Correlates of protection and disease in experimental human respiratory syncytial virus infection

A thesis submitted for the degree of Doctor of Philosophy

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Declaration

I hereby declare that this thesis is my own work and effort and was written by myself. Where other sources of information have been used or collaborations took place, they have been acknowledged.

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without a break. I have no doubt that I would not have been successful in completing the challenge studies without his unique contribution.

I am reliably informed that a PhD begins with a sense of great euphoria which rapidly descends into a sense of overwhelming followed by determined realism and at some point, usually around the 18 month mark, despondency and depression. I can confirm based on personal experience that this is a fair assessment and were it not for the arrival of Christopher (Chris) Chiu and Agnieszka (Aggie) Jozwik, I may have never recovered. Chris provided me with great scientific direction and support, not to mention collaborative work on the B cell experiments. Aggie's tireless dedication in the lab and assistance during the 2012 summer challenges enabled a great deal more to be accomplished. Throughout my PhD studies, my supervisor Peter Openshaw has supported and guided me, even when I have seemingly gone off and pursued an entirely different scientific agenda from what was originally intended. I am forever grateful for him giving me my first opportunity to do scientific research, and the Wellcome Trust for providing the funding for my project.

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Maximillian Shahin Habibi, 1st October 2014

For my wife Angela, love, light, and fire of my life.

Abstract

Understanding of respiratory syncytial virus (RSV) disease and vaccine development has been hampered by an incomplete understanding of the immune mechanisms of protection and disease in humans. Reinfection with antigenically similar strains of RSV occurs throughout life, and observational studies of natural infection do not permit analysis of events in the pre-symptomatic phase of disease. Experimental infection of healthy adults with a standardised inoculum was therefore used to study RSV upper respiratory tract (URT) disease. Pre-existing humoral immunity was assessed locally and systemically, and dynamics of URT symptoms, viral load and nasal mediators were analysed to identify factors involved in symptomatic infection.

Forty subjects were enrolled of whom 24 developed infection and 17 exhibited typical cold-like features. Peak symptoms and viral load occurred on days 7 and 8 respectively. Pre-inoculation RSV-specific nasal IgA was most clearly associated with resistance to infection, but infection-boosted levels waned after 6 months. The frequency of acute IgG and IgA antibody secreting cells increased after infection and virus-specific IgG^+ memory B cells (MBC) incremented around 4-fold. By contrast, IgA^+ MBC were undetectable pre- and post- infection whereas influenza-specific IgG^+ and IgA^+ cells were found at comparable frequencies in convalescent blood from patients recovered from H1N1 influenza, suggesting a specific defect in IgA memory after RSV infection. Nasal viral load correlated with levels of nasal IFN-γ, CXCL10, MIG, TNF and IL-6. Subjects exhibiting early pre-symptomatic IFN-α responses showed reduced symptom scores and lower overall viral loads.

Lifelong susceptibility to RSV infection thus reflects a specific defect in IgA memory to RSV, the cause of which needs to be determined. Symptomatic disease results from a failure of innate immune control of RSV replication in the pre-symptomatic stages of infection during the first 48 hrs of viral encounter. Viral replication leads to epithelial injury and inflammation reflected by a number of inflammatory mediators in nasal secretions. Mucosally-delivered vaccines could overcome the IgA memory defect and induce durable protection, which might be enhanced by inducing local IFN-α immediately after viral exposure.

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List of Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ADE	Antibody-dependent enhancement
AEC	3-Amino-9-ethylacarbazole
APRIL	A proliferation-inducing ligand (TNFSF13)
ASC	Antibody-secreting cells
AUC	Area under curve
BAFF	B cell activating factor (TNFSF13B)
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
cDNA	Complementary DNA
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CSR	Class-switch recombination
CX3C	C-X-3C chemokine (fractalkine)
CXCR4	C-X-C chemokine receptor 4
D10	DMEM + 10% FCS v/v
DAB	3,3'-Diaminobenzidine
DMEM	Dulbecco's modified Eagle medium with high glucose and added pyruvate
DMEM-LP	Dulbecco's modified Eagle medium with low pyruvate
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELF	Epithelial lining fluid
ELISA	Enzyme-linked immunosorbant assay
ELISpot	Enzyme-linked immunospot
FAM	6-Carboxyfluorescein
FCS	Fetal calf serum
FFP	Fit-factor protection
GC	Germinal centre
GMP	Good manufacturing practice

hMPV	Human metapneumovirus
HRP	Horseradish peroxidase
ICHNT	Imperial College Healthcare NHS Trust
ICRRU	Imperial Clinical Respiratory Research Unity
IFN	Interferon
Ig	Immunoglobulin
Ig	Immunoglobulin
IL	Interleukin
IP-10	Interferon-gamma-inducible protein-10 (CXCL-10)
kDa	Kilodaltons
LLPC	Long-lived plasma cell
LRTI	Lower respiratory tract infection
MALT	Mucosa-associated lymphoid tissue
MBC	Memory B cell
MHC	Major histocompatibility complex
MIG	Monokine induced by gamma interferon (CXCL-11)
mRNA	Messenger RNA
MSD	Meso Scale Discovery
MV	Measles virus
nAb	Neutralising antibody
NALT	Nasal-associated lymphoid tissue
NIAID	National Institutes of Health
NIH	National Institute of Allergy and Infectious Diseases
NK	Natural killer (cell)
NOD	Nucleotide-binding oligomerisation domain
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PBS-T	PBS + 0.05% Tween20 v/v
PCR	Polymerase chain reaction
PFU	Plaque-forming units
PPE	Personal protective equipment

PRR	Pattern recognition receptor
qPCR	Semi-quantitative (real-time) PCR
R10	RPMI + 10% FCS v/v
RIG-I	Retinoic acid inducible gene I
RLH	RIG-I-like helicase
rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute medium
RSV	Respiratory syncytial virus
RTI	Respiratory tract infection
SDF-1	Stromal-cell-derived factor 1
sIgA	Secretory IgA
SNP	Single nucleotide polymorphism
TAMRA	Tetramethylrhodamine
TD	T-dependent
Tfh	Follicular T-helper
TGF-β	Transforming growth factor-β
Th1	T-helper 1
Th17	T-helper 17
Th2	T-helper 2
TI	T-independent
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNFSF	Tumour necrosis factor superfamily
Treg	Regulatory T cell
URTI	Upper respiratory tract infection
rVV	Recombinant Vaccinia virus

1 Introduction

1.1 Immunity to infection

The concept of immunity to infectious disease has been a source of curiosity for centuries. In 430 BC whilst writing on the Plague of Athens, the historian Thucydides noted that,

> *"... more often the sick and the dying were tended by the pitying care of those who had recovered, because they knew the course of the disease and were themselves free from apprehension. For no one was attacked a second time, or not with a fatal result."*
>
> (Jowet 1900, page 138 (translated))

It was not until the 18th century AD however, that Edward Jenner, following his observations that milkmaids did not succumb to smallpox, was able to translate this concept to a reality in the form of vaccination. Since that time, our knowledge of microbe theory and the science of immunology have led to numerous successful vaccines.

