ secreting Th1 $CD4^+$ T cells (Varga et al., 2001; Varga et al., 2000). What is not clear however is how a small subset of $CD4^+$ T cells specific to a single dominant epitope within the G protein can develop into Th2 cytokine secreting $CD4^+$ T cells despite the presence of high numbers of IFN-γ secreting $CD4^+$ T cells. This data is supported by analyses of cytokine production by RSV stimulated T cell cultures (established from peripheral blood mononuclear cells) that show that, regardless of clinical severity, the responses are dominated by the production of IFN-γ

and that only low levels of IL-4 and IL-10 are detectable (Brandenburg et al., 2000). Collectively this data refutes an association between clinical severity and Th2-like T cell responses.

1.4.3 $CD8^+$ T cells

In murine models, $CD8^+$ T cells have been demonstrated to play a pivotal role in recovery from RSV infection (Alwan et al., 1992; Cannon et al., 1988). In the BALB/c mouse model, clearance of RSV is associated with a temporal increase in the number of virus-specific $CD8^+$ CTL's (Anderson et al., 1990) and, crucially, these cells appear to be essential to eliminate the virus completely (Ottolini et al., 1999). Regarding the major target antigens, it appears that the bulk of the class I restricted CTL responses are directed against a single immunodominant, H-$2K^d$-restricted peptide epitope found within the matrix 2 (M2) protein of RSV ($M2_{82\text{-}90}$) (Kulkarni et al., 1993a). This epitope is protective (Kulkarni et al., 1993b) and appears to be essential for host resistance to RSV infection but, recently, the protective efficacy of the CTL generated has been at the centre of some dispute. A recent study of the $CD8^+$ T cell response during RSV infection by Chang *et al.* (2002) demonstrated that the M2-specific $CD8^+$ T cells within the RSV-infected lung appear to have impaired effector activity including cytokine production and *ex vivo* cytolytic activity (Chang and Braciale, 2002), suggesting that RSV may suppress the host response to infection resulting in the impairment of immune memory. However, these findings were contradicted by a paper by Ostler and Ehl published some four months later (Ostler and Ehl, 2002), their data suggesting that the suppression described by Chang *et al.* does not take place. These opposing findings may be partially explained by the differing experimental methods used by both groups, especially in the techniques used to isolate the cells but this incident is not an isolated

one and demonstrates the inherent difficulties that are commonplace with RSV investigation, re-enforcing the need for further detailed study into the complex mechanisms of this virus.

In humans, it has been shown that RSV-specific HLA class I restricted $CD8^+$ CTL are present in the circulation (Bangham and McMichael, 1986; Bangham et al., 1986; Cannon and Bangham, 1989) and, as discussed above, it has been demonstrated that they are essential for the complete resolution of RSV infection. Several recent studies have demonstrated that $CD8^+$ T cells play an important role in directing the type of inflammatory infiltrate in the lungs of G protein sensitised mice that have been challenged with wild-type RSV. In mice where Th2-like G protein specific $CD4^+$ T cells were adoptively transferred in order to elicit pulmonary pathology, co-transfer of M2 specific $CD8^+$ T cells resulted in reduction of pulmonary eosinophilia (Alwan et al., 1994). Similarly, the insertion of an MHC class I restricted $CD8^+$ T cell epitope (derived from M2) into the G protein used to drive a memory $CD4^+$ T cell response, prevented the development of pulmonary eosinophilia following sensitisation and RSV challenge infection (Srikiatkhachorn and Braciale, 1997a). Deletion of $CD8^+$ T cells in F sensitised mice leads to the development of pulmonary eosinophilia following live RSV challenge infection and data from additional studies suggests that IFN-γ production by these $CD8^+$ T cells is required to prevent the induction of pulmonary eosinophilia by the F-specific $CD4^+$ T cells (Hussell et al., 1997). However, at present it is still unclear whether the IFN-γ produced by the $CD8^+$ T cells is having a direct effect on the differentiation of the F specific $CD4^+$ T cells or whether the IFN-γ is acting through another intermediate cell type that interacts with the $CD4^+$ T cells. As mentioned above it is still unknown why the high level of IFN-γ produced by G-specific

$CD4^+$ T cell effectors during challenge does not down-modulate the production of Th2 cytokines.

1.5 B cells, their subsets and functions

1.5.1 Follicular (Fo) or B-2 B cells

Follicular B cells (sometimes referred to as conventional or B-2 B cells) possess surface immunoglobulin that serves as an antigen receptor and plays two roles in their activation. Firstly it transmits signals directly to the cells interior when it binds antigen and, secondly, it delivers the antigen to intracellular sites where it is first degraded and then returned to the cell surface as peptides bound to MHC class II molecules (Janeway, 2001). The peptide:MHC class II complex can then be recognised by antigen specific $CD4^+$ T cells (Gray et al., 1986; MacLennan et al., 1990), triggering them to make molecules that cause the B cell to proliferate and its progeny to differentiate into antibody-secreting cells. Once established, these responses can run for an indefinite period without the need for recruitment of further B cells (Askonas et al., 1970; Gray et al., 1986; MacLennan et al., 1990) leading to the generation of memory and plasma cells. This process is referred to as a thymus dependent (TD) response but is not the only manner in which B cells can be activated. Certain antigens can activate B cells directly in the absence of T cell help and provide a means whereby antibodies can be produced rapidly (a thymus independent or TI response), however the changes in functional properties of antibody molecules that result from isotype switching, and the changes in the variable region of the B cell receptor (BCR) that occur during affinity maturation depend upon the interaction of antigen-stimulated B cells with $CD4^+$ T-cells and other cells in the peripheral lymphoid organs.

1.5.2 The germinal centre (GC) reaction

Both memory and plasma B cells are generated during the germinal centre (GC) reaction which is induced in secondary lymphoid tissues following immunisation with thymus-dependent (TD) antigens. Germinal centres themselves are highly complex cellular structures that encompass oligoclonal populations of antigen-specific T and B cells as well as antigen harbouring follicular dendritic cells (FDC's) and tingible body macrophages. Nascent GC's form after activated T and B cells migrate to the dendrites of the FDC which can be found clustered in lymphoid follicles. The FDC themselves do not process antigen but merely present them on their surface in the form of immune complexes - this display is essential as it is the driving force behind the GC response.

As the reaction ensues, the recently arrived GC lymphocytes proliferate considerably and begin to fill the reticular spaces of the FDC before acquiring new phenotypes and physiologies. Within twelve hours of intravenous challenge the production of switch transcripts can be detected (Toellner et al., 1996), these are by-products of B cells that have been specifically activated by T cells and give an indication of how rapidly the reaction in the GC occurs. It is during this time that the B cells acquire the affinity-enhancing point mutations within the variable regions of their immunoglobulin genes that serve to further augment the immune response and which are characteristic of memory and plasma B cells (Kelsoe, 1996). The continual accumulation of these nucleotide substitutions reflects the repeated cycles of proliferation, mutation and affinity selection that occur within the GC (**Figure 1.9**) and, if these actions result in a non-productive B cell, they are quickly directed down an apoptotic pathway and are efficiently dealt with by the resident macrophages. Alternatively, those B cells with an

affinity-matured receptor that recognises FDC-held antigen with high affinity are not directed down this pathway and are allowed to leave the germinal centres. If the GC reaction is interrupted or hindered in any way then there is significant reduction (if not loss) of the secondary antibody response and an impaired affinity maturation of serum antibody (Ahmed and Gray, 1996). The cells that survive the GC reaction and successfully exit the GC symbolise at least two different differentiation pathways that each share a common feature in that they may both produce long-lived cell populations.

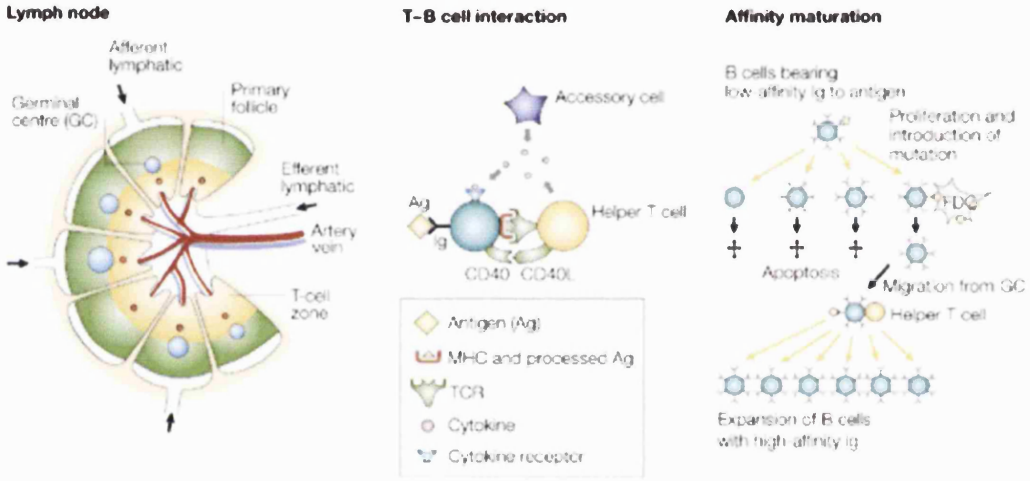


Figure 1.9: The generation of B cells with high affinity immunoglobulin via affinity maturation. Image taken from Kinoshita and Honjo, 2001 (Kinoshita and Honjo, 2001).

1.5.3 Plasma cells

In essence, plasma cells are cellular factories, devoted entirely to the production and export of a single product; soluble immunoglobulin (Ig). They are critical for the effective function of the adaptive immune system and are responsible for the majority of the antibody produced in response to infection. Because they are terminally differentiated, end-stage cells, they no longer divide and their lifespan can vary from

anything up to a few days to many months (Ehrich et al., 1949; Ho et al., 1986; Makela and Nossal, 1962; Schooley, 1961; Slifka et al., 1995). This variation in lifespan appears to be determined in part by their developmental history, their homing capacity and the ability of the spleen to support them (Calame, 2001; Sze et al., 2000).

Following the germinal centre reaction, centroblasts (clonally dividing antigen stimulated B cells) that have been selected to become plasma cells leave the GC and undergo terminal differentiation to become plasma cells that secrete high affinity antibodies. After terminal differentiation there are many phenotypic changes as numerous B cell specific surface proteins are down-regulated, including major histocompatability complex (MHC) class II, B220, CD19, CD21 and CD22. Following their exit from the GC, some plasma cells will home to the bone marrow where they will receive survival signals from stromal cells (Merville et al., 1996) whilst others will recirculate and migrate to the lymph nodes or the lamina propria of mucosal tissues. The plasma cells found within the bone marrow are responsible for the maintenance of the elevated levels of serum antibody found in immune animals and can be responsible for up to 90% of all IgG production (Janeway, 2001).

1.5.4 Memory B cells

The alternative fate of B cells leaving the germinal centre is to become memory B cells. These are small, resting B cells that can be recovered from both the blood and peripheral lymphoid tissues (Kelsoe, 2000). These cells do not secrete antibody during the primary response but are biased to become plasma cells upon subsequent challenge with the same antigen. In response to this secondary antigenic stimulation they undergo

a rapid and massive expansion, generating up to ten-fold more plasma cells than during a primary response (McHeyzer-Williams and Ahmed, 1999).

Several studies have commented that FDC's may retain antigen long after the GC response has ended, however this is a controversial issue and the role of this persistent antigen in the maintenance of the B cell memory compartment has been long debated (Manz and Radbruch, 2002). A recent study by Maruyama *et al.*(Maruyama et al., 2000) makes a strong case for the persistence of B cell memory in the absence of antigen but at present there is still not a full consensus of opinion on this matter.

1.5.5 Memory or Plasma?

What determines whether a B cell becomes a plasma cell or a memory cell? As with the issue of how memory is maintained, there is no consensus of opinion and many theories have been put forward. One study suggests that expression of high affinity antibody may favour plasma cell rather than memory cell development (Smith et al., 1997) and yet another study suggests that signals via CD40 favour the memory phenotype whereas the presence of IL-2 and IL-10 and the absence of CD40 ligand act to generate cells of a plasma phenotype (Arpin et al., 1995). At present there does not appear to be a single unifying concept to categorically show how the pathway to memory or plasma cell differentiation is taken.

1.5.6 B-1 B cells

B-1 B cells are a distinct population of B cells found predominantly in the peritoneal cavity. They are seldom found within the spleen (approximately 5%) and are extremely

rare, if present at all, in the lymph nodes (Forster et al., 1991; Hayakawa et al., 1985; Marcos et al., 1989). They possess characteristics that distinguish themselves from the conventional (B-2) population of B cells, being long lived (Hayakawa and Hardy, 1988), refractory to activation through B cell receptor (BCR) ligation (Bikah et al., 1996; Morris and Rothstein, 1993; Rothstein and Kolber, 1988a), induced to proliferate by treatment with phorbol esters (Rothstein and Kolber, 1988b) and, skewed towards reactivity with common bacterial and self-antigens. In addition B-1 cells contain fewer N-insertions than do B-2 cells (Kantor et al., 1997) and do not contain somatic mutations (Forster et al., 1988). Cell transfer studies have demonstrated that B-1 cells have a self-renewal capacity (Hayakawa et al., 1985; Hayakawa et al., 1986; Kantor et al., 1992; Kantor et al., 1995) and that this is an important characteristic that distinguishes B-1 cells from B-2 cells.

B-1 cells can be distinguished from other B cells by surface phenotype, they are CD43 positive, CD23 negative, express high levels of surface IgM and low levels of B220 and IgD ($CD43^+$, IgM^{hi}, $B220^{lo}$ (CD45), IgD^{lo}, $CD23^-$). They are also larger in size than conventional B cells and this is demonstrated during FACS analysis as increased side scatter (Berland and Wortis, 2002). In the peritoneal cavity (but not in the spleen) B-1 cells express low levels of Mac-1 and a subpopulation, B-1a cells, also express intermediate levels of CD5 on their surface (Hayakawa et al., 1983; Herzenberg et al., 1986; Kantor and Herzenberg, 1993). Exit from the peritoneal cavity however, results in the loss of both of these markers (Kantor et al., 1995).

B-1 and B-2 cell subsets differ in their exhibition of different V_H repertoires and Ig specificities (Forster and Rajewsky, 1987; Hayakawa et al., 1984; Lalor and Morahan, 1990; Mercolino et al., 1986; Su et al., 1991; Tarlinton et al., 1988; Tornberg and

Holmberg, 1995) with B-1 cells often recognising self-antigens such as phosphatidylcholine (Hardy et al., 1989; Pennell et al., 1989), immunoglobulin (Rhesus factor) (Burastero et al., 1988; Casali et al., 1987), DNA, membrane proteins on erythrocytes and thymocytes (Hayakawa et al., 1990) and common bacterial antigens such as phosphorylcholine (Kantor and Herzenberg, 1993; Mercolino et al., 1986). Originally B-1 cells were identified by their expression of CD5, however a second population of peritoneal B-1 cells that were $CD5^-$ but otherwise identical in surface phenotype to other B-1 cells, were discovered. It is now widely accepted that $CD5^+$ B-1 cells are referred to as B-1a cells and $CD5^-$ B-1 cells as B-1b cells.

The issue of the origin of B-1 cells is still under debate and has divided opinion between those who claim that B-1 cells derive from different precursors ('the lineage hypothesis') (Hardy and Hayakawa, 1994; Herzenberg, 2000; Kantor and Herzenberg, 1993) and those who are convinced that B-1 cells are not 'born' but are 'made' ('the induced differentiation hypothesis') (Berland and Wortis, 2002; Clarke and Arnold, 1998; Haughton et al., 1993). However, the difference in these opinions appears not to come from conflicting data but rather from their interpretation.

1.5.7 B-1 'natural' antibody

B-1 cells secrete large amounts of IgM, IgG3 and IgA and are considered to be a major player in natural immunity (Hardy, 1992; Herzenberg et al., 1986; Sidman et al., 1986; Tarakhovsky, 1997). Adoptively transferred B-1 cells in irradiated (Herzenberg et al., 1986; Kroese et al., 1989), unmanipulated neonatal (Forster and Rajewsky, 1987) or B cell depleted neonatal mice produce quantities of IgM similar to the levels observed in unmanipulated animals and, based on these studies, B-1 cells are thought to be the

primary source of natural IgM. This is an antibody produced without the presence of an exogenous antigen (Bos et al., 1988; Haury et al., 1997) that is polyreactive, weakly autoreactive and reacts with many pathogen associated carbohydrate antigens. These B-1 antibodies tend to have low affinities and broad specificities (Lalor and Morahan, 1990) which is in direct contrast with the high affinity binding and specificities of conventional B-2 antibodies. Consistent with a major role for B-1 cells in natural IgM production a number of IgM specificities have been identified in the B-1 repertoire. These include specificities for LPS (Su et al., 1991), phosphatidyl choline (PtC) (Cong et al., 1991; Hardy et al., 1989; Hayakawa et al., 1984), thymocytes (Hayakawa et al., 1990) and influenza virus (Baumgarth et al., 1999). It has been suggested that the size of the B-1 compartment is regulated by serum IgM levels (Boes et al., 1998; Ehrenstein et al., 1998) and this has been demonstrated by an increase in the number of B-1 cells in mice engineered so that they express membrane IgM but cannot produce secretory IgM.

Another interesting aspect of B-1 cells is that in uninfected mice you can find natural anti-influenza IgM that is derived exclusively from B-1 cells (Baumgarth et al., 1999). The titres of this antibody remain static and do not increase after infection which is in direct contrast to the B-2 derived IgM antibody titres which increase in response to the infection. Reconstitution experiments demonstrate that resistance to influenza virus infection is dependent on both B-1 and B-2 derived IgM (Baumgarth et al., 2000) and that in the absence of B-1 derived anti-influenza IgM there is a delayed IgG2a response that leads to an increase in mortality. Together this data suggests that natural IgM is necessary for a normal adaptive immune response to influenza virus infection (Baumgarth et al., 2000).

1.6 Antibody responses to RSV

The best studied component of RSV immunity to natural infection is that of the serum antibody response. Most full-term, newborn babies have RSV-specific neutralising antibodies that are obtained via transplacental transfer of maternal antibodies (Beem et al., 1964; Parrott et al., 1973). Initially these antibody titres are comparable to maternal levels but they decline quickly during the first few months of life to unprotective levels (Brandenburg et al., 1997) and, after seven months of age, any detectable neutralising antibody is usually as a result of natural infection (Beem et al., 1964). Natural infection however does not elicit a strong antibody response and, in infants, the antibody titres that develop are usually low. The reason for this is not clear, it may be due to the relative immaturity of the infant immune system or alternatively may be due to interference by maternal antibody (Brandenburg et al., 1997; Murphy et al., 1986a). Further evidence for this phenomenon of suppression by passively transferred antibody has been observed in animal models of RSV infection (Crowe et al., 1995; Murphy et al., 1988).

1.6.1 Maternal antibody

The phenomenon of suppression of antibody responses by maternal antibody has been demonstrated in studies with humans, showing that that the level of maternally derived serum RSV neutralising antibodies is inversely related to the titre of the convalescent RSV complement fixing antibodies (Parrott et al., 1973). In fact, antibody mediated suppression of vaccine candidates of all types is evident in experimental studies where the route of immunisation is parenteral (Belshe et al., 1982; Murphy et al., 1988; Murphy et al., 1991) but these suppressive effects (of passively acquired antibody) can

be partially overcome by the administration of immunogens by the intranasal route (Murphy et al., 1989) or by an increase in the dose of antigen (Murphy et al., 1991). In the light of these observations it has been suggested that passively acquired IgG antibodies may have a decreased ability to suppress the response to infection at mucosal sites (Crowe, 1998). Further confirming that the suppressive effect may be variable is the revelation that high levels of maternal antibodies may also suppress secretory IgA responses in the nasopharyngeal secretions of infants following primary infection (Yamazaki et al., 1994) and that this suppression appears to be to a much lesser degree than is seen with the suppression of IgG antibodies (Tsutsumi et al., 1995). This has also been shown to be the case in murine studies where passive transfer of RSV antibodies prior to primary infection can suppress induction of both systemic and mucosal antibodies of all isotypes (Crowe, 1998). Interestingly, animals immunised in the presence of RSV antibodies present a suppressed primary antibody response but, in response to a second infection, display a much enhanced secondary antibody response, the mechanism for this enhanced immunogenicity is presently unknown (Crowe et al., 1995).

1.6.2 Anti-RSV antibody responses

Humans develop antibodies to most of the RSV proteins but there are no unequivocal correlates of immunity to RSV infection. It was initially thought, from animal models, that antibodies generated against the surface proteins F and G were the most important for protective immunity (Walsh et al., 1987) but studies with RSV protein vaccines suggest that the presence of RSV neutralising antibody is much more important than the absolute titre of anti-F and/or anti-G antibody present in the serum (Tristram et al., 1994; Tristram et al., 1993).

The role of antibody in immunity to RSV infection is unclear. From studies in mice it appears that RSV-specific antibodies do not play a major role in the resolution of primary infection (Graham et al., 1993) (it is thought that $CD8^+$ T cells are responsible for this) although it has been shown that administration of physiological amounts of RSV-specific antibody during the peak of RSV replication can accelerate the resolution of the infection, indicating that antibody may play a role in immunity to infection. After the FI-RSV vaccine debacle the high titres of RSV-specific antibody generated after vaccination were considered to be a factor in the severity of the clinical outcome on subsequent exposure to the wild-type virus (Chin et al., 1969; Murphy et al., 1986b). This concept was strengthened by the fact that most severe RSV disease occurs between six weeks to six months of age when protection from maternal antibody should be highest, suggesting that anti-RSV antibody may contribute to bronchiolitis (Feigin and Cherry, 1998; Stein et al., 1999). However the relative sparing of severe RSV disease in infants less than six weeks of age, and the correlation between cord blood antibody titre and disease severity suggests otherwise and argues against the idea that local or systemic antibody production plays a major part in disease pathogenesis (Feigin and Cherry, 1998). It has now become clear that virus neutralising (VN) antibodies are in fact protective against RSV infection, provided sufficient titres are present (Prince et al., 1985b), and this has been demonstrated in studies where children with higher titres of maternal VN antibody were less likely to develop bronchiolitis than those with lower titres (Glezen et al., 1981; Holberg et al., 1991). In fact serum neutralising titres have been shown to correlate inversely with the risk of primary infection in children and young adults (Glezen et al., 1981; Hall et al., 1991) with the frequency of RSV lower respiratory tract infection being significantly less in children with RSV neutralising antibody titres greater than 1:100 (Law et al., 1997; McNamara and Smyth, 2002). This

data is corroborated by evidence from animal models (Hemming et al., 1985; Prince et al., 1985a; Suffin et al., 1979) (neonatal ferrets, cotton rats and owl monkeys) and also from the fact that intravenous administration of normal gamma globulin does not induce protection against severe RSV infection (Meissner et al., 1993) whereas administration of immune globulin with a high titre of VN antibodies against RSV (RSVIG) decreases the incidence of severe lower respiratory tract involvement in high risk children (Groothuis et al., 1993; Hemming et al., 1995; Siber et al., 1992).

The success of prophylaxis with RSV intravenous immunoglobulin (RSVIG) and the humanised monoclonal antibody Palivizumab (Welliver, 1998) further suggests that anti-RSV antibody is essential to prevent enhanced disease and is highly effective at preventing serious RSV illness in premature children and those with bronchiopulmonary dysplasia (1998).

1.6.3 The humoral immune response to natural infection

In children, natural infection with RSV results in the production of both serum and secretory antibody (McIntosh et al., 1978) and this is true of even the youngest children, although it must be noted that infant titres are reduced in comparison with older children and adults (Stein et al., 1999). During primary infection a classical antibody response is observed, with RSV-specific serum IgM detectable within a few days and IgG appearing in the second week. The serum IgA response in infants however is much more variable and in some cases does not occur (Feigin and Cherry, 1998). Throughout natural infection, infants respond more slowly than older children and adults, their response comprising RSV-specific IgG1 and IgG2 antibody and being biased toward the RSV F protein (Wagner et al., 1989). Also, in addition to serum antibody, RSV-

specific secretory antibody is generated upon primary infection but again, the issue of secretory antibody is controversial with many different groups reporting conflicting findings. What is known though is that during infection with RSV humans mount a secretory response with both secretory IgA and IgG present in the upper and lower respiratory tracts respectively (Shay et al., 1999). The presence of VN antibodies in mucosal secretions correlates well with protection against experimental infection in adults (Mills et al., 1971; Nadal and Ogra, 1990) and the appearance of IgA coincides with clearance of the virus (McIntosh et al., 1978). This is further supported by adult challenge studies with one such study by Mills *et al.* (Mills et al., 1971) finding that although most volunteers continued to shed virus after challenge, those with low pre-challenge nasal neutralising antibody levels shed greater amounts of virus and were more likely to develop upper respiratory tract symptoms. Similar findings were reported in studies by Hall *et al.* (Hall et al., 1991) and Watt *et al.* (Watt et al., 1990) but it must be noted that even though a trend towards a protective effect of nasal antibody was seen in these studies, many volunteers with even the highest anti-viral antibody levels could still be re-infected. It is clear therefore that protection is incomplete and that re-infection, especially of the upper respiratory tract is still possible even in the presence of high titres of VN antibody (Hall et al., 1991).

There are few published studies on RSV infections in the elderly and therefore it is not clear if there are specific groups within this population that are more susceptible (via immune senescence or other age associated factors) than others. What is clear from a recent study however is that the high incidence of RSV-associated morbidity and mortality may not be due to any significant defect in serum humoral immunity (Falsey et al., 1999). The fact that elderly people undergoing infection possess serum antibody

levels comparable to those that found in young healthy adults suggests that serum antibody may not be as important as local secretory antibody or other unknown factors.

1.6.4 Immunoglobulin A

IgA is often known as 'the mucosal antibody' as it is the most abundant immunoglobulin found in secretions (Burnett, 1986; Kerr, 1990). In humans IgA antibody predominates within the upper respiratory tract of the lung and has a ratio with IgG of 2.5:1 (Burnett, 1986). The majority of the IgA antibody in the lung is located in the proximal bronchial tubes but, further down the respiratory tract, within the alveolar spaces, the antibody hierarchy is reversed and IgG supersedes IgA as the dominant antibody isotype (Burnett, 1986; Merrill et al., 1985; Reynolds, 1988). Whereas IgG within the lung is often accepted to have originated from the serum (via passive transudation) there is evidence to suggest that this is not the case with IgA. The fact that the concentration of IgA (adjusted for total protein in the BAL fluid) is greater than that of serum under normal conditions suggests that a large percentage of IgA is produced locally in the lung and that elevated levels of IgA in the lower respiratory tract in response to pathogens are the result of local synthesis and not passive transudation from serum (Burnett, 1986).

Passive-transfer antibody experiments in mice have demonstrated the critical significance of virus-specific IgA in mucosal immunity against virus infection. Polyclonal anti-influenza IgA antibodies purified from the mucosal secretions of infected mice have been shown to protect naïve mice from virus infection when they are administered intranasally before virus challenge (Tamura et al., 1990). Following the intravenous transfer of influenza-specific IgA into naïve mice, polymeric IgA can be

detected in nasal secretions and the mice are protected from further challenge with influenza virus. This protection is specific to IgA though as repeat experiments where IgG antibodies were administered in place of IgA failed to provide protection (Renegar and Small, 1991a). Furthermore it was demonstrated that the protective mucosal immunity induced by influenza virus infection could be abrogated by intranasal administration of anti-IgA antiserum (Renegar and Small, 1991a; Renegar and Small, 1991b). Therefore virus specific IgA antibodies play an important role in the prevention of virus infection and viral clearance on the mucosal surfaces.

1.6.5 IgA1 and IgA2

IgA exists as both a dimer and a monomer (400 kD and 160 kD respectively) and, in humans, there two subclasses of IgA known as IgA1 and IgA2 (**Figure 1.10**). IgA1 accounts for almost 80% of the IgA antibody in the serum whereas in exocrine secretions the ratio of IgA1 to IgA2 is approximately 1:1 (Kerr, 1990). The two subclasses differ by the presence or absence of a thirteen amino-acid hinge region (**Figure 1.10**) (Kerr, 1990). This region, exclusively present in IgA1 has many O-linked glycosylation sites and is a target for at least two families of IgA1 bacterial proteases, the expression of which has been linked to pathogenicity. These proteases are able to cleave the hinge region of the IgA1 subclass with great specificity to produce Fab and Fc fragments (Kilian et al., 1996; Plaut and Bachovchin, 1994; Plaut and Wright, 1995). As these enzymes are produced by limited groups of pathogens that colonise mucous membranes they have been envisioned as virulence factors that promote infection by degrading IgA1 antibodies. The existence of IgA2 would appear to have evolved as a result of selective pressure on the immune system to possess a protease resistant subclass of IgA.

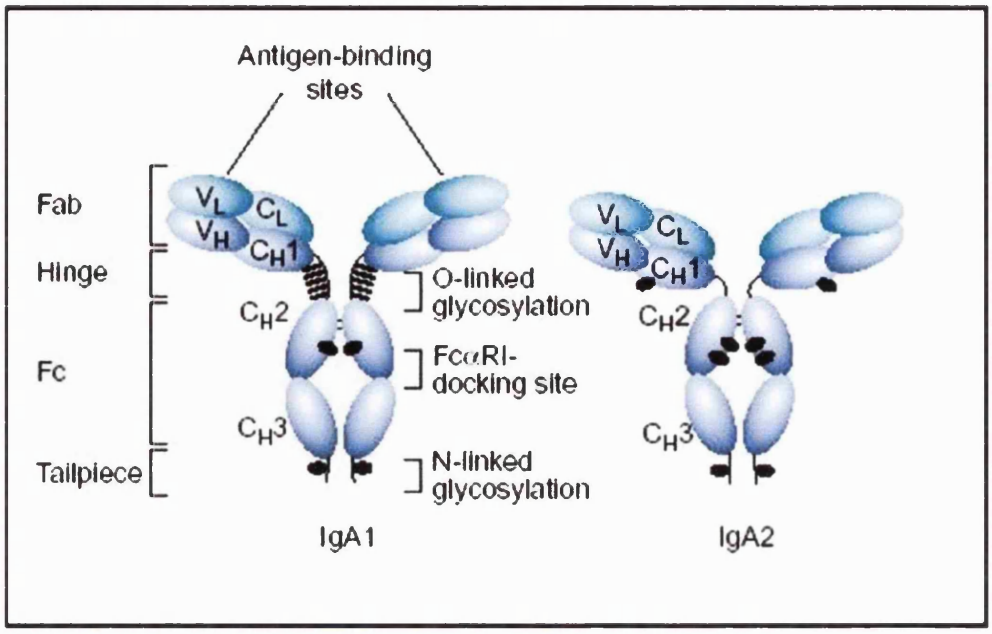


Figure 1.10: IgA isotypes; IgA1 and IgA2.

Diagram adapted from *Trends in Immunology* 2001, Vol 22, No.4, 205-211.)

1.6.6 sIgA and IgA functions in the lung

In most animal species IgA exists in the circulation as a polymer. In human serum however, IgA is predominantly monomeric and constitutes 15-20% of the total immunoglobulin. However, as stated previously, at mucosal sites IgA is the principal antibody class and it is here that IgA is expressed with an adjoining peptide known as the 'J-chain' which stimulates dimerisation (Kerr, 1990). Through binding to the polymeric immunoglobulin receptor (pIgR), expressed on the basolateral surface of mucosal epithelial cells, dimeric IgA can be actively transported through a cell (Mostov, 1994). At the apical membrane, the external domains of pIgR are cleaved off and the complex is released into secretions. The remainder of pIgR that binds covalently to the dimerised IgA is called the secretory component (SC) and the SC-IgA dimer complex is referred to as secretory IgA (sIgA) (**Figure 1.11**) (Kerr, 1990).

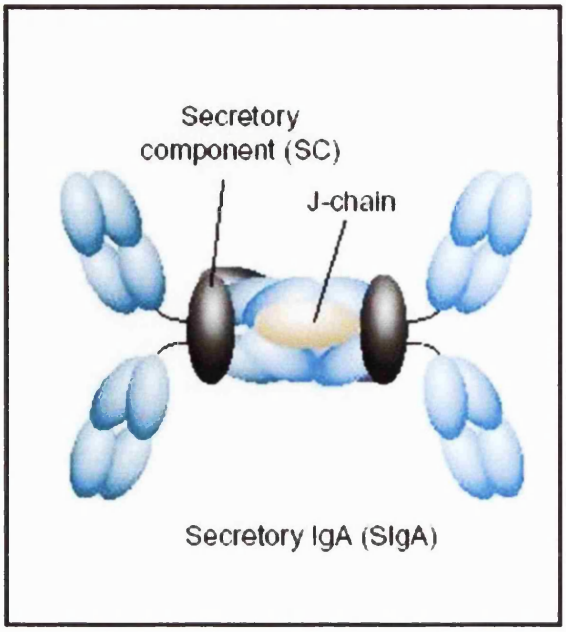


Figure 1.11: sIgA.

Diagram adapted from *Trends in Immunology* 2001, Vol 22, No.4, 205-211.)

Three main mechanisms have been proposed to explain how IgA participates in mucosal defence (Mazanec et al., 1993). Firstly, sIgA may act in a protective role physically inhibiting the adherence of pathogenic microorganisms and blocking the adherence of pathogens to epithelial cells in the airway lumen. This is possible through of the predominance of hydrophilic amino acids in the Fc region of IgA, and the abundant glycosylation of both sIgA and SC (Kerr, 1990), as such sIgA can surround microorganisms with a 'hydrophilic shell' that is repelled by mucosal surfaces (**Figure 1.12, panel A**).

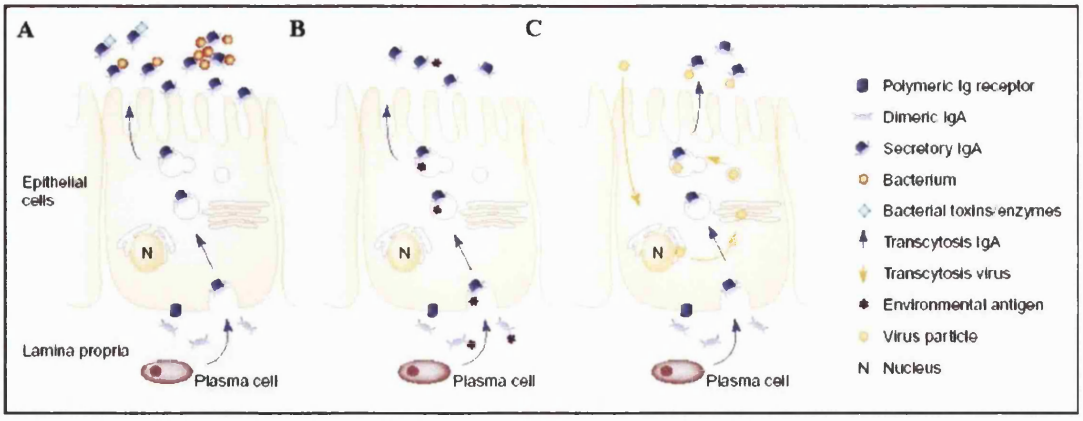


Figure 1.12: Proposed functions of IgA in the mucosal area. (A) 'Masking' of pathogenic receptors by IgA. (B) Transport of pathogens away from the lamina propria by IgA undergoing transcytosis. (C) Intracellular neutralisation of pathogens by IgA undergoing transcytosis. (Diagram adapted from *Trends in Immunology* 2001, Vol 22, No.4, 205-211.)

Secondly, whereas antibodies usually offer little protection against intracellular pathogens, normal transcytosis (**Figure 1.12, panel C**) of IgA from the basilar to apical regions of epithelial cells suggests that IgA is effective at neutralising intracellular pathogens and that this would be particularly effective at the intra-epithelial neutralisation of viruses (Lamm, 1997; Mazanec et al., 1992; Rojas and Apodaca, 2002).

Finally, IgA located in proximity to the lamina propria may trap pathogens or antigenic material that have been absorbed from the airway lumen to the basilar region of the epithelium and, in this manner, IgA may complex with the pathogen and transport it by transcytosis back to the airway lumen where it can be expelled from the body (**Figure 1.12, panel B**). This immune elimination role of IgA might provide an effective means of ridding the mucosa of excessive immune complexes (van Egmond et al., 2001) and support for this theory has been obtained in pIgR/SC deficient mice (Johansen et al., 1999).

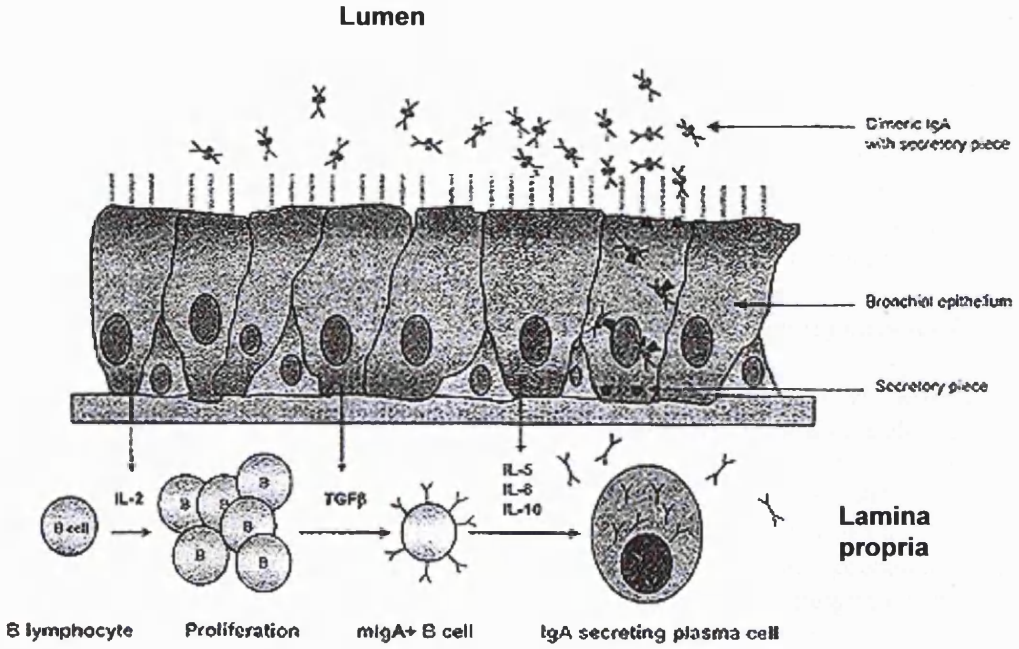


Figure 1.13: A representation of the origins of murine secretory dimeric IgA within the lumen via transcytosis from the lamina propria. (Diagram adapted from *Clin. and Exp. Allergy* 1999, Vol 29, 1597-1605.)

1.6.7 Immunoglobulin E

A role for IgE in RSV bronchiolitis and the subsequent development of wheeze is a matter of ongoing debate. RSV-specific IgE has been documented after infection and its presence in nasopharyngeal secretions has been associated with RSV bronchiolitis (Welliver et al., 1981) however the relationship between RSV-specific IgE and disease has not yet been determined. The suggestion that the severity of RSV infection is influenced by the induction of Th2 type T cells may lend some credibility to the concept of IgE mediated pathology as Th2 cytokines such as IL-4, IL-5 and IL-13 are all able to upregulate IgE production (Del Prete et al., 1988). Additionally, observations by Haeberle *et al.* (Haeberle et al., 2001) suggest that RSV infected mice with gene

deletions for the chemokine MIP-1α have significantly less lung inflammation and less severe clinical disease than intact mice. This is significant as MIP-1α has been linked to human B cell synthesis of IgE, independent of IL-4 production (Kimata et al., 1996) and these observations may explain how RSV-specific IgE can be observed in the absence of Th2 responses (Welliver, 2003). At present the link between bronchiolitis and IgE is still tenuous but the findings described above suggest that IgE may play a more important role in disease pathology that has been previously anticipated; however more substantial research will need to be undertaken in order to definitively demonstrate a role for IgE in RSV bronchiolitis.

1.7 The mucosal-associated lymphoid tissue (MALT)

The mucosal associated lymphoid tissue or MALT is a collective term to describe the set of lymphoid tissues that are diversely distributed throughout the anatomy and yet share many morphological and functional similarities. MALT consists of aggregates of lymphocytes found in the lamina propria and the submucosa of the mucosal membranes. These aggregates can vary in size and form, from solitary lymphoid nodules (follicles) to large accumulations such as those seen in the tonsils, lungs, Peyers patches (PP) and the appendix (Guy-Grand and Vassalli, 1993). The MALT is a major inductive site for B and T cells and has been shown to continuously supply mucosal sites with both effector T cells as well as IgA producing plasma cells (Kiyono et al., 1992). The provision of IgA producing plasma cells is particularly notable by the predominance of local dimeric IgA at the mucosal surface. This immunoglobulin originates from plasma cells in the lamina propria and is actively transported across the mucosal membranes in order to be expressed as secretory IgA (sIgA) (Brandtzaeg, 1987).

The MALT can be further compartmentalised according to the location of the lymphoid tissue, for example in the gut the specialised MALT here is known as the gut-associated lymphoid tissue or GALT. Of particular relevance for this thesis is that two of the MALT compartments can be found within the respiratory tract, the first being the nasal-associated lymphoid tissue (NALT) and the second being the bronchus-associated lymphoid tissue (BALT).

1.7.1 The nasal-associated lymphoid tissue (NALT)

The immune system of the upper and lower respiratory tracts can be divided into three important parts: the epithelial compartment (comprising immunocompetent cells in and at the surface of the epithelium and underlying connective tissue), the lymphoid structures (associated with the mucosal and bronchial epithelium – NALT and BALT), and the lymph nodes which drain the respiratory epithelium. Of these the NALT may be considered highly important as it represents the first lymphoid contact with any inhaled pathogen (Sminia and Kraal, 1999).

The concept of NALT was first introduced in 1987 (Mair et al., 1987; Spit et al., 1987) and has since been described by many different authors, most notably Asanuma *et al.* (Asanuma et al., 1995; Asanuma et al., 1997) whose group was responsible for devising the technique required to extract and isolate NALT in the murine system.

1.7.2 Ontogeny

In the mouse, the NALT exists as a paired aggregate situated on the floor of the nasal cavity at the entrance to the nasopharyngeal duct. It is located immediately underneath

the nasal epithelium and is complemented by a great number of ciliated epithelial cells, a few goblet cells and numerous M cells, both alone and in clusters. The NALT has a more activated appearance than the BALT with better developed follicles and marked intraepithelial infiltration by lymphocytes and this may be attributable to the strategic location of the NALT – the mucosa that lines the nasal cavity being one of the first sites of contact for inhaled antigen. The NALT is present at birth as a small accumulation of B and T-lymphocytes and intermittent macrophages (Hameleers et al., 1989; van der Ven and Sminia, 1993) and during the first few days post partum the number of lymphocytes increases. After seven days the first signs of formation of B cell follicles and T cell areas can be seen (Kuper et al., 1992) and in the latter areas the first high endothelial venules (HEV) occur. Four-weeks after birth the NALT has fully developed and resembles adult NALT (van der Ven and Sminia, 1993) which is considered the murine equivalent of the Waldeyers' ring in humans (Kuper et al., 1992).