1.1.1 Aims of vaccination

The core aim of any vaccine is to induce protection from disease caused by the specific pathogen in question. This protection can be thought of as a specific immune response to defend against the pathogen. In the case of active immunisation, such a response must ideally be long-lasting, if not lifelong (Beverley 2002). Whilst a robust immune response might be most effective for pathogen clearance, there will be a proportionate cost in terms of host tissue damage. This is especially the case for viruses, which replicate inside host cells that must be destroyed to eliminate the infection. Thus an additional aim of vaccination must be to induce protection that is sufficient but not so vigorous as to lead to further pathology. Infectious disease is the leading global cause of mortality in children under five years (Liu et al. 2012), hence those most in need of vaccination are young children and infants, whose immunity may be immature therefore unable to mount a response to the full range of protective antigens. Thus a final aim for vaccination must be to protect those most at risk from disease.

1.1.2 Correlates of protection from infectious disease

Whilst a full understanding of the mechanisms of immune protection would likely enhance the development of any vaccine, at the time of writing, the majority of successful vaccines have been developed empirically (Pulendran and Ahmed 2011). In this case, a measurable correlation of protection from disease is crucial to predict vaccine efficacy. For most currently available vaccines this correlate is serum antibodies (Plotkin 2001; Plotkin 2008). The protective capacity of antibodies is demonstrated by the observations that passive transfer of immunoglobulin can protect against numerous infections (Zeitlin et al. 2000), and the protection of infants by transplacentally-acquired maternal IgG. However, the majority of vaccine-preventable infections induce disease via invasion or release of toxin by the pathogen into the bloodstream, affording antibodies in the serum an opportunity to act. For intracellular bacteria and viruses, appropriate cell-mediated immunity is also likely to be critical for protection and recovery from disease.

1.1.3 Longevity and durability of humoral responses

In the apparent absence of reinfection, antibody levels in serum can be shown to have half-lives of many years, far exceeding the half-life of individual immunoglobulins (Amanna, Carlson, and Slifka 2007). Thus they must be continually produced by plasma cells, the fully-differentiated antibody secreting cells of the B cell lineage. These highly activated cells typically undergo apoptosis very readily, thus for serum antibodies to be maintained these cells must be continually replenished from a pool of precursors or must be able to acquire the ability to survive for many years. Three explanatory hypotheses have been proposed: 1) plasma cells are maintained by continual polyclonal stimulation and differentiation of circulating naïve and or memory B cells (referred to as bystander stimulation, Bernasconi, Traggiai, and Lanzavecchia 2002), 2) B cells are continually activated and plasma cells produced in germinal centres by persisting antigen in the form of immune complexes captured on follicular dendritic cells (referred to as antigen persistence) (Zinkernagel and Hengartner 2006), or 3) plasma cells acquire the ability to migrate to and survive in immunological niches such as the bone marrow (referred to as long-lived plasma cells, Slifka et al. 1998; Slifka,

Matloubian, and Ahmed 1995). Limited evidence exists to support all of these to varying degrees and it may be that elements of each are relevant in different situations.

1.2 Respiratory syncytial virus

Respiratory syncytial virus (RSV) was first isolated from a chimpanzee with a coryzal illness in 1956 (Blount, Morris, and Savage 1956). Initially named chimpanzee-coryzal agent, subsequent research revealed this virus to be the same as that isolated from children with severe acute respiratory tract infection (RTI) (Chanock and Finberg 1957). Renamed for its cytopathic effect in tissue culture, epidemiological study revealed RSV to be an important cause of respiratory tract infection in children and the commonest aetiological agent of bronchiolitis (Chanock et al. 1961).

1.2.1 Virology

Human RSV is an enveloped, nonsegmented negative-strand RNA virus, of the order *Mononegavirales*, family *Paramyxoviridae*. Within this family are two subfamilies; *Paramyxoviriniae*, which includes measles, mumps, and parainfluenza viruses, and *Pneumovirinae*, which includes human RSV (of genus *Pneumovirus*) and the related human metapneumovirus (hMPV, of genus *Metapneumovirus*). The *Pneumovirus* genus also includes bovine RSV and Murine pneumonia virus (formerly known as Pneumonia virus of mice, PVM). Of interest, Ebola virus (EBOV), the cause of a current major outbreak of severe disease in West Africa, is also of the order *Mononegavirales*, family *Filoviridae,* which is most closely related to *Paramyxoviridae* family. RSV and EBOV thus share similar genomic organisation, replication strategy, and nucleocapsid structure yet cause profoundly different disease (Pringle 1991).

Figure 1.1 shows the gene maps of human RSV and hMPV and sequence-relatedness of the two genera based on the nucleoprotein (N) gene. Human RSV has a single serotype with two antigenic subgroups A and B. Virion particles appear irregular and spherical 100 – 350 nm in diameter on electron microscopy (see Figure 1.2). Filamentous forms up to 10 μm in length exist and may predominate (Jeffree et al. 2003). The nucleocapsid exhibits helical symmetry. Table 1.1 lists the

proteins of the *Pneumovirinae*. The envelope contains three transmembrane virally encoded surface glycoproteins: G (major attachment protein), F (fusion protein), and SH (small hydrophobic protein). The matrix (M) protein forms a layer on the inner side of the envelope. The viral RNA is associated with four proteins: N (nucleoprotein), P (phosphoprotein, polymerase co-factor), M2-1 (transcription processivity factor), and L (large polymerase).

Name	Size (kDa)	Function
Nonstructural-1 (NS1)	13.8	Non-structural proteins; interfere with type I interferon induction and signalling
Nonstructural-2 (NS2)	14.5	
Nucleoprotein (N)	45	Major nucleocapsid protein
Phosphoprotein (P)	33	Polymerase cofactor; nucleocapsid protein
Matrix (M)	25	Inner surface of envelope; important in virion morphogenesis
Small hydrophobic (SH)	7.5–60	Transmembrane envelope surface protein; exists as oligomers with porin-like structure; function unknown
Glycoprotein (G)	90	Major attachment protein and protective antigen; transmembrane envelope surface protein; extensively glycosylated and highly variable but contains conserved central domain
Fusion (F)	70	Fusion protein and protective antigen; transmembrane envelope surface protein; mediates fusion in penetration and syncytium formation; highly conserved among strains and between subtypes A and B
M2 ORF 1 protein (M2-1)	22	Transcription elongation factor; enhances transcription of viral RNA; nucleocapsid protein
M2 ORF2 protein (M2-2)	11	Shifts balance of RNA synthesis from transcription to replication of viral genome
Large (L)	250	Large polymerase; nucleocapsid protein

Table 1.1 Proteins of the *Pneumovirinae* in genomic order. Approximate size in kDa as determined by polyacrylamide electrophoresis under reducing and denaturing conditions (Collins and Crowe 2007).