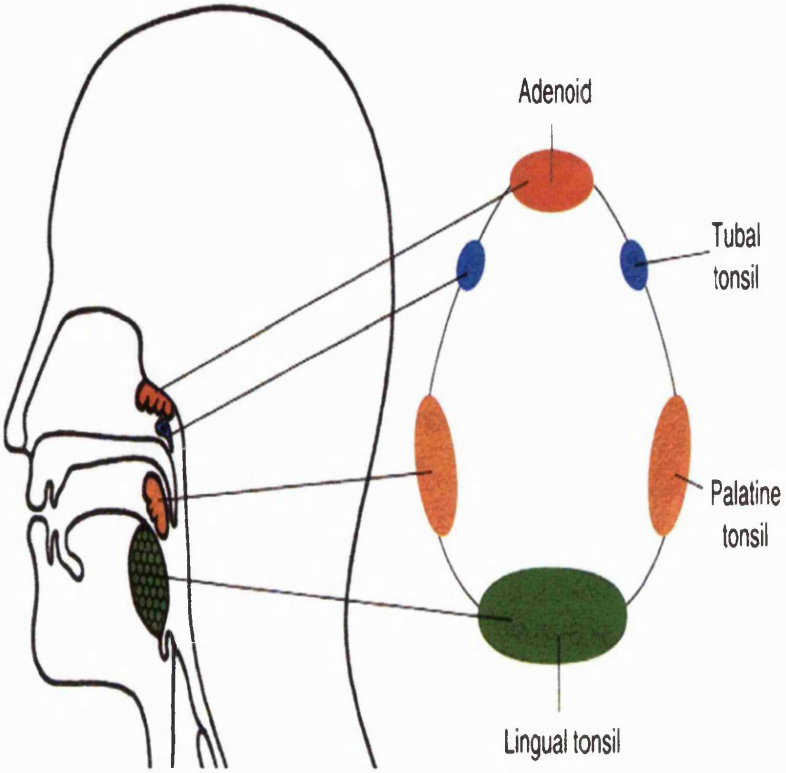


Figure 1.14: The Waldeyers' ring and its associated lymphoid structures in humans.

1.7.3 NALT composition

The NALT is composed of a loose reticular network in which lymphocytes, macrophages and DC's are embedded. The lymphocytes are organised into follicles (B cell areas) and interfollicular areas (T cell areas) of approximately the same size (Sminia and Kraal, 1999) with numerous HEV located in the T cell areas. Within these T cell areas it has been noted that there are more cells of the $CD4^+$ phenotype than that of the $CD8^+$ phenotype (the ratio being approximately 2:1) and that virtually all of the T cells express the α/β TCR (Koornstra, 1997; McDermott and Snider, 1997). Interestingly the NALT contains a relatively higher proportion of T cells when compared with the Peyers patches of the gut. There are further differences within the $CD4^+$ T cell population, the NALT contains more $CD45RB^{hi}$ and less $CD45RB^{lo}$ T cells

when compared with the PP indicating that NALT $CD4^+$ population contains more naïve and fewer memory cells than the T cell populations of the Peyers patches (Ogra, 1999).

The majority of NALT B cells have been demonstrated to be IgM^+ with very few IgG^+ or IgA^+ cells detectable; further analysis by co-staining with IgD suggests that most NALT B cells are both IgM^+/IgD^+ naïve B cells (Sminia and Kraal, 1999). Plasma cells, in particular IgA plasma cells, occur predominantly at the abluminal side of the NALT in the surrounding connective tissue and almost all of the lymph vessels within the NALT are located at the posterior aspect of both these B and T cell areas (Koornstra, 1997; Sminia and Kraal, 1999). In addition to B and T cells the NALT also contains various types of accessory cell including MHC class II positive interdigitating DC's which are found in the T cell areas and also follicular dendritic cells (FDC) which can be seen in the B cell areas. Monocytes and macrophages are also scattered throughout the NALT (van der Ven and Sminia, 1993) and tingible body macrophages can be observed within the B cell follicles (Koornstra, 1997).

The NALT compartment also contains microfold or M cells. These are non-ciliated cells with an irregular outline that possess numerous finger like villi and often enclose lymphocytes. They are found in the epithelium above the lymphoid aggregates (Gebert et al., 1996; Trier, 1991) and their function is to transport antigen from the mucosal lumen to the underlying lymphoid tissue. M cells are not MHC class II positive under normal (non-pathological) conditions and since the lysosomal system is bypassed during antigen transport, M cells do not actually process antigen (Keren, 1992). M cells are also found in the PP and BALT and have been shown to be identical to those found in the NALT (Kuper et al., 1992).

1.7.4 Antigen uptake

The nature of the interaction between antigen, nasal mucosa and NALT depends upon the composition, dose and frequency of exposure to the antigen. Small soluble antigens mainly penetrate the nasal epithelium to interact with intraepithelial and submucosal lymphocytes, DC's and macrophages whilst particulate antigens can be taken up specifically by M cells above the NALT. Because of this selective uptake, soluble and particulate antigens drain to different lymph vessels, with soluble antigens draining first to the superficial CLN and then to the posterior CLN and particulate antigens draining preferentially to the posterior CLN only (Kuper et al., 1992; Tilney, 1971).

1.7.5 Immediate and recall responses in the NALT

Intra-nasal (i.n.) immunisations with equal amounts of antigen as given by the intra-gastric route display earlier and stronger mucosal IgA responses than Peyers patches in almost all mucosal sites including saliva, vaginal wash, gut wash and bronchial wash (Wu and Russell, 1993) indicating that the NALT is more effective at the dissemination of immune cells. More importantly NALT cells proliferated in response to antigen re-stimulation *in vitro* up to six months and longer after an initial immunisation indicating that the NALT can retain long term memory (Liang et al., 2001; Wu et al., 1996).

1.7.6 Antibody responses in the NALT

Intra-nasal immunisation produces a response by plasma cells in the NALT that is predominantly of the IgA isotype. In addition, intra-nasal immunisation also elicits a

systemic immune response that can be observed as an enhancement of the IgG response (Wu et al., 1997; Wu and Russell, 1993). However, the exact mechanism for how this systemic enhancement of the immune response occurs is not clear. Investigations on the draining lymph nodes of the NALT have demonstrated that the superficial and central cervical lymph nodes (CLN) are locations where the augmentation of the IgG response is strongly suggested (Wu et al., 1997) although this has yet to be conclusively proven.

As described earlier, IgA plasma cells can be found in murine NALT and it is thought that their generation through B cell isotype switching, differentiation and maturation occurs at this location. This is supported by the observations that TGF-β and dominant type-2 cytokine mRNA's (including IL-5, IL-6 and IL-10) are present in NALT cell populations after *in vitro* re-stimulation with antigen (Wu et al., 1996). However, the murine NALT is not exclusively populated with IgA plasma cells and contains both IgG1 and IgG2a plasma cells suggesting that IgG isotype switching also occurs at this location but to a much lesser extent (Wu et al., 1996).

Complementing this, it has been noted that IgG B cell responses were not enhanced until the cells had passed through the draining lymph nodes of the NALT. The IgG plasma cell responses appear to be boosted on transit through the superficial and central CLN and this effect appears to be mediated by IL-4 and IFN-γ (Wu et al., 1996) both of which are readily detectable in these lymphatic vessels.

1.7.7 NALT and non-NALT

Almost all of the studies described above consider NALT in the mouse solely as the lymphoid structures situated on the floor of the nasal cavity at the entrance to the

nasopharyngeal duct. However, previous techniques to isolate NALT cells were not entirely accurate as the treatment process often harvested cells from other parts of the nose. These often included the septum and lateral walls, the turbinates and other less well organised lymphoid tissues located along the lacrimal duct and within the lamina propria and nasal epithelium (Kuper et al., 1992). To overcome this, an improved method devised by Asanuma *et al.* (1997) was used to differentiate between the lymphocytes located in the organised NALT and those in the non-NALT areas of the nasal cavity (Asanuma et al., 1997). This technique allowed the delineation of two distinct populations of lymphocytes within the nasal cavity and subsequent work suggests that the organised NALT and non-NALT compartments may function as inductive and effector sites of mucosal immune responses respectively (Asanuma et al., 1997).

The non-NALT nasal lymphocyte population of normal BALB/c mice contains two to three times more cells than the NALT lymphocyte population. The non-NALT is particularly enriched in both T and B cells and when analysed using markers for CD4, has marginally more cells of the $CD4^+$ phenotype than the NALT (Asanuma et al., 1997). Studies using influenza virus demonstrated that the non-NALT nasal lymphocyte population does respond to infection, as seen by an increase in cell number coupled with an upregulation of IFN-γ production and an expansion of the plasma cell population (Asanuma et al., 1997). However, although this response resembles that of the NALT, the overall data suggests that the non-NALT nasal lymphocyte population differs sufficiently both quantitatively and qualitatively from the NALT lymphocyte fraction and should be considered as distinct. In our studies we have investigated both populations and have used the abbreviation O-NALT to describe the organised NALT and the abbreviation D-NALT to describe the diffuse or non-NALT.

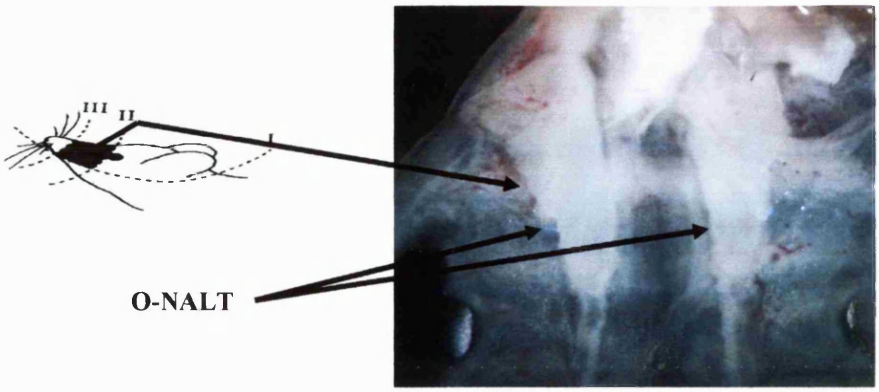


Figure 1.15: A schematic demonstrating the location of the paired lymphoid aggregates of the O-NALT in the mouse. Diagram adapted from Asanuma *et al.* (Asanuma et al., 1997).

1.8 Historical approaches to RSV vaccine design

One of the key priorities of the World Health Organisation's global program for vaccines is an RSV vaccine (Crowe, 1995), unfortunately in the last forty years little progress towards this goal has been made. The major factor which retarded advancement was a now infamous vaccine trial carried out during the 1960's where a potential vaccine candidate was used in a clinical trial with unexpected and unfortunate results.

The vaccine in question was formulated by Pfizer and utilised formalin treatment to inactivate the Bennett strain of RSV (FI-RSV). The final concentration factor of the vaccine was 100x and therefore the preparation was known as 'Lot 100'. This vaccine was then administered as two or three intra-muscular injections at intervals of one to

three months and was received by children and infants between two months and seven years of age (Chin et al., 1969; Fulginiti et al., 1969; Kapikian et al., 1969; Kim et al., 1969). Initial results were encouraging and the FI-RSV vaccination elicited high rates of seroconversion amongst the children (Fulginiti et al., 1969) with high anti-F titres as determined by ELISA (Murphy et al., 1986a). Unfortunately, upon subsequent natural exposure to the wild-type virus the vaccine failed to protect the children and exaggerated the clinical response in those children who were RSV naïve before vaccination. Within one group the hospitalisation rate of the vaccinees was 16 times greater than those of the controls and, sadly, two infants died following wild-type RSV infection. A cellular infiltrate of eosinophils, mono- and polymorphonuclear cells was observed in the lungs of the deceased children and, of the infants who presented this pathology, RSV was readily detectable in their lower respiratory tracts (Kim et al., 1969). This inflammatory infiltrate in the lungs of the vaccinated children suggests an immunopathological basis for the enhanced disease observed and stored lymphocytes from FI-RSV vaccinees showed a greater proliferative response to RSV antigen than did those obtained from children naturally infected with wild-type RSV (Kim et al., 1976). Analysis of the vaccinees' antibody profiles demonstrated poor anti-G and virus-neutralising responses (Murphy et al., 1986a) and also demonstrated that serum from vaccinated children had poor fusion inhibiting antibody, suggesting the possibility that the fusion protein may have been altered during the formalin treatment (Murphy and Walsh, 1988).

Several hypotheses have been suggested to account for the occurrence of the enhanced pathology but one of the major obstacles in the way of these investigations has been the lack of an appropriate system for testing safety. Small experimental animal models like mice exist but are only semi-permissive for RSV replication and because of this

relatively few cells in the lung become infected. Only monkeys (in particular the African green monkey) and humans are permissive to RSV and exhibit RSV disease, however, the cost and ethical constraints associated with primate use make them a largely unused tool and the mouse has thus become the primary focus for RSV work. It is possible to re-create the 'enhanced disease' pathology seen in the 1960's vaccinees by priming mice with FI-RSV and then challenging them with wild-type RSV, the resulting response being similar to that seen in humans - Th2 dominated and displaying a relative increase in IL-4 (Graham et al., 1993), IL-10 and IL-13 cytokine mRNA (Waris et al., 1996). In addition FI-RSV priming also exerts a strong and rapid upregulation of eotaxin and monocyte chemotactic protein 3 (MCP-3) relative gene expression, both of which are potent lymphocyte and eosinophil chemoattractants (Power et al., 2001) and may result in increased inflammation and bronchioconstriction (Tang and Graham, 1995; Waris et al., 1996). Conversely, mice primed with wild-type RSV demonstrate an alternative response, Th1 cells dominate with increased levels of γ-IFN mRNA (Graham et al., 1993). To substantiate the potential link between the 'enhanced disease' and the T cell subsets, IL-4 and IL-10 were depleted using a combination of antibodies and this eliminated the enhanced pulmonary pathology (Connors et al., 1994), as did selective depletion of $CD4^+$ T cells (Connors et al., 1992) supporting the hypothesis that the enhanced disease might be caused by the induction of Th2 memory cells (Graham et al., 1993). The Th2 theory is particularly appealing as such a response is often seen to allergic antigens in patients with asthma (Robinson et al., 1992; Wierenga et al., 1991) and RSV is often considered to be a potential trigger for this condition. Recently a new theory pertaining to enhanced RSV disease has been suggested. Polack *et al.* (Polack et al., 2002) have shown that bronchioconstriction appears to be mediated by immune complexes and further confirming this, they show that disease pathology may be abrogated in complement-component C3 and B cell

deficient mice but not in controls. This hypothesis has also been applied to the post-mortem lung tissue sections taken from children with RSV enhanced pathology and evidence of complement activation was readily detectable. Taken together all these studies suggest that the scope of RSV enhanced pathology may be far wider and more complex than was first anticipated, highlighting the multifactorial nature that this disease and its pathology present, a fact that is readily apparent when it comes to new vaccine approaches.

In the years that followed the 1960's trial there have been many attempts to create a new RSV vaccine but most without success. Popular methods have included using low temperature adapted RSV (Kim et al., 1971), temperature sensitive (*ts*) mutants and chemical mutagenesis. However, none of these approaches have proven acceptable, with many vaccines unable to establish a protective immune response, and those that did being subject to problems with the strain of RSV utilised being under-attenuated and/or reverting to wild-type (Belshe et al., 1993). Future prospects include subunit vaccines based upon the F protein, alternative chimeric constructs expressing RSV proteins, and technologies incorporating reverse genetics (Collins et al., 1999b) and other emerging concepts.

To be effective an RSV vaccine must protect against lower respiratory tract disease in the very young, as the peak of severe disease and mortality generally occurs in infants younger than three months (Kim et al., 1973b). These infants however often have high titres of maternally derived antibody which presents a major hurdle to vaccine design by providing a form of immunosuppresion (Collins et al., 2001; Murphy et al., 1994). Another aspect that must be kept in mind is that of the relative immaturity of the infant immune system, as the immune system is not fully developed in the infant, candidate

vaccines must be carefully designed in order to avoid overwhelming it. For example - certain attenuated strains of virus which may be safe in adult humans with fully developed immune systems may not be sufficiently attenuated when presented to an immature immune system and their administration could lead to the onset of disease and its associated consequences of pathology and potentially, mortality.

As with all vaccine strategies the ideal vaccine must accommodate all the aspects mentioned above but, primarily, be safe for use in infancy, ideally negating the effect of immunosuppressive maternal antibody and also accounting for the relative immaturity of the infant immune system (Kovarik and Siegrist, 1998; Murphy et al., 1986a). Ideally, an RSV vaccine should induce protective levels of neutralising antibodies as well as $CD8^+$ RSV-specific cytotoxic T cells and, most importantly, mirror the pattern of $CD4^+$ T cell responses seen after wild-type RSV infection.

1.9 Aims and objectives

As discussed, RSV is a major source of morbidity and mortality in both the extremely young and old. Unfortunately, protection from natural RSV infection is only partial and appears to be cumulatively acquired over successive infections; consequently there is a real need for an effective RSV vaccine candidate. To date, most research related to protective immunity to RSV has focussed mainly on the systemic immune response during infection and little emphasis has been placed on the mucosal (local) humoral responses within the respiratory tract. As a result of this there is an important gap in our knowledge of how the immune system reacts to RSV at the site of infection which may elucidate alternative options for future vaccine strategies.

Recent studies of influenza virus infections in this laboratory have shown that following a single infection, a significant frequency of influenza-specific antibody forming cells are generated in the nasal-associated lymphoid tissue (NALT) and are retained here for the lifetime of an animal. It is currently unknown if such a role exists for the NALT during the immune response to RSV infection. This project aims to investigate the humoral immune response to RSV infection in the mouse model as a prerequisite to developing an RSV vaccine whilst, at the same time paying particular attention to the role, if any, of the NALT.

Within this aim there are several objectives that we wish to consider:

- Firstly, we want to establish whether the mouse is an appropriate model to investigate the development of protective immunity to RSV.
- Secondly, we want to examine the antigen-specific B cell response in the NALT and contrast it with the B cell response observed in both the local draining lymph nodes of the respiratory tract and also in specific systemic tissues.
- Thirdly, we want to dissect how the humoral immune system responds to initial and subsequent infections of RSV.

The approaches taken to achieve these aims utilised a mixture of conventional and specialised assays and are depicted on the following page.

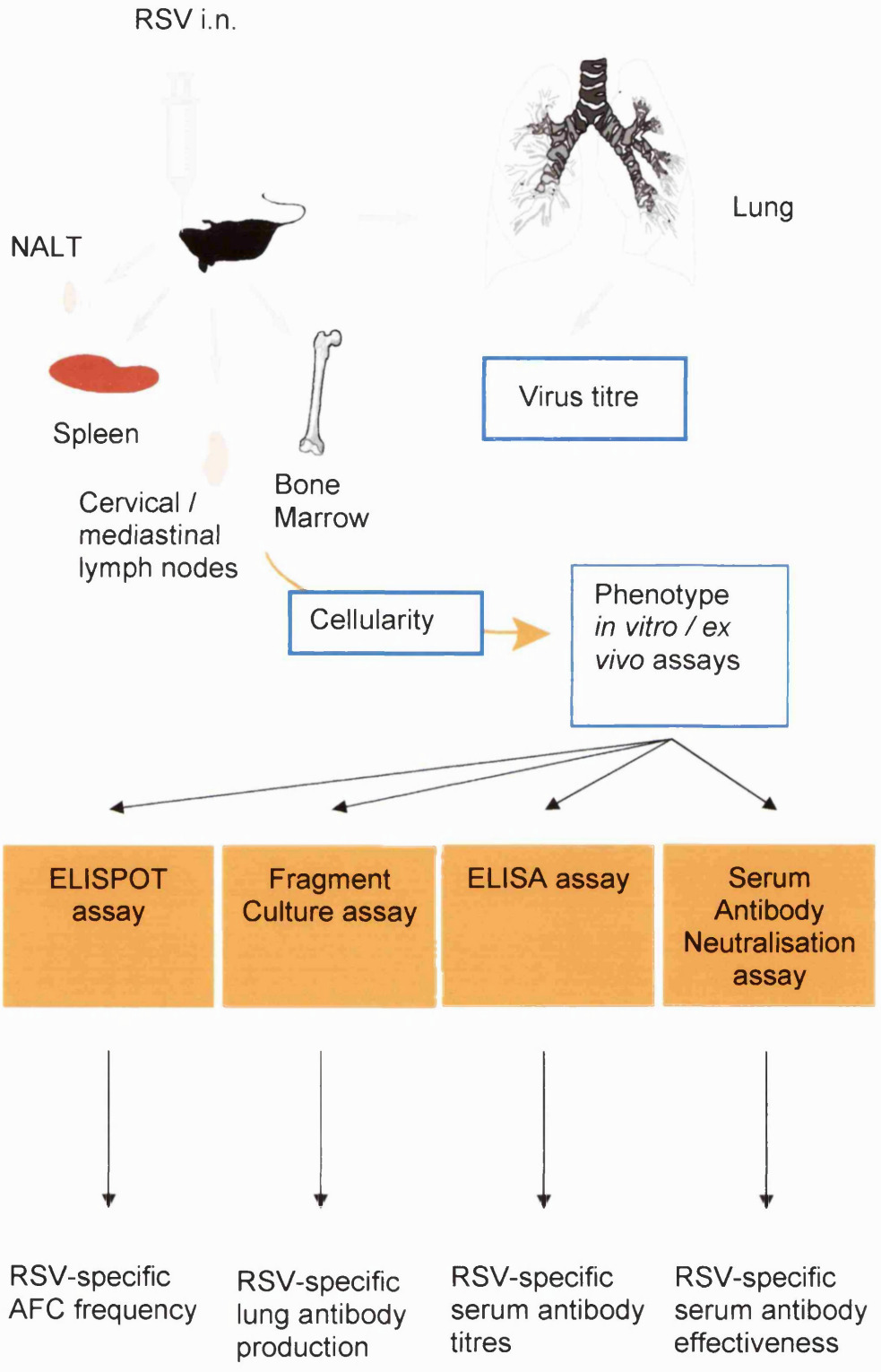


Figure 1.16: A diagrammatic summary of the experimental work undertaken throughout this thesis.

Chapter Two: Materials and Methods

2.1 Animals

Inbred female BALB/c mice were obtained from Charles River UK (Margate, Kent). All mice were held under specific pathogen free conditions and were used between 8-12 weeks of age.

2.2 Infection and Sampling

2.2.1 Intranasal immunisation

Two different anaesthetics, Avertin and Ketamine-Xylazine were used in this study. Avertin comprises 2% 2,2,2-tribromoethanol (Sigma) and 2% tert-amyl-alcohol (Sigma), formulated in distilled water. Dosing was at the rate of 100µl per 10-15g body weight (approx. 250-300µl/mouse). Ketamine-Xylazine is formulated to achieve final concentrations of 9mg/ml Ketamine and 2mg/ml Xylazine and a single dose of 150-200µl/mouse is sufficient to anaesthetise safely.

Mice were anaesthetised by intra-peritoneal (i.p.) immunisation and reflex action was observed by gently pinching the footpad of the mouse. Upon cessation of the reflex action mice were then immunised intranasally. This was achieved by orientating the mouse in a supine posture with the head uppermost and by then administering droplets of the viral diluent through the nostrils using a pipette. RSV at a concentration of

approximately 10^7 plaque forming units/ml was used for all immunisations and, to achieve lower respiratory tract penetration 50µl was administered to each mouse.

As controls for the RSV studies, mock immunisations of mice were performed. In these immunisations the mice were prepared exactly as described above for virus immunisation but were instead treated intranasally with 50µl of supernatant from the cell line used to grow the experimental virus stock.

2.3 Extraction and preparation of NALT

Both the organised (O) and diffuse (D) nasal-associated lymphoid tissues (NALT) were obtained from the head of the mouse and the following method was adapted from that initially described by Asanuma (Asanuma et al., 1997). Using a razor blade, an incision was made laterally behind the eyes, and the posterior portion of the head was then removed as was the lower jaw and surrounding muscle tissue. The cheeks and eyes were then extricated prior to the removal of the fore-teeth and nose and finally the brain was extracted and the soft palate stripped from the roof of the mouth.

2.3.1 O-NALT

The O-NALT exists as a paired lymphoid aggregation and is located bilaterally on the posterior side of the palate. This tissue was extracted under a dissection microscope (Leica MZ6) using fine-point forceps and then pressed between two sterile frosted glass slides to release the lymphocytes. The resulting homogenate was passed through gauze (50µm) to remove debris and the cells pelleted by centrifugation (Sorvall RT7+) at 328g for 10 minutes. The pellet was then re-suspended in fresh IMDM medium (100U/ml penicillin, 100mg/ml streptomycin, 5 x 10^{-5}M 2-mercaptoethanol and 15% FCS).

2.3.2 D-NALT

Once the palate had been removed from the roof of the mouth the remaining part of the skull, containing the nasal passages/turbinates, was homogenised and placed in culture media containing 4mg/ml collagenase A (Roche). This was incubated for thirty minutes at 37°C on a blood tube rotator. The resulting digest was filtered using a 70μm cell strainer (Becton Dickinson), spun at 328g for a further five minutes before being re-suspended in 2ml of media. A Percoll (Amersham Pharmacia) density gradient was prepared by layering 2mls of a 40% solution carefully onto 2mls of a 75% solution. The digest was then carefully layered onto the top of the Percoll and was centrifuged at 582g for twenty minutes. The interface between the 40% and 75% concentrations was then harvested and re-suspended in culture media before being centrifuged at 328g for a further five minutes. The resulting pellet was re-suspended in an appropriate volume of tissue culture media.

2.4 Extraction and preparation of all other tissues

Spleen and lymph nodes were prepared by homogenising the tissues between two sterile frosted glass slides. The homogenates were subsequently filtered through gauze (50μm), centrifuged at 328g for five minutes and re-suspended in fresh tissue culture media.

Bone marrow was prepared by harvesting the hind limbs of the mice and removing the surrounding muscle tissue and skin. The marrow was flushed out with media using a 1ml syringe and was gently homogenised. The homogenate was then centrifuged at 328g for five minutes and the pellet re-suspended in red blood cell lysis buffer (Sigma)

for another five minutes. Following this the white cells were pelleted through a cushion of FCS (472g, 5min) before finally being re-suspended in fresh culture media.

2.5 Production of RSV

T150 tissue culture flasks (Falcon, **Table 2.5**) were seeded with 2 x 10^6 BS-C-1 (American Type Culture Collection, **Table 2.8**) cells in complete DMEM (10% FCS, 2-mercaptoethanol, penicillin/streptomycin, sodium pyruvate). After two days the cells were 90% confluent which corresponded to a concentration of approximately 1 x 10^7 cells per flask. Stock RSV was then centrifuged at 1700g for five minutes to remove cell debris before being diluted in serum-free DMEM to allow for a multiplicity of infection (m.o.i.) of 0.05 to 0.01 pfu/cell (in a 5ml inoculate). The media from the flasks was removed and the inoculate placed onto the cells and left for two hours at 33°C in a 10% CO_2 incubator. 30mls of DMEM +2% FCS per flask was then added and the flasks incubated for 4 to 6 days until general cytopathic effects (CPE) were observed (syncytia). On observation of CPE, the media was removed from the flasks and replaced with 5ml of serum-free DMEM either with or without 10% stabilisation buffer (1M MgSO4, 50mM HEPES, 150mM NaCl, pH 7.5, 0.22μ filtered) depending upon whether the virus was to be used for infection of animals (stock), or for use in *in vitro* assays (sucrose purified). The flasks were then frozen at -70°C until needed.

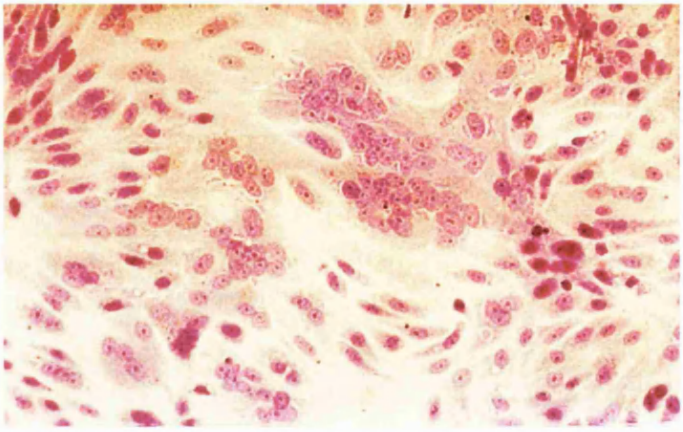


Figure 2.1: An example of RSV induced cytopathic effect in cell culture.

Linda Stannard, University of Cape Town, Virology Laboratory, Yale-New Haven Hospital (http://www.virology-online.com/general/Tests.htm).

2.6 Stock RSV titration

A 24-well plate was seeded with BS-C-1 cells at 5 x 10^4 cells per well and was incubated for three days at 37°C. Following this, the viral aliquots to be tested were centrifuged at 2660g at 4°C for five minutes. After centrifugation, serial ten-fold dilutions of the virus (kept on ice to retain infectivity) were made in serum-free DMEM. The media from the 24 well plate was then discarded and 200µl of each virus dilution was added and then left to incubate for at least two hours at 33°C. When the incubation was complete each well was supplemented with 2ml of DMEM + 2% FCS and incubated for a further 48 hours at 33°C. The medium was then gently discarded (to avoid damaging the fragile infected cells) and the cells were fixed using 2ml/well of 4% paraformaldehyde (pH 7.4 in PBS) at 4°C for one hour. Following this, the cells were rinsed twice with 2ml/well PBS before being blocked with 500µl/well 4% milk protein at 37°C for an hour. The cells were then rinsed, once with PBS + 0.05% Tween-20 and

twice with PBS before the addition of 200µl of goat anti-RSV capture antibody per well (Chemicon, **table 2.3**, 1/400 in 0.1% BSA) for two hours at room temperature. The wells were then washed three times with PBS + 0.05% Tween-20 and a 200µl volume of alkaline phosphatase-labelled rabbit anti-goat IgG antibody (Sigma, **table 2.3**, 1/200 in PBS + 0.05% Tween-20 + 0.5% BSA) was then added to each well and incubated for two hours at room temperature. Subsequently the plates were washed with PBS + 0.05% Tween-20 before the addition of 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) (200µl/well) at a concentration of 1mg/ml in 10% diethanolamine buffer (100mM Tris pH 9.5, 100mM NaCl, 5mM $MgCl_2$, 10% diethanolamine). The plates were then left to develop for 15 to 60 minutes until colour developed and were then washed to stop the reaction before counting the plaques. Virus titres were then expressed as plaque forming units per ml (pfu/ml) and were calculated by multiplying the number of blue plaques observed in a given dilution by the dilution factor of that sample and then, as 200µl of each virus sample was added per well, by five to give a pfu value per ml.

2.7 RSV Micro titration

RSV was also titred according to a protocol provided by Dr. Andrew Easton (University of Warwick). The cell line BS-C-1 (in DMEM +10% FCS) was used to seed a 96 well flat-bottomed plate (Falcon, BD) at a concentration of 2 x 10^4 cells/well. The plates were incubated overnight at 37°C by which time they were roughly 90% confluent. Seven ten-fold serial dilutions of the virus were made in a separate 96-well plate in serum-free DMEM. The media from the cultured cells was then discarded and 50µl of the appropriate virus dilution was added per well (triplicate wells per dilution). The plate was incubated for two hours at 33°C and, after incubation, 100µl of DMEM (+2%

FCS) was added to each well and further incubated for two to three days at 33°C. The media from the plate was then discarded and the plate washed once in PBS. The cells were fixed with 200µl of 4% paraformaldehyde (Sigma) for twenty minutes at 4°C and the plates were then washed three times with PBS before being blocked with DMEM +10% FCS at room temperature. After blocking the cells were washed with PBS again and 50µl/well of goat anti-F antibody (Chemicon, **table 2.3**), diluted 1/500 in PBS + 0.05% Tween-20 was added and incubated for forty-five minutes at room temperature. After incubation the plates were washed with PBS and 50µl/well of alkaline-phosphatase-labelled, polyclonal rabbit anti-goat Ig antibody (Sigma, **table 2.3**, 1/1000 in PBS + 0.05% Tween-20) was added. After incubation for another forty-five minutes at room temperature the plates were washed again with PBS and 100µl of BCIP (Sigma, 1 mg/ml in diethanolamine substrate buffer) was added per well. The plates took one to three hours to fully develop. Virus titres were then expressed as plaque forming units per ml (pfu/ml) and were calculated by multiplying the number of blue plaques observed in a given dilution by the dilution factor of that sample and then, as 50µl of each virus sample was added per well, by twenty to give a pfu value per ml.

2.8 Use of rollerbottles for large scale growth of RSV

As a large scale alternative to growing virus in the T150 flask we used the pleated surface rollerbottle (Becton Dickinson) which has a surface area (1450cm^2) approximately ten-times that of a single flask. We seeded rollerbottles with 1 x 10^7 BS-C-1 cells in 300ml DMEM + 10% FCS (Gibco) and the flasks were then gassed with 10% CO_2 in air (British Oxygen Company, BOC) for 15 seconds and this process was repeated daily. Rollerbottles were then incubated on a rotating mechanism at 37°C for at least seven days (based on a rate of cell number doubling every two days) before the

media was removed and the cells infected with RSV at a m.o.i. of 0.05 in serum-free DMEM (Gibco). Following this the rollerbottles were transferred to a 33°C incubator for two hours before the addition of 300ml of DMEM + 2% FCS. They were then returned to the 33°C incubator for at least another six days or until widespread CPE was observed. Upon observation of CPE the media was removed and replaced with 5ml of serum free DMEM + 10% stabilisation buffer (as described in **Section 2.5**) and the rollerbottles were frozen at -80°C until required.

2.9 RSV Purification

To provide concentrated (high titre) stocks of virus free from cellular debris, RSV was purified by the method of M'biguino & Menezes (Mbiguino and Menezes, 1991). Sorvall centrifuge tubes were sterilised with 70% ethanol and allowed to dry prior to purification. The sucrose (BDH) gradients were prepared by layering 8ml of 20% sucrose over 8ml 50% sucrose. The previously infected BS-C-1 flasks were then thawed at room temperature and the cells collected, pooled and homogenised by gentle pipetting. The harvest was centrifuged for ten minutes at 910g (4°C) to remove the cell debris and the supernatant was then layered carefully onto the sucrose gradient and was centrifuged at 13440g, 4°C, for four hours. Following centrifugation, the virus was removed from the 20%-50% interface (typically 2-3mls volume) and the purified virus was then aliquoted in volumes less than 500µl and frozen at -70°C immediately for further use in RSV-specific ELISPOT or ELISA assays.

2.10 Titration of purified RSV

Purified RSV was titrated using an anti-RSV F hybridoma cell line, 130-8F (donated by Dr. R. Tripp, CDC, Atlanta). ELISPOT plates were coated with various concentrations of purified RSV and were left overnight at 4°C. The wells were then washed three times with PBS (200µl/well) and blocked with PBS/2%FCS (200µl/well) for one hour. After blocking the plates were washed again and specific numbers of the 130-8F cell line (ranging from approximately 50 cells/well to 500 cells/well) were added. After incubation at 37°C for four hours the plates were washed several times with PBS and plaques were detected using IgG1-specific goat anti-mouse reagents (Southern Biotechnology Associates Inc., **table 2.3**) diluted 1:500 in PBS/5%BSA (Sigma). These were added to the wells for three hours at room temperature. The plates were then washed first with PBS, then PBS/0.1% Tween 20 and finally PBS again before being developed with BCIP (1mg/ml in diethanolamine substrate buffer). The plates were incubated for up to an hour (depending on the rate of reaction) or until plaques developed and the reaction was then stopped by the removal of the substrate and washing with PBS. Finally, the plates were left to air dry for several days (to reduce background) before counting any plaques.

The optimum coating concentration of RSV was determined by counting the plaques and selecting the concentration with the most accurate numerical representation of the hybridomas.

2.11 Inactivation of RSV with beta-propiolactone (BPL)

This method of RSV inactivation was a modification of the method described by LoGrippo *et al.* (LoGrippo, 1957; LoGrippo and Hartman, 1955). Briefly, 0.5M Na_2HPO_4 was made in distilled water and one part of this solution was mixed with eighteen parts of RSV stock solution. The beta-propiolactone (BPL) solution was made on ice in a chemical extraction hood by gradually adding the correct amount of the aqueous BPL into distilled water with gentle shaking to achieve the final BPL concentration of 0.1%. One part of the 0.1% BPL was then added into the above virus solution and the mixture was transferred into a clean container to ensure thorough mixing of the BPL and the virus solution. The mixture was incubated in a 37°C water-bath for two hours and the pH of the solution tested and adjusted to 7.3-7.4 with 10% sodium bicarbonate if necessary.

2.12 ELISPOT Assay

For RSV-specific assays, plates (Millititre-HA 96, Millipore) were coated with a pre-determined concentration of purified RSV (made up in PBS, 100µl/well) and left overnight at 4°C. The wells were washed three times with PBS (200µl/well) and then blocked (PBS/2%FCS - 200µl/well) for one hour. After blocking the plates were washed again and kept at 4°C until needed.

Tissues for the assay were collected in Isocoves Modified Dulbecco's Medium (IMDM, GibcoBRL) containing 15% FCS (GibcoBRL). These tissues were prepared as described previously (**Sections 2.3 & 2.4**) and 10^5 cells (5 x 10^5 cells for the bone marrow) and two serial five-fold dilutions were added to duplicate wells of the coated

96 well plate. Plates were then incubated for four hours at 37°C (or overnight) in a humidified 5% CO_2 atmosphere. Following this incubation the plates were washed once with PBS, once with PBS/0.1% Tween 20 (Sigma) and finally three times with PBS. Plaques were detected with isotype-specific goat anti-mouse reagents (Southern Biotechnology Associates Inc., **table 2.3**) Each conjugate was diluted 1:500 in PBS/5%BSA (Sigma) and was added to the plates for three hours at room temperature. Following incubation, the plates were again washed, first with PBS, then PBS/0.1% Tween 20 and finally PBS again before being developed with the substrate BCIP (1mg/ml in diethanolamine substrate buffer). The plates were then incubated for up to an hour (depending on the rate of reaction) or until plaques developed and the reaction was then stopped by removal of the substrate and washing with PBS. Finally, the plates were left to air dry for several days (to reduce background) before counting any plaques.

2.13 Titration of RSV in the lung

A 24-well plate was seeded with BS-C-1 cells at 5 x 10^4 cells per well and was incubated for three days at 37°C. At the end of the incubation, lung samples were mashed in 'v' shaped eppendorf tubes before being homogenised with a 1ml pipette tip that has had the end cut off. Following this the suspension was homogenised again, this time with an intact 1ml pipette tip before centrifuging the suspension at 2660g for five minutes at 4°C. After centrifugation, six four-fold serial dilutions of the lung homogenate (kept on ice to preserve any virus present) were made in serum-free DMEM. The media from the 24 well plate was then discarded and 200µl of each lung dilution was added and then left to incubate for at least two hours at 33°C. When the incubation was complete each well was then supplemented with 2ml of DMEM + 2% FCS and incubated for a further 48 hours at 33°C. The medium was then gently

discarded (to avoid damaging the fragile infected cells) and the cells were fixed using 2ml/well of 4% paraformaldehyde (pH 7.4 in PBS) at 4°C for one hour. Following this, the cells were rinsed twice with 2ml/well PBS before being blocked with 500µl/well 4% milk protein at 37°C for an hour. The cells were rinsed again, once with PBS + 0.05% Tween-20 and twice with PBS before the addition of 200µl of goat anti-RSV capture antibody per well (Chemicon, 1/400 in 0.1% BSA, **table 2.3**) for two hours at room temperature. The wells were then washed three times with PBS + 0.05% Tween-20 and 200µl volumes of alkaline phosphatase-labelled rabbit anti-goat IgG antibody (Sigma, 1/200 in PBS + 0.05% Tween-20 + 0.5% BSA) were then added to each well and incubated for two hours at room temperature. Subsequently the plates were washed with PBS + 0.05% Tween-20 before the addition of 200µl/well of BCIP (1mg/ml in diethanolamine substrate buffer). Plates were then left to develop for 15 to 60 minutes until colour developed and were then washed to stop the reaction before counting the plaques. Lung virus titres were then expressed as plaque forming units per ml (pfu/ml) and were calculated by multiplying the number of blue plaques observed in a given dilution by the dilution factor of that sample and then, as 200µl of each virus sample was added per well, by five to give a pfu value per ml.

2.14 Serum antibody RSV neutralisation assay

This method is an adapted version of the virus titration described previously (**Section 2.6**). In order to ascertain the neutralisation ability of murine serum antibody, serum samples from RSV-infected mice were mixed with known quantities of RSV and the samples then assayed for virus infectivity. Serum samples were diluted four-fold from 1:4 to 1:64 and were then co-incubated with known volumes and concentrations (pfu/ml) of RSV for five minutes at room temperature. The samples and a positive control of

RSV not mixed with sera, were then placed into appropriate wells of a 24-well tissue culture plate containing 90% confluent monolayers of BS-C-1 cells. The plates were then incubated at 33°C for two hours before being supplemented with 2ml of DMEM + 2% FCS per well and returning to the incubator for a further 48 hours at 33°C. Following this the cells were fixed and stained exactly as described in Section 2.6.

The RSV-specific plaques detected in the serum sample and positive control wells were then counted. The reduced number of RSV-specific plaques in the serum samples could then be expressed as a percentage of the total number of RSV-specific plaques obtained in the positive controls thus allowing the calculation of the serum dilution which caused a 50% reduction in RSV-specific plaque formation. This value was then expressed as the effective dose at which there was a 50% reduction in RSV-specific plaque formation (ED50).

2.15 Fragment Cultures

Antibody secreted from lung tissue following infection of mice with RSV was acquired using the fragment culture assay. The anatomical right lung was sectioned (1mm) and two duplicate adjacent sections were washed six times in sterile PBS containing Penicillin/Streptomycin (100U/ml penicillin and 100mg/ml streptomycin - GibcoBRL). The sections were transferred to a 24 well plate (Nunc) containing 1ml of RPMI media (GibcoBRL) (supplemented with penicillin/streptomycin and 10% FCS). The plates were then placed in a 37°C incubator and gassed with 95% O_2 daily. Five days later the supernatants were harvested and frozen at -20°C for subsequent analysis of virus specific antibody by ELISA.

2.16 ELISA assay

ELISA plates (Nunclon) were coated with 50µl/well of purified RSV diluted in carbonate buffer at pre-determined concentrations (1/20 – 1/30) 24 hours prior to use. They were then washed three times with 200µl PBS and blocked with 200µl/well PBS + 1% BSA for one hour at 37°C. Following blocking, the plates were washed once with PBS + 0.05% Tween-20. Sera and standards were then diluted in PBS + 0.05% Tween-20 + 0.5% BSA and 50µl of each dilution were added to the appropriate well in duplicate. Plates were then incubated for two hours at room temperature before being washed four times in PBS + 0.05% Tween-20. Isotype-specific, alkaline phosphatase-conjugated antibodies (Southern Biotechnology Associates) were diluted 1/2000 in PBS + 0.05% Tween-20 + 0.5% BSA and 50µl/well was added and incubated for two hours. Following this the plates were washed four times in PBS + 0.05% Tween-20. p-Nitrophenyl phosphate tablets (Sigma) were diluted in diethanolamine buffer (see **Section 2.6**) to a final concentration of 0.33mg/ml and dispensed at a volume of 100µl per well. Approximately 15 to 60 minutes later, or when the colour developed the reaction was stopped with the addition of 50µl/well of 3M NaOH. Plates were then read at 405 nm on a SpectraMax 340 microplate reader (Molecular Devices) and values were calculated using SoftMax software (version 3.1 – Molecular Devices). The ELISA data was obtained using standard endpoint calculations with the endpoint titre being the last dilution that is greater than the cut-off for the assay, the cut-off being three times the O.D. of the negative control specific for that sample.

2.17 Fragment culture amplification

For the fragment cultures it was necessary to amplify the ELISA signals in order to observe the low titres of antibody. To do this we used the AmpliQ system (Dako) and followed the provided protocol.

2.18 FACS Analysis

The procedure used for FACS staining is as follows and all steps unless otherwise stated, were carried out on ice. The harvested tissues were prepared, counted and adjusted to concentrations between the range 5 x 10^6 and 1 x 10^7 cells/ml. A 100µl volume of cells was then added to each FACS tube and supplemented with 4ml of PBS/BSA (0.1%) solution. The tubes were centrifuged at 328g for seven minutes at 4°C, the supernatant was aspirated and 100-150µl of primary antibody (appropriately diluted in PBS/BSA 0.1% solution) was added to each tube. The tubes were gently vortexed and incubated on ice for thirty minutes. The cells were then washed in PBS/BSA (0.1%) and in some cases were incubated with secondary antibody (appropriately diluted in PBS/BSA 0.1% solution) on ice for a further thirty minutes. The cells were then finally washed and re-suspended in 300-500µl of PBS/BSA (0.1%) and data was acquired using a FACScalibur (Becton Dickenson) running CellQuest Pro software (version 4.0.2 - Becton Dickenson). Data analysis was performed using WinMDI (version 2.8 – Joe Trotter, Scripps Institute).

2.19 Antibodies

Table 2.1: Primary monoclonal antibodies used in flow cytometric analysis

Target Molecule	Clone	Isotype	Conjugate	Supplier	Titre
Mouse CD4	GK1.5	Rat IgG2b, κ	PE	PharMingen	1/400
Mouse CD4	H129.19	Rat IgG2a, κ	FITC	PharMingen	1/400
Mouse CD5	53-7.3	Rat IgG2a, κ	PE	PharMingen	1/400
Mouse CD5	53-7.3	Rat IgG2a, κ	Biotin	PharMingen	1/400
Mouse CD8a	53-6.7	Rat IgG2a, κ	PE	PharMingen	1/400
Mouse CD8a	53-6.7	Rat IgG2a, κ	FITC	PharMingen	1/400
Mouse CD8a	53-6.7	Rat IgG2a, κ	Biotin	PharMingen	1/400
Mouse CD43	S7	Rat IgG2a, κ	PE	PharMingen	1/400
Mouse CD43	S7	Rat IgG2a, κ	FITC	PharMingen	1/400
Mouse CD43	S7	Rat IgG2a, κ	Biotin	PharMingen	1/400
Mouse CD45R/B220	RA3-6B2	Rat IgG2a, κ	PE	PharMingen	1/400
Mouse CD45R/B220	RA3-6B2	Rat IgG2a, κ	FITC	PharMingen	1/400
Mouse CD45R/B220	RA3-6B2	Rat IgG2a, κ	Biotin	PharMingen	1/400

Table 2.2: Secondary antibodies used in flow cytometry

Name	Conjugate	Source	Titration
Streptavidin	QR	Sigma	1/400
Streptavidin	PE	Sigma	1/400
Streptavidin	FITC	Caltag	1/400
Streptavidin	Cy5	Caltag	1/400

Table 2.3: Antiseras used in ELISPOT and ELISA assays

Target molecules	Type	Conjugate	Source
Mouse Ig (H+L)	Goat antisera	Alkaline Phosphatase	Southern Biotechnology Associates (SBA)
Mouse IgA	Goat antisera	Alkaline Phosphatase	Southern Biotechnology Associates (SBA)
Mouse IgG1	Goat antisera	Alkaline Phosphatase	Southern Biotechnology Associates (SBA)
Mouse IgG2a	Goat antisera	Alkaline Phosphatase	Southern Biotechnology Associates (SBA)
Mouse IgG2b	Goat antisera	Alkaline Phosphatase	Southern Biotechnology Associates (SBA)
Mouse IgG3	Goat antisera	Alkaline Phosphatase	Southern Biotechnology Associates (SBA)
Mouse IgM	Goat antisera	Alkaline Phosphatase	Southern Biotechnology Associates (SBA)
Respiratory Syncytial Virus (RSV) - Polyclonal	Goat antisera	Purified	Chemicon International
Respiratory Syncytial Virus – F protein	Goat antisera	Purified	Chemicon International
Goat IgG	Rabbit antisera	Alkaline Phosphatase	Sigma

2.20 General reagents and consumables

Table 2.4: Tissue culture reagents used in these studies

Reagent	Supplier	Catalogue Number
Crystal violet	Sigma	C3886
Dimethyl sulphoxide (DMSO)	Sigma	D-2650
Dulbecco's MEM with Glutamax-1	Gibco	32430-027

Dynabeads M-450, goat anti-mouse	Dynal	110-06
Dynabeads M-450, sheep anti-rat	Dynal	110-08
Foetal bovine serum – lot 40Q1282F	Gibco	10106-169
Glutamax-1	Gibco	35050-038
HEPES Buffer 1M	Gibco	15630-056
Iscove's modified Dulbecco's medium with Glutamax-1	Gibco	31980-022
MEM Amino acids (50X)	Gibco	11130-036
MEM Non-essential amino acids (100X)	Gibco	11140-035
Penicillin/Streptomycin	Gibco	15140-122
Phosphate Buffered Saline (PBS)	Gibco	14200-067
RBC Lysing buffer	Sigma	R7757
RPMI 1640 with Glutamax-1	Gibco	72400-021
Sodium pyruvate MEM 100mM	Gibco	11360-039
Trypan blue 0.4%	Gibco	15250-061
Trypsin EDTA (1X) in HBSS	Gibco	25300-054
Virus purification – serum free media (VP-SFM)	Gibco	11681-020

Table 2.5: Plastics used in these studies

Type of plastic	**Supplier**	**Catalogue Number**
15ml round conical tube	Falcon	2096
24 well plates	Falcon	3043
25cm^2 tissue culture flasks	Falcon	9025
50ml conical tube	Falcon	2070
75cm^2 tissue culture flasks	Falcon	9076

96-well filtration plate	Millipore	MAHA N45 50
96-well flat-bottomed plate	Nunclon	163321
96-well U-bottomed plate	Nunclon	163320
150cm^2 tissue culture flasks	Falcon	90151
Cell strainer 40μm	Falcon	2340
Microlance 3 needle 25GA x 5/8"	Beckton Dickinson	300600
Microlance 3 needle 26GA x 3/8"	Beckton Dickinson	300300
Pleated surface 'TufRol' Roller Bottles	Beckton Dickinson	353079

Table 2.6: Biochemical reagents used in these studies

Reagent	**Supplier**	**Catalogue Number**
Beta-propiolactone (BPL)	Sigma	P5648
2,2,2 tribromoethanol	Sigma	T4840-2
2-mercaptoethanol 50mM	Gibco	31350-010
5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP)	Sigma	B8503
Albumin, bovine	Sigma	A4503
Collagenase A	Roche	1088793
Crystal violet	Sigma	C3886
Diethanolamine	Sigma	D8885
Ficoll Paque plus	Amersham Pharmacia	17-1440-02
Ketamine	Sigma	K2753
Magnesium chloride	Sigma	M8266
Magnesium sulphate-7-hydrate	BDH	101514Y
Milk powder	Marvel	Off shelf

Paraformaldehyde	Sigma	P6148
Percoll	Amersham Pharmacia	17-0891-02
P-nitrophenyl phosphate tablets	Sigma	P9389
Polyoxyethylene sorbitan monolaurate (Tween 20)	Sigma	P2287
Sodium chloride	BDH	102415K
Sodium hydroxide	BDH	102524X
Sucrose	BDH	102744B
tert-amyl alcohol (2-methyl-2-butanol)	Sigma	A1685
Trizma hydrochloride	Sigma	T5941
Xylazine	Sigma	X1251

Table 2.7: Reagent kits used in these studies

Reagent kit	**Supplier**	**Catalogue number**
AmpliQ ELISA kit	DAKO Ltd	K6245

Table 2.8: Cell lines used in experiments

Clone name	**Target molecule**	**Supplier**
130-8F	RSV Fusion protein	Gift from Dr. Ralph Tripp CDC, Atlanta
30-H-12	Thy 1.2	ATCC
53-6-72	CD8	ATCC
BS-C-1	N/A	ATCC
RA3-6B2	CD45R/B220	ATCC
Vero	N/A	Institute for Animal Health, Compton, UK

Chapter Three: Clearance of respiratory syncytial virus from the murine lung.

3.1 Introduction

The respiratory tract is the primary site of entry for many viral pathogens with several viral agents initiating replication on the bronchiopulmonary mucosa and then disseminating systemically without causing local clinical disease (Welliver and Ogra, 1988). This is not true of all viruses though and for a large number of them the respiratory tract is the main focus for disease.

Classic studies (Graham et al., 1991b; Taylor et al., 1984) have demonstrated that inoculation of BALB/c mice with high doses of RSV leads to viral replication of high titre in the lung, however, it has been suggested that RSV does not infect the mouse and that it is merely the presence (without infection) of the virus in the lung that is detected during many studies. Since it is also unclear as to what degree the lung of a previously immunised animal is protected from subsequent infections we quantified the number of virus particles as a function of time during primary, secondary and tertiary exposures.

We collected lungs from RSV infected mice at selected time points after primary, secondary and tertiary infection. The virus present in the lungs was then isolated and titrated on a 90% confluent cell monolayer (see Materials and Methods). After incubating for forty-eight hours at 33°C the numbers of RSV plaque forming units were counted using specific anti-RSV antibodies (Materials and Methods – **Table 2.3**).

The results shown at each time point were obtained from four individual BALB/c mice and are displayed both individually and as the mean of the four mice.

3.2 Results

3.2.1 Comparison of virus titres in the lung following primary and subsequent exposures to RSV

Following primary RSV infection virus is readily detectable in the lungs of mice. The virus titre on day three is $2.7 \pm 1.1 \times 10^3$ plaque forming units per millilitre (pfu/ml) but this rapidly rises to a peak of $11.4 \pm 8.3 \times 10^3$ pfu/ml two days later. By day seven the virus titre is declining and viruses could no longer be detected ay day ten (**panel A, Figure 3.1**). Pursuant to secondary challenge with the same dose of RSV, the titres seen were much reduced, approximately one log lower in magnitude (**panel B, Figure 3.1**). Additionally, it should be noted that following secondary infection one of the four mice at each time-point did not clear the virus as well as the other three. When immunised with a third dose of RSV the virus titres observed at day two post-infection were $5.7 \pm 3.4 \times 10^2$ pfu/ml (**panel C, Figure 3.1**).

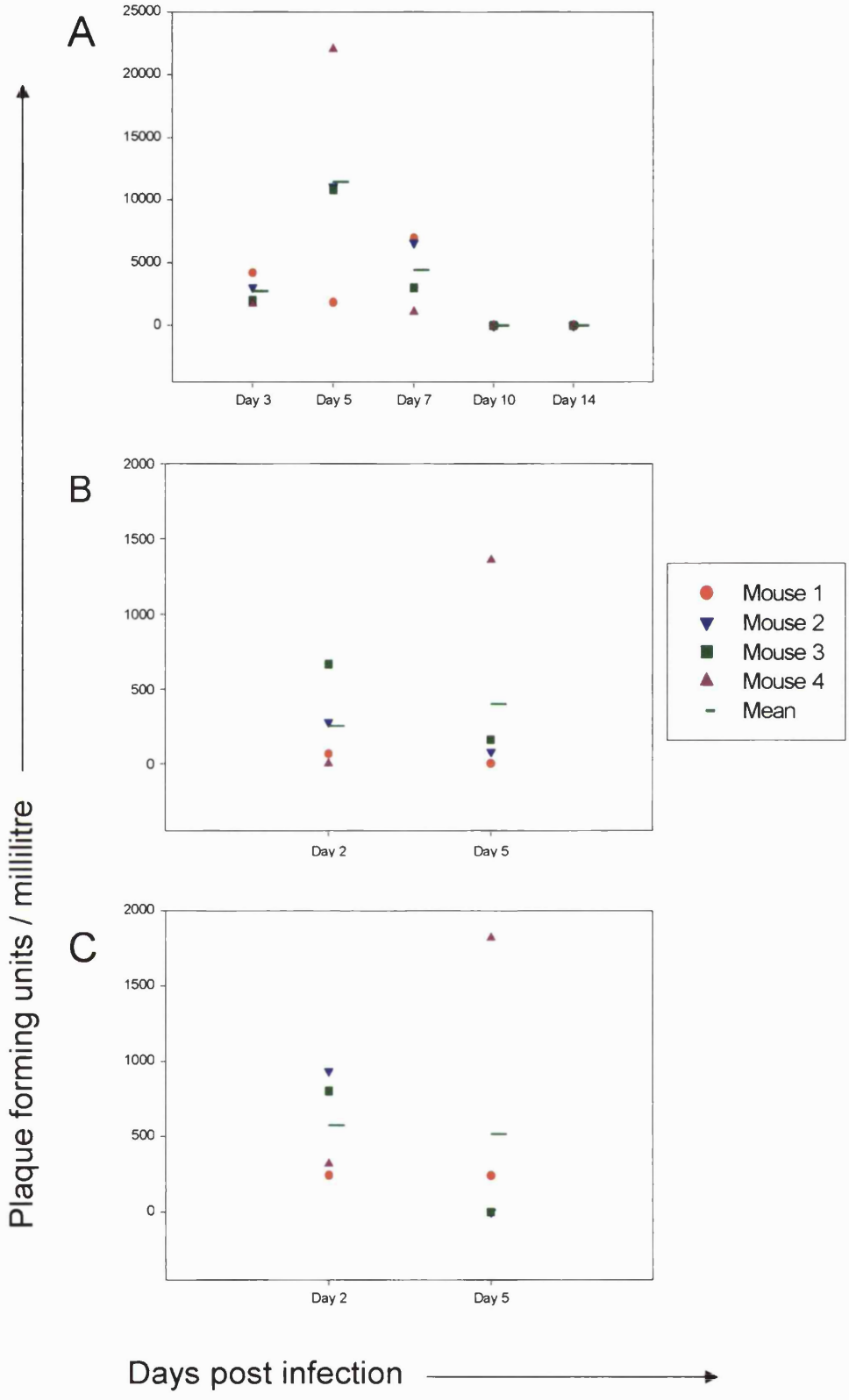


Figure 3.1 Plots representing the amount of RSV (measured in plaque forming units per millilitre) detected in murine lungs on selected time-points following primary, secondary and tertiary infection with live RSV. **A** represents virus titres following primary infection; **B** represents virus titres following secondary infection, and **C** represents virus titres following tertiary infection. This experiment was performed once and each time-point displays four individual mice and the mean of those mice.

3.3 Discussion

In infants, reinfection with RSV has been reported to occur within weeks of recovery from primary infection (Henderson et al., 1979). In adults with high levels of circulatory neutralizing antibodies, upwards of 25% immune individuals could be re-infected with RSV of the same serotype within two months of natural infection (Hall et al., 1991). These and related observations suggest that the duration of protective immunity to challenge with RSV infection is short-lived.

Although the experiments in this chapter were only performed once the presence of virus in the lungs, up to and beyond five days post-exposure, strongly suggests that RSV actively replicates in the lung during primary infection. This compellingly suggests that any immunological responses detected in the lung occur as a result of virus replication and are not merely due the residual presence of the initial inoculate. During primary infection peak titres are achieved on day five and the virus appears to be completely cleared from the lung five days later. Secondary and tertiary challenges demonstrate a significant decrease in lung viral titre in comparison with those observed during primary infection, however virus is still detectable up to five days post-challenge.

Primary clearance of viruses from the lungs is normally achieved via $CD8^+$ T-cells and, during this first exposure of the virus to the immune system, virus–specific antibody is generated in order to protect the lungs from subsequent exposure. However, the presence of virus (although at low titre) in the lungs after secondary and tertiary challenge suggests that, in this case, protection from RSV infection may be impaired. It has previously been shown that there is an impairment of both the pulmonary CD8+ T-effector response and the development of pulmonary CD8+ memory during primary

RSV infection (Chang and Braciale, 2002) and this may account for the data observed in these experiments. This study also suggested that, in addition to RSV suppressing the development of peripheral $CD8^+$ T cell memory in the lungs, it may also inhibit the function of virus-specific $CD4^+$ T cell effectors, possibly influencing both cellular and humoral immunological memory in this compartment. This would be highly significant as it could impact upon the strength and durability of the humoral immunologic response and the generation and maintenance of B cell memory at this site, substantiating our data observed during the secondary and tertiary challenge experiments. However, at this point this hypothesis is only speculation but later on in this thesis (**chapter seven**) we will show data demonstrating very poor RSV-specific local antibody responses during RSV infection.

Alternatively, infection of the epithelial cells of the respiratory tract with RSV may suppress B-cell effector functions through the secretion of inhibitory host soluble factors (RANTES, MCP -1, MIP1α, IL-8) (Olszewska-Pazdrak et al., 1998; Preston et al., 1995; Thomas et al., 2000). If RSV mediated antibody suppression were taking place then the reduced capacity of the lung to clear infectious RSV would explain why virus is so readily detectable following secondary and tertiary infection. It would also suggest that innate immunity has a more substantial role to play in the immune response to RSV in the lung and that alone it is insufficient to protect against infection. This concept will be fully discussed later on in the context of the data as a whole (**chapter eight**).

An alternative explanation as to why clearance of RSV from the lung may be impaired could be due to the virus itself somehow circumventing the antiviral products of immune cells such as cytokines. The action of interferons (IFNs) on virus-infected cells

and their surrounding tissues elicit an antiviral state that is characterized by the expression and antiviral activity of IFN-stimulated genes, in turn, viruses encode proteins that can counteract the host response and support efficient viral replication, thereby minimizing the therapeutic antiviral power of IFNs (Katze et al., 2002). Studies on the bovine form of RSV show that both the NS1 and NS2 non-structural proteins have the ability to cooperatively antagonize the antiviral effect of IFN (Gotoh et al., 2001). Whilst this not yet been demonstrated with the human form of RSV, it may well take place as the ability to circumvent IFN is a common strategy of immune evasion in several viruses including influenza, hepatitis C, herpes simplex and vaccinia (Katze et al., 2002).

The presence of live RSV within the lungs of mice during secondary and tertiary infection may provide a significant clue as to why RSV is able to re-infect humans so frequently and with such apparent ease. Alteration or suppression of immune processes in this location may play a key role in explaining why protection is incomplete and would warrant further detailed study of the mechanisms underlying them. Indeed the characterisation of RSV-specific $CD8^+$ responses such as those described previously by Chang and Braciale (Chang and Braciale, 2002) have already demonstrated intriguing new insights into the immune response to RSV and, combined with the data presented in this thesis, developments in functional genomics, bioinformatics and other emerging technologies, may go some way to explaining how RSV is able to avoid generating protective immunity in the host.

Although the mouse is only considered to be semi-permissive for RSV infection we, and others (Graham et al., 1991b), have demonstrated the presence of live virus in the lungs of mice during primary infection. However, the isolation of live virus following

secondary RSV challenge is significant as it has not been reported before. This data provides evidence that infection does take place *in vivo* and further indicates that any immune responses observed during these challenge studies are attributable to the action of a live, replicating pathogen.

In conclusion we have shown that infection and replication of the lung with RSV does occur in mice and that live virus is present in the lung for up to five days following secondary and tertiary challenge. This data would seem to parallel the human situation where successive re-infection is a hallmark of the disease and so, even with its limitations as a disease model, the mouse may more accurately represent RSV infection than was previously thought.

Chapter Four: Phenotyping and characterisation of lymphoid tissues following RSV infection and treatment with BPL-RSV.

4.1 Introduction

Humans and higher primates are the most permissive species for infection with RSV (Chanock et al., 1957; Morris et al., 1956), however, because of the problems associated with the infamous 1960's vaccine debacle many groups are understandably reticent to undertake any research involving the administration of any form of RS virus to children. As an alternative the mouse model provides a simple and accessible tool for research (Prince et al., 1979; Taylor et al., 1984) although its role in the immunology of RSV is contentious. Although mice are only semi-permissive to infection with RSV it is possible to infect them, given a sufficient dose, and this has been demonstrated in several studies (Graham et al., 1988). However, relatively little is known about the humoral response of mice to RSV, especially in the context of the nasal-associated lymphoid tissue (NALT).

As described previously in this thesis the NALT can be distinguished into two compartments, the organised (O-NALT) and diffuse (D-NALT) (Asanuma et al., 1997). It has been suggested that the O-NALT acts as a mucosal inductive site, where naïve B and T-cells are clonally selected and expanded upon antigen contact, and the D-NALT is thought to act as an effector site where these activated B and T-cells relocate to after antigen-priming in order to express their effector functions. However, little is known

about the immunological potential of these inductive and effector sites in the respiratory tract. Studies with pathogens other than RSV have demonstrated that the NALT is capable of acting as an inductive site for virus-specific humoral and cellular immune responses (Wu et al., 1996; Zuercher et al., 2002) but these studies did not distinguish between the two classes of NALT described by Asanuma *et al.*., 1997 (Asanuma et al., 1997) and so the data must be interpreted carefully.

In order to examine the characteristics of these key sites the following study was designed to investigate the cellular dynamics and phenotypic differences within the NALT and other selected tissues during the course of an infection(s) with RSV. In order to show that any effects observed were attributable to actions of a live, replicating virus control groups utilising mock infections and beta-propriolactone inactivated RSV were used when necessary.

Mice aged between 8-12 weeks were immunised with either live RSV, inactivated RSV (BPL-RSV) or cell supernatant (mock infection) and analysed as a function of time.

BALB/c mice were immunised intranasally once with either live-RSV A2 (1×10^7 pfu/ml) or cell supernatant (mock infected) and the cellularities of the nasal associated lymphoid tissue (NALT), the cervical lymph nodes (CLN) and the mediastinal lymph nodes (MLN) enumerated and compared as in **Figure 4.1**. The phenotype of the tissues in these mice was also determined by FACS analysis using the cellular markers CD4, CD8 and B220 to identify the T and B cell populations as seen in **Figures 4.2 – 4.5**.

The second group were immunised intranasally either three times (at six-week intervals) with live-RSV A2 (1×10^7 pfu/ml) to give a primary, secondary and tertiary response or

with a combination of BPL-inactivated RSV followed by a further two challenges with live-RSV (again at six-week intervals) and the cellularities of the O-NALT, D-NALT, CLN, MLN, spleen and bone marrow compared as in **Figures 4.6 – 4.11**. Naïve mice aged between 8-12 weeks receiving no treatment were used as a control during this study.

As the cell numbers in the nasal tissues are consistently low, all of the tissues in this series of experiments are pooled from four mice and, in the case of the CLN, the four most superficial lymph nodes were taken on each occasion. The data is thus displayed as the mean of these four mice and, as this experiment was only performed once, there are no applicable statistics displayed. For the studies that utilised FACS, the data was obtained from gates located on the lymphocyte populations.

4.2 Results

4.2.1 The comparative effect of primary RSV and mock infection on the cellularity of the NALT and draining lymph nodes of the neck and lung in the mouse.

4.2.1.1 Nasal tissues

In the O-NALT, higher total nucleated cell counts were observed in RSV-infected mice than those immunised with media (mock control). Over the course of the experiment this difference in cell number between the RSV and mock infected mice was maintained although both mice possessed similar cellular dynamics (**Figure 4.1**). In the D-NALT the RSV infected mice had higher numbers of cells compared with mock treated mice and on day fourteen, the cellularity in the D-NALT of the RSV infected mice was 1.57 x 10^6 total nucleated cells, a value almost ten-fold higher than that observed in the D-NALT of the mock infected mice at 2.20 x 10^5 total nucleated cells (**Figure 4.1**).

4.2.1.2 Draining lymph nodes

The RSV infection resulted in an apparent increase in the total number of nucleated cells within the CLN over the time-course of the experiment. Since this was not seen in the mock treated mice, who showed a variable response with no discernable trend, it suggests that the increase in cellularity may be directly related to the live RSV infection. (**Figure 4.1**). Within the MLN, six days after treatment, the cellularities of both the RSV and mock treated mice are very similar, and this observation can be applied for the reminder of the experiment (**Figure 4.1**).

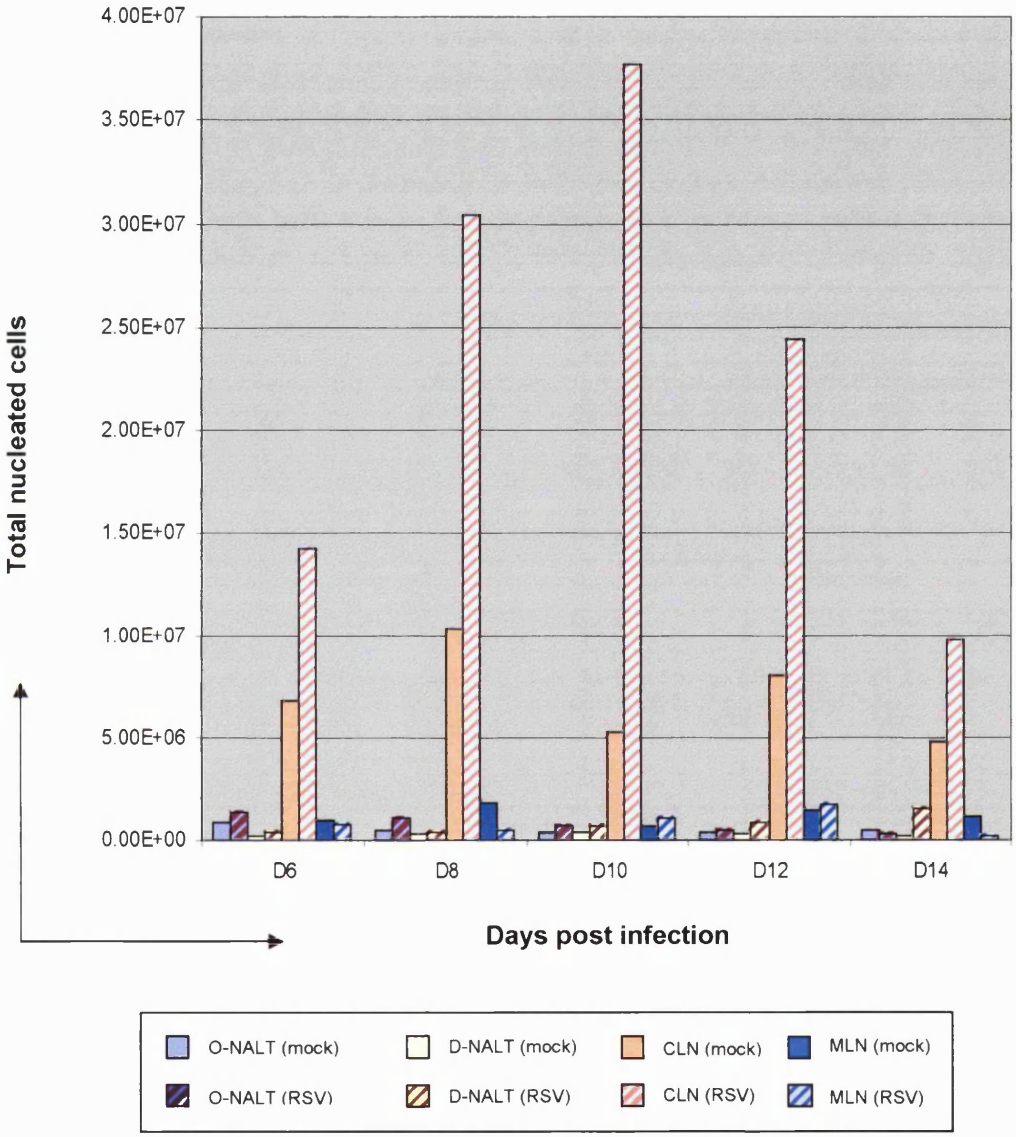


Figure 4.1 A graph of the total nucleated cell numbers of selected tissues (O-NALT, D-NALT, MLN and CLN) taken from mice immunised with either live-RSV A2 (1 x 10^7 pfu/ml) or with supernatant from the uninfected cell line used to cultivate RSV (mock). The values displayed represent pooled tissues from four BALB/c mice. This experiment was performed once.

4.2.2 Distribution of B and T-lymphocyte populations within the NALT following primary RSV infection

4.2.2.1 O-NALT

From the first graphs (**panels A & B, Figure 4.2**) it is apparent that there are few, if any phenotypic differences between the O-NALT from RSV infected mice and the control (mock immunised) mice. In the naïve mouse, the distribution of lymphocytes in the O-NALT is approximately 70% B cells, approximately 25% $CD4^+$ T cells and approximately 5% $CD8^+$ T cells, giving a CD4:CD8 ratio of 5:1 within this tissue. The size of the lymphocyte subpopulations within the RSV infected group does not change greatly and, given that similar distributions occur in both the T and B subpopulations of the media control mice, this suggests that these changes are non-specific and as such cannot be solely attributed to infection of the mouse with RSV.

4.2.2.2 D-NALT

B cells are also the prominent lymphoid subpopulation in the D-NALT and represent more than 25% of the total lymphocyte population in the naïve mouse. No apparent changes within the D-NALT were observed after primary infection with live RSV. Again the media control group closely matched the distribution in the RSV infected group (**panels A & B, Figure 4.3**). The inherent difficulties stemming from the processing of D-NALT tissue makes it difficult to draw firm conclusions from this data as variations in lymphoid cell subpopulation may be attributable to the extraction process and not from any immune mediated processes.

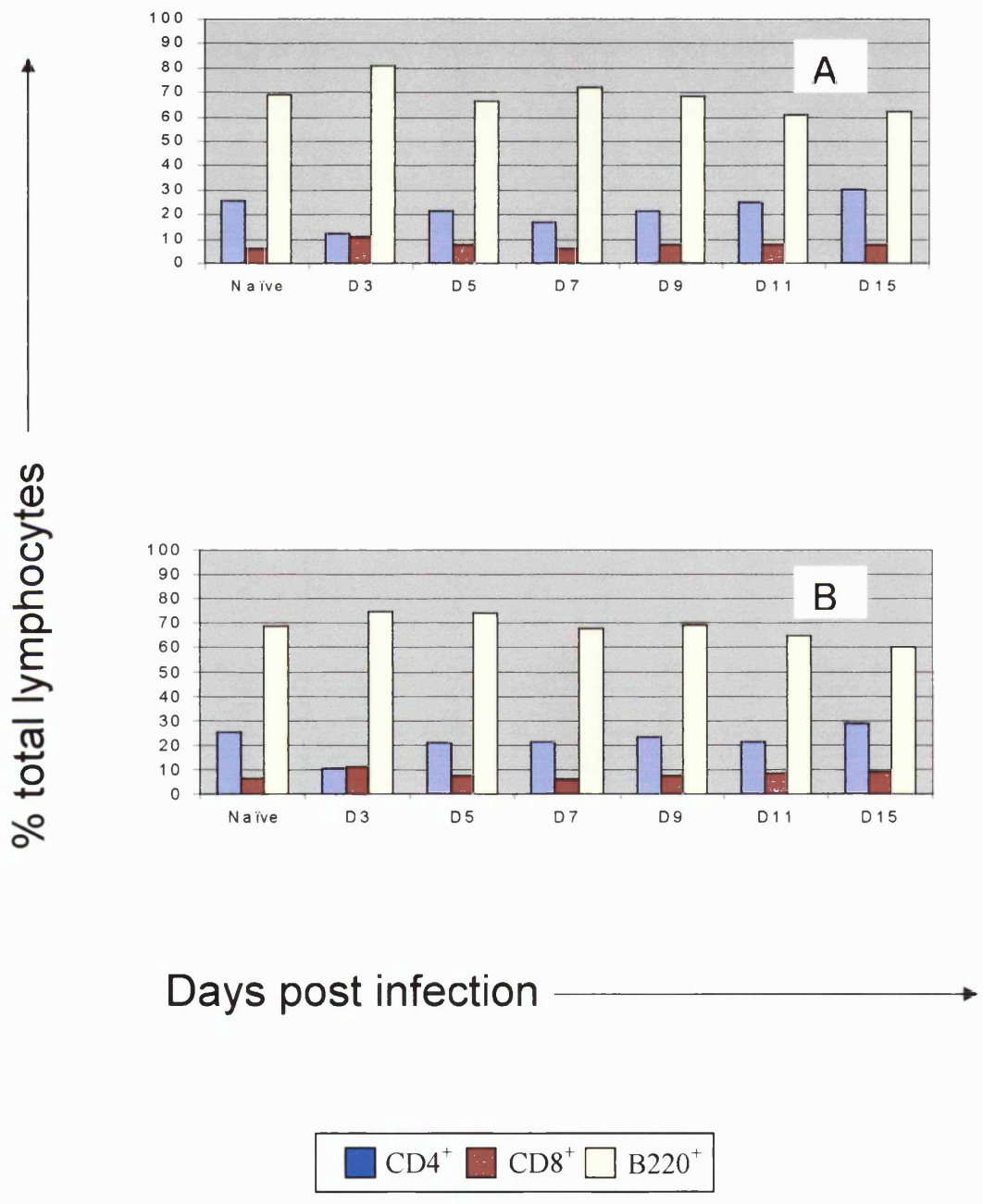


Figure 4.2 The percentage of cells positive for the markers CD4, CD8 and B220 following primary infection with live-RSV in the O-NALT. Data was obtained from both RSV infected and mock treated mice. **A** represents mock treated mice and **B** represents RSV infected mice.

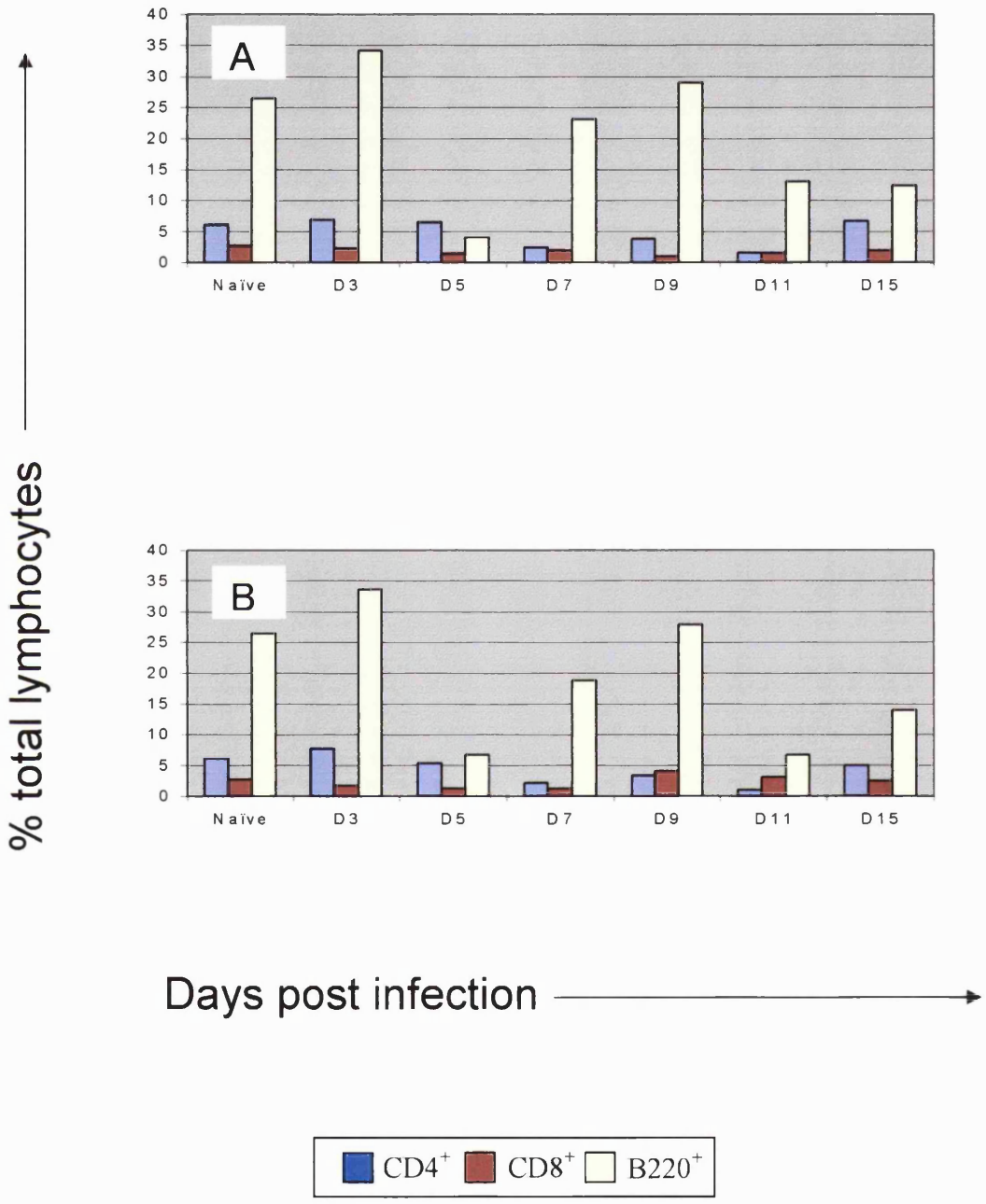


Figure 4.3 The percentage of cells positive for the markers CD4, CD8 and B220 following primary infection with live-RSV in the D-NALT. Data was obtained from both RSV infected and mock treated mice. **A** represents mock treated mice and **B** represents RSV infected mice.

4.2.3 Measurement of B and T-lymphocyte populations within the draining lymph nodes of the lung and neck following primary RSV infection

This series of experiments were performed only once and as such the figures quoted in this section are representative of single experiments only and therefore cannot be statistically verified at present.

4.2.3.1 CLN

In the naïve mouse, this tissue consisted predominantly of T cells with over 50% of the total lymphocyte population consisting of $CD4^+$ T cells (**panel B, Figure 4.4**). $CD8^+$ T cells were also present but only represented 20% giving a CD4/CD8 ratio of approximately 2.5:1. B cells accounted for approximately 26% of the lymphoid population. Although the CLN showed the greatest influx of cells in response to RSV infection, the general composition of the lymphocytes did not change compared to the mock treated controls (**panel A, Figure 4.4**). It must be noted that during the day seven and nine time points the virus infected group did differ from the controls in that they had comparatively lower numbers of B cells, however as this experiment was only performed once we cannot draw firm conclusions from this

4.2.3.2 MLN

In the draining lymph node of the lungs, the size of the lymphocyte subpopulations in the naïve mouse were as follows: B cells accounted for 38%, $CD4^+$ T cells accounted for 21% and $CD8^+$ T cells accounted for 11%. Following virus infection the T lymphocyte populations did fluctuate but these changes were observed in both the live

RSV and mock treated mice. B cell levels remained constant (**panel B, Figure 4.5**) and as with the T lymphocyte populations, any changes observed in the mock treated controls were very similar to those observed in the RSV infected mice (**panel A, Figure 4.5**).

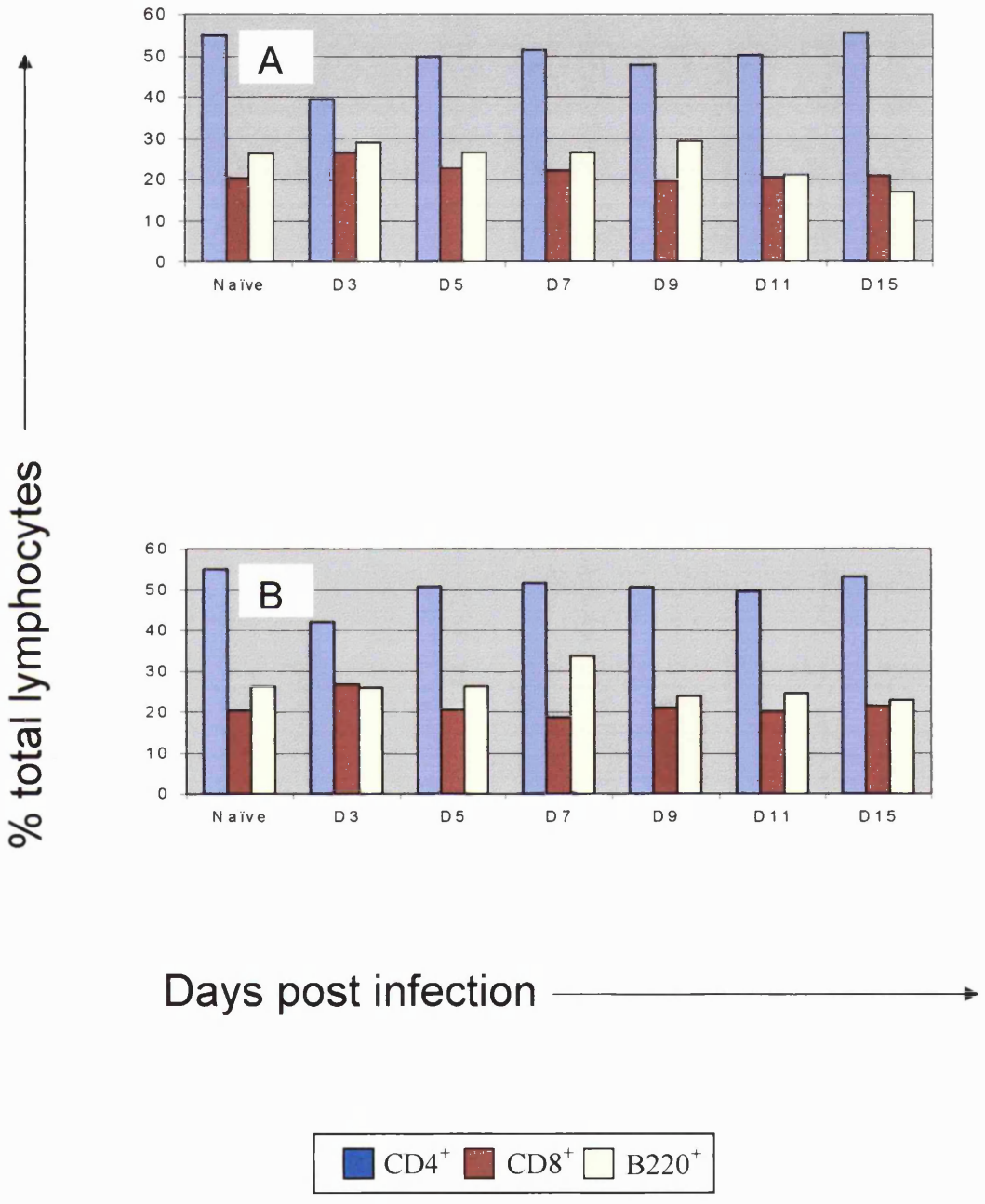


Figure 4.4 The percentage of cells positive for the markers CD4, CD8 and B220 following primary infection with live-RSV in the CLN. Data was obtained from both RSV infected and mock treated mice. **A** represents mock treated mice and **B** represents RSV infected mice.