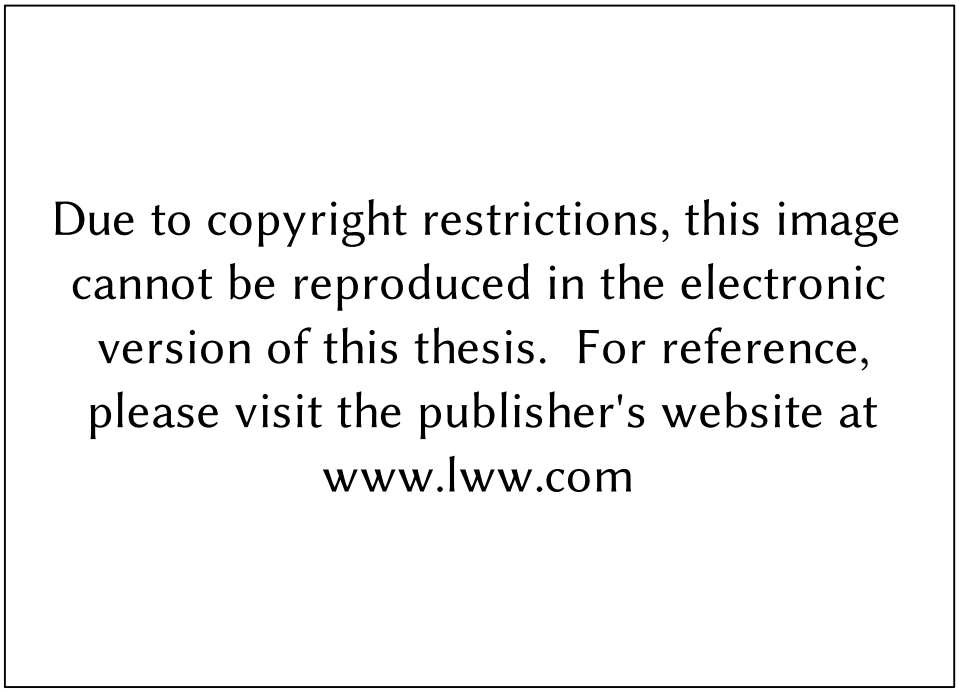


Figure 1.1 Gene maps and encoded proteins (boxes) of human RSV and hMPV with proteins of similar function aligned. Known major protective antigens indicated by grey boxes (top). Dendrogram illustrating relatedness among members of *Pneumovirus* and *Metapneumovirus* genera based on sequence of nucleoprotein (N) gene. Scale bar indicates 5% nucleotide difference between pairs (bottom). BRSV: bovine RSV, AMPV: avian metapneumovirus, SV5: simian virus 5, NDV: Newcastle disease virus. Figures taken with permission from Fields Virology, 5th Edition, Copyright Lippincott Williams and Wilkins 2007 (www.lww.com).

The transmembrane fusion protein, as its name suggests, directs membrane fusion of virion envelope and host cell during viral invasion and between adjacent host cells in syncytia formation (see Figure 1.2). The F protein is essential for viral replication and this likely explains why it is also the most conserved of the RSV encoded proteins between strains and subtypes. By contrast, the other surface proteins G and SH, are dispensable for infection *in vitro*, with the majority of the antigenic variation between strains concentrated in the highly glycosylated G protein (Techaarpornkul, Barretto, and Peeples 2001; Melero et al. 1997). The degree and nature of glycosylation depends on the host cell. Approximately 20% of the total G protein expressed in RSV-infected cells *in vitro* is expressed as a secreted form that lacks the N-terminal 65 amino acids (Hendricks, McIntosh, and Patterson 1988), however due to rapid secretion accounts for up to 80% of G protein in cell culture. The secreted form potentially contributes to inflammation by interaction with chemokines (see below) and may absorb anti-G-protein antibody shielding the virion from

immune recognition. The central domain of G includes a totally conserved 13 amino acid region which was thought to function in viral attachment, however subsequent studies showed that deletion of this domain did not cause significant reduction in replication in HEp-2 cells or mice (Teng and Collins 2002). The central domain also includes a region with sequence homology with the CX3C chemokine fractalkine, and peptides containing this sequence demonstrated leucocyte chemotactic activity *in vitro* (Tripp et al. 2001). This domain has also been demonstrated to modulate innate immunity by inhibiting the transcription factor nuclear factor (NF) -κB (Polack et al. 2005). As stated, G protein can be deleted (ΔG) without affecting replication *in vitro*, however this is cell line specific. ΔG strains produced in Vero cells replicate poorly in HEp-2 cells and are highly attenuated in humans (Karron et al. 1997a). Together, this suggests a co-ordinated role for F and G proteins in attachment to human cells, and an important role for G protein in modulation of host immunity to facilitate infection.

The small hydrophobic (SH) protein is an integral membrane protein of 64 amino acids that exists as oligomers (Collins and Mottet 1993) and might be a channel-forming porin-like protein, however the relevance of this in RSV replication is unclear. SH-deletion (ΔSH) mutants are fully-viable in tissue culture, moderately attenuated in mice (Bukreyev et al. 1997) and chimpanzees (Karron et al. 1997a), and deletion of SH did not increase attenuation of a vaccine candidate suggesting SH is not required for efficient replication in humans (Karron et al. 2005). The matrix (M) protein is a 256 amino acid non-glycosylated internal component of the virion that interacts with F protein during virus particle morphogenesis (Teng and Collins 1998).

The four RNA-associated proteins (N, P, L, and M2-1) are all found in the nucleocapsid. RSV nucleoprotein (N) binds tightly to both genomic RNA and the antisense replicative RNA intermediate in the nucleocapsid. Phosphoprotein (P), together with the large polyermase (L), form a complex with N protein that has transcriptase activity (Collins et al. 1996), however in the absence of M2-1 protein, transcription terminates prematurely and viral genes are not significantly expressed. Thus all 4 nucleocapsid proteins are required for RNA transcription. The M2 gene

contains a second open reading frame that codes for a 90 amino acid protein (M2-2) whose function appears to be to promote genomic RNA replication over transcription to mRNA (Bermingham and Collins 1999). Deletion of M2-2 results in attenuation in rodents and chimpanzees (Teng et al. 2000).

The non-structural proteins NS1 and NS2 are not found in significant quantity in the virion but rapidly and abundantly synthesised following host cell invasion. Both inhibit induction of type I interferon via inhibition of interferon regulatory factor 3 (Spann et al. 2004; Spann, Tran, and Collins 2005), although the effect is primarily seen with NS1 (discussed further in 1.2.5.2.4). By contrast, Ns2 is able to interfere with signalling and amplification of the interferon response via the interferon α/β receptor and JAK/STAT pathway (Ramaswamy et al. 2004). Strains lacking either of these two genes (ΔNS1, ΔNS2) grow slowly in cell lines competent for type I interferon production and are attenuated in chimpanzees. However, growth of ΔNS1 and ΔNS2 mutants in Vero cells, which lack the genes for interferon– α and –β, is reduced suggesting an additional function beyond suppression of interferon.