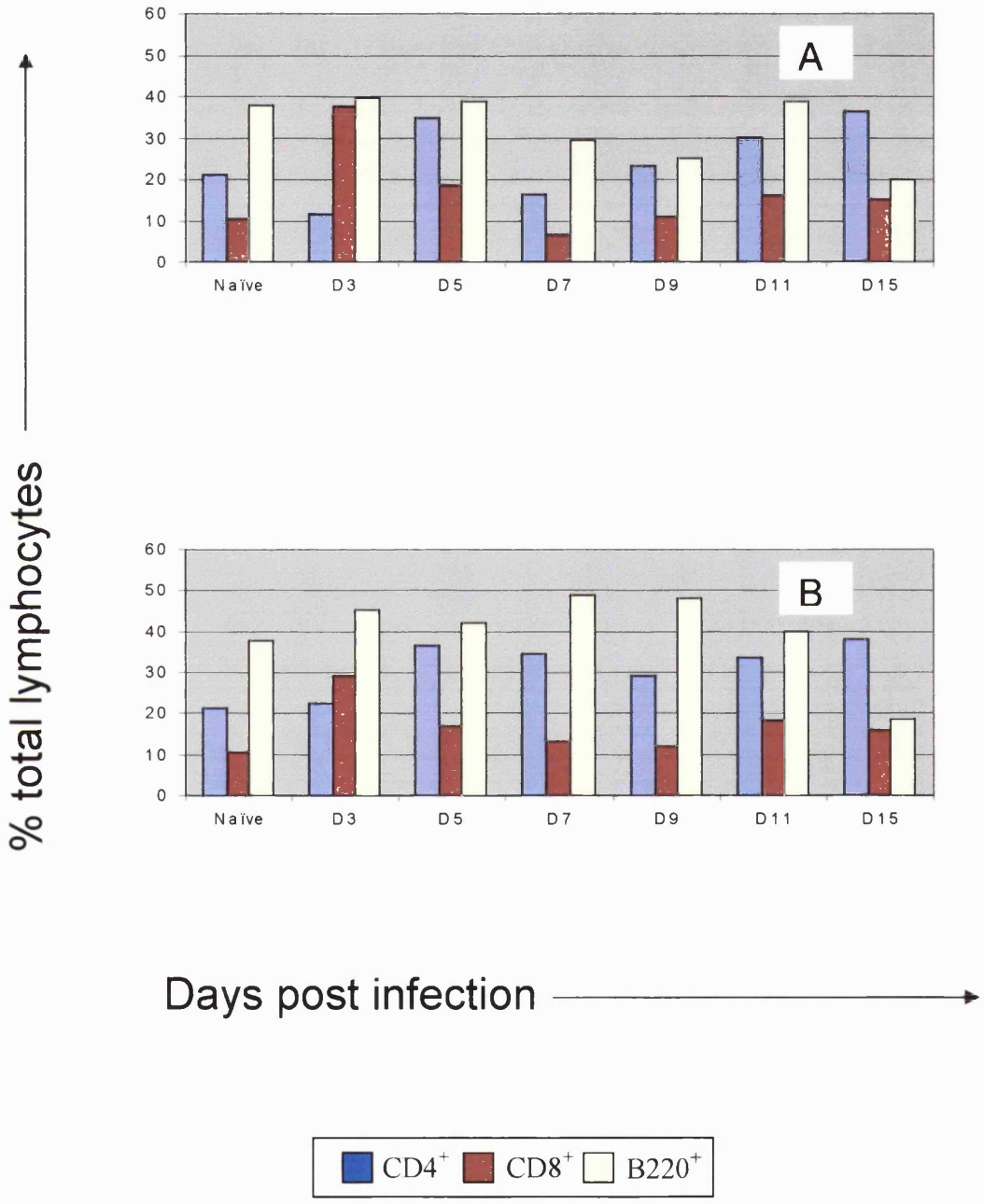


Figure 4.5 The percentage of cells positive for the markers CD4, CD8 and B220 following primary infection with live-RSV in the CLN. Data was obtained from both RSV infected and mock treated mice. **A** represents mock treated mice and **B** represents RSV infected mice.

4.2.4 The effect of live RSV on the cellularity of murine lymphoid organs following primary, secondary and tertiary immunisation.

This series of experiments were performed only once and as such the figures quoted in this section are representative of single experiments only and therefore cannot be statistically verified at present.

Primary immunisation

4.2.4.1 Nasal tissues

In O-NALT, following primary immunisation, there appears to be a modest increase in cell number on day six post-infection (3.40×10^6) but by day ten they have returned to levels close to those observed in the naïve mouse (**panel A, Figure 4.6**). Although it is difficult to accurately enumerate cell numbers in the D-NALT, compared to the naïve mouse (in this experiment), a drop in D-NALT cell number after primary infection with RSV can be seen on day six (3.72×10^5). There appears to be some recovery two days later but from this point onwards the cell numbers remain relatively unchanged (**panel A, Figure 4.6**).

4.2.4.2 Draining lymph nodes

Possessing higher cell numbers than most of the other tissues examined, the CLN cellularity increases in number to a peak of 4.12×10^7 around day eight before declining shortly after (**panel A, Figure 4.6**). The cellularity of the MLN also peaks on day eight

(7.20×10^6) with cell numbers remaining at a level more than double that of the naïve mouse at 1.00×10^6 until at least fourteen days post-infection (**panel A, Figure 4.6**).

4.2.4.3 Spleen and bone marrow

Very little change in cellularity is observed during the course of the infection in the spleen or bone marrow (**panel B, Figure 4.6**).

Secondary immunisation

4.2.4.4 Nasal tissues

Within the O-NALT, two days after secondary immunisation, the cell numbers are similar to those seen on day six of the primary experiment at 3.75×10^6 and are greater in comparison to the naïve mouse at 8.50×10^5. Over the course of the secondary immunisation the cellularity of the O-NALT does not appear to change significantly but still remains elevated in comparison to the naïve mouse (**panel C, Figure 4.7**). Conversely, the cellularity of the D-NALT on day two (1.70×10^6) is slightly less than that seen in naïve mice (2.39×10^6) and by day five it has decreased further, varying throughout the secondary challenge time course (**panel C, Figure 4.7**).

4.2.4.5 Draining lymph nodes

On day two, the cellularity in the CLN is similar to values observed in both the naïve mouse and on day six during the primary experiment, however this value decreases after this point and, by day thirteen, has dropped to a value less than half of the naïve cell

count at 1.00 x 10^7 total nucleated cells (**panel C, Figure 4.7**). With regard to the MLN, two days after secondary challenge the cell count is comparable to that of the naïve value and a minor increase in cellularity is observed over days seven-to-nine which returns to low levels on day thirteen (**panel C, Figure 4.7**).

4.2.4.6 Spleen and bone marrow

Over the course of the secondary experiment the cell number within the spleen varies slightly but there appear to be no significant changes taking place (**panel D, Figure 4.7**). The same is true for the bone marrow, the data showing an almost static cellularity profile for the majority of the experiment (**panel D, Figure 4.7**).

Tertiary immunisation

4.2.4.7 Nasal tissues

Pre-challenge (D-1), cell numbers in the O-NALT are higher (2.10 x 10^6) than for naïve mice but, following tertiary immunisation there is no major change in cell number (**panel E, Figure 4.8**). Within the D-NALT, very low cell numbers (3.70 x 10^5) are observed pre-challenge (D-1) which remain at these low levels for the reminder of the experiment (**panel E, Figure 4.8**).

4.2.4.8 Draining lymph nodes

Prior to tertiary immunisation (D-1), CLN cell numbers have recovered after the decrease seen during the secondary immunisation at a value of 2.10 x 10^7 total nucleated cells but, on day nine the numbers again decline and by day forty-four have fallen by almost 50% to 1.10 x 10^7 total nucleated cells (**panel E, Figure 4.8**). After tertiary immunisation the cellularity in the MLN doubles to 1.39 x 10^6 total nucleated cells and remains at this level until day twenty-nine where it returns to pre-challenge values (**panel E, Figure 4.8**).

4.2.4.9 Spleen and bone marrow

Prior to tertiary immunisation the cell numbers in the spleen appear to be higher than those seen in the naïve mouse and, following tertiary immunisation, increase to a peak of 5.20 x 10^8 total nucleated cells on day nine. The cell numbers then remain at this approximate level until the end of the experiment (**panel F, Figure 4.8**). Following tertiary infection there is no significant change in bone marrow cell number observed for the duration of the experiment (**panel F, Figure 4.8**).

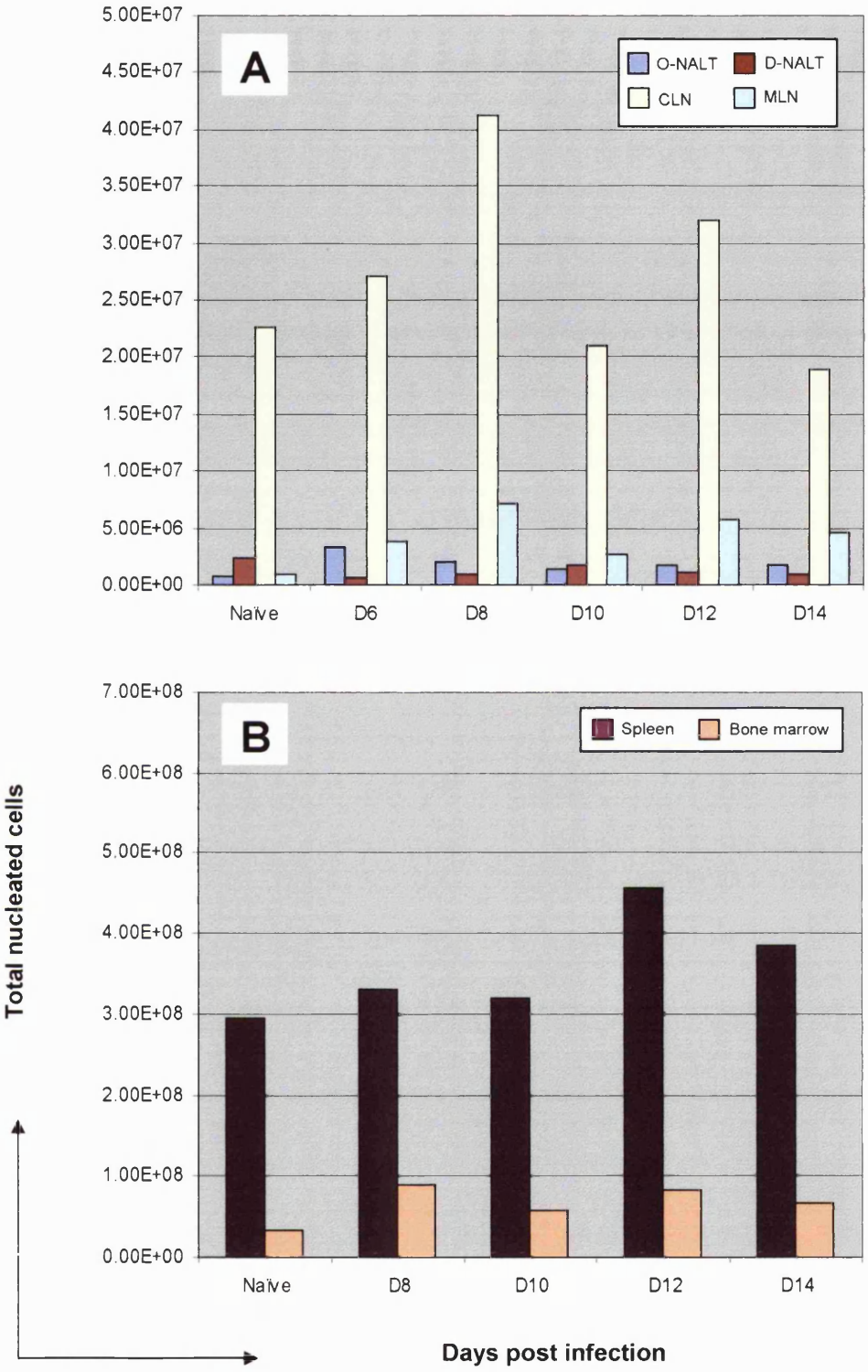


Figure 4.6 Analysis of the total cell numbers found within selected tissues after primary immunisation of BALB/c mice with RSV. **A** represents the cellularities of the O-NALT, D-NALT, CLN and MLN; **B** represents the cellularities of the spleen and bone marrow. All values represent pooled groups of four BALB/c mice.

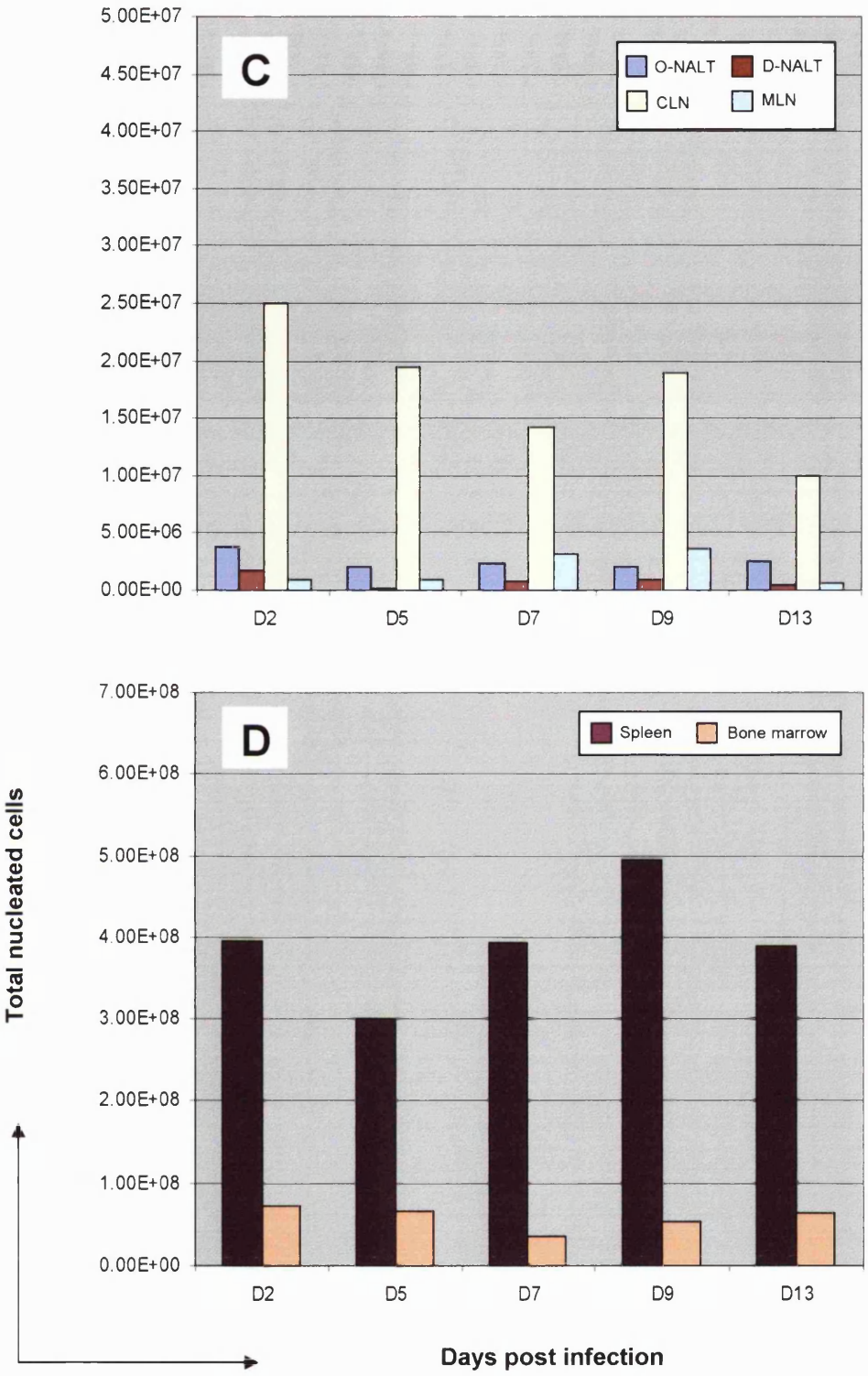


Figure 4.7 Analysis of the total cell numbers found within selected tissues of BALB/c mice after two immunisations with live RSV. **C** represents the cellularities of the O-NALT, D-NALT, CLN and MLN; **D** represents the cellularities of the spleen and bone marrow. All values represent pooled groups of four BALB/c mice. Challenges were made at six-week intervals.

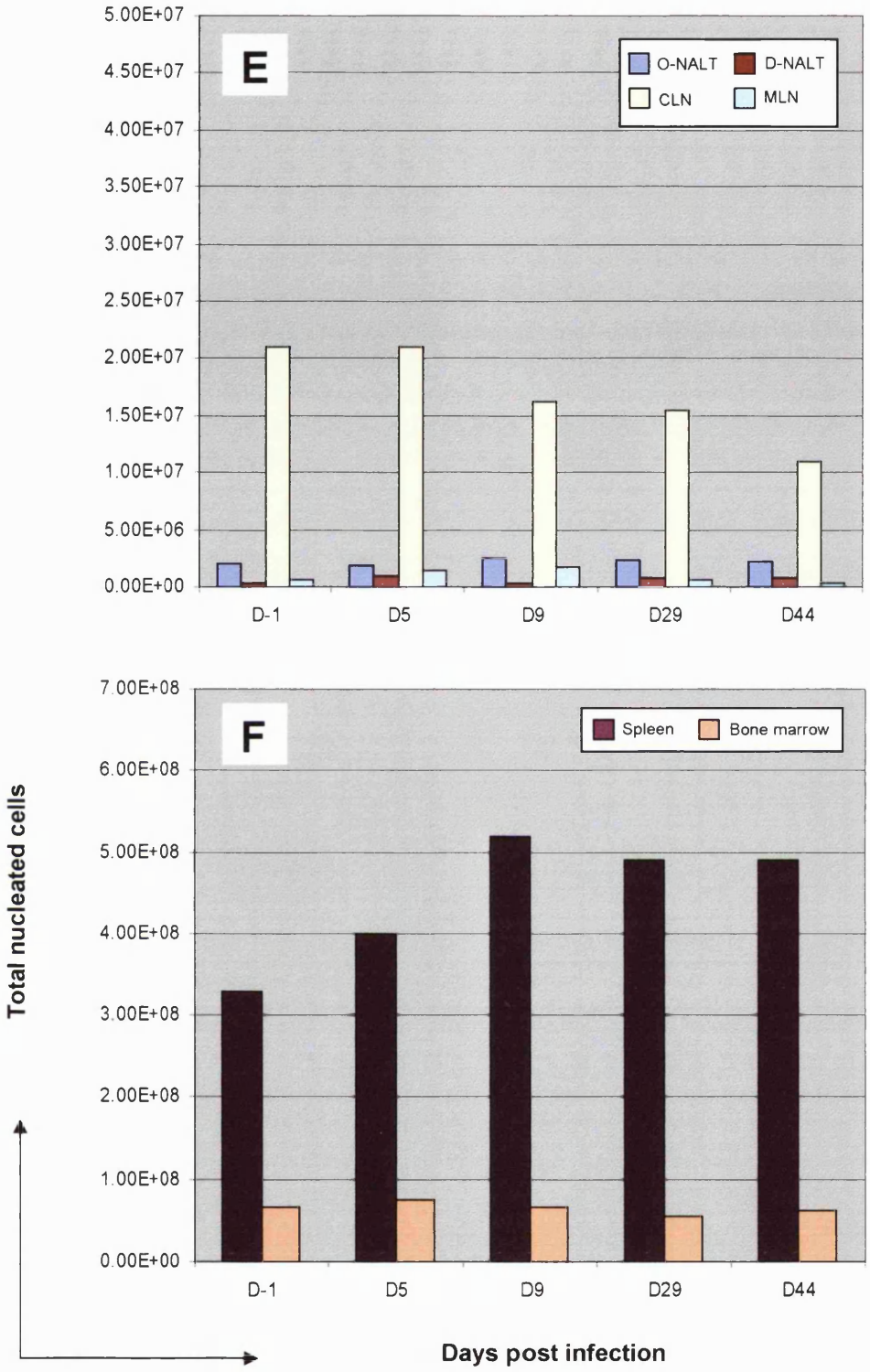


Figure 4.8 Analysis of the total cell numbers found within selected tissues of BALB/c mice after three immunisations with live RSV. **E** represents the cellularities of the O-NALT, D-NALT, CLN and MLN; **F** represents the cellularities of the spleen and bone marrow. All values represent pooled groups of four BALB/c mice. Challenges were made at six-week intervals.

4.2.5 The effect of BPL-inactivated RSV on the cellularity of murine lymphoid organs following challenge with live RSV.

This series of experiments were performed only once and as such the figures quoted in this section are representative of single experiments only and therefore cannot be statistically verified at present.

Primary immunisation with BPL-RSV

4.2.5.1 Nasal tissues

No significant changes in cell number within the O-NALT were observed throughout the experiment (**panel G, Figure 4.9**). Similar results were seen in the D-NALT where, compared to naive mice, the cellularity following primary immunisation is similar, if not slightly decreased, for the duration of the experiment (**panel G, Figure 4.9**).

4.2.5.2 Draining lymph nodes

Following primary immunisation there are no detectable trends in cell numbers in the CLN (**panel G, Figure 4.9**). Whilst much lower in comparison to the CLN, the MLN cell numbers appear relatively static during primary immunisation and are very similar to naïve levels (**panel G, Figure 4.9**).

4.2.5.3 Spleen and bone marrow

After primary immunisation the cell numbers of the spleen vary slightly with no major changes (**panel H, Figure 4.9**). Within the bone marrow, the cell numbers initially increase, and appear to be more than double the numbers seen in the naïve mouse. The cellularity is then maintained at this approximate level for the remainder of the experiment (**panel H, Figure 4.9**).

Secondary immunisation with live RSV

4.2.5.4 Nasal tissues

Following secondary immunisation (first exposure to live RSV) the O-NALT cellularity (2.59×10^6) is almost double the value seen at two days post-infection during the primary immunisation. However, the cell numbers do not vary much during the secondary immunisation and remain at this approximate level (**panel I, Figure 4.10**). This is not the case with the D-NALT where, after secondary immunisation the cell numbers are severely decreased, dropping to numbers almost ten-fold less than seen in the naïve mouse. The cell recovery at day two post infection is 3.40×10^5 total nucleated cells compared with 2.39×10^6 total nucleated cells in the naïve mouse and these numbers then remain at this low level for the remainder of the secondary experiment (**panel I, Figure 4.10**).

4.2.5.5 Draining lymph nodes

Day two post-immunisation, the CLN cell numbers (2.31×10^7) are comparable to the naïve mouse and remain at this approximate level until the end of the experiment (**panel**

I, Figure 4.10). Immediately following secondary immunisation the MLN cellularity on day two is 2.47 x 10^6 total nucleated cells, more than twice the naïve value, and this increased cellularity is maintained throughout the experiment (**panel I, Figure 4.10**).

4.2.5.6 Spleen and bone marrow

An increase (compared with the naïve mouse) in cell number was observed in the spleen two days after secondary immunisation (4.91 x 10^8) and this elevated value is maintained for the remainder of the experiment (**panel J, Figure 4.10**). The cellularity of the bone marrow after secondary immunisation appeared comparable to that observed during the primary immunisation with similar cell numbers which were maintained with little variation for the rest of the experiment (**panel J, Figure 4.10**).

Tertiary immunisation with live RSV

4.2.5.7 Nasal tissues

One day prior to tertiary immunisation (D-1), the O-NALT cellularity (3.40 x 10^7) is elevated compared with naïve mice (8.5 x 10^5) but following tertiary immunisation there is no significant change in cellularity observed for the remainder of the experiment (**panel K, Figure 4.11**). Within the D-NALT the cell numbers are still very low and, no significant changes in cellularity are observed during the course of the time-course (**panel K, Figure 4.11**).

4.2.5.8 Draining lymph nodes

Cell numbers in the CLN prior to tertiary immunisation (D-1) are initially low compared with the naïve mouse and during previous immunisations. Following tertiary immunisation however there is an increase in cellularity on day nine (2.48×10^7) that declines to pre-challenge levels by the end of the experiment (**panel K, Figure 4.11**). Within the MLN, tertiary immunisation appears to significantly boost cell numbers (in comparison to those observed prior to tertiary immunisation), with an almost ten-fold increase in cell number (compared with D-1) being seen on day five (4.50×10^5). However, this effect is transient and by day nine and certainly by day forty-four the cell numbers have decreased and are present at a similar level (5.30×10^5) as seen prior to tertiary immunisation (**panel K, Figure 4.11**).

4.2.5.9 Spleen and bone marrow

Within the spleen there was an initial a decrease in cell number following tertiary immunisation compared with the pre-challenge time point (D-1). However the cell numbers recovered quickly and by day forty-four had reached a cellularity of 5.59×10^8 total nucleated cells towards the end of the experiment, a value almost double that of the naïve mouse (**panel L, Figure 4.11**). No major changes in bone marrow cellularity were observed during the tertiary immunisation (**panel L, Figure 4.11**).

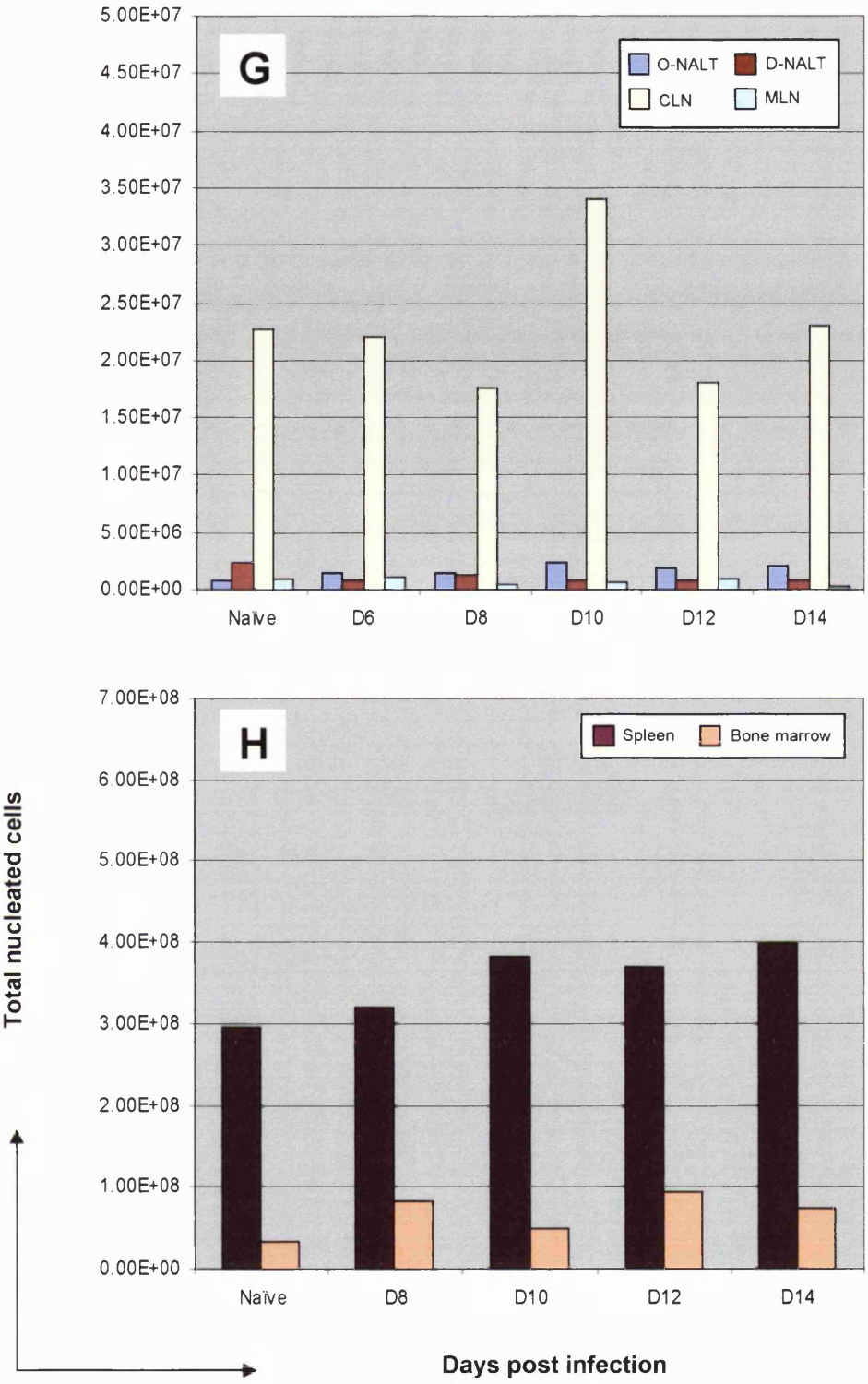


Figure 4.9 Analysis of the total cell numbers found within selected tissues after primary immunisation of BALB/c mice with BPL-inactivated RSV. **G** represents the cellularities of the O-NALT, D-NALT, CLN and MLN; **H** represents the cellularities of the spleen and bone marrow. All values represent pooled groups of four BALB/c mice.

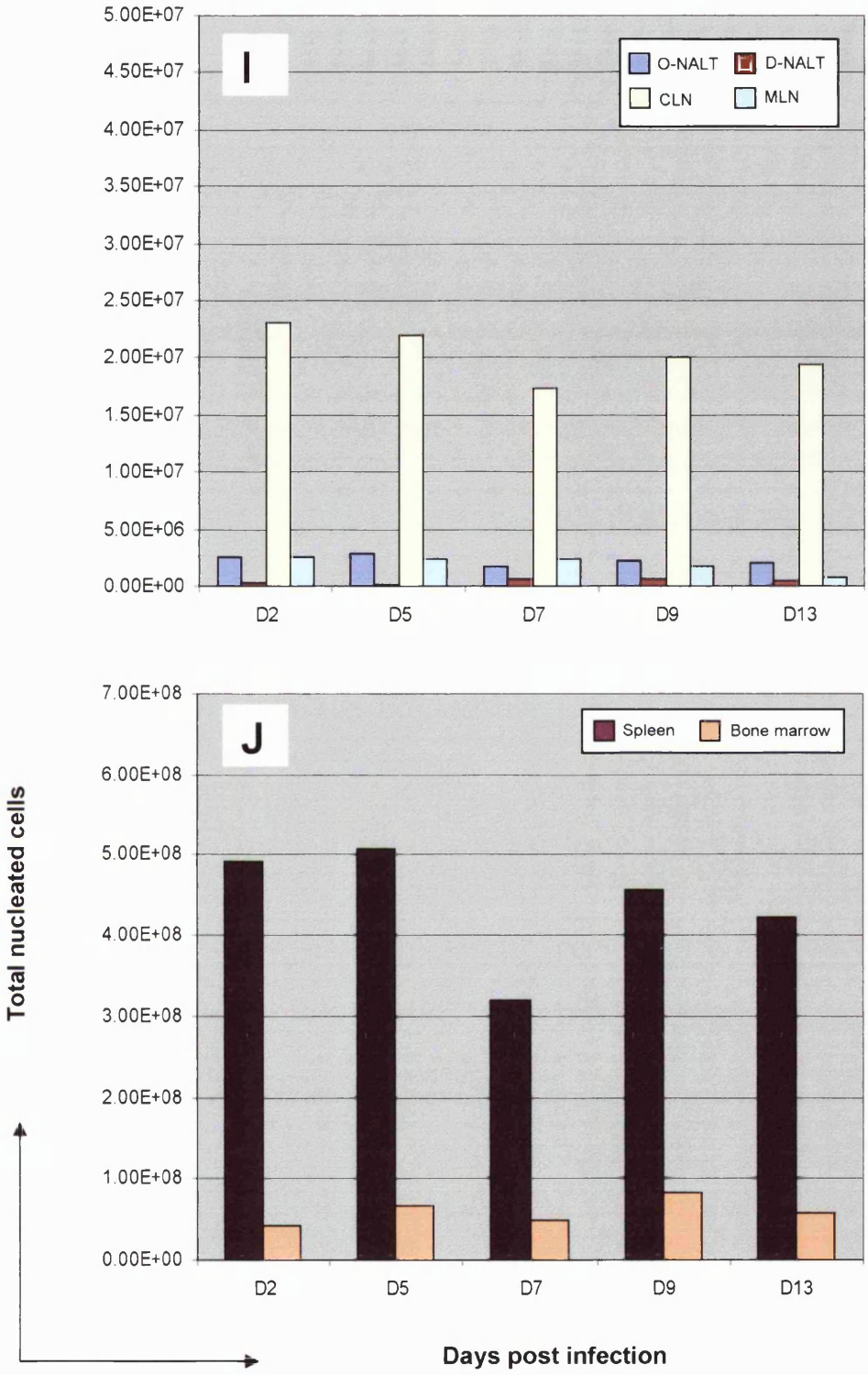


Figure 4.10 Analysis of the total cell numbers found within selected tissues after priming of BALB/c mice with BPL-RSV and challenge with live RSV. **I** represents the cellularities of the O-NALT, D-NALT, CLN and MLN; **J** represents the cellularities of the spleen and bone marrow. All values represent pooled groups of four BALB/c mice. Challenges were made at six-week intervals.

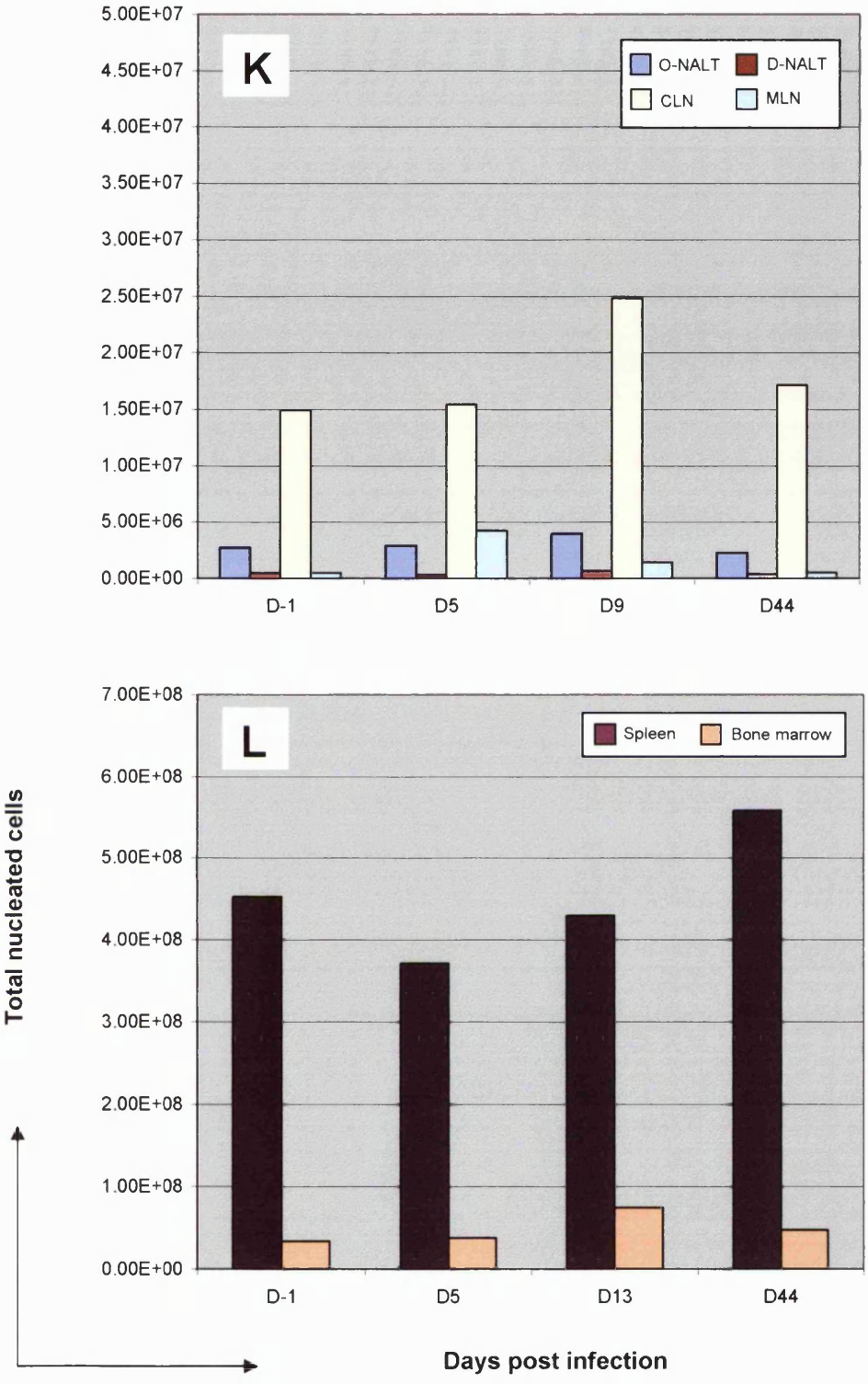


Figure 4.11 Analysis of the total cell numbers found within selected tissues after priming of BALB/c mice with BPL-RSV and challenging twice with live RSV. **K** represents the cellularities of the O-NALT, D-NALT, CLN and MLN; **L** represents the cellularities of the spleen and bone marrow. All values represent pooled groups of four BALB/c mice. Challenges were made at six-week intervals.

4.3 Discussion

The data presented in this chapter is from preliminary studies that were only performed once, it is not therefore possible to draw firm conclusions. However the results may propose a possible hypothesis as to what events are taking place and this could be confirmed if necessary at a later date by repeating the experiments.

The cellularity and phenotyping data obtained for both the RSV infected and mock treated mice was very similar and the few very minor differences observed could equally well be attributed to the limitations of the experimental design (the requirement for pooled samples in order to acquire enough cells for analysis). The cellularities in the tissues of the RSV infected group did not vary greatly in comparison with the values observed in the naïve mouse. It should be noted perhaps that we did not have age-matched naïve mice for the comparison of secondary and tertiary infection which would have been a more appropriate control. The use of BPL-RSV as a comparative control for these studies demonstrated that a lack of virus replication did not have any notable effect upon RSV-specific anti-viral cellular responses at any time, the cellularities in these BPL studies being very similar to those observed using solely live RSV.

One possible explanation may be that all three experimental groups (live RSV, mock treated, BPL inactivated RSV) are undergoing non-specific inflammation. This could be due to the retention of either cellular debris or soluble factors (secreted by the cell line) in the supernatants of all the preparations or potentially by the retention of cellular protein fragments on the virions themselves which were obtained during the virus budding process. This does not exclude RSV-specific cellular responses, in fact we know from later studies in this thesis (**chapter five**) that they definitely do occur,

however these RSV-specific immune responses do not result in substantial changes in the total numbers of lymphocytes in the relevant lymph nodes or affect the composition of B/$CD4^+$/$CD8^+$ cells. Also, although there were no apparent differences in the cellularity of the naïve and RSV challenged mice during secondary and tertiary infections this may be misleading as the naïve mice were not age-matched and possibly any differences that take place may be more apparent with age-matched controls.

One possible explanation is that the virus is able to modulate cellular trafficking of lymphocytes. Potential inhibitory effects of the virus on cellular trafficking may then contribute to the perceived unresponsiveness observed to RSV infection but this concept will be discussed more thoroughly later on in the final chapter.

Another possibility is that the virus itself is somehow promoting a state of unresponsiveness. Over the years many viruses have evolved complex strategies in order to avoid the immune system (Katze et al., 2002; Vossen et al., 2002) and in the case of RSV this apparent unresponsiveness to the effects of live replicating virus may be significant in the context of the poor degree of protection observed following infection. It may also imply that some form of immune evasion is being utilised by the virus to avoid triggering a reaction, such as circumventing the host IFN response (Gotoh et al., 2001). However, this is only speculation and further specific studies on the interaction of the virus with each aspect of the host immune system would be necessary to draw firm conclusions.

Regarding the data shown here, the lack of distinction between any of the cellular or phenotypic responses due to mock treatment or infection with live RSV may have some significance regarding the lack of protection observed during RSV infection. The results

obtained here during RSV infection are in stark contrast with data observed during influenza virus infection which generates clear-cut changes in cellularity and lymphoid cell distribution (Liang et al., 2001). What may be more significant though is the fact that a single inflection with influenza virus is sufficient to protect against further re-infection whereas the same is not true of RSV infection suggesting that the differences in cellular and phenotypic responses between the two respiratory viruses may potentially contribute to the level of protection afforded.

Chapter Five: Characterisation of the humoral immune response to RSV.

5.1 Introduction

Protection from infection with respiratory viruses is a complex issue. Work by Hyland *et al.*., demonstrated that infection of the mouse with respiratory viruses such as Sendai stimulates a permanent humoral immune response (Hyland et al., 1994). With regards to the type of humoral immunity generated it is widely accepted that primary infection stimulates the production of virus-specific serum and secretory (mucosal) antibodies. However it is highly contentious as to which type is accountable for the limited protection observed after primary infection with RSV.

Studies on the antibody response to RSV infection in the mouse model are relatively few and of these, the focus of attention is almost always upon the serum. However, the significance of local immunity in respiratory viral infections, in particular virus-specific IgA, has been demonstrated previously in passive-transfer antibody experiments in mice with influenza virus (Renegar and Small, 1991b). Polyclonal anti-influenza IgA antibodies purified from the mucosal secretions of infected mice have also been shown to protect naïve mice from virus infection when they are administered intranasally before virus challenge (Tamura et al., 1990). Whether this is the case with RSV is not presently known.

During primary infection with RSV, virus-specific serum and secretory (mucosal) antibodies are generated and it is widely accepted that, whilst they are not responsible

for the termination of RSV replication after primary infection (Anderson et al., 1990), they are essential for optimum protection upon re-challenge (Graham et al., 1991a). What is not clear is whether the overall protection observed is derived from the action of local or systemic antibodies.

The literature suggests that RSV-specific secretory antibodies tend to protect against upper respiratory tract infections whereas serum antibodies appear to protect primarily against infection of the lower respiratory tract (Falsey and Walsh, 1998; Holberg et al., 1991). This role of local immunity in the protection of the upper respiratory tract against RSV is proposed by the observation that passive transfer of neutralising antibodies offers protection but does not significantly affect viral replication in the upper respiratory tract (Brandenburg et al., 2001; Crowe, 2001; Openshaw, 2002; Staat, 2002; Varga et al., 2001; Weisman, 2002; Wright et al., 2002). In cotton rats for example, the administration of monoclonal antibodies against the F and G proteins provides almost complete protection of the lower respiratory tract, but not of the upper respiratory tract, against RSV challenge (Murphy et al., 1991). In contrast cotton rats that were given live RSV (via the respiratory tract) were resistant to re-challenge for 6 to 12 months, suggesting that local immune responses inhibited reinfection.

In humans both secretory IgA and IgG are present in the upper and lower respiratory tracts respectively (Shay et al., 1999) and it is thought that the IgG within the lung originates from the serum via passive transudation. With regards to IgA though there is evidence to suggest that this is not the case and that it is locally produced in the respiratory tract (Burnett, 1986). Further studies have shown that the presence of virus neutralising (VN) antibodies in mucosal secretions correlates well with protection against experimental infections (Mills et al., 1971; Nadal and Ogra, 1990) and that the

appearance of IgA coincides with clearance of the virus (McIntosh et al., 1978) however no detailed studies on the local humoral response to RSV in the mouse model have been performed.

Previous work in this laboratory using the influenza mouse model have identified a population of long-lived, virus-specific, IgA antibody forming cells (AFC) within the D-NALT (Liang et al., 2001). This population was present at high frequency for up to eighteen months after a single exposure to influenza virus and, speculatively, it may be involved in the immune mechanisms that are so effective in preventing reinfection with homologous influenza virus. How this population may be involved with the complete protection that is seen after primary influenza virus infection is unknown but the fact that the data is so striking suggests that mucosal immunity may be a more appropriate field in which to study protective antibody responses to mucosal pathogens, in particular RSV.

In this study we set out to characterise the antibody forming cell frequency in peripheral lymphoid tissues in response to respiratory syncytial virus infection. In particular we looked at the nasal associated lymphoid tissue (NALT), a murine structure analogous to the 'Waldeyers' ring' in humans, as it represents the first site of physical contact with inhaled antigens such as RSV. The tissue itself can be further categorised into inductive and effector sites, the O-NALT and D-NALT respectively, and we have studied each separately. In addition we examined the lymph nodes that drain the neck and lung, the cervical and mediastinal lymph nodes and, to conclude our studies, we also examined two of the major lymphoid tissues, the spleen and the bone marrow. Finally, to complement these studies we have also examined the antibody response when non-replicating RSV is used to 'prime' the mice before challenging them with live RSV.

Using the ELISPOT assay the frequency of RSV-specific, antibody forming cells (AFC) within each tissue can be determined. The mice in this study received either one, two or three 50µl intra-nasal immunisations with RSV-A2 (1 x 10^7 pfu/ml) at six week intervals and were then culled at specific time points post-infection. To confirm that the RSV-specific AFC response generated was attributable to the effect of replicating antigen we compared it with beta-propiolactone (BPL) inactivated RSV treated mice which were used as controls. For the BPL-RSV studies the mice initially received a primary immunisation of BPL-inactivated RSV-A2 (equivalent to 1 x 10^7 pfu/ml) and mice were subsequently challenged twice with live RSV at six week intervals. The AFC data presented from the experiments using solely live RSV are representative of three independent experiments. The BPL-RSV series of experiments were only performed once.

5.2 Results

With the exception of the BPL studies, which were only performed once, the data presented in this chapter has been taken from one study which is representative of a series of three repeats. Although there are no statistics displayed for the live RSV data, during each repeat the results were similar and followed the same trends indicating that the data presented here is consistent and fully represents the AFC response in these tissues.

5.2.1. The frequency and isotype specificity of RSV-specific antibody forming cells (AFC's) in the mucosal tissues following primary, secondary and tertiary infection with live RSV

5.2.1.1 O-NALT

Following primary infection, the antibody forming cell (AFC) frequency in the O-NALT was dominated by IgA. On day six, the O-NALT contained 58.8 IgA-specific AFC per 5 x 10^5 nucleated cells which increased to a maximum of 140.9 IgA-specific AFC per 5 x 10^5 nucleated cells on day eight. At this time the IgA AFC encompassed 74% of the total RSV-specific AFC. By day ten the frequency of IgA-specific AFC had declined sharply and by day twelve was reduced to 7.7 AFC per 5 x 10^5 nucleated cells.

After this point the IgA-specific AFC remained at this low level for the remainder of the primary time-course. In addition to IgA a few IgM AFC were observed (days six and eight), however these cells disappeared shortly afterwards. There was evidence of RSV-specific IgG2a and IgG2b AFC being present towards the end of the primary time-course, but these levels were low (<9 AFC per 5 x 10^5 nucleated cells) compared with the earlier IgA responses (**panel A, Figure 5.1**). The secondary immunisation with the same dose of RSV produced few RSV-specific AFCs with very low frequencies of IgA and IgG2a AFC observed on day nine. Of these, only IgG2a remained on day thirteen at extremely low frequency (6.7 AFC per 5 x 10^5 nucleated cells) (**panel B, Figure 5.1**). Six-weeks after secondary challenge (day 42) no RSV-specific AFC were detected and, following tertiary challenge only an extremely low number of RSV-specific IgA were observed (day 5) with no other isotypes detected at any other time (**panel C, Figure 5.1**).

5.2.1.2 D-NALT

The frequency of RSV-specific AFCs in the D-NALT following primary infection was similar to that of the O-NALT in that the response was dominated by IgA and the kinetics were similar. IgA frequency peaked on day eight and accounted for a high proportion (81%) of all the AFC detected at this time point. The frequency of RSV-specific IgA AFC observed on day eight reached 180 AFC per 5 x 10^5 nucleated cells before declining in the following days. As seen in the O-NALT, IgM AFC were also observed during the first two time-points, peaking on day six with 34.2 AFC per 5 x 10^5 nucleated cells before quickly waning. During the primary infection RSV specific AFCs of all isotypes had declined by day ten. However, fourteen months after primary immunisation with RSV, low numbers of RSV-specific AFC remained detectable. The main isotypes of AFC detected at this time were IgA, IgG1 and IgG2a and were present at frequencies of just less than 7 AFC per 5 x 10^5 nucleated cells. At eighteen months post-infection. the RSV-specific AFC were still present albeit at the low levels observed four months previously (**panel A, Figure 5.2**).

Following secondary infection the D-NALT response was quite different to that of the O-NALT. The main response appeared around day nine with the appearance of IgA and IgG2a AFC (although some IgM was evident on day five) with maximal numbers of 253.1 and 61.7 AFC per 5 x 10^5 nucleated cells appearing on day thirteen respectively. This response was not long-lived however as six-weeks after secondary infection (day 42) no RSV-specific AFC of any isotype were detected (**panel B, Figure 5.2**). Tertiary infection was dominated by IgA AFC, peaking on day five post-infection, although at a

lower frequency than was observed at the peak of either the primary or secondary infection (128.4 AFC per 5 x 10^5 nucleated cells).

The acute phase of the tertiary response was very short-lived and had declined below the levels of detection by day nine. However, one-month post-tertiary immunisation (day 29), low numbers of IgA, IgG1 and IgG2a RSV specific AFCs were observed and, in total, represented 56.8 AFC per 5 x 10^5 nucleated cells. This response was not long-lived though and no AFC of any isotype could be detected at forty-four days post tertiary infection within the D-NALT (**panel C, Figure 5.2**).

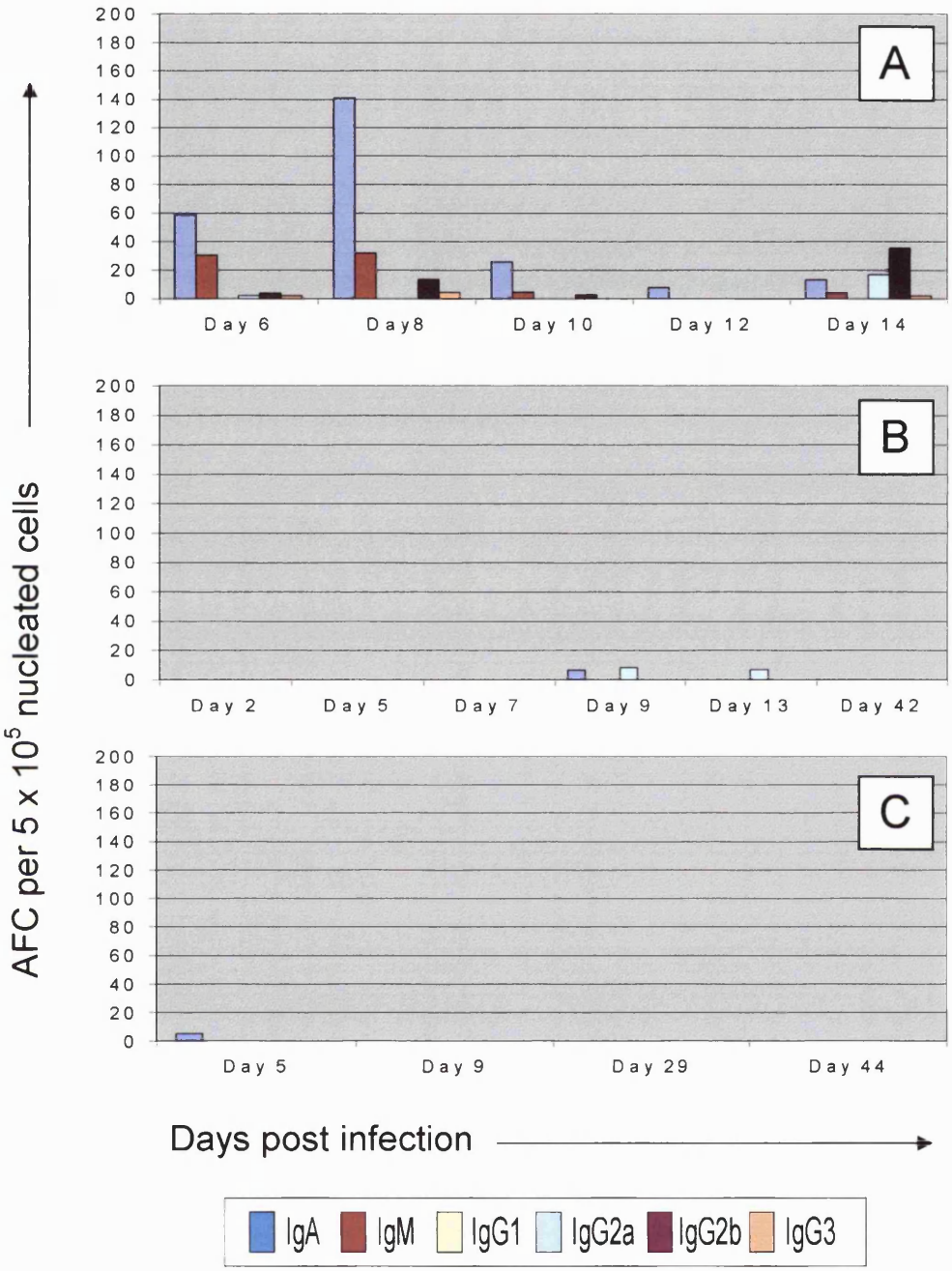


Figure 5.1 The frequency of RSV-specific AFC within murine O-NALT during the primary, secondary and tertiary response to infection with live-RSV. Chart **A** represents the primary response; **B** represents the secondary response and **C** represents the tertiary response. The period of time between each successive infection was six weeks. This data is representative of a total of three experiments.

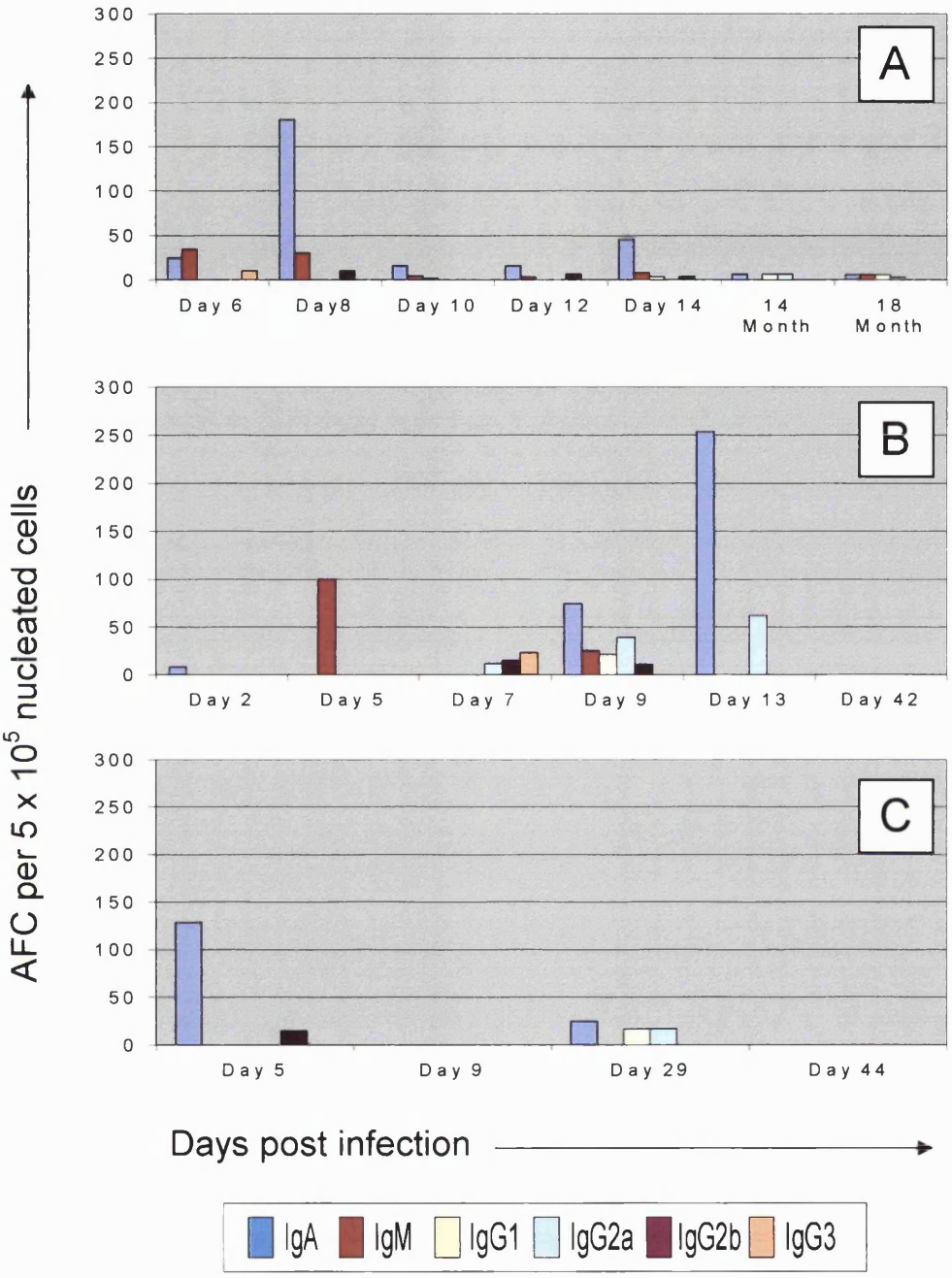


Figure 5.2 The frequency of RSV-specific AFC within murine D-NALT during the primary, secondary and tertiary response to infection with live-RSV. Chart **A** represents the primary response; **B** represents the secondary response and **C** represents the tertiary response. The period of time between each successive infection was six weeks. This data is representative of a total of three experiments.

5.2.2 The frequency and isotype specificity of RSV-specific antibody forming cells (AFC's) in the systemic tissues following primary, secondary and tertiary infection with live RSV

5.2.2.1 CLN

The draining lymph nodes of the neck, the cervical (CLN), demonstrate a primary response that consists of two stages. The first stage involved the appearance of predominately IgA and IgM AFC on day six, rising to a peak on day eight. At this peak, IgA and IgM AFC together account for 82% of the total AFC observed on day eight at frequencies of 24.2 and 41.6 AFC per 5 x 10^5 nucleated cells respectively. These cells then begin to disappear and by day twelve were almost undetectable. IgA and IgM AFC were displaced by the appearance of RSV-specific AFC of the IgG isotypes on day eight. These remained at levels of approximately 1-13 AFC per isotype throughout the time-course, and by day fourteen these isotypes accounted for 72% of the total RSV-specific AFC, at frequencies of 10.7 and 13.7 AFC per 5 x 10^5 nucleated cells respectively (**panel A, Figure 5.3**). Following secondary RSV infection no virus-specific AFC were observed until day five at which point IgG2a AFC appeared and increased in frequency peaking at day seven. At this stage IgG2b AFC were also present and almost matched the IgG2a AFC in magnitude (20.3 and 17.7 AFC per 5 x 10^5 nucleated cells respectively). None of these AFC were detected two days later (day nine) and no significant numbers of RSV-specific AFC were observed for up to six-weeks post-secondary infection (day 42) (**panel B, Figure 5.3**). Following tertiary infection a variety of RSV-specific AFC isotypes were detected on day five at very low levels and these low numbers were detected throughout the remainder of the tertiary time-course (**panel C, Figure 5.3**).

5.2.2.2 MLN

In general, the mediastinal lymph node (MLN) displayed higher frequencies of RSV-specific AFC's than was seen within the CLN following primary infection. As expected from a normal immune response, IgM AFC were observed early from day six (161.7 AFC per 5 x 10^5 nucleated cells), and were maintained at approximately this level until day twelve when the numbers began to wane. During this time-course a mixed IgG response was observed. Isotypes of note included a strong IgG2a and IgG2b response over days eight, ten and fourteen of which the peak occurred on day eight with 95.7 IgG2a and 56.8 IgG2b AFC per 5 x 10^5 nucleated cells respectively. Taken together, at their peak on day eight, these two isotypes accounted for nearly 40% of all the AFC observed which was almost equal to the proportion of IgM AFC observed (41%). This is in contrast to day fourteen where they accounted for 84% of all the RSV-specific AFC detected at levels of 63.4 and 60.5 AFC per 5 x 10^5 nucleated cells respectively (**panel A, Figure 5.4**). Secondary infection elicited an RSV-specific response that was dominated by three isotypes of AFC; IgG1, IgG2a and IgG2b and by day seven, significant frequencies of these three isotypes were observed, with IgG2a being the greatest (275.5), followed by IgG2b (142.6) and then IgG1 (74.2 AFC per 5 x 10^5 nucleated cells). Together all three isotypes accounted for 93% of all the virus-specific AFC observed. These high frequencies were not maintained, and by day thirteen had declined quite substantially. They did not disappear completely though as, at a long-term time-point six-weeks after secondary infection (day 42) which was one day prior to tertiary infection, low-levels of IgG2a and IgG2b were still observed (**panel B, Figure 5.4**). Following tertiary infection, the frequencies of IgG2a and IgG2b on day five were comparable to the peaks observed during the secondary infection at 217.4 and 195.7 AFC per 5 x 10^5 nucleated cells respectively. However, they began to decline two

days later and were present at much reduced frequencies later on. Additionally high numbers of IgA were observed on day five (197.8 AFC per 5 x 10^5 nucleated cells) although these too had declined two days later and were not detected again (**panel C, Figure 5.4**).

5.2.2.3 Spleen

Virus-specific AFC (less than 20 total AFC per 5 x 10^5 nucleated cells) were detected in the spleen following primary infection and, of those observed, most were of the IgM isotype with low numbers of IgA (**panel A, Figure 5.5**). After secondary infection the AFC frequency was still very low but IgM and IgA were accompanied by some IgG isotypes demonstrating that isotype-switching had occurred within the spleen (**panel B, Figure 5.5**). Tertiary infection did not appear to provide a significant boost to the frequency of RSV-specific AFC in the spleen with the frequencies of splenic IgA and IgG AFC being comparable to those observed six-weeks after secondary infection (day 42) (**panel C, Figure 5.5**).

5.2.2.4 Bone marrow

Following primary infection with RSV the number of RSV-specific AFC's in the bone marrow were just above background levels (< 5 AFC per 5 x 10^5 nucleated cells) with some isotypes of AFC appearing just above the threshold of detection.

At fourteen months post-primary immunisation the bone marrow contained all of the isotypes of virus-specific AFC assayed for, albeit at low frequencies. At this time point the bone marrow contained mainly IgM (10 AFC per 5 x 10^5 nucleated cells), IgG2a (7 AFC per 5 x 10^5 nucleated cells) and IgA (5 AFC per 5 x 10^5 nucleated cells) AFCs. All

of the other isotypes were present at frequencies just below 2 AFC per 5 x 10^5 nucleated cells. Four months later the RSV-specific AFC isotypes were still detectable and present at approximately 4 AFC per 5 x 10^5 nucleated cells per isotype (**panel A, Figure 5.6**).

Following secondary immunisation an increase in AFC frequency was seen from day seven onwards for all of the isotypes, reaching a level of approximately 80 AFC per 5 x 10^5 nucleated cells in total. By day thirteen three of the isotypes (IgA, IgM and IgG2a) had increased in frequency reaching peaks of 18, 23 and 27 AFC per 5 x 10^5 nucleated cells respectively. This was not maintained though as six-weeks post-secondary infection (one day prior to tertiary infection – day 42) all of the isotypes of AFC had returned to lower levels (approximately 20 AFC per 5 x 10^5 nucleated cells in total), similar to those frequencies seen early on in the secondary infection (**panel B, Figure 5.6**). The effect of tertiary infection on bone marrow AFC was apparent with faster kinetics and slightly higher frequencies of almost all of the isotypes (with the exception of IgG3). IgA, IgM and IgG2a were again the three isotypes most boosted with numbers of IgA and IgM peaking on day nine at 22 and 25 AFC per 5 x 10^5 nucleated cells respectively, and IgG2a at 28 AFC per 5 x 10^5 nucleated cells on day twenty-nine. Forty-four days after tertiary infection with live RSV there were still detectable levels of virus-specific AFC present in the bone marrow with IgA, IgM, IgG2a and IgG2b all present at approximately 10 AFC per 5 x 10^5 nucleated cells each (**panel C, Figure 5.6**).

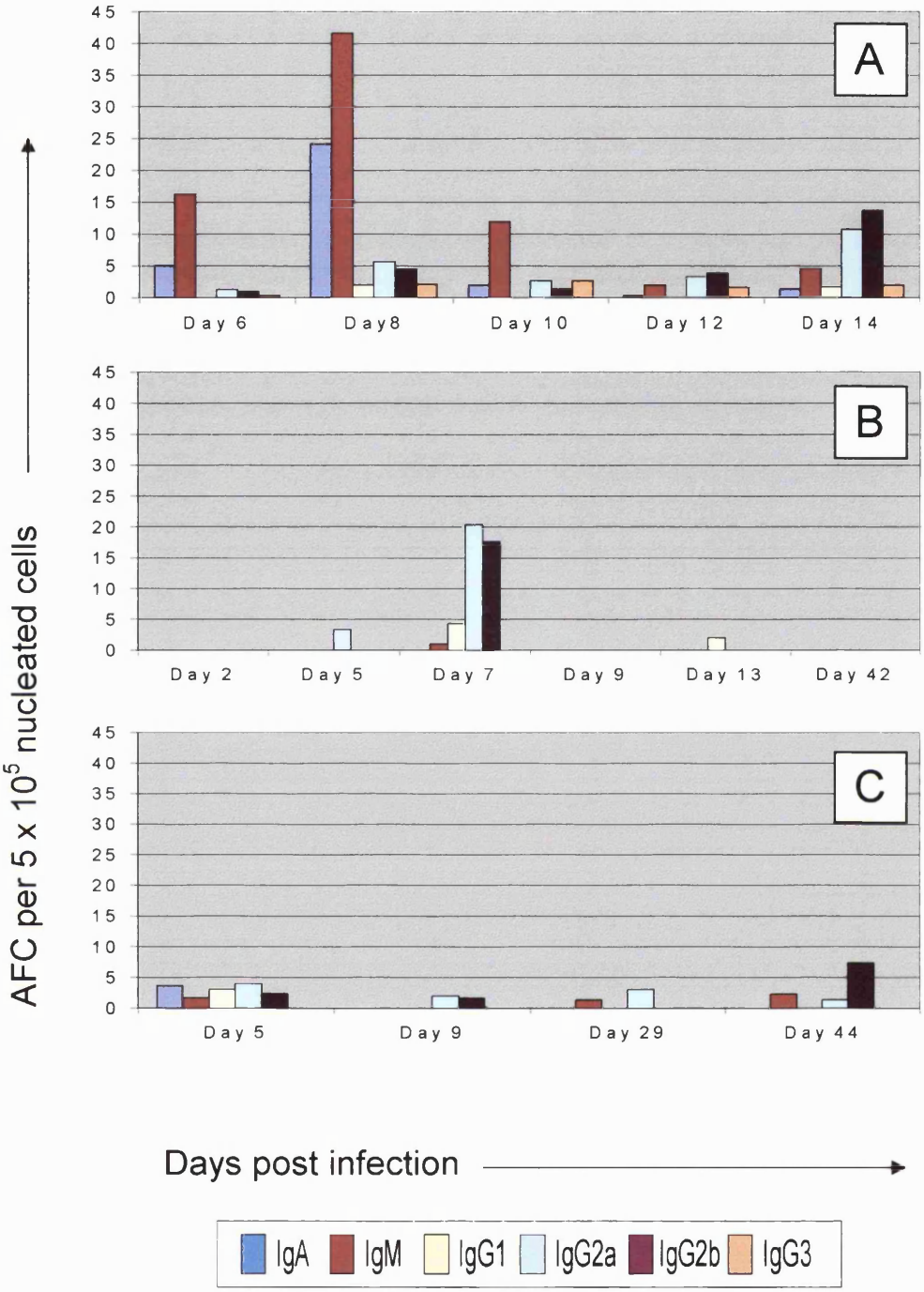


Figure 5.3 The frequency of RSV-specific AFC within murine CLN during the primary, secondary and tertiary response to infection with live-RSV. Chart **A** represents the primary response; **B** represents the secondary response and **C** represents the tertiary response. The period of time between each successive infection was six weeks. This data is representative of a total of three experiments.

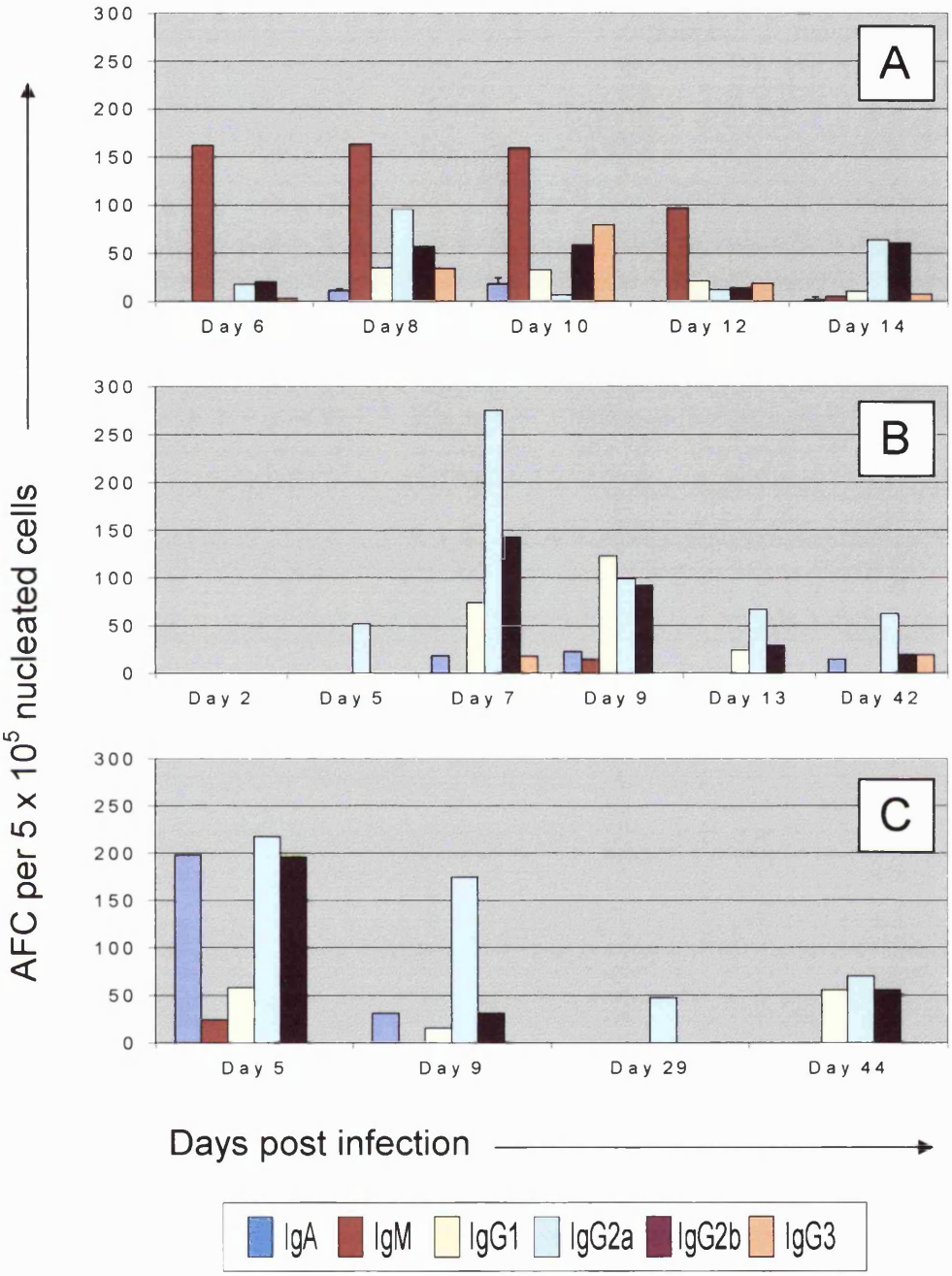


Figure 5.4 The frequency of RSV-specific AFC within murine MLN during the primary, secondary and tertiary response to infection with live-RSV. Chart **A** represents the primary response; **B** represents the secondary response and **C** represents the tertiary response. The period of time between each successive infection was six weeks. This data is representative of a total of three experiments.

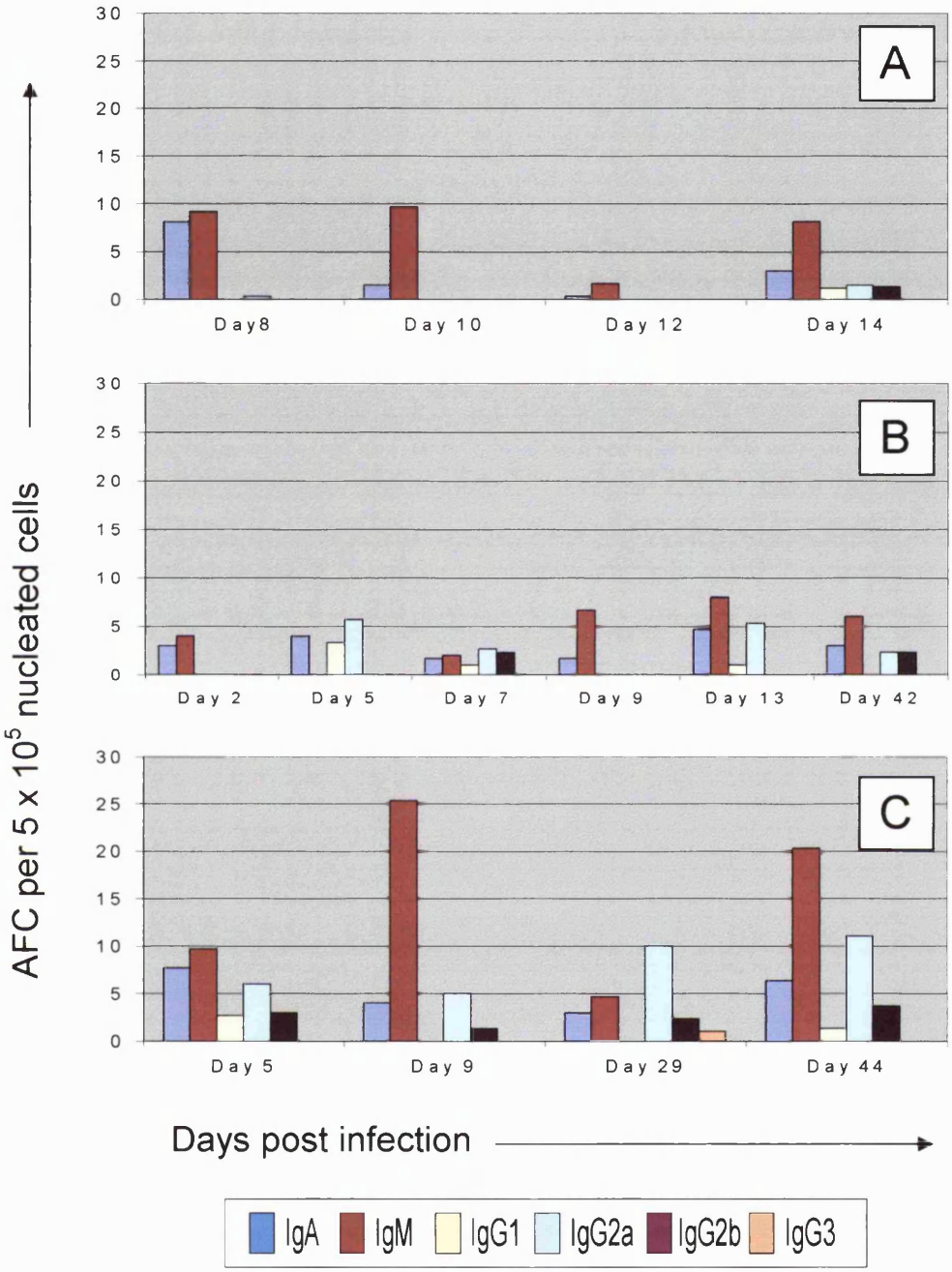


Figure 5.5 The frequency of RSV-specific AFC within murine spleen during the primary, secondary and tertiary response to infection with live-RSV. Chart **A** represents the primary response; **B** represents the secondary response and **C** represents the tertiary response. The period of time between each successive infection was six weeks. This data is representative of a total of three experiments.

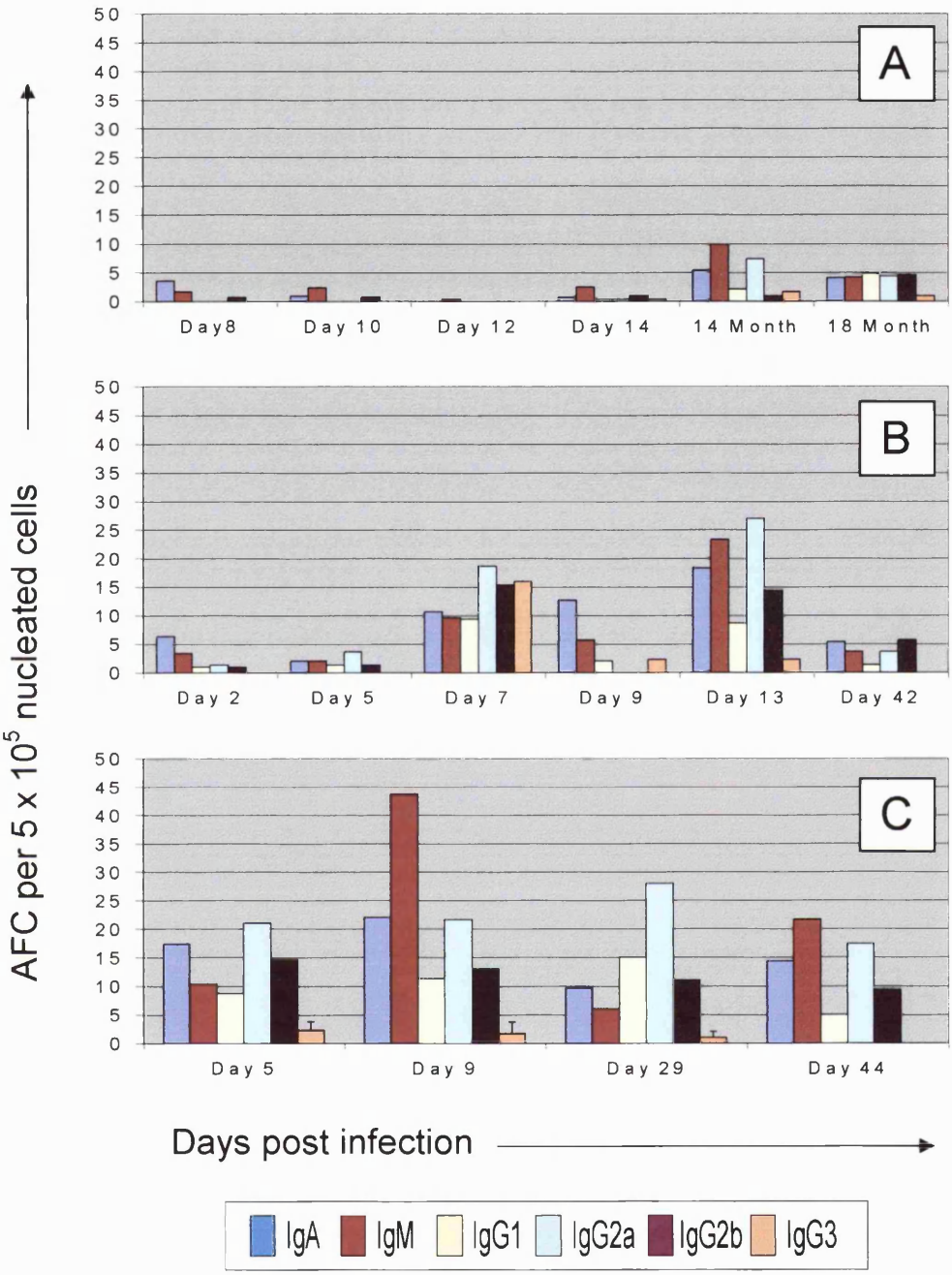


Figure 5.6 The frequency of RSV-specific AFC within murine bone marrow during the primary, secondary and tertiary response to infection with live-RSV. Chart **A** represents the primary response; **B** represents the secondary response and **C** represents the tertiary response. The period of time between each successive infection was six weeks. This data is representative of a total of three experiments.

5.2.3 The frequency and isotype specificity of RSV-specific antibody forming cells (AFC's) in the mucosal tissues following primary BPL-treated RSV administration, followed by secondary and tertiary challenges with live RSV

Beta-propiolactone (BPL) was used in these experiments to inactivate RSV. This was done to observe the immune response of the mouse to non-replicating RSV antigen in order to show that the immune response to the virus is specific to its ability to replicate.

5.2.3.1 O-NALT

Following primary immunisation with BPL-inactivated RSV no RSV-specific antibody response was detectable in the O-NALT (**panel A, Figure 5.7**). Subsequent challenge with live RSV generated a response only at nine days post infection and was restricted to that of a single isotype, IgA (10 AFC per 5 x 10^5 nucleated cells). By day thirteen the IgA frequency had more than doubled (28 AFC per 5 x 10^5 nucleated cells) and small numbers of immunoglobulin switched AFC began to appear, mainly IgG2b (21 AFC per 5 x 10^5 nucleated cells). These levels did not persist as, one day prior to tertiary immunisation (six-weeks post secondary immunisation – day 42), we could not detect any RSV-specific AFC (**panel B, Figure 5.7**). Exposure of the mouse to a second dose of live RSV (tertiary immunisation) boosted the IgA response to levels comparable with day thirteen on the secondary immunisation; this response was short-lived though and had disappeared eight days later (**panel C, Figure 5.7**). Low frequencies of IgM and IgG2a were observed on day five but appear to be transient and expressed only at very low levels (< 7 AFC per 5 x 10^5 nucleated cells).

5.2.3.2 D-NALT

The D-NALT response to the administration of BPL-inactivated RSV was similar to that observed with the O-NALT with few discernible RSV-specific AFC detected (**panel A, Figure 5.8**). Challenge with live RSV brought about a faster response detectable on day seven and consisting of IgM and IgG2b and IgG3, at frequencies of 33, 16 and 55 AFC per 5 x 10^5 nucleated cells respectively. Of these only IgM remained by day thirteen. IgA appeared on day nine (26 AFC per 5 x 10^5 nucleated cells) and trebled in frequency by day thirteen (104 AFC per 5 x 10^5 nucleated cells) but could not be detected six-weeks later (one day prior to tertiary immunisation – day 42) suggesting that these frequencies were not maintained long-term (**panel B, Figure 5.8**). As with IgA, no other isotypes were observed prior to tertiary immunisation (day 42). Following tertiary challenge, on day five, very high numbers of IgA (232 AFC per 5 x 10^5 nucleated cells) were seen but these waned rapidly and had dropped by 80% by day thirteen (**panel C, Figure 5.8**).