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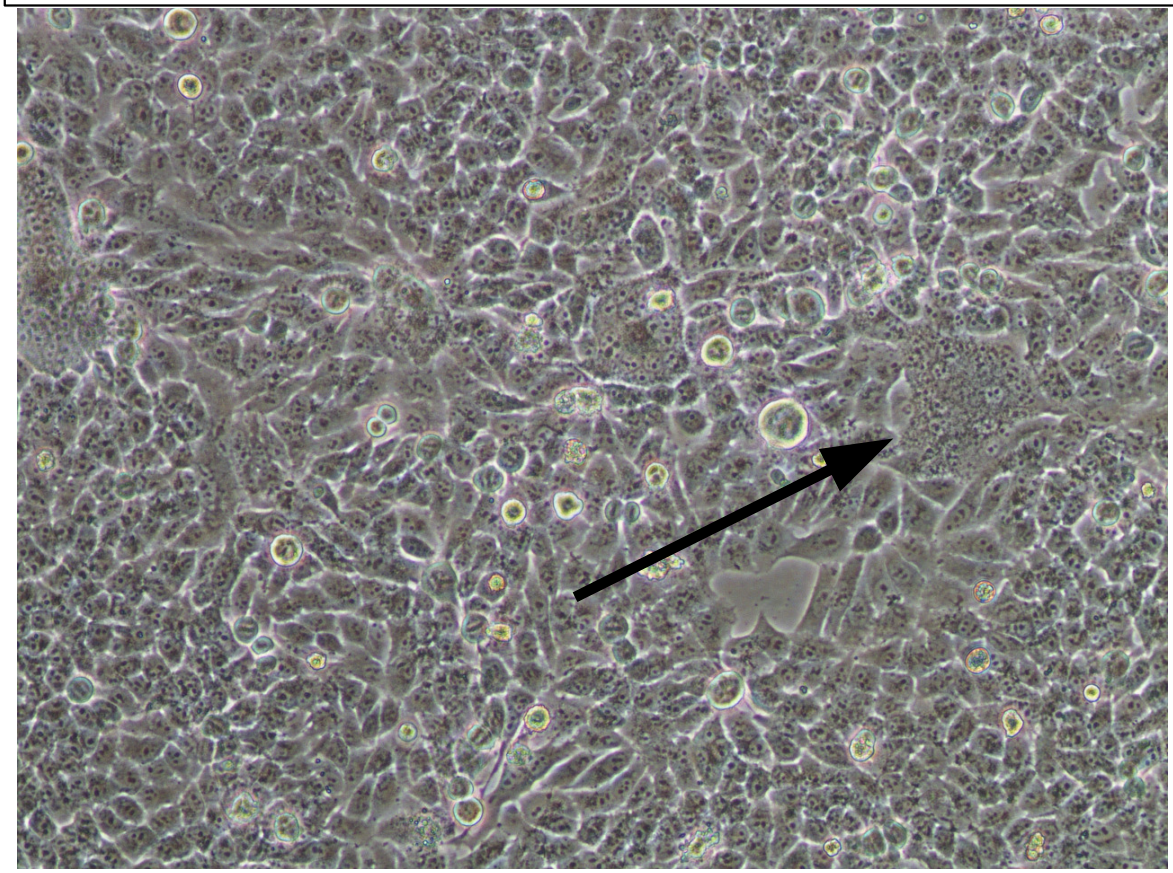


Figure 1.2 Scanning electron micrograph (top) showing mock (a) and RSV infected cells (b). Note irregular shape and presence of filamentous forms (VF). Magnification 30,000x, mv = microvilli. Reprinted from Virology, 306(2), Jeffree *et al.*, Distribution of the attachment (G) glycoprotein and GM1 within the envelope of mature respiratory syncytial virus filaments revealed using field emission scanning electron microscopy, Page 257, Copyright 2003, with permission from Elsevier. HEp-2 cells 48 hours after infection with RSV (bottom). Characteristic syncytia (fused, multinucleated cells) are seen (arrow). Author's own image. 10X magnification.

1.2.2 Epidemiology

RSV is the main aetiological agent of bronchiolitis, a severe inflammation of the lower respiratory tract. Bronchiolitis is the single most important cause of severe acute RTI in infants occurring in large epidemics during the winter in temperate regions of the world. The seasonal pattern in other

regions is less well studied, however a recent study found more heterogeneous patterns of monthly incidence in tropical regions (Bloom-Feshbach et al. 2013 and Figure 1.3). Nair *et al* published a comprehensive analysis of the global burden of RSV disease in 2010 (Nair et al. 2010). In this study, the annual global incidence in children < 1 year was estimated to be 48.5 per 1000, however the estimated incidence in less developed countries (59.1 per 1000) was more than twice as high as that in industrialised countries (24 per 1000). Similarly, the case fatality ratio of severe RSV-associated RTI was 0.3% in industrialised countries compared with 2.1% in less developed countries. The authors estimated that nearly 34 million episodes in children <5 years occurred in 2005, compared with nearly 14 million episodes of pneumococcal pneumonia in the same age group, thus RSV infection accounted for approximately 22% of all acute lower RTI, resulted in 3.4 million hospital admissions, and between 66,000 and 199,000 deaths. The burden of morbidity and mortality in the elderly is increasingly being recognised with one study implicating RSV infection in hospitalisation for 10.6% of pneumonia, 11.4% of chronic obstructive pulmonary disease (COPD) and 5.4% of congestive cardiac failure (Falsey et al. 2005).

Nearly all children are infected with RSV during the first two years of life (Glezen et al. 1986), with older siblings being a frequent source of virus infecting infants sharing the same household (Hall et al. 1976; Munywoki et al. 2013). Most children infected with RSV usually experience a self-limiting febrile upper RTI. However, some children, especially those under 12 months of age, develop bronchiolitis, which may be followed by recurrent childhood wheeze (often diagnosed as asthma).