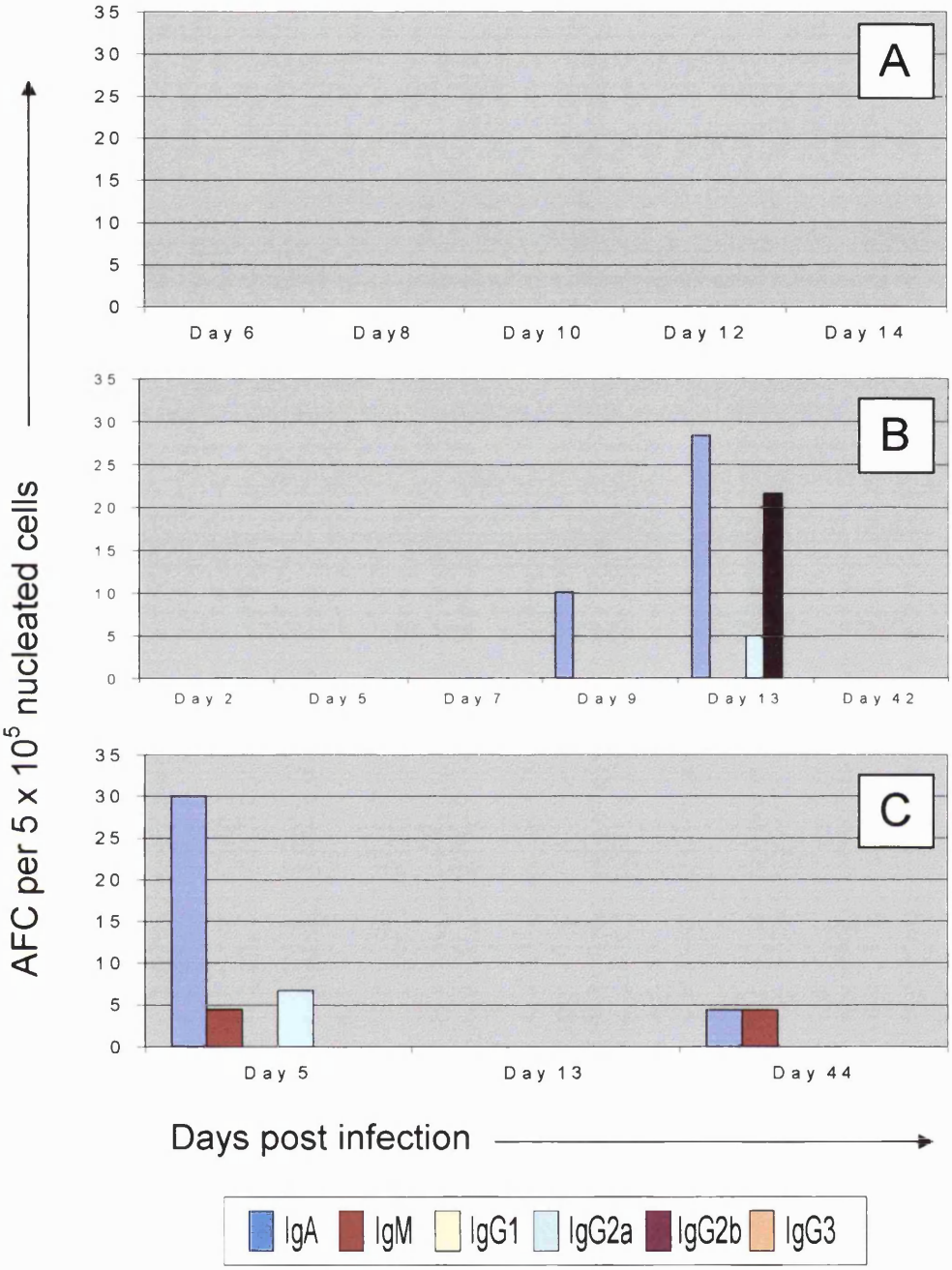


Figure 5.7 ELISPOT analysis of the frequency of RSV-specific AFC within murine O-NALT following primary immunisation with BPL-inactivated RSV and subsequent challenges with live RSV. Chart **A** represents the response following BPL-RSV immunisation; **B** represents the response following challenge with live RSV and **C** represents the response following a second challenge with live RSV. The period of time between each successive immunisation was six weeks. This data is representative of a total of three experiments.

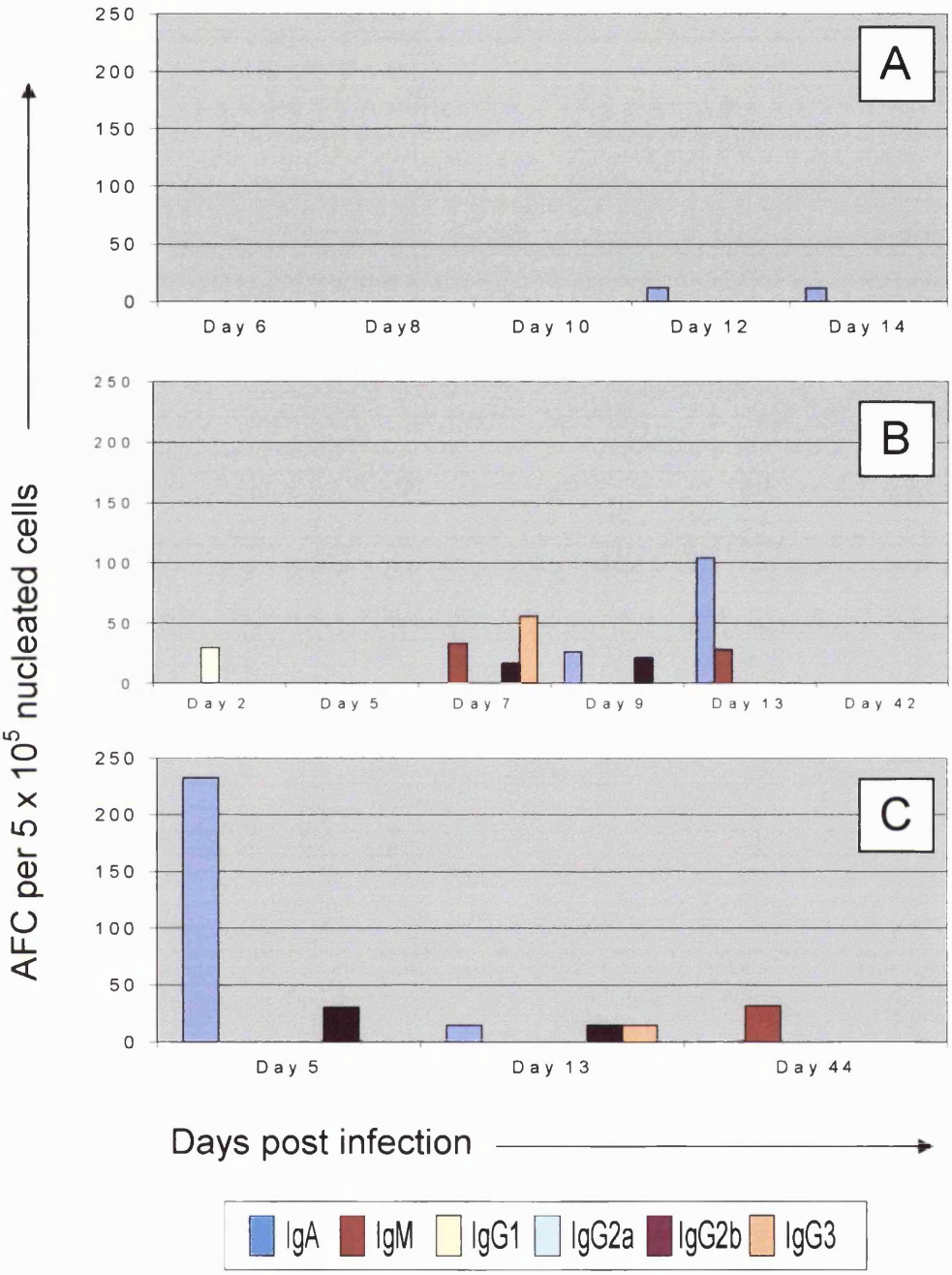


Figure 5.8 ELISPOT analysis of the frequency of RSV-specific AFC within murine D-NALT following primary immunisation with BPL-inactivated RSV and subsequent challenges with live RSV. Chart **A** represents the response following BPL-RSV immunisation; **B** represents the response following challenge with live RSV and **C** represents the response following a second challenge with live RSV. Primary, secondary and tertiary immunisations were made at six week intervals. This data is taken from a single experiment.

5.2.4 The frequency and isotype specificity of RSV-specific antibody forming cells (AFC's) in the systemic tissues following primary BPL-treated RSV virus administration, followed by secondary and tertiary challenge with live RSV

5.2.4.1 CLN

No significant responses to BPL-inactivated RSV in the CLN were observed during the primary immunisation (**panel A, Figure 5.9**). Challenge with live RSV elicited a response first observed on day seven as a very low frequency of IgG1 AFC. By day nine this response had increased ten-fold to a frequency of 22 AFC per 5 x 10^5 nucleated cells. It did not persist however, and by day thirteen had completely abated. Low numbers of IgG2a and IgG2b appeared on day nine (10 and 7 AFC per 5 x 10^5 nucleated cells respectively) but declined in frequency four days later and were not present at the six-week, pre-tertiary immunisation (day 42) time-point (**panel B, Figure 5.9**). No RSV-specific AFC of any isotype were observed on day forty-two. Tertiary immunisation resulted in accelerated kinetics and comparatively high frequencies of IgA, IgG1, IgG2a and IgG2b on day five, with values in the range of 10 – 15 AFC per isotype, per 5 x 10^5 nucleated cells. These frequencies rapidly waned though and were greatly reduced down to very low frequencies (> 4 AFC per 5 x 10^5 nucleated cells) by day thirteen (**panel C, Figure 5.9**).

5.2.4.2 MLN

In the MLN there was no significant primary response to BPL-inactivated RSV (**panel A, Figure 5.10**) and, as with the CLN, challenge with live RSV first evoked a detectable response seven days post challenge. On day seven there was a large increase

in frequency of IgG1 to 159.8 AFC per 5 x 10^5 nucleated cells but this decreased two days later and had almost disappeared by day thirteen. Also on day seven, the frequency of IgG2b rose to 34 AFC per 5 x 10^5 nucleated cells before doubling two days later and waning by day thirteen. More strikingly on day nine there was a considerable IgG2a response of 295 AFC per 5 x 10^5 nucleated cells, however this was only transient and did not persist past this single time-point (**panel B, Figure 5.10**). No AFC were observed six-weeks after secondary immunisation (one day prior to tertiary immunisation - day 42) but, post tertiary challenge, a broad range of AFC of different isotypes were detected. The frequencies of the responding AFC varied with IgG2a demonstrating the highest response (182 AFC per 5 x 10^5 nucleated cells) followed by both IgG1 and IgG2b (81 and 75 AFC per 5 x 10^5 nucleated cells respectively) and then IgA (60 AFC per 5 x 10^5 nucleated cells). None of the responding isotypes persisted past day thirteen and those of note were all at their greatest frequency on day five (**panel C, Figure 5.10**).

5.2.4.3 Spleen

In the spleen, the primary response to BPL-inactivated RSV almost entirely consisted of low numbers of RSV-specific IgM AFC's and, from its peak on day eight, (13 AFC per 5 x 10^5 nucleated cells) the frequency of this response progressively waned, dropping to a third of its peak value on day fourteen at 3 AFC per 5 x 10^5 nucleated cells (**panel A, Figure 5.11**). The response to challenge with live RSV was also dominated by IgM from the onset and followed a similar trend to the primary immunisation with BPL-RSV. However some IgA was present in low frequencies from day seven onwards and this persisted until day thirteen when it reached a peak value of 7 AFC per 5 x 10^5 nucleated cells. By day nine some IgG2a and IgG2b RSV-specific AFC appeared although at very

low frequencies and, by day thirteen, IgG1 and IgG3 AFC's were also present albeit at low frequencies. Of all of these isotypes only IgM persisted and was present six-weeks after the challenge with live RSV (one day prior to tertiary immunisation - day 42) at 7 AFC per 5 x 10^5 nucleated cells (**panel B, Figure 5.11**). After tertiary immunisation IgA, IgG1 and IgG2a could be detected at very low frequencies (< 16 AFC per 5 x 10^5 nucleated cells in total) but these responses were not present on day thirteen. IgM however remained at a frequency similar to that seen pre-challenge (day 42) but between day thirteen and day forty-four increased five-fold to 34 AFC per 5 x 10^5 nucleated cells (**panel C, Figure 5.11**).

5.2.4.4 Bone marrow

In the bone marrow the primary response to BPL-inactivated RSV was mixed, with no single AFC isotype dominating. On day eight all of the isotypes except IgA were detectable, albeit at very low frequency (< 4 AFC per isotype, per 5 x 10^5 nucleated cells), and were maintained at this level until day ten when very low numbers of IgA appeared. On day twelve however, no responses were detectable and two days later barely detectable numbers (< 2 AFC per 5 x 10^5 nucleated cells) of all the isotypes were observed (**panel A, Figure 5.12**).

Challenge with live-RSV stimulated a slightly more robust response compared to the primary. This response peaked on day seven and consisted of all six isotypes of AFC assayed for, with IgM and IgG1 having the highest frequencies of 15 and 10 AFC per 5 x 10^5 nucleated cells respectively. These frequencies quickly decreased two days later with all the IgG disappearing, leaving only IgM and IgA at low levels (9 and 2 AFC per 5 x 10^5 nucleated cells respectively). Six-weeks after secondary immunisation (day 42)

IgM and IgA were still present at low frequencies (**panel B, Figure 5.12**). Tertiary immunisation stimulated an increase in frequency of all of the isotypes with IgM and IgG2b having the highest values of 21 and 22 AFC per 5 x 10^5 nucleated cells respectively on day thirteen. By day forty-four however, only IgA and IgM AFC's could still be detected (**panel C, Figure 5.12**).

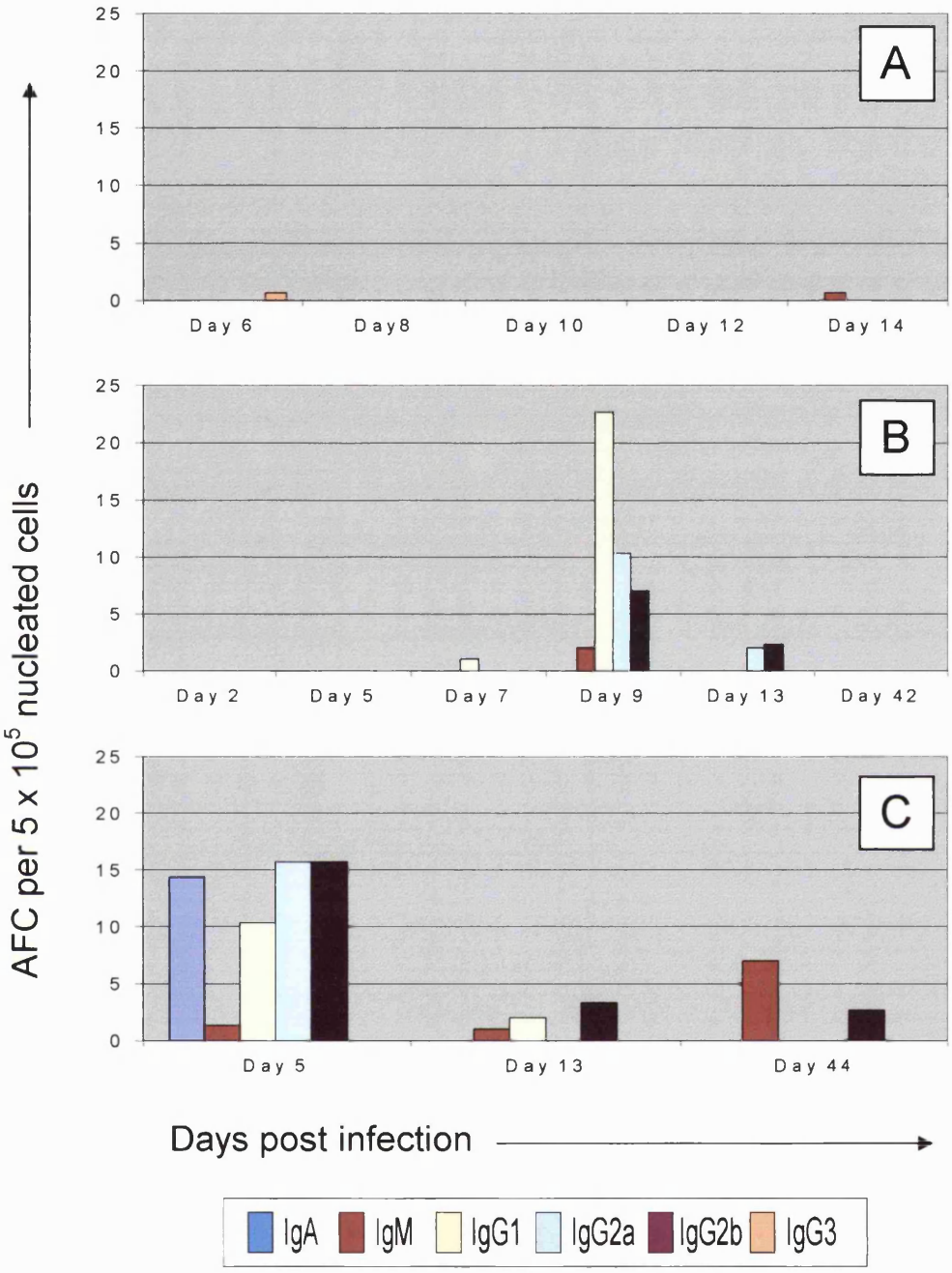


Figure 5.9 ELISPOT analysis of the frequency of RSV-specific AFC within murine CLN following primary immunisation with BPL-inactivated RSV and subsequent challenges with live RSV. Chart **A** represents the response following BPL-RSV immunisation; **B** represents the response following challenge with live RSV and **C** represents the response following a second challenge with live RSV. The period of time between each successive immunisation was six weeks. This data is taken from a single experiment.

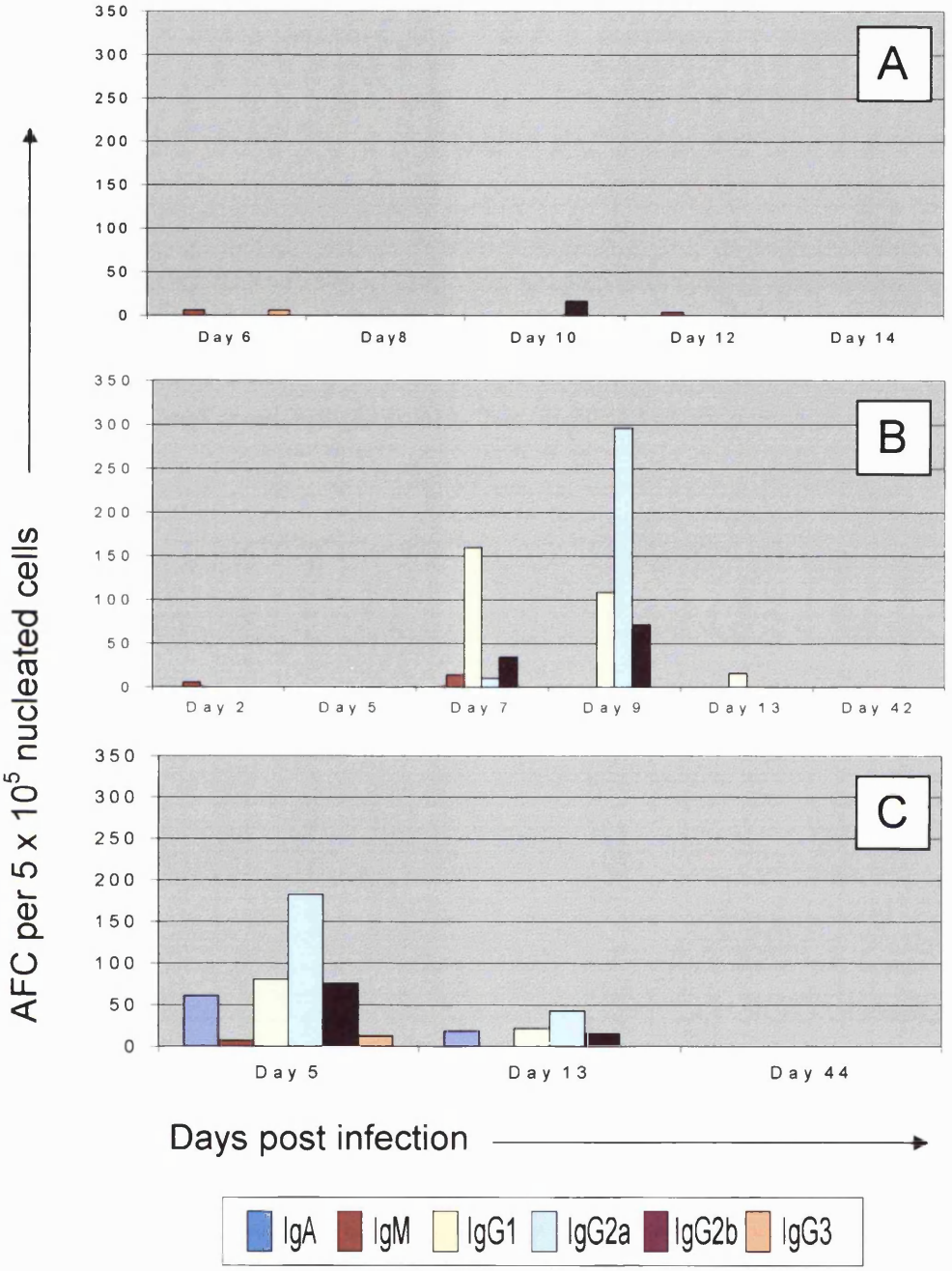


Figure 5.10 ELISPOT analysis of the frequency of RSV-specific AFC within murine MLN following primary immunisation with BPL-inactivated RSV and subsequent challenges with live RSV. Chart **A** represents the response following BPL-RSV immunisation; **B** represents the response following challenge with live RSV and **C** represents the response following a second challenge with live RSV. The period of time between each successive immunisation was six weeks. This data is taken from a single experiment.

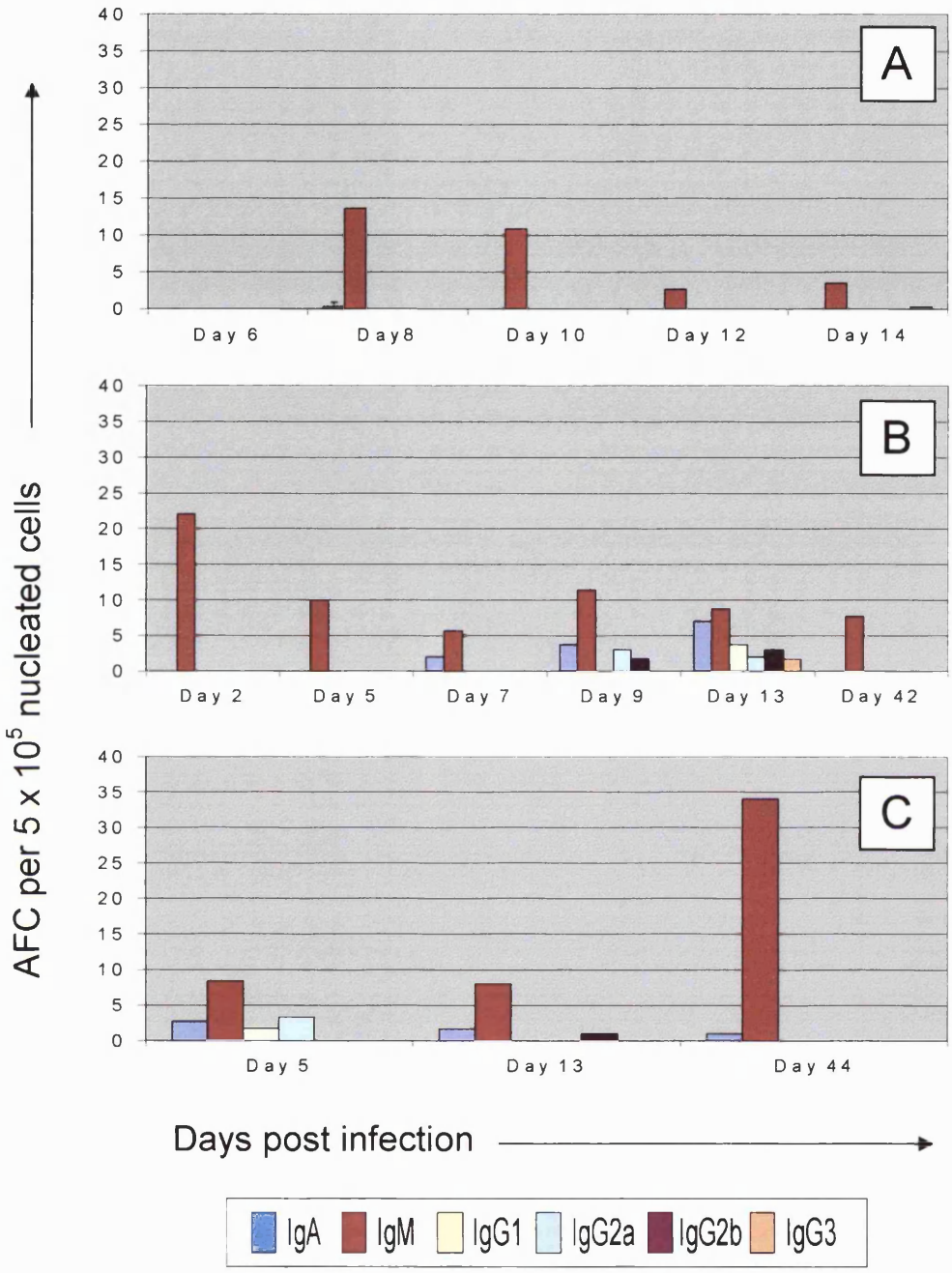


Figure 5.11 ELISPOT analysis of the frequency of RSV-specific AFC within murine spleen following primary immunisation with BPL-inactivated RSV and subsequent challenges with live RSV. Chart **A** represents the response following BPL-RSV immunisation; **B** represents the response following challenge with live RSV and **C** represents the response following a second challenge with live RSV. The period of time between each successive immunisation was six weeks. This data is taken from a single experiment.

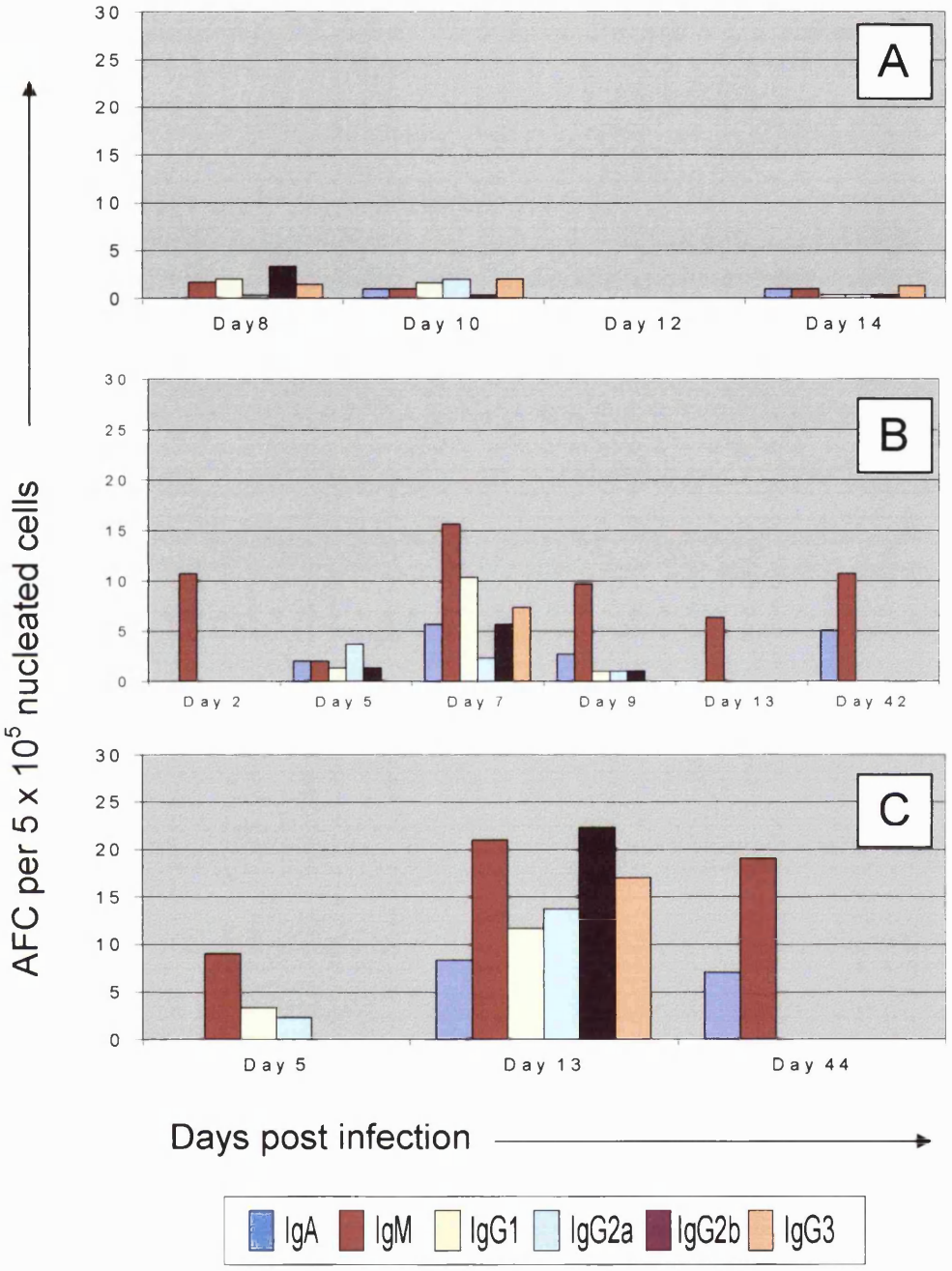


Figure 5.12 ELISPOT analysis of the frequency of RSV-specific AFC within murine bone marrow following primary immunisation with BPL-inactivated RSV and subsequent challenges with live RSV. Chart **A** represents the response following BPL-RSV immunisation; **B** represents the response following challenge with live RSV and **C** represents the response following a second challenge with live RSV. The period of time between each successive immunisation was six weeks. This data is taken from a single experiment.

5.3 Discussion

5.3.1 Live RSV study

In the live RSV experiments both the O-NALT and D-NALT primary responses were dominated by IgA but were very short-lived, not lasting more than twelve days (**panel A, Figures 5.1 & 5.2**). Both the diffuse nasal associated lymphoid tissue (D-NALT) and the bone marrow are sites for production of virus-specific antibody following influenza virus infection of mice (Hyland et al., 1994; Liang et al., 2001). To see if this was also the case for RSV we quantified the virus-specific AFC in the relevant lymphoid tissues following primary and secondary infections. At both fourteen and eighteen months, the RSV-specific D-NALT AFC frequencies were consistently low (less than 7 AFC per 5 x 10^5 nucleated cells) and, when compared to the influenza virus data it is obvious that the numbers of RSV-specific plasma cells in the D-NALT are significantly reduced. On secondary challenge the profile of the D-NALT did not resemble that of a classical secondary response and surprisingly the antibody did not appear much earlier (**panel B, Figure 5.2**). This was not repeated during tertiary challenge however as IgA was seen at high frequency as early as day five whereupon shortly afterwards, almost all of the RSV-specific AFC responses declined to almost undetectable levels (**panel C, Figure 5.2**). The secondary and tertiary responses of the D-NALT are in contrast to those of the O-NALT as during both challenges there was no significant response in the O-NALT at any time (**panels B & C, Figure 5.1**).

In the draining lymph nodes of the neck, the cervical lymph nodes (CLN), the response was dominated by IgM and IgA in the primary infection (**panel A, Figure 5.3**). The kinetics of the IgA response were similar those seen in the NALT but in the CLN the

frequency was approximately five-fold lower. There was also evidence of class-switching as indicated by the presence of many of the IgG isotypes during the secondary and tertiary responses (**panels B & C, Figure 5.3**) but, overall, these responses were fairly low in magnitude and only appear to have been transient in their appearance. The converse can be applied to the mediastinal lymph nodes or MLN; here the response was extremely strong with the primary infection being dominated by IgM and a gradual shift towards the IgG isotypes being readily apparent (**panel A, Figure 5.4**). The secondary response was almost exclusively represented by IgG antibodies with the IgG2a, IgG2b and IgG1 isotypes predominating at very high frequencies (>100 AFC per 5 x 10^5 nucleated cells). Tertiary challenge resulted in the accelerated appearance of AFC's and, during this response it was the classical anti-viral isotype, IgG2a (Coutelier et al., 1987) that dominated throughout (**panel C, Figure 5.4**). In the spleen, the primary response was low and represented mainly by IgM and, to a small extent, IgA. Secondary and tertiary challenge with RSV did not appear to boost the magnitude of the response but did result in the accelerated appearance of AFC's along with some of the IgG isotypes (mainly IgG2a), again demonstrating evidence of class-switching (**panel B, Figure 5.5**). Finally, the AFC response in the bone marrow was very interesting as, although it was low in frequency during the primary infection, it could still be detected, albeit still at this low frequency, during the long-term time-points (**panel A, Figure 5.6**). These long-term AFC were of mixed isotype and, unlike in the influenza virus C57BL/6 mouse model, neither IgG2a nor IgG2b dominated, however it must be noted that, much like in the D-NALT, the RSV-specific responses in the bone marrow were much lower in magnitude than the virus-specific AFC's observed following influenza virus infection. Secondary challenge with RSV did result in an increase in the kinetics and magnitude of the response with the frequency of most isotypes of AFC almost doubling for the period between seven and thirteen days post-

secondary infection. Additionally, tertiary infection stimulated a similar mixed isotype response that was consistently elevated in comparison to the primary and secondary responses. Here the majority of isotype frequencies were between 10 and 20 AFC per 5 x 10^5 nucleated cells, demonstrating a small but potentially significant boost in the bone marrow antibody response (**panel C, Figure 5.6**).

5.3.2 BPL-RSV studies

In the studies where the primary immunogen was BPL-inactivated RSV, with the exception of some IgM in the spleen and some very low frequencies of mixed isotypes in the bone marrow, no RSV-specific antibody responses were observed in any of the tissues following primary immunisation. This suggests that viral replication is essential to initiate and sustain RSV-specific antibody responses within these tissues. The antibody responses after secondary and tertiary challenges with live RSV varied between tissues but, appeared similar to those responses seen upon primary and secondary immunisation respectively with live RSV suggesting that non-replicating RSV is insufficient to 'prime' the immune system for subsequent challenge. There were some variations in isotype generated however, for instance following the first exposure of the BPL primed mice to live RSV (secondary immunisation), IgG1 was observed in the CLN and yet was not observed during at the same stage in the RSV only experiments and this was the case for several of the other tissues. However, as this particular experiment was only performed once and was not repeated, no firm conclusions can be drawn. From the available data though, it appears that BPL-inactivated RSV alone is insufficient to generate an immune response in the same manner as live RSV.

Classically, protection from respiratory viruses is mainly credited to the action of antibody; however with RSV the basis of the limited protection observed in humans is not clear cut. Whilst it is accepted that antibodies in general are responsible for the partial protection observed during RSV infection, their origin is of much debate. As mentioned previously, some studies suggest that resistance to infection correlates well with high levels of RSV-specific neutralising IgA in the nasal passages (Mills et al., 1971; Watt et al., 1990), and yet others present data to the contrary (Hall et al., 1991). The discovery that the D-NALT is a major source of virus-specific antibody following influenza virus infection (Liang et al., 2001) suggests that mucosal antibodies may be more significant that was once thought. In those studies the anti-viral response is extremely long-lived and, twelve-to-eighteen months after a single exposure, the frequency of virus-specific AFC's can be observed at approximately 150 (total) per 5 x 10^5 nucleated cells. Conversely, in these studies using RSV, we found far fewer RSV-specific AFC's in the D-NALT, the total frequency being less than 20 per 5 x 10^5 nucleated cells fourteen-to-eighteen months post infection. Furthermore, this response is not maintained following subsequent infections with live RSV, indicating that the local response to RSV in the D-NALT may be severely retarded in comparison with that seen during influenza virus infection or that it does not play such an important role in protective immunity to RSV.

In addition to the local antibody responses we also investigated the humoral responses within the bone marrow during RSV infection as previously it has been suggested that this is the major source of serum specific antibody following respiratory virus infections (Hyland et al., 1994). Again, prior studies utilising influenza virus have shown that a single exposure is sufficient to generate significant numbers of AFC's in the bone marrow (Liang et al., 2001) (approximately 40 AFC's per 5 x 10^5 nucleated cells) that

are maintained at a steady state, however the response to RSV is not as well defined. Whilst the bone marrow does contain AFC's specific to RSV, their frequencies are very low and decline rapidly compared with the numbers of virus specific AFC's observed during influenza virus infection. However, secondary and tertiary challenge with RSV resulted in increased the number of bone marrow AFC's to frequencies that paralleled those observed in mice infected with only a single dose of influenza virus; suggesting that successive infections may result in cumulative protection at least with regard to the bone marrow and the serum antibody that is derived from it.

The data presented in these studies demonstrates that, when compared with influenza virus, RSV infection elicits significantly lower frequencies of virus-specific AFC's within the D-NALT and bone marrow. The fact that a single intranasal influenza virus infection confers protection whereas a single intranasal RSV infection does not implies that RSV may not be eliciting sufficient numbers of AFC within either the D-NALT or the bone marrow to provide adequate protection from re-infection, at least in the mouse model. The gradual accrual of AFC within the bone marrow may be reflected in humans undergoing RSV infection as serum antibody wanes following primary infection only to return at higher titres following consecutive re-infections; however the propensity towards being re-infected with RSV still remains, implying that serum antibody alone is insufficient for protection. The lack of a robust, sustained AFC response in the D-NALT even after three infections with live RSV suggests that defective local responses may be a key factor in the hosts' inability to generate protective immunity to RSV. Unfortunately, the mechanisms that underlie this inability to generate a protective response are not known. As mentioned previously, RSV has been shown to modulate CD8+ T cells in the lung (Chang and Braciale, 2002) and so it may be possible that there is a similar suppressive effect on CD4+ T cells that may hinder development of

the humoral immune response to RSV and the subsequent generation of B cell memory. Another possibility is that through the infection of epithelial cells in the respiratory tract, RSV may elicit a host of inhibitory soluble factors that suppress B cell effector function such as interferon alpha and interleukin-8 (Olszewska-Pazdrak et al., 1998; Preston et al., 1995; Thomas et al., 2000) although this has yet to be conclusively proven.

Another intriguing possibility that may account for the poor AFC responses in the D-NALT and bone marrow may be the inability of the cells to traffic and home to these tissues. The discovery of chemokine motifs within the G protein of RSV (Tripp et al., 2001) provides a viable means by how this could possibly be explained, however the implications of this potential immunomodulation are far-reaching and may have significance for much of the data described in this thesis and so will be explored in more detail in the final discussion.

This lack of an effective local response to RSV may be a key factor in the hosts' inability to withstand re-infection and this deficiency does not appear to be compensated for by the increased frequencies of bone marrow AFC's observed over multiple infections. Of equal significance is the fact that the responses reported in this chapter can be attributed to the action of live replicating virus. The data generated from the BPL-inactivated RSV studies was crucial as it validated the results observed in the live RSV experiments. The inability of BPL-inactivated RSV to elicit primary immune responses similar to those seen during primary infection with live RSV, or the lack of a 'secondary-type' RSV-specific response upon challenge with live virus, categorically demonstrate that the data obtained with live RSV can only be achieved with a live replicating virus. This is important as it demonstrates that the specific immune responses are taking place as a result of viral replication and not through a form of

bystander activation brought about by exposure of the immune system to inert virus particles.

In conclusion, RSV infection does not appear to stimulate as vigorous a local humoral response as influenza virus. The reduced frequencies of AFC's in the D-NALT suggest that a defect in immunity at this point may be significant and could possibly explain the reduced capacity of the host to withstand subsequent RSV infection. Additionally, the slow incremental bone marrow AFC response suggests that the systemic humoral responses may not reach optimal efficiency until the host has undergone several exposures, however this will be discussed in the following chapter.

Chapter Six: Serum antibody responses to immunisation with live and inactivated RSV

6.1 Introduction

One of the most studied aspects of immunity to RSV is that of the serum antibody response, however, the concept of protective immunity against RSV infection is a complex one and the importance of serum antibody has been the subject of much controversy.

Serum and secretory antibodies are thought to play an important role in protection against infection and re-infection with regard to RSV, but the protection is often incomplete and is frequently insufficient under natural conditions (Brandenburg et al., 1997).

As discussed in the previous chapter, the literature suggests that RSV-specific secretory antibodies tend to protect against upper respiratory tract infections whereas serum antibodies appear to protect primarily against infection of the lower respiratory tract (Falsey and Walsh, 1998; Holberg et al., 1991). Upon infection with RSV, antibodies are developed against all the viral proteins but those directed against the F and G surface glycoproteins appear to be the most important for protective immunity (Walsh et al., 1987). In cotton rats for example, the administration of monoclonal antibodies against the F and G proteins provides almost complete protection of the lower respiratory tract, but not of the upper respiratory tract, against RSV challenge (Murphy et al., 1991).

However, of primary concern is the lack of information regarding the serum responses in the mouse. To date, it is not clear what role (if any) serum antibodies play in the immune protection of the mouse from RSV infection. As the ability to re-infect the same host is a hallmark of RSV, the situation is further complicated by the fact that there have been no studies investigating the serum responses in the mouse following multiple infections. Therefore, in this chapter we set out to characterise the serum antibody responses to RSV during several infections. Furthermore we assayed the efficacy of the antibody produced by investigating how effective it is at neutralising a known quantity of virus. This is important as high titres of serum antibody are not necessarily protective if they are unable to neutralise the virus. Finally, we set out to observe whether the serum responses generated after infection with RSV could be attributed to the actions of live, replicating virus by 'priming' the mice with inactivated RSV before exposing them to live virus.

To achieve these aims, serum samples were taken at corresponding time points post-infection and the effect of multiple infections of RSV was considered by infecting the mice with a second and third dose of RSV at six-week intervals after the primary infection. The sera were assayed for RSV-specific antibody by ELISA and, as previous studies have clearly demonstrated that the primary isotype of antibody associated with RSV neutralising activity are those of the IgG subclass (Wagner et al., 1989), we chose to study the RSV-specific IgG1, IgG2a and IgG2b responses only. ELISA serum antibody endpoints were calculated from the data obtained using the methods described in Materials and Methods and the data was controlled by coating ELISA plate wells with cell lysates of the cell line used to grow the stock RSV.

The RSV-specific antibody component was assayed for its effect on the infectivity of a known concentration of RSV (as described in the Materials and Methods section) and the data expressed as the reciprocal value of the dilution of sera at which fifty percent of the RSV plaques were neutralised.

Finally the effect of non-replicating inactivated RSV and its modulation of the serum antibody response upon subsequent natural infection was examined using beta-propriolactone (BPL) treated RSV. These studies were performed by immunising the mice primarily with BPL-RSV and then challenging on two further occasions (both at six-week intervals) with live RSV.

Unless otherwise stated, all the data values from experiments in this chapter are expressed as the results of four individual mice, the mean of that group and, where possible, the standard deviations within those groups.