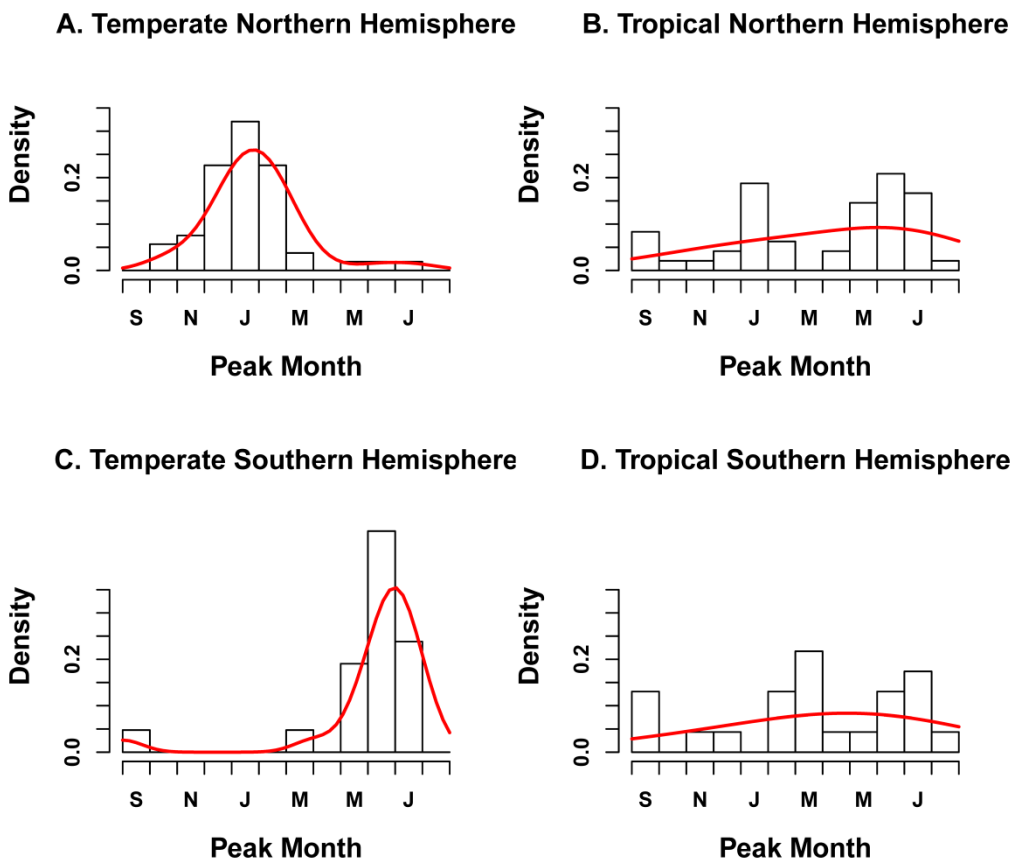


Figure 1.3 Peak month of RSV detection by geographical region. Figure taken from Bloom-Feshbach *et al.* Latitudinal Variations in Seasonal Activity of Influenza and Respiratory Syncytial Virus (RSV): A Global Comparative Review. PLoS One 8:e54445, doi:10.1371 /journal.pone.0054445.g005.

There is no known animal reservoir for human RSV, although non-human primates and various small mammals can be infected experimentally. The route of infection is via the nose or conjunctiva; the mouth is not a recognise portal of entry (Hall et al. 1981). Three methods of spread are possible: 1) large particles (> 10 µm in size) or droplets, 2) self-inoculation of the nose or eyes after touching contaminate surfaces (fomites), and 3) small particle (< 10 µm in size) aerosols. Nosocomial spread of RSV is well-documented (Ditchburn et al. 1971; Hall et al. 1978) and studies have sought to determine the mechanism of spread and effectiveness of control measures. In an experimental study of transmission (Hall and Douglas 1981), young adult volunteers were divided into three groups and deliberately exposed to RSV-infected infants in three different ways: 1) 'cuddlers', who cared for the infant over 2-4 hours in the usual manner (e.g. feeding, nappy changing, and playing), 2) 'touchers', who touched potentially contaminated surfaces with their

hands, whilst the infant was not in the room, and then gently rubbed their eyes and noses, and 3) 'sitters', who did not touch the infant or any surfaces, but read a book at a distance of more than six feet away from the infant for three hours. The cuddlers wore gowns, but no gloves or masks, the sitters wore gloves and gowns, but no masks. Cuddlers could be exposed by any of the three routes, touchers only by fomites, and sitters only by small particle aerosols, which are able to remain airborne and travel distances over six feet as opposed to large droplets, which are not (Wells 1934; Duguid 1946). All volunteers underwent daily nasal washes and had baseline and 4-week serology to determine RSV infection by viral isolation (culture in HEp-2 cells) from nasal wash or seroconversion respectively. Five out of seven cuddlers and three out of ten touchers developed a symptomatic upper respiratory infection, confirmed as RSV by viral culture; none of fourteen sitters developed symptoms or had an asymptomatic infection as determined by culture or serology. One toucher had an asymptomatic infection.

Some controversy exists over whether RSV is transmitted by small droplet (i.e. < 10 μm) aerosols. Older reports suggested that RSV survival was impaired in artificially-generated aerosols (Rechsteiner and Winkler 1969). Two more recent published studies have examined room air collected by specially-designed air samplers (Lindsley, Schmechel, and Chen 2006) where RSV infected children have been nursed; RSV RNA was detectable in both studies, supporting the possibility that transmission could occur under some circumstances (Lindsley et al. 2010; Aintablian, Walpita, and Sawyer 1998), however live RSV (i.e. virus that can be cultured) has not been documented. The proportion of samples with detectable RSV RNA decreased considerably as the distance of the air sampler from the patient reached > 1 m, and RSV RNA was found in the larger (> 4 μm) aerosol particle sizes. These observations suggest that RSV RNA, but not necessarily infectious virus, may be present in aerosols in the vicinity of children with RSV infection, however the proportion of RSV RNA decreases with increasing distance and decreasing particle size. Transmission of RSV by aerosol was not demonstrated.

Taken together, this evidence would support that RSV is transmitted by large droplets and fomites; spread thus requires close contact with infected persons or contact between contaminated hands to nasal or conjunctival mucosa. In terms of survival of secretions in the environment, there is evidence that hard surfaces (e.g. counter tops) provide the longest survival times; survival on skin being considerably lower (Hall, Douglas, and Geiman 1980). Prevention of transmission in the hospital environment is obviously imperative, and based on knowledge of transmission and interventional studies, cohorting (not necessarily single-room isolation, which is rarely possible during RSV season due to numbers of admissions) of infected infants and strict hand washing appear to be the most effective measures (Hall et al. 1978; Isaacs et al. 1991). Regular decontamination of hard surfaces would also reduce transmission, based on this evidence.

1.2.3 Clinical disease

The typical incubation period for an RSV RTI is 3-5 days (DeVincenzo et al. 2010c). Primary RSV infection in infants most commonly presents as a winter upper RTI, frequently associated with otitis media. Viral shedding begins up to the day before and continues for an average of 7 days after the onset of symptoms (DeVincenzo et al. 2010c). Lower respiratory tract symptoms follow in about 40% of cases and may include tachypnoea, hyperinflation, recession, crackles, and expiratory wheezing, characteristic of a clinical diagnosis of bronchiolitis (Openshaw and Tregoning 2005). Microscopically, infantile bronchiolitis is characterised by intense inflammation involving the small airways. Bronchoalveolar lavage (BAL) reveal neutrophils to be the predominant leucocyte (Smith et al. 2001; McNamara et al. 2003). There is increased mucus production and sloughing of epithelial cells, leading to airway obstruction, collapse, hyperinflation, and the typical clinical signs. In severe cases, hypoxia and respiratory failure ensues, which can require assisted ventilation or result in death. Infections usually resolve in a week however, and tend to be more severe in children aged 8 to 30 weeks. Hospitalisation rates vary; frequency in children born at term aged less than 12 months with no underlying medical conditions in Europe and the US are typically 30 per 1000 children per year; rates in premature neonates and those with bronchopulmonary dysplasia are considerably higher (Simoes and Carbonell-Estrany 2003). Length of stay varies by country with

durations of 4 days typical in the UK and the US with up to 9 days in other parts of Europe (Behrendt et al. 1998). Among hospitalised children, mortality in developed countries is very low with rates of 0.3% reported (Behrendt et al. 1998; Nair et al. 2010). Risk factors for death are low birthweight, increasing birth order, low Apgar score at 5 minutes, young maternal age, unmarried mother, and tobacco use during pregnancy (Holman et al. 2003).