6.2 Results

6.2.1 RSV-specific serum antibody response following infection and both secondary and tertiary challenge with live RSV

6.2.1.1 RSV-specific serum IgG1

Following primary immunisation with live RSV (**panel A, Figure 6.1**), no IgG1 was observed in any mice during the acute phase of infection until day ten (656.3 ± 482.3). At ten-months post immunisation this level had declined and was present at low titre (91.8 ± 89.0). Secondary challenge with live RSV resulted in increased IgG1 titres on

day two (600.7 ± 466.8), some six-fold higher than the value seen at ten-months (day 300) post-infection. The serum titre continued to rise peaking at 2635.1 ± 1270.3 on day nine and this level of antibody was maintained for up to six-weeks following secondary immunisation, as demonstrated by the titre one day before tertiary immunisation (day 42), 4161.9 ± 3540.3 (**panel B, Figure 6.1**). Following tertiary immunisation, although the titres vary, serum antibody levels of IgG1 are maintained up until at least day forty-four post-tertiary challenge, at which time point the titre was found to be 7594.9 ± 2821.8 (**panel C, Figure 6.1**).

6.2.1.2 RSV-specific IgG2a

An RSV specific IgG2a serum response is apparent on day eight post-primary infection (1208.9 ± 1018.9) and increases rapidly to a peak of 3249.4 ± 636.3 six days later, on day fourteen. Ten months (day 300) later this titre remains at a comparable level (3325.0 ± 3353.0), with some mouse to mouse variation (**panel A, Figure 6.2**). Following secondary immunisation with live RSV the antibody response increases over the course of the experiment to a peak of 5780.0 ± 1081.7 on day nine and, prior to tertiary challenge (six weeks after secondary immunisation – day 42), the titre was found to have increased greatly to 13,032.8 ± 4549.5 (**panel B, Figure 6.2**). Following tertiary immunisation however there does not appear to be any further boosting of the response and the titre remains at an approximate level between 12,000 to 13,000 throughout this experiment (**panel C, Figure 6.2**).

6.2.1.3 RSV-specific IgG2b

After primary infection with live RSV serum IgG2b antibody responses were apparent at a titre of 360.7 ± 239.3 on day eight. The titre increased rapidly reaching a peak of 2032.5 ± 386.5 six days later on day fourteen. By ten-months post-infection (day 300), the titre had reduced slightly but was still present at a value comparable to day ten (650.0 ± 264.6) demonstrating the presence of RSV-specific IgG2b, long-term, after a single immunisation (**panel A, Figure 6.3**). Following secondary immunisation with live RSV the titre increased slowly over the following week to reach a high of 5066.6 ± 4522.3 on day nine. These levels were maintained until at least six-weeks after secondary immunisation, (one day prior to tertiary immunisation - day 42) when the titre was found have reached a peak of 5275.5 ± 4069.7 (**panel B, Figure 6.3**). Tertiary immunisation resulted in a further boost in titre to 8014.4 ± 6603.9 but this was short-lived and afterwards the serum antibody titre had returned to pre-tertiary challenge levels. The titre was then maintained at pre-challenge values until day forty-four at which time point the titre reduced by approximately 90% to 1141.5 ± 2058.6 (**panel C, Figure 6.3**).

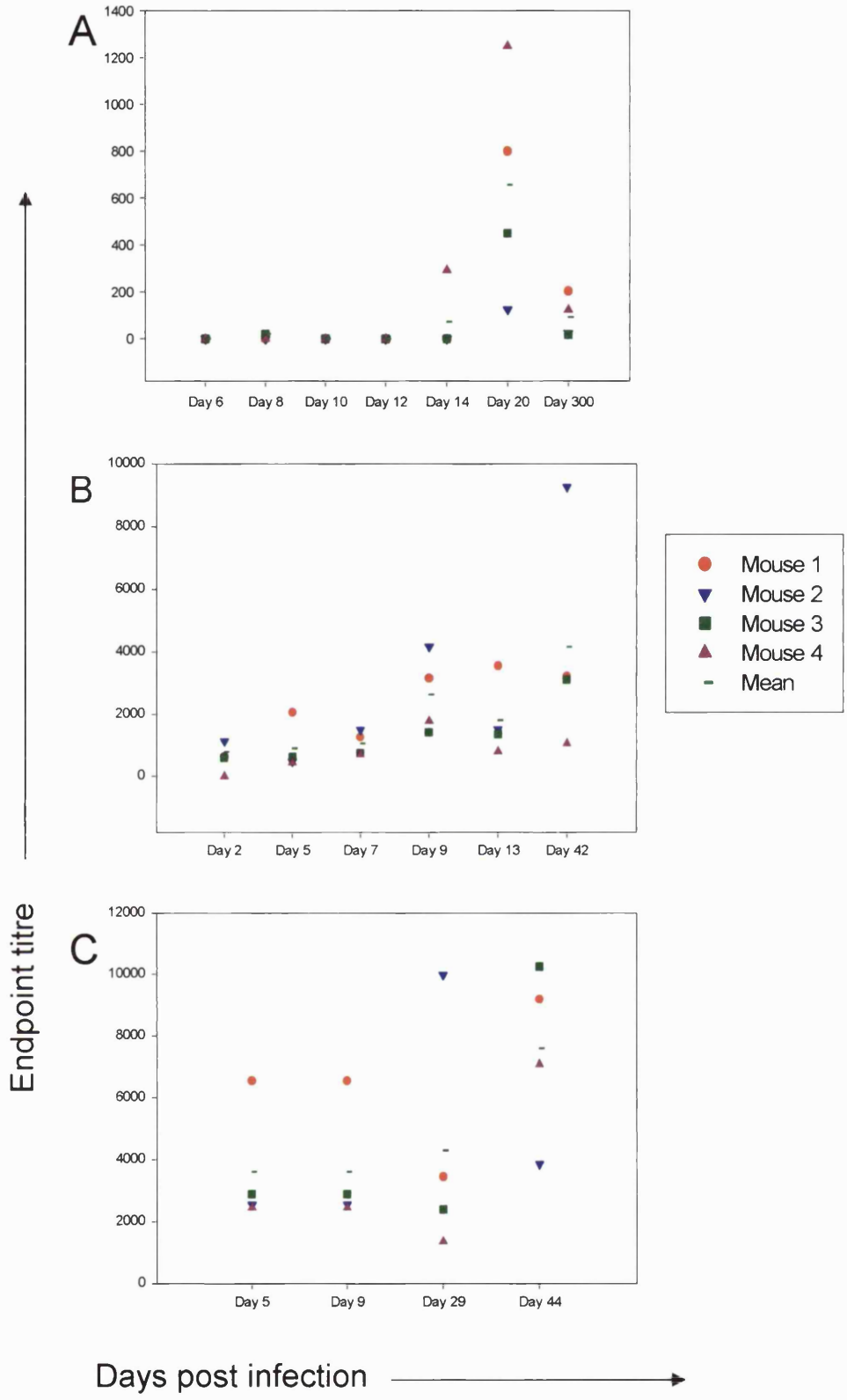


Figure 6.1 RSV-specific serum IgG1 antibody endpoint plots representing serum data taken from four mice and analysed individually following primary, secondary and tertiary infection with live RSV. **A** represents primary serum titres; **B** represents secondary serum titres and **C** represents tertiary serum titres. Each time-point displays four individual mice and the mean of those mice.

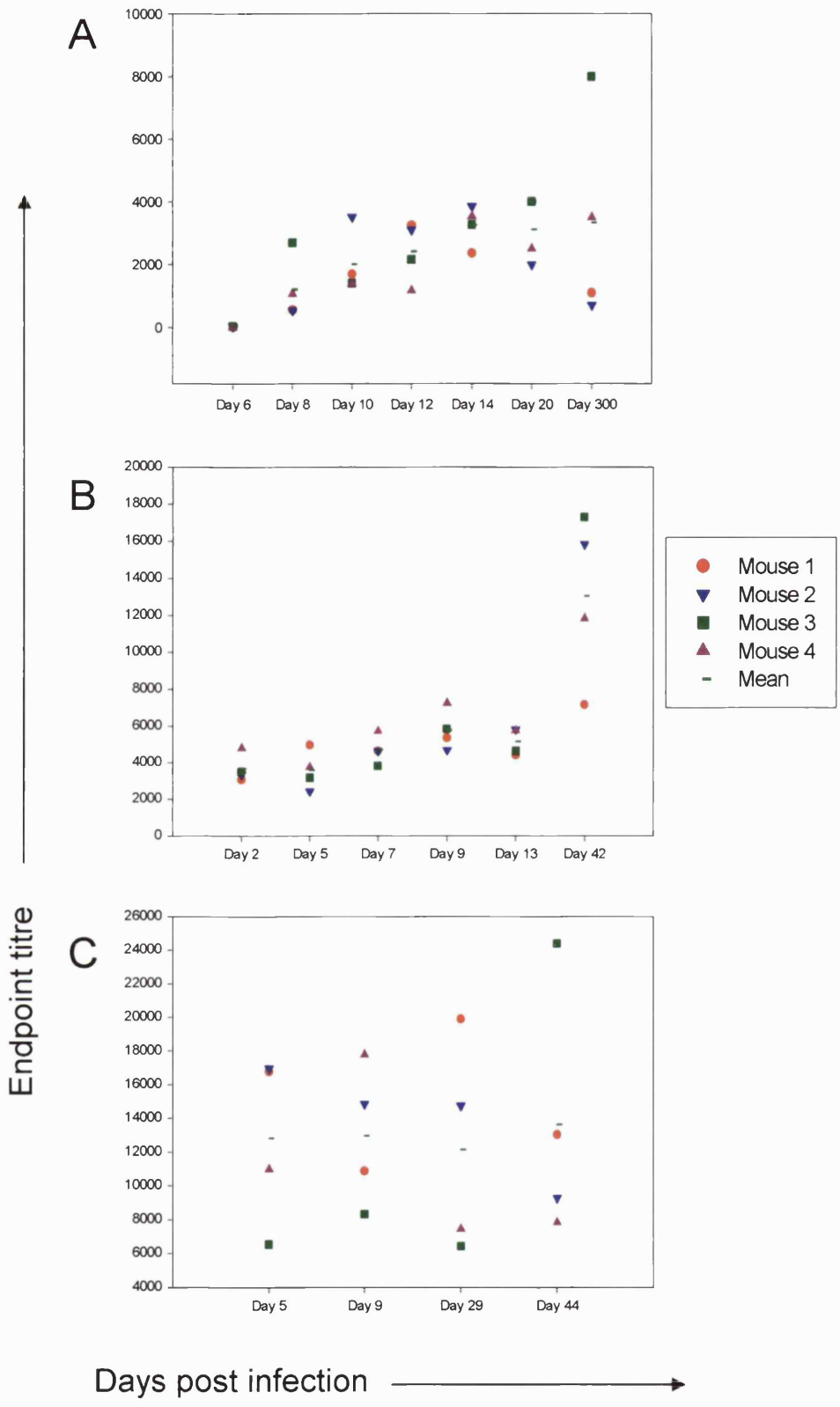


Figure 6.2 RSV-specific serum IgG2a antibody endpoint plots representing serum data taken from four mice and analysed individually following primary, secondary and tertiary infection with live RSV. **A** represents primary serum titres; **B** represents secondary serum titres and **C** represents tertiary serum titres. Each time-point displays four individual mice and the mean of those mice.

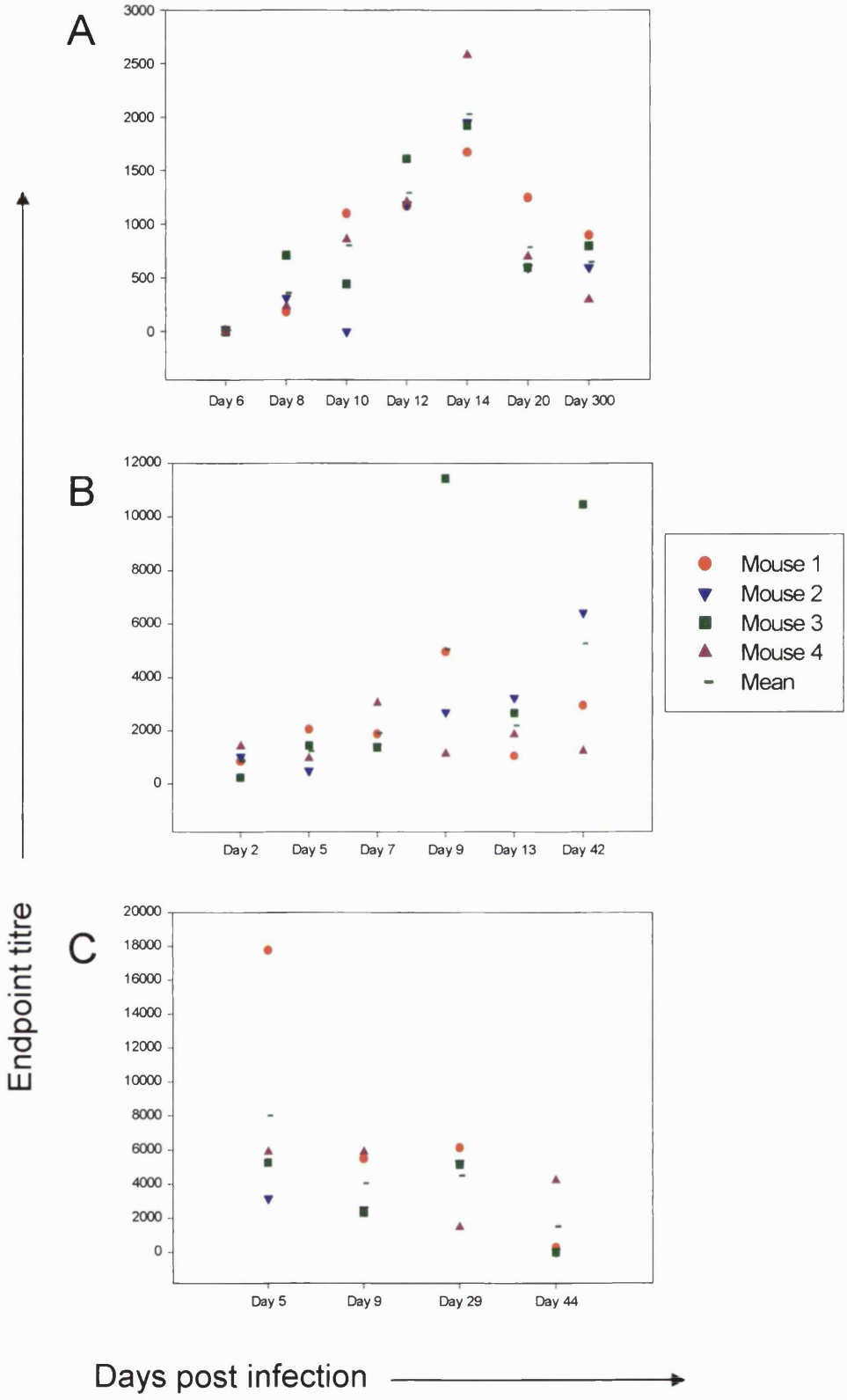


Figure 6.3 RSV-specific serum IgG2b antibody endpoint plots representing serum data taken from four mice and analysed individually following primary, secondary and tertiary infection with live RSV. **A** represents primary serum titres; **B** represents secondary serum titres and **C** represents tertiary serum titres. Each time-point displays four individual mice and the mean of those mice.

6.2.2 RSV-specific serum antibody response following primary administration of BPL-treated RSV and subsequent challenges with live RSV

6.2.2.1 RSV-specific IgG1

No antibody was observed during the acute phase following primary immunisation with BPL-inactivated RSV and, at ten-months post-primary immunisation (day 300), only very low titres (191.4 ± 278.6) were detectable, and in just two of the four mice examined (**panel A, Figure 6.4**). Following challenge with live RSV, antibody gradually increased from day seven onwards (**panel B, Figure 6.4**). RSV-specific serum titres then peaked on day thirteen at 3087.7 ± 1189.1. Six-weeks later, on the pre-challenge (day 42) time point, the titre had dropped by more than 50% to 1120.5 ± 374.8 but, following a second challenge with live virus, RSV-specific serum antibody responses were then boosted to a level of 3153.6 ± 656.8 by day thirteen. This titre of RSV specific serum antibody appears to be maintained until at least day forty-four at which point the titre was 2447.6 ± 3068.4 (**panel C, Figure 6.4**).

6.2.2.2 RSV-specific IgG2a

After primary immunisation with BPL-inactivated RSV, serum titres were low, reaching a maximum titre of 30.4 ± 40.1 ten-months after immunisation (day 300)(**panel A, Figure 6.5**). By day nine after challenge with live virus, titres had reached 2042.0 ± 797.5 and peaked at 2933.4 ± 3242.8 on day thirteen (**panel B, Figure 6.5**). Six-weeks after challenge (day 42) the serum titre dropped five-fold to 559.8 ± 203.9 but, following a second challenge with live RSV, was boosted to 2216.1 ± 2321.8 and

3507.9 ± 2154.1 on days five and thirteen respectively, Forty-four days post-infection the serum titre had reached a maximum of 3818.1 ± 3441.0 (**panel C, Figure 6.5**).

6.2.2.3 RSV-specific IgG2b

Acute serum titres following primary immunisation with BPL-inactivated RSV were < 25 and, after ten-months (day 300), a maximum titre of 106.0 ± 123.8 was observed (**panel A, Figure 6.6**). Following challenge with live RSV, the RSV-specific titres increased to 3524.5 ± 3363.1 on day nine and gradually declined shortly afterwards (**panel B, Figure 6.6**). At the pre-challenge time-point (day 42), some six-weeks after challenge with live virus, RSV-specific serum antibody titres were ten-fold less than those observed on day thirteen (308.3 ± 269.2). The effect of a second challenge with live virus was to elevate the titres to a peak of 1532.7 ± 786.1 after thirteen days which was then maintained at a similar level until day forty-four (**panel C, Figure 6.6**).

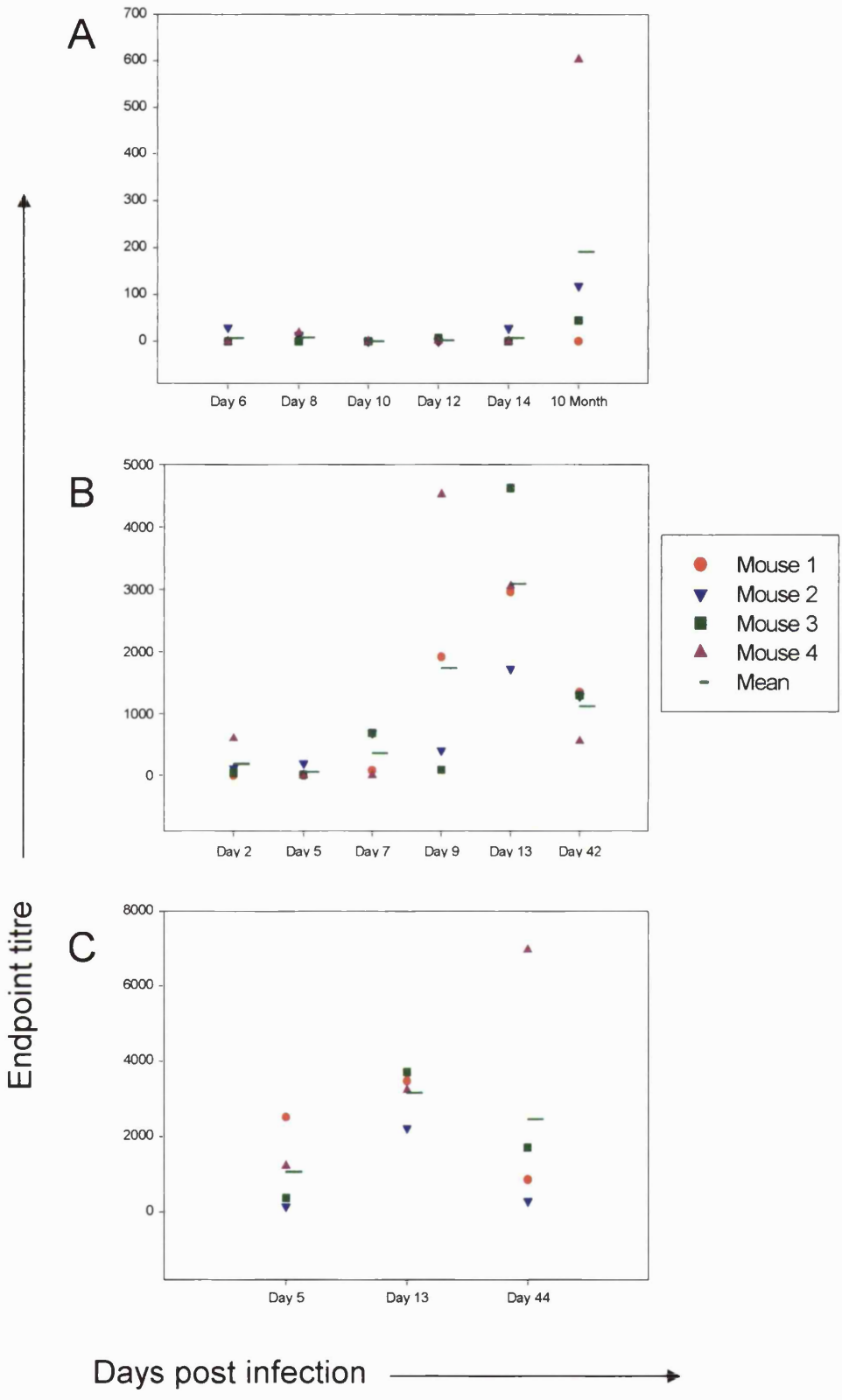


Figure 6.4 RSV-specific serum IgG1 antibody endpoint plots representing serum data taken from four mice and analysed individually following primary immunisation with BPL-RSV and challenge with live RSV. **A** represents serum titres after BPL-RSV priming; **B** represents serum titres after challenge with live RSV and **C** represents serum titres after a second challenge with live RSV. Each time-point displays four individual mice and the mean of those mice.

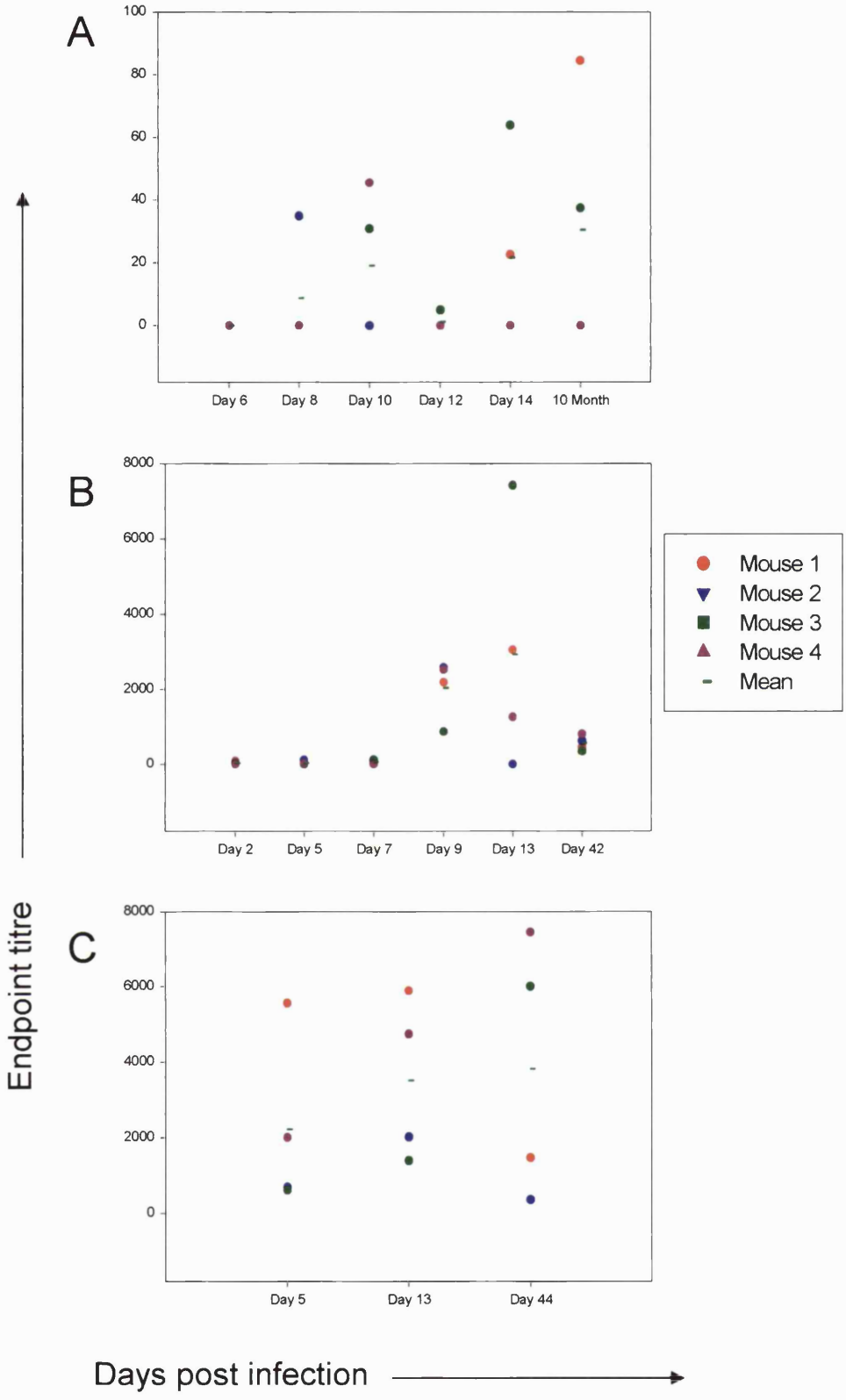


Figure 6.5 RSV-specific serum IgG2a antibody endpoint plots representing serum data taken from four mice and analysed individually following primary immunisation with BPL-RSV and challenge with live RSV. **A** represents serum titres after BPL-RSV priming; **B** represents serum titres after challenge with live RSV and **C** represents serum titres after a second challenge with live RSV. Each time-point displays four individual mice and the mean of those mice.

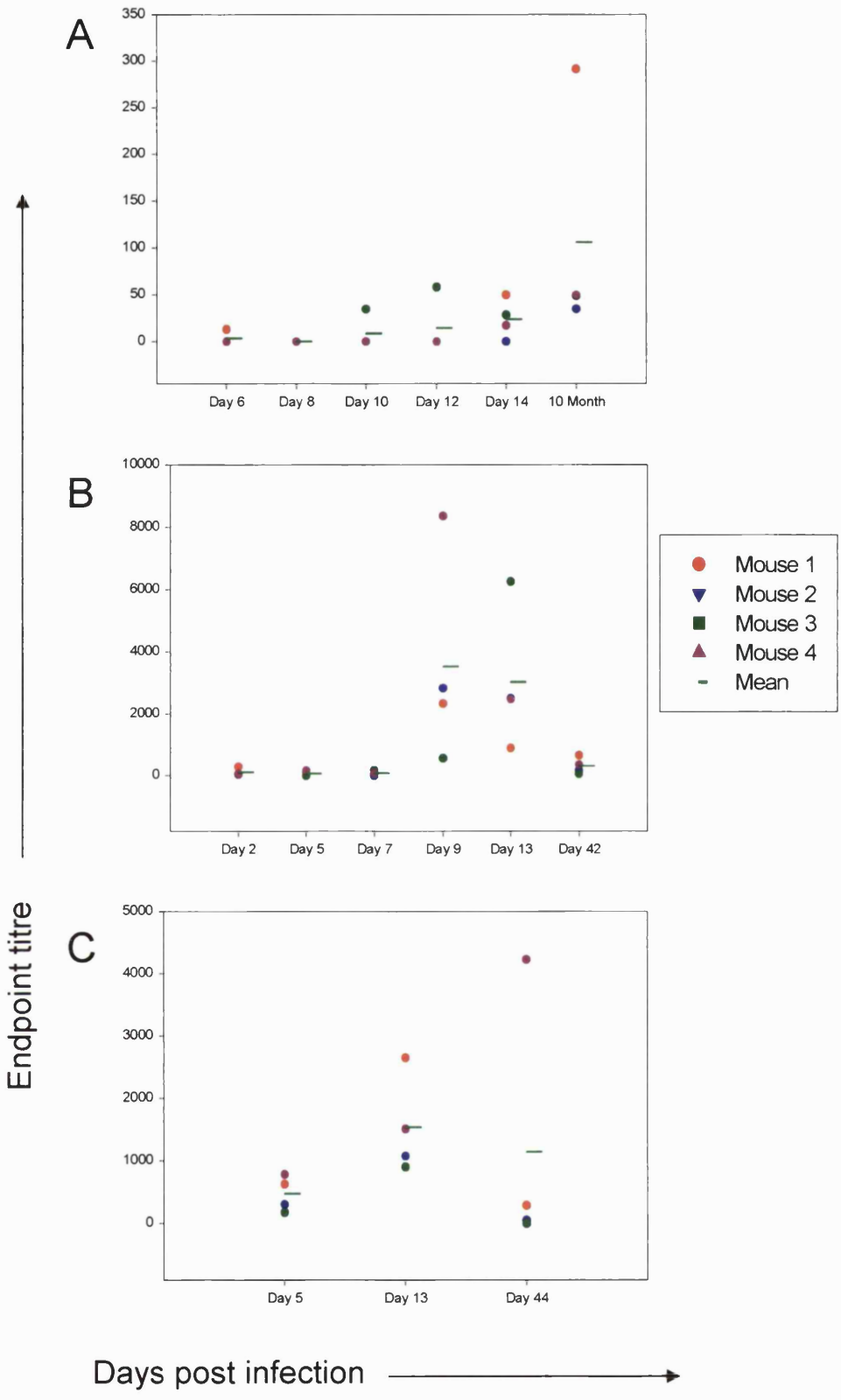


Figure 6.6 RSV-specific serum IgG2b antibody endpoint plots representing serum data taken from four mice and analysed individually following primary immunisation with BPL-RSV and challenge with live RSV. **A** represents serum titres after BPL-RSV priming; **B** represents serum titres after challenge with live RSV and **C** represents serum titres after a second challenge with live RSV. Each time-point displays four individual mice and the mean of those mice.

6.2.3 Virus neutralisation by serum antibody after primary exposure to RSV and subsequent secondary and tertiary challenge with live RSV

Ten days after the mice are first infected with RSV they have ED50 values ranging from 5 to 19. By day fourteen however, there is much less deviation within the group and the ED50 values have dropped to 4.0 ± 1.0 and appear to be maintained at this level which is clearly seen on day three-hundred (**panel A, Table 6.1**). Secondary infection elicits a neutralising response on day seven (9.0 ± 3.0) that is similar to that seen early on in the primary infection, but unlike then, this effectiveness does not decline and by day forty-two has shown an increased ability of the sera (in all but one of the mice) to neutralise RSV (**panel B, Table 6.1**). Following tertiary infection with RSV, the ED50 values on day nine are more than double those seen on the day seven time-point during secondary infection. It should be noted that these values are of a similar magnitude as those seen just prior to tertiary challenge on day forty-two and as such may represent a peak level of neutralising ability which cannot be increased with a third infection but is maintained for at least a month as shown by the value on day twenty-nine (**panel C, Table 6.1**).

A Primary	ED50 Titre		
	Day 10	Day 14	Day 300
Mouse 1	5.0	4.0	4.0
Mouse 2	11.0	5.0	3.0
Mouse 3	5.0	4.0	4.0
Mouse 4	19.0	4.0	3.0
Mean	**10.0**	**4.0**	**3.0**
SD	**7.0**	**1.0**	**1.0**

B Secondary	ED50 Titre		
	Day 7	Day 13	Day 42
Mouse 1	11.0	11.0	11.0
Mouse 2	11.0	10.0	83.0
Mouse 3	5.0	8.0	>130.0
Mouse 4	11.0	<5.0	<5.0
Mean	**9.0**	**10.0**	**47.0**
SD	**3.0**	**2.0**	**51.0**

C Tertiary	ED50 Titre	
	Day 9	Day 29
Mouse 1	83.0	8.0
Mouse 2	6.0	83.0
Mouse 3	19.0	8.0
Mouse 4	21.0	11.0
Mean	**32.0**	**28.0**
SD	**34.0**	**37.0**

Table 6.1 ED50 values for sera taken from RSV infected mice. **A** represents mice undergoing a primary infection with RSV, **B** represents mice undergoing a secondary infection with RSV and **C** represents mice undergoing a tertiary infection with RSV. The values represent the reciprocal maximum dilutions of murine sera required to achieve 50% neutralisation of a known concentration of RSV virus.

6.3 Discussion

These multiple challenge studies demonstrate that RSV-specific IgG2a is the most prevalent antibody isotype in the serum, regardless of primary immunogen (live RSV or BPL-inactivated RSV), although some variation in the ratio of antibody isotypes was noted for the two groups. For those mice primed with live RSV the hierarchy of the antibody isotypes was clear with greater amounts of IgG2a produced, followed by lesser amounts of IgG2b and then IgG1. However, for those mice primed with BPL-inactivated RSV the peak titres of IgG2b and IgG1 were similar. Overall, the antibody titres observed following BPL-RSV were much reduced compared with those observed during the live RSV studies, which was consistant with the low numbers of AFC seen in Chapter 5. However, when the BPL-primed mice were subsequently challenged with live RSV the titres of RSV-specific antibody in these mice then followed a pattern similar to that seen in the mice who had only received live-RSV once. In all of the studies there was a significant boost effect noted after each challenge.

The neutralising ability of the serum antibody (as measured by ED50) was initially low and declined towards the end of the primary infection. However, following secondary challenge the neutralising titre increased gradually reaching its highest observed effectiveness forty-two days post-secondary infection. This titre did not increase further after tertiary challenge.

The generation of RSV-specific serum antibody was observed over the same time-period as the AFC frequency studies described in chapter five and this enabled us to directly compare the kinetics and frequencies of the AFC study with the serum antibody data. As discussed previously the majority of virus-specific serum antibody is thought

to be derived from plasma cells within the bone marrow (Hyland et al., 1994) and this appears to be borne out by the parallel increases observed in serum antibody titre and bone marrow AFC frequency following each successive re-infection. The serum antibodies generated against RSV included IgG1 and IgG2b but were primarily of the IgG2a class which has been attributed as 'the' anti-viral isotype in the mouse (Coutelier et al., 1987) and, although present at moderate titres during the primary infection, were significantly boosted upon secondary and tertiary infection. These results agree with previous studies that demonstrate that long-lived serum antibody responses are only elicited after the second or third infection with live RSV (Wagner et al., 1989) as it was clearly observed from our data that ten-months after primary infection most antibody titres had declined considerably.

The BPL-RSV primed mice again demonstrated clearly that inactivated RSV is unable to initiate an immune response by itself and, as the subsequent challenges with live RSV were no different to those observed in primary and secondary live-RSV infections it is apparent that live-virus is necessary to stimulate an RSV-specific serum antibody response.

From studies using recombinant vaccinia viruses it has been established that only the F and G viral proteins are able to induce neutralising antibodies that are effective against RSV (Connors et al., 1991) implying that not all antibody generated will neutralise. We found only a transient presence of neutralising antibody during the primary infection but upon subsequent infections a gradual increase in titre was observed that eventually reached a value almost five-fold that seen during the primary infection. This titre could not be boosted by additional infections suggesting that the maximum neutralising potential of the circulating antibody is achieved after two infections. It is possible that

the virus is exerting some influence over epitope usage although it is not clear how. Another possibility is that there may be generation of viral escape mutants but, while these have been documented in the case of other viruses such as human immunodeficiency virus (HIV) (Burns and Desrosiers, 1994), this immune evasion strategy has never been described for RSV and, given that the virus does not replicate so extensively in the mouse, it is highly unlikely to occur. In comparison with other more pronounced respiratory viruses such as influenza virus (De Jong et al., 2000; Hay et al., 2001; Scholtissek, 1995) the degree of antigenic shift and drift for RSV (Cane and Pringle, 1995; Garcia-Barreno et al., 1990; Sullender, 2000) is extremely reduced and would seem to suggest that, although possible, the generation of RSV particles with dramatically altered antigenic structures in such a short time is highly unlikely. Overall, the serum data fits in well with the situation observed in humans where a gradual protection appears following more than one infection but, as we have seen in chapter three, high titres of neutralising serum are still insufficient to prevent re-infection.

With regard to the experimental design of this study it should be made clear that during the process of viral replication, RSV progeny virions bud from the infected cell in order to infect neighbouring cells. As a consequence of this some cellular proteins may be retained by the new virus particles and may generate an immune response themselves when the virus is used to infect mice. To avoid this, virus stocks for infection and for *in vitro* assays are usually grown in different cell lines and in serum-free media. However, when this has not been possible we have demonstrated that the immune response to these cellular proteins is not significant by coating ELISA plates with cell lysate and running samples known to possess RSV-specific antibody on these plates. When this assay was performed at no point did the total (all isotypes) serum antibody response to the cellular proteins in the experimental sera exceeded an endpoint titre of 1:30.

The experiments described in this chapter were only performed once and therefore there are limitations as to the interpretations that can be made from the data. Each mouse in the experimental group (n=4) was analysed separately and in spite of the natural variation, the overall values are consistant. It would be desirable to repeat the studies to confirm the findings and generate statistics, however in order to overcome the problem of mouse-to-mouse variation a great number of mice would need to be assayed which unfortunately was not possible within the time constraints of this thesis. The data presented here does appear to portray a representative picture of the RSV serum antibody response that, whilst not definitive, is still valid and correlates exceptionally well with the data presented previously on the bone marrow AFC responses.

Chapter Seven: Antibody responses to live RSV infection in the lung

7.1 Introduction

Pulmonary antibody responses have been evaluated in a number of model systems (Moore et al., 2001) but there have been no detailed studies performed on the mouse. Antigen exposure in these models results in the generation of antigen-specific B cells within the lung and, in addition to this, long-lived memory B cells may reside within the localised areas of the lung where the initial insult was encountered (Bice et al., 1987).

In humans infection with RSV elicits both a secretory and serum response with both IgA and IgG detectable in the upper and lower respiratory tracts respectively (Shay et al., 1999). The lower respiratory tract is a key compartment in the immunological battle with RSV as progression from the upper respiratory tract to this location is often associated with disease exacerbation leading to increased morbidity and mortality.

Historically it has been suggested that RSV-specific secretory antibodies protect against upper respiratory tract infections whereas serum antibodies protect against infection of the lower respiratory tract (Falsey and Walsh, 1998; Holberg et al., 1991; Reynolds, 1986).

However, the origins of the antibody classes found within the lung are a matter of debate, whereas it is widely accepted that some of the IgG within the lung originates from the serum (via passive transudation) there is evidence to suggest that this is not completely the case with IgA and that a large percentage of IgA is produced locally (Burnett, 1986).

The aim of this study was to investigate these main antibodies within the lungs of BALB/c mice during the course of several consecutive RSV infections.

This was achieved through the use of the 'fragment culture' technique (see Materials and Methods) where small sections of lung are taken *ex vivo* and subjected to high concentrations of oxygen in order to maintain the viability of the AFC within. These cells in the lung fragment then continue to secrete antibody into the supernatant over a five-day period and the supernatants are then assayed by ELISA for RSV-specific antibody.

7.2 Results

7.2.1 The RSV-specific antibody response and isotype specificity in lung fragment cultures following primary, secondary and tertiary infection with live RSV

7.2.1 RSV-specific IgA

No RSV-specific IgA was detected in the lung fragment cultures up to fourteen days post primary infection with live RSV (**panel A, Figure 7.1**). This was also the case after

the secondary challenge, with no readily detectable IgA up to, and including, day thirteen (**panel B, Figure 7.1**). Six weeks after secondary challenge (day 42) however, some IgA was observed but at extremely low levels and this response did not persist after the mice were given a third dose of live RSV (**panel C, Figure 7.1**).

7.2.2 RSV-specific IgG1

Twelve days after primary infection, no virus-specific IgG1 antibody was detected in the lung fragment culture supernatant (**panel A, Figure 7.2**). Two days later, antibody responses were observed but at very low titres (< 20).

Seven days after secondary infection with live-RSV, RSV-specific antibody titres were still very low (22.1 ± 44.2) but, six days later, had risen approximately eight-fold to a higher titre (190.5 ± 108.9). Six-weeks after the secondary infection (day 42) the supernatant titres of the lung samples were variable with supernatants from one mouse containing no antibody whilst two others possessed titres comparable with day thirteen (153.6 and 102.2) and one had a titre greater than 2000, demonstrating considerable mouse-to-mouse variation (**panel B, Figure 7.2**).

Following tertiary infection, RSV-specific antibody titres in the supernatant were observed at a level similar to those seen during secondary infection. The supernatant titres remained at a level between 125.0 and 234.0 for the duration of the tertiary infection (**panel C, Figure 7.2**).

7.2.3 RSV specific IgG2a

A lung supernatant titre of 126.8 ± 91.7 was observed on day twelve following primary infection, however, two days later this supernatant antibody titre had declined significantly, almost disappearing (**panel A, Figure 7.3**). The lung fragment culture titres after secondary immunisation were much higher than those seen following the primary response with a value of 323.3 ± 187.0 noted on day seven which doubled to 667.6 ± 515.8 two days later. This trend continued as time progressed and six-weeks after secondary immunisation (one day prior to tertiary immunisation (day 42)) the antibody levels had risen approximately five-fold to 3598.4 ± 2599.5 (**panel B, Figure 7.3**). Following tertiary immunisation a slight increase in titre was noted on day nine (4951.4 ± 2908.3) but this was short-lived and on day twenty-nine the titre had dropped by over 70%. By day forty-four, only low titres of RSV-specific antibody could be detected (407.2 ± 300.7) (**panel C, Figure 7.3**).

7.2.4 RSV-specific IgG2b

After primary infection with live RSV the RSV-specific lung supernatant IgG2b titres were extremely low (< 12) and were not detectable fourteen days post-infection (**panel A, Figure 7.4**). After secondary infection a supernatant titre of 145.2 ± 133.8 was seen on day seven which more than doubled six days later. The titre then continued to increase until at least six-weeks after secondary immunisation (day 42) at which time it had risen to 4274.7 ± 6981.8 (**panel B, Figure 7.4**). Whilst it does appear that the trend in increased antibody is true over the secondary infection, the data on day forty-two is skewed as one of the four mice appeared to react excessively to the antigen, producing a massive titre of antibody. Tertiary infection did not appear to result in a further increase

in lung supernatant antibody titre although it must be noted that the titres present here were higher than those seen during both the primary infection, and during the initial stages of the secondary infection. The response appeared to decline sharply between day nine (1169.8 ± 889.3) and twenty-nine (362.7 ± 242.0) however by the last time point of the experiment the titre appeared to have stabilised at a value of 362.7 ± 242.0 some forty-four days after tertiary immunisation (**panel C, Figure 7.4**).

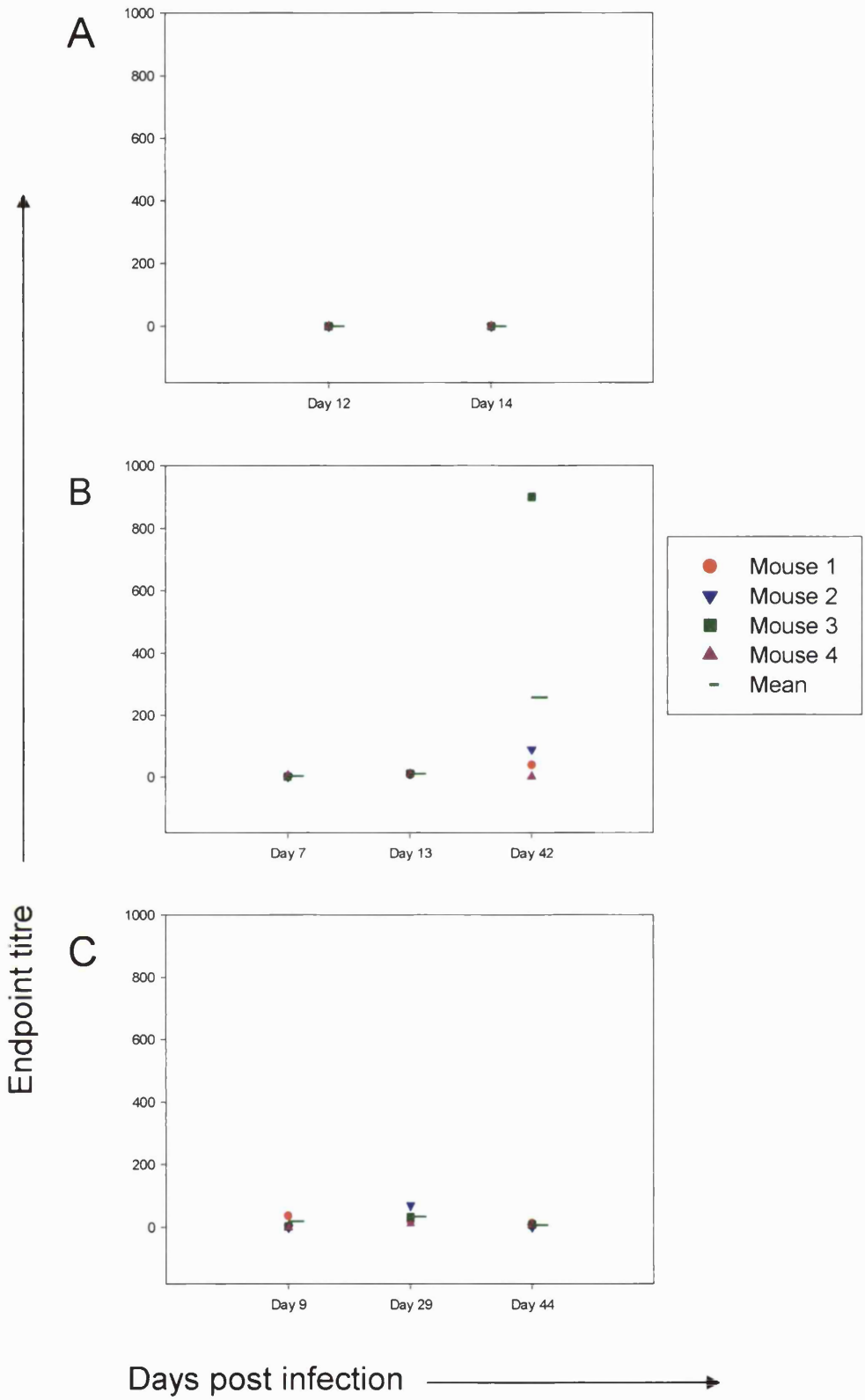


Figure 7.1 Plot representing RSV-specific IgA antibody titres from lung fragment culture. The individual IgA mouse endpoint titres and their collective mean values are shown following primary, secondary and tertiary infection with live RSV. **A** represents primary titres; **B** represents secondary titres and **C** represents tertiary titres. Each time-point displays four individual mice and the mean of those mice.

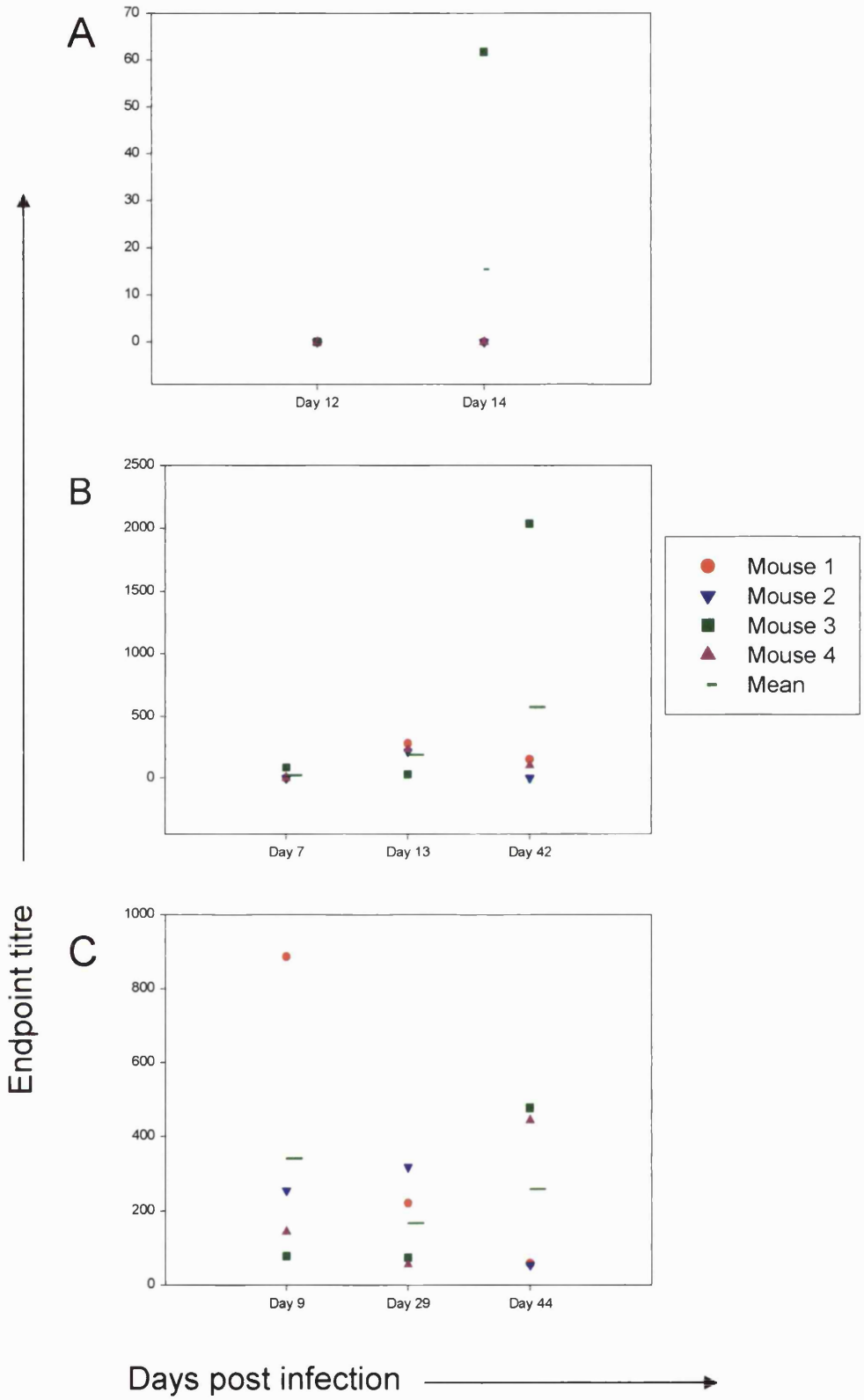


Figure 7.2 Plot representing RSV-specific IgG1 antibody titres from lung fragment culture. The individual IgG1 mouse endpoint titres and their collective mean values are shown following primary, secondary and tertiary infection with live RSV. **A** represents primary titres; **B** represents secondary titres and **C** represents tertiary titres. Each time-point displays four individual mice and the mean of those mice.

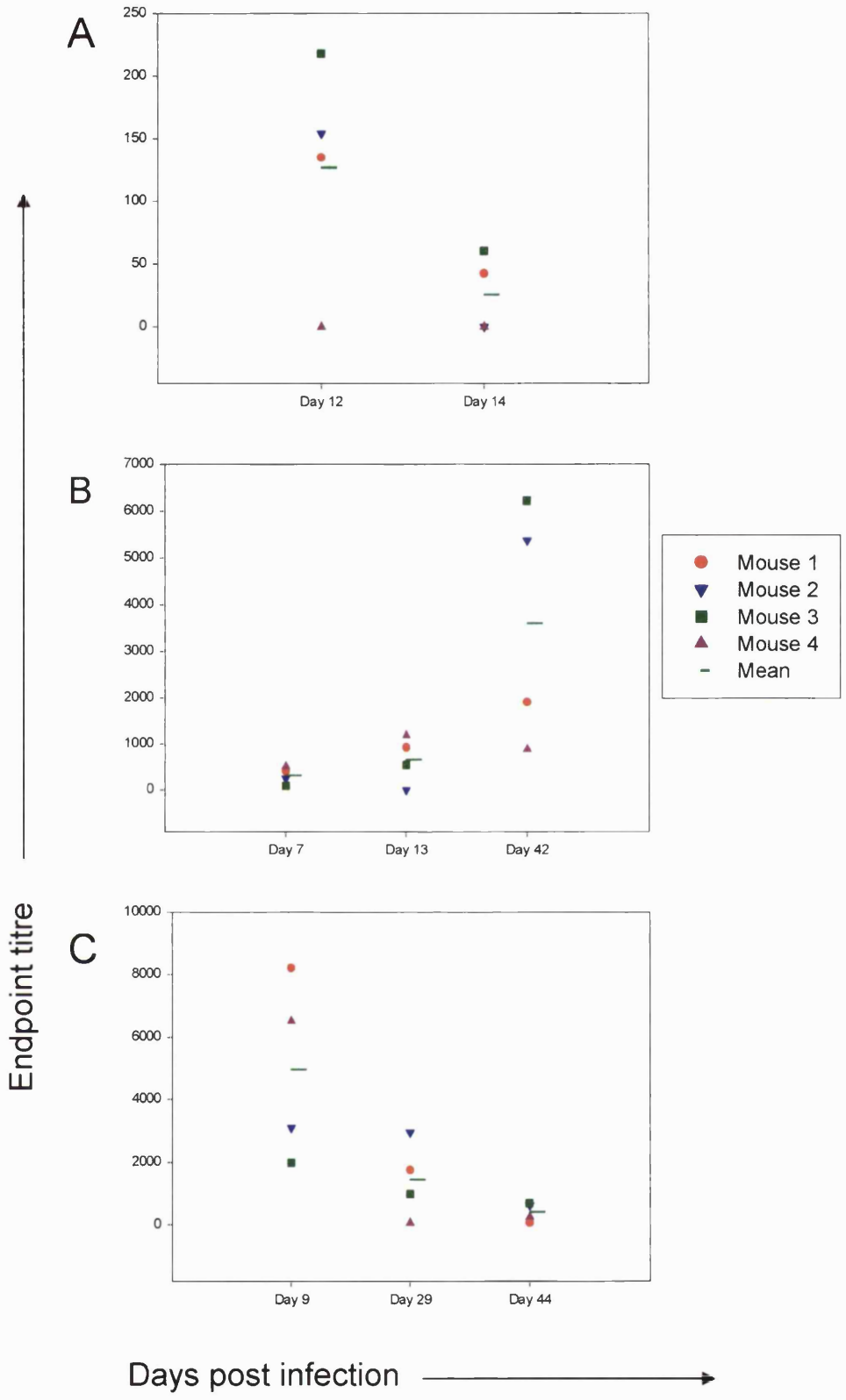
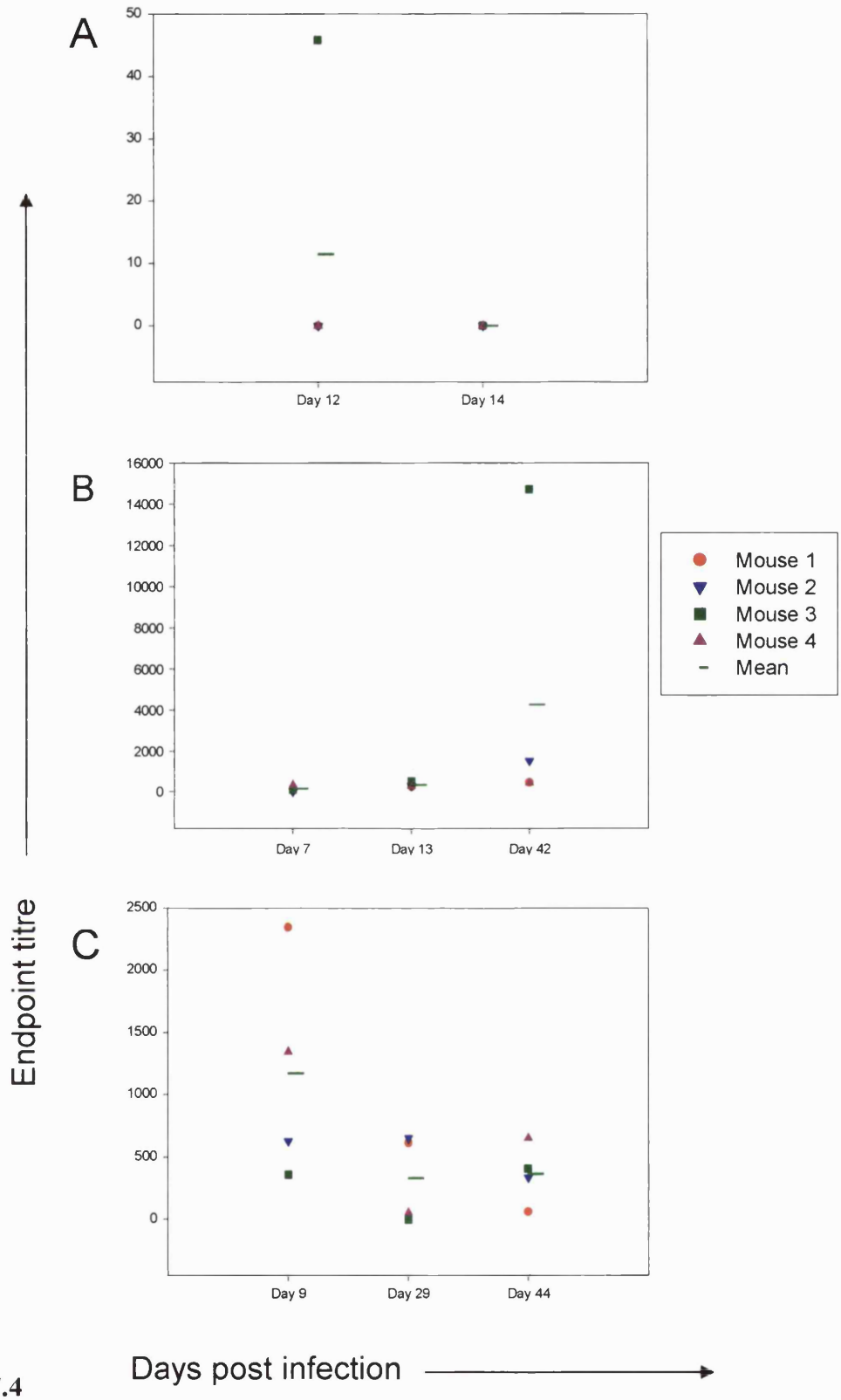


Figure 7.3 Plot representing RSV-specific IgG2a antibody titres from lung fragment culture. The individual IgG2a mouse endpoint titres and their collective mean values are shown following primary, secondary and tertiary infection with live RSV. **A** represents primary titres; **B** represents secondary titres and **C** represents tertiary titres. Each time-point displays four individual mice and the mean of those mice.



7.4

Figure 7.4 Plot representing RSV-specific IgG2b antibody titres from lung fragment culture. The individual IgG2b mouse endpoint titres and their collective mean values are shown following primary, secondary and tertiary infection with live RSV. **A** represents primary titres; **B** represents secondary titres and **C** represents tertiary titres. Each time-point displays four individual mice and the mean of those mice.

7.4 Discussion

During the primary infection the titres of the anti-RSV IgG antibodies (IgG1, IgG2a, IgG2b) in the lung fragment cultures were initially low (less than ~150) and were primarily of the IgG2a isotype, however, more surprising was the complete lack of IgA during this time given that it is thought that long lived IgA AFC are thought to reside in the lung tissue [Wilkes, 2001 #209]. All of the IgG isotypes assayed increased in titre during the secondary and tertiary infections with IgG2a and IgG2b being the most prominent, their tertiary peak titres having increased to values more than ten-fold higher than those observed following primary infection. With regard to IgG1, although the titres did increase after secondary and tertiary infections they still remained approximately ten-fold lower than those of IgG2a and IgG2b. This was not the case with IgA though which exhibited only a marginal and extremely short-lived response late on during the secondary infection but otherwise was not present for the entire duration of the study.

In distinction to the systemic response, AFC present in the submucosa of the airways give rise to the local antibody response and that these AFC become detectable in the respiratory tract-associated tissues five-to-seven days after infection. In agreement with this, studies with influenza have demonstrated the presence of high numbers of virus-specific AFC in the lung following primary infection (Jones and Ada, 1986) and in these studies IgM, IgG and IgA AFC's were all detected at high frequency (IgM>1000 AFC/10^6 cells, IgG>600 AFC/10^6 cells & IgA>400 AFC/10^6 cells) and were present up to eleven months post-infection.

Although the studies in this chapter have not assayed the frequencies of RSV-specific AFC's in the lung it is clear from the antibody titres that the frequencies of active, antibody secreting AFC's in this study are much reduced in comparison with influenza virus. Whilst some RSV-specific antibodies are produced in response to primary infection it is apparent that multiple infections are necessary in order to sustain a strong RSV-specific antibody response and that, even so, these responses are short-lived and low in titre compared with those observed in the serum.

It is commonly accepted that IgG is associated with lower respiratory tract antibody responses and that a significant proportion of this antibody is derived via passive transudation from the serum (Renegar and Small, 1991b). We cannot therefore exclude the possibility of some serum antibody being present via transudation; however the contribution to the overall titre would be very low as it has been previously shown that in passively immunised mice, serum antibody does not appear to contribute to the antibody recovered from lung fragment cultures (personal communication, Dr Julia Tree).

Conversely, it is also commonly accepted that IgA is often exclusively associated with upper respiratory tract responses (Renegar and Small, 1991b) and yet during studies with influenza, virus-specific IgA AFC were readily detected in the lung at significant frequency (Jones and Ada, 1986). The lack of locally produced IgA during multiple infections with RSV then is intriguing and may potentially be a factor that contributes to the ability of the virus to re-infect so frequently.

The lack of local IgA and the poor IgG responses could be explained by the AFC within the lung not functioning effectively (although before this could be considered their

presence in the lung must be experimentally confirmed) or that there are simply not enough AFC trafficking to the lung in order to carry out an effective response to the infection.

The data from these studies suggests that few, if any, RSV-specific AFC are present within the lung during RSV infection and the lack of a sustained antibody response implies either that local contributions from the lung do not play a role in protection from RSV or that they are simply ineffective in their current form. The fact that that live virus can still be isolated from the lung two-to-five days after secondary and tertiary infection (**chapter three**) further emphasises the importance of local antibody at this site especially as there are significant titres of serum antibody present (**chapter six**).

Additionally, the observation that the local lung antibody titres, although low, can be boosted upon successive infections suggests that repeated exposure to the virus has a cumulative effect on the local antibody response, potentially mirroring the gradual accrual of immunity to RSV seen in humans (Glezen et al., 1986; Hall et al., 1991; Henderson et al., 1979; Panuska et al., 1995). However, it would appear that even after these multiple exposures to RSV the local lung antibody titres do not reach sufficient levels to prevent re-infection in contrast with the immune response seen with influenza virus.

As the concept of the common-mucosal immune system (CMIS) is built upon the trafficking of cells from inductive sites to effector sites (Brandtzaeg, 1987; Brandtzaeg et al., 1999a; Brandtzaeg et al., 1999b; Butcher, 1999; Cyster, 2003; Lamm and Phillips-Quagliata, 2002) it may be the case with RSV that B cells activated at locations such as the NALT are simply not reaching the lung in order to take effect. It is well

known that, with regard to cell trafficking, the NALT has unique characteristics that differ from those classically described in the Peyer's patches (Csencsits et al., 1999) and the discovery that the RSV G protein interacts with CX3CR1, the specific receptor for the CX3C chemokine fractalkine (Tripp et al., 2001) may be one possible way in which the virus subverts the host trafficking system for its own benefit. This has yet to be proven however and is discussed in more detail in chapter eight.

Although this is only preliminary data the results observed here appear to correlate well with the data already presented and what little we know about the mode of action of RSV. As a follow on experiment it would be very interesting to track RSV-specific B cells from the NALT in order to monitor their progress during the immune response and this could be achieved using congenic mice. Further studies involving the interaction of chemokines and AFC's, in particular the effect that the interaction of the RSV G protein and CX3CR1 has with regard to trafficking, would be extremely interesting.

Chapter Eight: Discussion and future perspectives

The last thirty years has seen little progress towards the development of a vaccine for RSV and, at present, there are still many aspects of the biology of this unique virus that are far from well understood. In the studies described above we have investigated an area of RSV immunology that has not been studied in detail and may provide clues to the question of why natural immunity to RSV is incomplete.

Historically, efforts to develop a protective RSV vaccine have met with little success and the fact that the main targets of any such vaccine are infants of less than six months of age (Crowe, 2001) complicates matters. The relative immaturity of the infant immune system coupled with the well-documented inhibitory effect of passively acquired maternal antibody (Crowe, 1998; Murphy et al., 1988; Murphy et al., 1991) presents a significant obstacle to the development of a viable, effective and most importantly, safe, vaccine. In addition, due consideration must also be made with respect to the potential predisposition of vaccinees for the enhanced pulmonary disease seen upon natural exposure to RSV during the now infamous FI-RSV study (Kim et al., 1969).

Protection against respiratory pathogens is generally mediated via antibody responses and, whilst this may be true for RSV, this response appears insufficient to prevent infection. In particular, in children, RSV specific antibody is present in the serum during infection but declines in titre shortly afterwards. A higher titre of serum antibody is generally detected following challenge, but this too declines rapidly establishing a pattern that is repeated for all subsequent exposures (Henderson et al., 1979). The fact that serum antibody is generated upon infection and that the titres increase with each re-

infection suggests that there is a gradual build up of resistance to infection with RSV (Henderson et al., 1979; Kaul et al., 1981; Welliver et al., 1980) but the short-lived nature of these responses may be a key factor in the lack of protection so commonly seen. However, the association of RSV specific serum antibody with protection is unclear with some studies suggesting a correlation between RSV-specific neutralising nasal IgA rather than RSV specific neutralising serum antibody levels (Mills et al., 1971; Watt et al., 1990) and other studies suggesting that the level of RSV specific nasal neutralising IgA is irrelevant (Hall et al., 1991). Despite these conflicting studies, the fact that RSV is a pathogen restricted to the respiratory tract suggests that the role of secretory antibody must not be disregarded as it may be critical in the protection of the host from further infection.

From the studies by Benner *et al.* (Benner et al., 1974a; Benner et al., 1974b; Benner et al., 1974c; Benner and van Oudenaren, 1975; Benner and van Oudenaren, 1976; Benner and van Oudenaren, 1977; Benner et al., 1977a; Benner et al., 1977b; Benner et al., 1977c) we know that plasma cells, once generated, migrate to the bone marrow where they become the major source of specific antibody found in the serum long after exposure to respiratory viruses such as influenza and Sendai virus (Hyland et al., 1994). In the influenza virus mouse model, our laboratory has previously observed a population of influenza virus-specific AFC within the bone marrow (approximately 40 AFCs/$5x10^5$ total nucleated bone marrow cells) eighteen months after a single exposure (Hyland et al., 1994; Liang et al., 2001). This contrasts with our results for RSV where the bone marrow of RSV infected mice contained much lower frequencies of RSV-specific AFCs at a similar time point after infection. However, the fact though that there are detectable, albeit low, levels of virus-specific AFC's at this location, suggests that specific plasma cells have seeded to the bone marrow and have undergone terminal

differentiation. More interestingly, after successive re-infections with RSV, the bone marrow RSV-specific AFC population demonstrated an increase in frequency after each challenge and, following tertiary exposure to RSV, had attained a frequency almost equal to that seen following primary influenza virus infection. The necessity for three successive exposures in order to achieve levels comparable to those seen after a single influenza virus infection is not clear but may be due to differences in the initiation of the immune response within the periphery or may even be due to certain immunomodulatory properties of the virus itself (Olszewska-Pazdrak et al., 1998; Preston et al., 1995; Thomas et al., 2000). This incremental increase in bone marrow specific AFC frequency following RSV infection serves to reinforce the concept that there is a gradual build up of RSV immunity over a period of subsequent infections. Complementing this, the kinetics of the AFC response correlates well with the increases seen in RSV-specific serum antibody after secondary and tertiary boosting. These findings also agree with studies carried out in humans where long-lived serum antibody responses are generated only after two or three infections with RSV (Wagner et al., 1989).

With regard to the composition of the serum, virus neutralising antibodies are a key component in the immune protection of the host from RSV infection (Prince et al., 1985b) and in our *in vitro* studies we observed an initial decrease in the ability of the serum to neutralise virus following primary infection (**table 6.1, Chapter 6**) that was overcome following secondary infection, suggesting that repeated exposure is essential to generate neutralising serum antibody responses.

The isotype specificity of the antibody produced in the serum was also examined and it was observed that, in line with the observations by Coutelier *et al.* (Coutelier et al.,

1987), the RSV-specific serum response was dominated by antibodies of the IgG2a isotype, the classical anti-viral isotype. The fact that there appears to be no major defect in the serum compartment either in specificity, neutralising ability or titre suggests that there may be some other element that is responsible for the high susceptibility of the host to reinfection with RSV. The success of prophylaxis with RSV intravenous immunoglobulin (RSVIG) and the humanised monoclonal antibody Palivizumab (Welliver, 1998) at preventing enhanced disease would seem to suggest that serum antibody plays an important role, however, it is clear that serum antibody is not responsible for the initial stages of immune defence following primary infection and, in reality, it may act in a 'damage limitation' role once the initial innate defences have been breached. The question remains as to where and what these defences really are.

To answer this question we investigated the role of the nasal tissues, specifically the nasal associated lymphoid tissue (NALT). The strategic significance of the lymphoid tissue in the nasal passages is cannot be understated. Antigens of all forms are continuously inhaled and therefore the NALT is being constantly exposed to pathogens. Indeed the result of this 'first contact' of antigen with the NALT may prove to be crucial to the immune systems' response as a whole, due in part to the potential communicative nature of the tissues within the common mucosal immune system. It is possible that the result of this initial contact may shape both the local and systemic immune response to RSV. With regard to future vaccination strategies, it is well established that mucosal immunisation is preferable to parenteral immunisation as it elicits both mucosal and systemic responses (Sminia and Kraal, 1999) and the NALT provides a potential gateway with which this aim can be achieved. Highlighting the importance of the nasopharyngeal lymphoid tissue in this context is the observation that children who had undergone tonsillectomy or adenoidectomy possessed diminished

poliovirus specific IgA antibody titres compared with those children that had intact nasopharyngeal lymphoid tissue (Ogra, 1971). This evidence for the immune function of the NALT and the fact that nasal IgA has been demonstrated to directly mediate local anti-influenza virus immunity in the mouse model (Renegar and Small, 1991a) underlines the potential importance of this lymphoid tissue during respiratory infection. Recently, in this laboratory, we identified the D-NALT as the source of the virus-specific antibody following influenza virus infection (Liang et al., 2001) and showed that twelve-to-eighteen months after a single exposure to influenza virus, the frequency of virus-specific AFCs in the D-NALT is 150 total AFCs per 5 x 10^5 D-NALT cells. The high frequency of these plasma cells suggests that antibody is produced in locally significant amounts, however, when this data is compared and contrasted with our RSV studies, an RSV-specific population of AFC can clearly be seen in the D-NALT, yet at a much reduced frequency (less than 20 total AFC's per $5x10^5$ D-NALT cells). More significantly this response is not maintained, even after two further challenges with live RSV. This observation draws more speculation, especially in light of the polarised reactions for RSV and influenza virus upon subsequent natural challenge – influenza virus eliciting complete and effective sterilising immunity whilst RSV provides little if any protection at all. It also begs the question 'Is it possible that the D-NALT AFC population acts as a crucial immune obstacle to re-infection with airborne viruses?' The obvious response to this hypothesis would be to study animals in which the D-NALT tissue is not present, however at present no experimental mice have been described as having a D-NALT deficient phenotype.

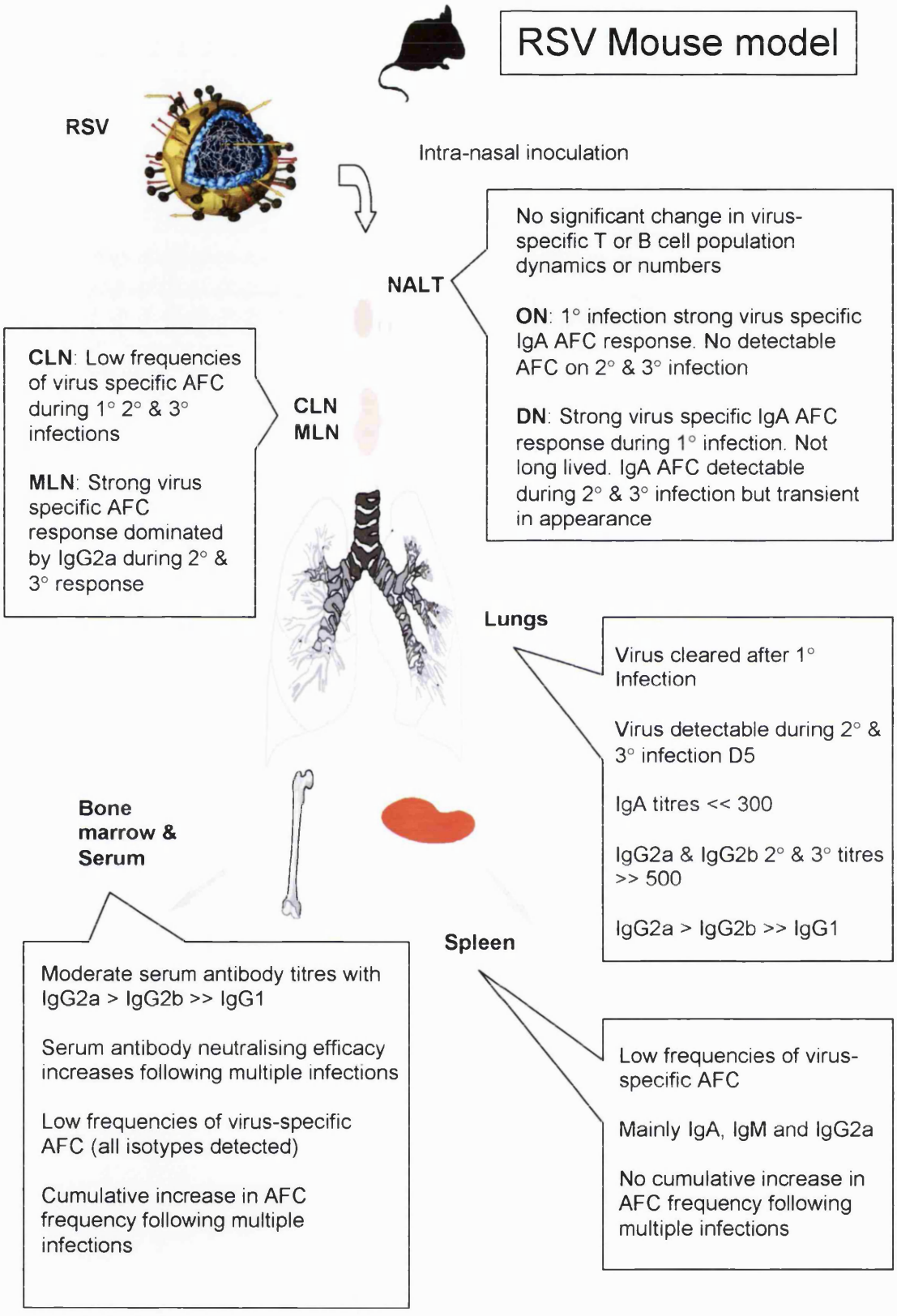


Figure 8.1 Summary of the data presented in this thesis for the murine immune responses to intra-nasal infection with respiratory syncytial virus.

Whilst it is known that many IgG and IgM AFC home to the bone marrow, it has been shown that within mucosal associated lymphoid tissues many activated B cells switch to IgA in preference to IgG and that these IgA AFC's preferentially travel to mucosal sites (Benner et al., 1981; Benner et al., 1977a; Lamm and Phillips-Quagliata, 2002). These early studies also demonstrated that this preferential homing to mucosal sites rather than to bone marrow also takes place with IgA AFC's that have been transferred from another donor (Rudzik et al., 1975). Additionally these studies have provided evidence for the compartmentalisation of the mucosal surfaces (Lamm and Phillips-Quagliata, 2002), in particular, when cells have been transferred from mesenteric lymph nodes, IgA AFC's could be detected within 24h in the gut and several other mucosal tissues, whereas following transfer of bronchial lymph node cells, IgA AFC's were found preferentially in the lung (McDermott and Bienenstock, 1979). It must be stated that this preferential homing of IgA AFC's to mucosal sites is not absolute though as during our previous studies with influenza virus, large numbers of influenza-specific IgA AFC's can also be detected in the bone marrow following infection (Liang et al., 2001).

One potential theory that may explain the lack of RSV-specific IgA AFC in the D-NALT may come from recent work with chemokines. Chemokines and chemokine receptors are chemoattractant proteins that direct the movement of other types of lymphoid cells within lymphoid tissues (Cyster, 1999) and are divided into subfamilies based upon specific sequence motifs defined by cystine residues separated by a number of other amino acids (C, C-C, C-X-C and C-X3-C). Chemokines may interact with specific, shared or promiscuous receptors and each category has multiple members, the exception being the CX3C subgroup which comprises only of fractalkine (Fkn) (Bazan et al., 1997; Hesselgesser and Horuk, 1999; Ward and Westwick, 1998; Wong and Fish, 2003).

Initially CX3CR1 was thought to be a receptor (expressed on many different types of cells) that interacted exclusively with fractalkine (Combadiere et al., 1998; Imai et al., 1997) however it has since been demonstrated that CX3CR1 is also a receptor for RSV. Tripp *et al.* (Tripp et al., 2001) discovered that the conserved CX3C region within the G glycoprotein of RSV shared a high degree of homology at residues aa169-191 with the chemokine domain (aa 33-55) of fractalkine and this newly discovered interaction may serve to illustrate new ways in which RSV may subvert the immune system. Fractalkine is a transmembrane mucin-chemokine hybrid molecule expressed on activated endothelium that has been shown to be involved with the trafficking of monocytes, $CD8^+$ T cells and NK cells (Fong et al., 1998). It has been suggested that during RSV infection the G glycoprotein may mediate chemotaxis and compete with fractalkine for binding to both the CX3CR1 receptor and glycosaminocglycans (GAGs) on cells in order to subvert or alter Fkn-mediated immune responses (Fong et al., 1998; Schall, 1997), the outcome of which may potentially have far-reaching effects on the trafficking of AFC within the mouse model of RSV infection by preventing an appropriate protective immune response from taking place. The lack of RSV-specific IgA AFC within the D-NALT upon secondary and tertiary infection may be due to memory B cells not homing back to the NALT and this could potentially explain the differences observed between RSV and influenza infection. Although the previous hypothesis is only speculation there is some further evidence that this chemokine mimicry by the G glycoprotein may have an effect on B cells as studies with cp52 RSV (a B1 derived mutant lacking the G and SH genes) demonstrate a striking increase in the $B220^+$ bronchio-alveolar lavage (BAL) cell population during infection when compared to the wild-type (Haynes et al., 2003).

In addition it has also been suggested that G glycoprotein mediated subversion or alteration of the Fkn mediated response could slow virus clearance (Tripp et al., 2001) and this could potentially contribute to the continued virus presence in the lungs of RSV infected mice following secondary and tertiary infection. The presence of RSV-specific antibody in the fragment cultures from the lung sections indicated that RSV-specific AFC are indeed present at this location but the low titres and short duration of this response would suggest that local contributions from the lung itself do not play a role in protection from re-infection with RSV. The fact that live RSV can be isolated from the lungs of mice 2-5 days after secondary and tertiary exposure, even in the presence of high titres of RSV-specific serum antibody, merely serves to reinforce the potential importance of the local nasal antibody responses. The problems associated with the lack of a strong local antibody response may therefore be enhanced by the immunomodulatory properties of the G glycoprotein further retarding clearance of the virus at this location. As it is well established that the G protein is unnecessary for RSV infection (Bukreyev et al., 1997; Jin et al., 2000b; Karger et al., 2001; Schlender et al., 2002; Techaarpornkul et al., 2001; Whitehead et al., 1999) and is considered highly likely to be responsible for the immunopathology associated with RSV infection (Johnson et al., 1998; Openshaw, 1995) then it may be necessary to consider future vaccine candidates that lack G or do not retain the chemokine motif, especially in light of the potential effect that the chemokine motif may have on lymphocyte trafficking.

It could also be suggested that if the local antibody responses are deficient there is much more emphasis placed upon the RSV-specific CD8+ T cells in the lung which makes the impairment described by Chang and Braciale (Chang and Braciale, 2002) far more significant. Following primary infection with respiratory viruses, CD8+ T cells are usually responsible for viral clearance but, during subsequent re-infections, it is usually

virus-specific antibody that is responsible for protection. If however, as we have shown in this thesis, there is an impairment of the local antibody responses then the task of protection and clearance would fall to RSV-specific CD8+ T cells; but if what Chang and Braciale reported is true, this would mean that RSV may be able to re-infect with ease by virtue of the suppressed CD8+ T cell activity in the lung, effectively side-stepping both the humoral and cellular arms of the immune system. Clearly this is speculation but, if correct, it could explain how RSV is able to re-infect so consistently, however further study would be required to test this.

The experimental study of RSV is difficult, and whereas there are several more suitable models than the mouse in which to study this virus they are often subject to major complications associated with their usage, such as being prohibitively expensive (bovine), ethically dubious (chimpanzee, human) or suffering from a lack of reagents (cotton rat). Although the mouse model of RSV has been much maligned in recent years with criticisms levelled at it concerning the lack of disease observed during RSV infection and whether or not the mouse is truly susceptible to infection with the virus, we, and others (Graham et al., 1991b) have clearly shown that viral infection and replication does take place. However, in order to reinforce our claims and demonstrate that the data obtained in these studies was due to the immune response to replicating virus, we repeated our live RSV challenge studies using BPL inactivated RSV as the primary immunogen.

We chose BPL because of its proven track record in vaccine development (Barth et al., 1984; Jiang et al., 1986; King, 1991; Parker, 1975; Perrin and Morgeaux, 1995) and primarily because, unlike formalin, it does not modify viral epitopes (Chaplin et al., 1989). With regard to the effect of BPL-RSV in the mouse, the responses observed were

not at all similar to those seen with live RSV as was demonstrated by the background numbers of virus-specific AFCs and low specific serum titres. As expected, this may be attributed to the lack of viral replication which may act as a limiting factor in the scope of the immune systems' response to vaccination. In other words this lack of replication may restrict the amount of time the immune system gets to 'see' the antigen and it may simply be cleared before the full range of antibody generation mechanisms can be fully implemented, effectively demonstrating that replication of RSV does occur in the mouse and that our data has not been obtained as a result of 'residual exposure'.

One of the final studies performed was that of the phenotypic analysis of the T and B-lymphocyte subsets during primary infection with RSV. This was carried out to investigate the size of lymphoid cell populations during infection. Several changes in the cell numbers of the B and T cell subsets were observed during the course of the experiment, however there did not appear to be any significant changes in cell number when compared with mock infected or naïve mice or when using BPL-RSV as an immunogen. Additionally, the phenotypic analysis demonstrated that the size of the T and B cell populations during RSV infection were almost identical to those of the mock controls. As the mock infection consisted only of supernatant from the non-infected cell line used in our laboratory to grow RSV, this suggests that any inflammation taking place may be non-specific as a result of factors in this preparation.

Whilst it is generally accepted that the mouse is not an ideal model for human RSV infection, due to the lack of significant morbidity and mortality upon infection, it is possible that the results from these humoral studies may actually reflect the behaviour of the virus within the human host. The very nature of RSV within the human host and its propensity for not evoking a strong protective response by the immune system

suggests that it may be a reflection of the same degree of unresponsiveness that occurs in the mouse.

As is the case with many studies on RSV we have potentially raised more questions than we have answered. Overall, we have established that the RSV mouse model, whilst not ideal, does provide useful data that can be used to further our knowledge of the immune interactions of virus and host. The NALT antibody response to RSV infection has, to date, not been investigated and may yet prove to be a key site to be considered in the development of vaccines. The observation that the local immune response is clearly lacking, when compared to infections with influenza virus, suggests that it may be more significant than was originally thought. The concept of the D-NALT and the poor frequency of AFC seen within it during RSV infection again may play some role in disease pathogenesis and this, coupled with the ability of re-infection to boost bone marrow AFC responses, would seem to provide at least some evidence that ties in with the epidemiology of RSV infection. The fact that RSV-specific antibody can be detected in the lung goes further to suggest that there may be an immune response to RSV but that it is insufficient, a fact that may be supported by the relatively low (compared with influenza) serum titres and the presence of live virus in the lung up to five days after secondary and tertiary infection. However, the case of RSV infection is a finely balanced one and the FI-RSV incident demonstrates just how easy it is to tip that balance.

Future prospects / Did we achieve the aims?

In this thesis we set out to investigate and characterise the local immune response to RSV in the mouse model. Primarily we wanted to examine the nasal associated lymphoid tissue (NALT) because of its potential significance in the role of immunity to respiratory pathogens but, additionally, we wanted to take a snapshot of the whole immune response (local and systemic) in the mouse as many of the previous studies have just focussed on the systemic aspects of immunity to this disease.

Although we may have raised more questions than answers I believe we have achieved the aims set out for this project. The data we have generated will hopefully play a significant role in our greater understanding of how RSV interacts with the immune system and hopefully it will be a springboard for future work in examining the interaction of the NALT with RSV. The work undertaken in this thesis is by no means complete in terms of the bigger picture and there are many experiments that should be undertaken in the future, particularly with regard to the mode of action of T cells, whether the B cells are acting in a T dependent or independent fashion, the trafficking of the immune cells, the role played by the common mucosal immune system and, of course, further study on the NALT (particularly histology) during infection with RSV.

Publications from this thesis

1. **Singleton, R., Etchart, N., Hou, S. and Hyland, L.** (2003). Inability to evoke a long-lasting protective immune response to respiratory syncytial virus infection in mice correlates with ineffective nasal antibody responses. *J Virol* **77**, 11303-11.

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