Whilst notable for its role in severe bronchiolitis in infants, humans remain susceptible to RSV throughout life. RSV is associated with acute exacerbations of both asthma and COPD, and has been implicated in adult hospitalisation for pneumonia and exacerbations of congestive cardiac failure (Guilbert and Denlinger 2010; Falsey et al. 2006; Falsey et al. 2005). Immunosuppressed persons, especially those receiving allogeneic stem-cell transplants, may experience severe disease, and RSV infection may have a role in graft rejection (Avetisyan et al. 2009; McCarthy et al. 1999). Healthy, working-age adults experience a mild upper RTI typically resulting in nasal congestion, fever, sore throat, and non-productive cough. Whilst it is difficult to get an accurate estimate of the incidence of RSV infection in this group due to lack of available sensitive diagnostic tests and less frequent contact with healthcare providers, one study has suggested a prevalence of 7% (Hall, Long, and Schnabel 2001). Illness is not distinguishable from other viral RTI on clinical grounds, although RSV RTI has been reported to be prolonged, with cough and wheeze reported more commonly, compared with non-RSV RTI (Hall, Long, and Schnabel 2001; O'Shea et al. 2005).

1.2.4 RSV-induced wheezing and relationship to subsequent asthma

As stated, RSV bronchiolitis frequently manifests with wheezing and there exists an association between infant bronchiolitis and asthma diagnosis in later childhood (Sigurs et al. 2000). The reasons for this association are unknown, but a non-randomised trial suggested that prevention of RSV infection by passive antibody therapy reduces the risk of long-term respiratory morbidity (Simoes et al. 2007). Observational studies however cannot determine whether RSV infection is the cause of wheeze or simply an indicator of a pre-existing disposition to wheeze (Stensballe et al. 2009). In a recent study involving 429 healthy pre-term infants, the efficacy of palivizumab to

prevent wheezing episodes was evaluated in a double-blind randomised controlled trial (Blanken et al. 2013). Infants receiving palivizumab had a relative reduction in wheezing days during the first year of life of 61% (absolute reduction 2.7%). The benefit was associated with prevention of RSV infection regardless of family history of atopy, however the ability of palivizumab to prevent later asthma was not investigated and as such a causal link between RSV infection and development of asthma remains controversial.

1.2.5 Immunity to RSV

1.2.5.1 Background

The pathological appearances of severe RSV bronchiolitis are mucus production and leucocyte infiltration of the airways; see Figure 1.4 and (Johnson et al. 2007). Whilst mucus is an important barrier defence, overproduction can impair gas exchange and cause obstruction, especially in the small airways of infants. Likewise, whilst a robust immune cell response may be efficient for viral clearance, an inappropriate cellular response can lead to collateral tissue damage and worse pathology. RSV-induced immunopathology has been extensively studied in mice (Cannon, Openshaw, and Askonas 1988; Graham et al. 1988; Graham et al. 1991; Openshaw and Tregoning 2005; Openshaw 2013). Whilst the mouse is a well-characterised and convenient model offering unparalleled possibilities for host and virus manipulation, it is not a natural host for RSV. Inbred laboratory-reared mice are profoundly different from genetically diverse human beings living in heterogeneous environments combined with confounding comorbidities. Furthermore, not all features of human RSV disease are readily reproduced in the mouse, thus observations from mice infected with human RSV must not be over-interpreted.

1.2.5.2 Innate immunity

1.2.5.2.1 Epithelial cells

The conducting airway epithelium is comprised of ciliated cells and secretory goblet cells that provide mucocilliary clearance. Not only is the airway epithelium a physical barrier, as RSV specifically targets epithelial cells it also is the first point of pathogen recognition and initiation of

the immune response via chemokines and cytokines that can recruit specific leucocyte populations. As stated in section 1.2.1, the exact mechanism of host-cell invasion is unclear, however RSV interacts with many host cell proteins, contributing to early inflammation. RSV G, the major attachment protein, has structural homology with CX3C chemokine (fractalkine), and can mediate chemotaxis of cells expressing receptors for this chemokine (Tripp et al. 2001). Polymorphisms of this receptor have been associated with more severe disease (Amanatidou et al. 2006). Tumour necrosis factor (TNF)-α and interleukin (IL)-6, both potent pro-inflammatory cytokines, are produced by alveolar macrophages and airway epithelial cells (AECs) following RSV infection; high levels have been found in the lower airways of infants admitted with bronchiolitis (McNamara et al. 2004).

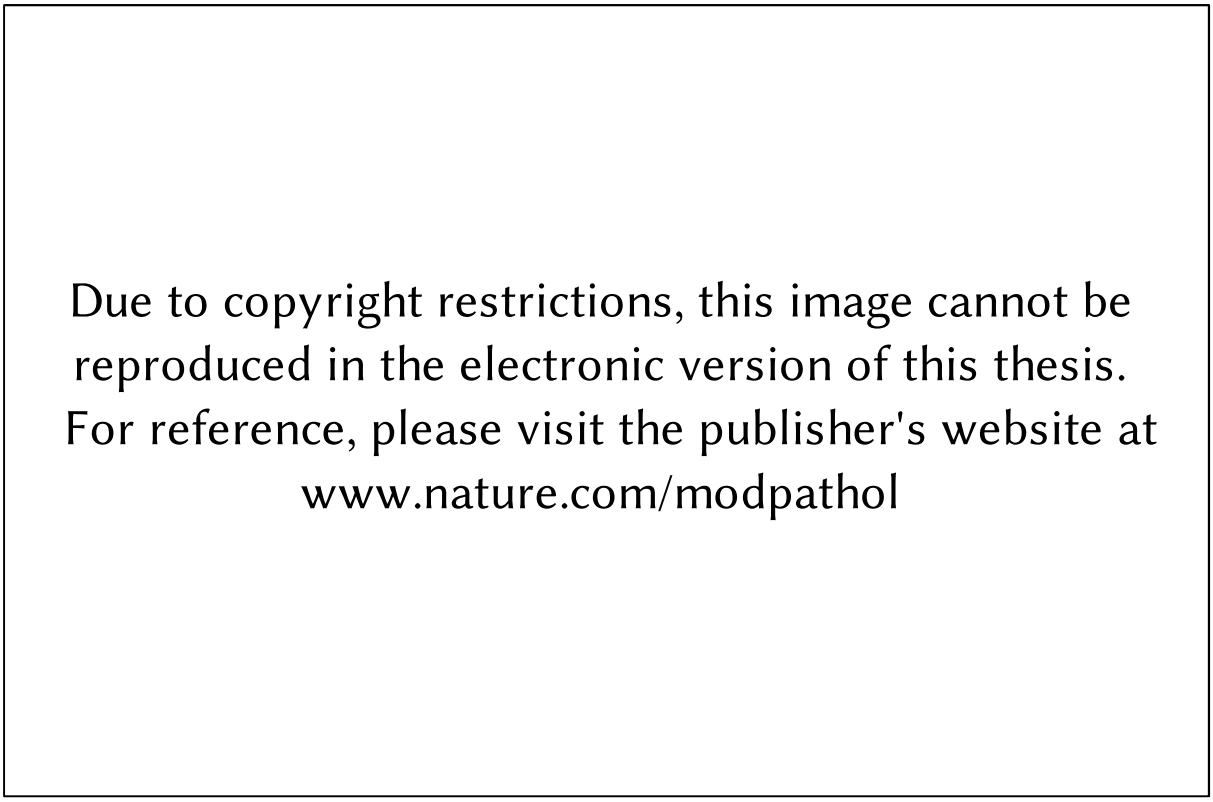


Figure 1.4 Immunohistochemical staining identifying RSV antigen (stained brown) in ciliated airway epithelial cells. (a) Bronchiolar epithelium showing circumferential infection at the epithelium surface. (b) Bronchiolar epithelium showing infection of ciliated cells (arrow). (c) Intraluminal debris staining positive for RSV, a = arteriole. (d) Syncytia (arrow) with RSV antigen in the membrane. Magnification: a = × 25, b-d = × 250. Reprinted with permission from Macmillian Publishers Ltd. Johnson *et al.* 2007. Modern Pathology 20:108-119. Copyright 2007 Nature Publishing Group.

Neutrophils are the predominant leucocyte found in the bronchoalveolar lavage (BAL) and infiltrate the small airways in severe bronchiolitis. Their recruitment depends on chemokines including CXCL8 (IL-8) and produced by AECs and macrophages in response to RSV; levels of this chemokine correlate with disease severity (McNamara et al. 2005). Other chemokines including CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), and CXCL10 (IP-10) are also produced by RSV-infected epithelial cells and have been correlated with disease severity in human infants (Miller, Bowlin, and Lukacs 2004; McNamara et al. 2005). A small study reported by Noah *et al* (Noah and Becker 2000) found elevated levels of MIP-1α, RANTES, and MCP-1 in nasal lavage during the period of viral shedding from human adults experimentally infected with RSV. The chemokine milieu can influence the type of adaptive response by recruiting different T-helper (Th) cell subsets via specific chemokine receptors (Rossi and Zlotnik 2000). BALB/c mice primed with a recombinant Vaccinia virus expressing RSV-G (rVV-G) develop Th2-biased responses accompanied by increased MCP-1, IP-10,

and CCL11 (eotaxin) and pulmonary eosinophilia in BAL (Openshaw, Clarke, and Record 1992; Culley et al. 2006). However it is not clear whether susceptibility to severe RSV disease in humans depends on different chemokine profiles.

1.2.5.2.2 Toll-like receptors

Toll-like receptors (TLR) are a family of pattern recognition receptors (PRR) that are able to recognise evolutionarily conserved structures of pathogens such as bacterial peptides, viral nucleic acids, and fungal glucans. TLR4 is a cell-surface receptor that classically senses bacterial lipopolysaccharide (LPS), however it can also bind structurally-unrelated RSV F protein using CD14 as a co-receptor (Kurt-Jones et al. 2000). TLR4 is activated early in RSV-infected in mice (Kurt-Jones et al. 2000), and peripheral blood mononuclear cells (PBMC) from infants with bronchiolitis upregulate TLR4 expression (Gagro et al. 2004). Production of pro-inflammatory cytokines following RSV infection of bronchial epithelial cells can be inhibited by disruption of RSV-TLR4/CD14 interaction (Numata et al. 2010). Two single-nucleotide polymorphisms (SNP, Asp299Gly and Thr399Ile) in the TLR4 ectodomain have been associated with severe RSV bronchiolitis (Tal et al. 2004; Awomoyi et al. 2007) further suggesting that RSV-F-TLR4 interactions may be important in pathogenesis. However, more recent studies found no evidence for increases in RSV disease severity with these SNP, thus this remains controversial. TLR3 is an endosomal receptor that senses dsRNA, a replicative intermediate in RSV's life-cycle. Polyinosinic:polycytidylic acid (poly I:C) mimics dsRNA and is a synthetic TLR3 ligand with potent antiviral activities (Ashkar et al. 2004; Gill et al. 2006). RSV infection can increase subsequent responses to poly I:C *in vitro* via upregulation of TLR3 expression (Klein Klouwenberg et al. 2009). RSV-infected mice have been shown to upregulate TLR3 expression at day 8 of infection and TLR3 expression has been inversely related to disease severity, however these observations have not been confirmed in humans.

1.2.5.2.3 RIG-like helicase receptors and NOD-like receptors

Additional families of PRR include retinoic acid-inducible gene (RIG) -like helicase receptors (RLH) and nucleotide-binding oligomerisation domain (NOD) -like receptors (NLR). RIG-I and MDA-5 are cytosolic RLH able to sense dsRNA that are present in most cells including epithelial cells and macrophages. In a study of infants with bronchiolitis, RSV was the most frequent pathogen identified and mRNA levels of both RLH were upregulated in nasal wash where virus was detected compared with no virus detected (Scagnolari et al. 2009). However when infants with RSV as the only virus identified were considered separately, the differences were not significant. RSV infection of A549 cells, a human lung adenocarcinoma cell line, results in activation of the transcriptional regulators, NF-κB and interferon regulatory factor (IRF) -3, as early as twelve hours post-infection (Liu et al. 2007), generating IFN-β, and the pro-inflammatory chemokines IP-10 and CCL5 (RANTES). Liu and colleagues further demonstrated that siRNA knockdown of RIG-I significantly inhibited early expression of these cytokines whereas silencing of TLR3 did not, and that TLR3 upregulation following RSV depended on IFN-β induced by RIG-I signalling. NOD2 is able to sense ssRNA and RSV infection of A549 cells results in upregulation of NOD2 expression, IRF-3 activation, and IFN-β production within two hours (Sabbah et al. 2009). Silencing of NOD2 expression reduced early (< 6 hours) IRF-3 activation and IFN-β production but later time points (10 hours) were not affected. The authors speculated that this might be because signalling via RIG-I is more important later during infection.

Thus numerous cellular innate immune receptors are triggered by RSV infection and play a role in the ensuing immune response. RSV infection in infants may modulate innate immune responses and hence predispose to allergy and airways disease. A better understanding of and how to influence the innate immune response to RSV will advance vaccine development.

1.2.5.2.4 Interferon

Interferons (IFN) are specialised cytokines produced by host cells in response to pathogens. They are divided into three types based on receptor utilisation. Type I IFN includes IFN-α and IFN-β as

well as several minor members. These IFN are produced by most cell types including epithelial cells, act through the IFN-α receptor (IFNAR), and are essential for a robust antiviral response (Sadler and Williams 2008). Type II IFN, represented by IFN-γ alone, binds to the IFN-γ receptor (IFNGR) and is produced mainly by activated T cells and natural killer (NK) cells. IFN-γ is a key effector of the Th1 response and important in the defence against intracellular parasites and viruses. Type III interferon includes three gene products of IFN-λ (IFN-λ1, IFN-λ2, and IFN-λ3) and shares many of the antiviral functions of type I IFN (Mordstein et al. 2010). RSV infection of A549 cells and nasal epithelial cells results in production of type I and type III IFN (Spann et al. 2004; Okabayashi et al. 2011). The main mechanism of RSV-induced type I IFN production is thought to involve binding of dsRNA replicative intermediates to cytoplasmic RIG-I, promoting the induction of IRF3 and NF-κB (Bitko et al. 2007; Liu et al. 2007). Once released, type I interferons mediate their effects through their receptor linked to a Janus family kinase-signal transducer and activator of transcription (JAK-STAT) signalling cascade (Sadler and Williams 2008). Phosphorylation of IFNAR results in release of phosphorylated STAT1 and STAT2, which associate with IRF-9 to form transcriptional activator complex interferon stimulated gene factor 3 (ISGF3). On translocation to the nucleus, this complex activates transcription of type I IFN-inducible genes including MxA, 2',5'-oligoadenylate synthase (2,5-OAS), and protein kinase R (PKR), which establish an antiviral state in host cells (Katze, He, and Gale 2002).

RSV, like all *Paramyxoviridae*, has evolved means to disrupt the innate immune response. However unlike related paramyxoviruses, the pneumoviruses have additional non-structural proteins (NS1 and NS2, see section 1.2.1), which are rapidly synthesised following host cell invasion and can block the activity of type I interferon via a variety of mechanisms. NS1 can bind and inhibit IRF3 and degrade TNF receptor-associated factor 3 (TRAF3), suppressing IFN synthesis (Swedan, Musiyenko, and Barik 2009; Swedan et al. 2011; Ren et al. 2011). NS2 binds to RIG-I and inhibits its interaction with the NF-κB activator protein, mitochondrial antiviral signalling (MAVS), thereby preventing IFN gene induction (Ling, Tran, and Teng 2009). Both NS proteins promote degradation of STAT2, thus

targeting IFN response as well as IFN production (Lo, Brazas, and Holtzman 2005, 2; Ramaswamy et al. 2006).

1.2.5.2.5 NK cells

Natural killer (NK) cells are important innate effector cells that can rapidly respond to pathogens and provide early signals including IFN-γ to activate other parts of the immune system. During primary infection of BALB/c mice, NK cells are detectable in the lung by day 1 and peak at day 3-4 (Anderson et al. 1990), corresponding to the peak of lung viral load (Openshaw and Tregoning 2005). Lung NK cells are the most abundant early source of IFN-γ (Hussell and Openshaw 1998); production of IFN-γ by NK cells is vital for later CD8 T cell recruitment to the lungs and can inhibit lung eosinophilia (Hussell and Openshaw 2000). NK cells can therefore influence subsequent T cell subset development and their depletion during RSV infection has been shown to promote Th2-driven pathology (Kaiko et al. 2010). However, NK cells may contribute to pathology under certain conditions. Mice inoculated with a recombinant RSV expressing murine IL-18 demonstrated enhanced disease associated with increased lung NK number and activation (Harker et al. 2010). Another group have suggested that IFN-γ production by NK cells may contribute to lung pathology as their depletion or blocking of IFN-γ in mice prior to and early on in RSV infection was demonstrated to reduce lung inflammation (Li et al. 2012). Further to this, a murine model of neonatal sensitisation with RSV showed an influx of NK cells and granulocytes into the airways following adult reinfection, accompanied by weight loss (Harker et al. 2014). NK cell depletion during reinfection attenuated weight loss but had no effect on the T cell response. Thus NK cells may have a dual role of early viral defence and the potential to cause inflammation.

1.2.5.2.6 Dendritic cells

Naïve T cells require activation by mature antigen-presenting cells (APC) to proliferate and acquire effector functions. Dendritic cells (DC) are 'professional' APC, and are the primary inducers of adaptive immune responses. DC express many PRR, enabling them to recognise microbial products and in addition to cytokine secretion, provide the necessary co-stimulation to naïve T cells, thus

determining differentiation toward Th1, Th2, or other phenotypes. Several subsets of DC have been characterised in humans and mice (Neyt and Lambrecht 2013). In the resting state, conventional DC (cDC) located below the basement membrane of the lung epithelium extend projections into the lumen to enable sampling of antigen from the airway (reviewed in (Lambrecht and Hammad 2003)). These cDC express the integrin CD103 and are capable of cross-presentation of exogenous antigen to CD8 T cells (del Rio et al. 2010). Conventional DC located in the lamina propria mainly express CD11b and are known as myeloid cDC. A third subset expresses Siglec H but neither CD103 nor CD11b; these DC are known as plasmacytoid DCs (pDC) and specialise in production of type I IFN in response to viral nucleic acids (Reizis et al. 2011). Under pro-inflammatory conditions, additional monocyte-derived $CD11b^+$ DCs (moDC) are recruited to the lungs. Both cDC subsets can promote antiviral immunity via activation of CD_4 and CD8 T cells (Lambrecht and Hammad 2012). Conventional DC encountering inhaled antigen in the absence of inflammation migrate to the draining lymph nodes and are able to activate T cells, however the response is directed toward tolerance, thereby maintaining lung homoeostasis. If however, inflammatory signals, such as bacterial LPS, are present, cDC upregulate their co-stimulatory signals and are able to promote efficient effector T cells. Thus the signals provided by cDC are key to the initiation and direction of the subsequent T cell response.

Lung DC have been well-studied in murine models of RSV, and numbers expand during inflammation and persist for several weeks (Beyer et al. 2004). This expanded DC pool exhibits an activated phenotype with increased expression of major histocompatibility complex (MHC) class II and co-stimulatory surface molecules CD40, CD80, and CD86. The precise role of this population in the antiviral response is unclear, as the expansion occurs when cytotoxic T cell responses are already established (Schwarze 2008). However the presence of an expanded pool of activated DC could potentially permit sensitisation to subsequent inhaled allergens. By contrast, numbers of pDC in the lung increase early in the course of RSV infection (Smit, Rudd, and Lukacs 2006). Type I IFN production by these cells is important for control of viral replication as their depletion results in higher RSV viral loads and increased lung pathology (Wang, Peters, and Schwarze 2006). The role of

DC in human RSV is less well studied. One study has demonstrated productive infection of human moDC, *in vitro* resulting in diminished capacity to activate CD_4 T cells (de Graaff et al. 2005). A more recent study found that following RSV infection, human moDC failed to downregulate chemokine receptors CCR1, CCR2, and CCR5, which maintain residence in peripheral tissues, and upregulate CCR7, which directs migration to the lymph nodes (Le Nouën et al. 2011, 7). Taken together, these findings suggest an ability of RSV to interfere with the initiation of adaptive immune responses, however much work needs to be done to define precise mechanisms.

1.2.5.3 Adaptive immunity

1.2.5.3.1 Humoral immunity

Antibodies have a unique role in preventing virus infection via direct neutralisation of infectivity (e.g. blocking attachment or fusion), or promoting phagocytosis of virus particles by opsonisation. Fusion-inhibition appears to be the most effective means to prevent paramyxovirus infections (Merz, Scheid, and Choppin 1980) and the major protective antigens of RSV for neutralising antibodies are thus the F protein and the major attachment G protein (Olmsted et al. 1986). The protective effect of maternal antibody and the efficacy of palivizumab suggest that antibody-mediated protection is a key determinant of RSV immunity. Neonatal antibody responses are typically poorly functional, and analysis of neonatal RSV antibody gene segments demonstrates reduced levels of somatic mutation compared with those of older children and adults (Williams et al. 2009). Elderly persons with low serum IgG and mucosal IgA neutralising antibody levels are more susceptible to winter RSV infections (Walsh and Falsey 2004) and observations from adult experimental infection have suggested that serum neutralising antibody levels correlate partially with resistance to viral challenge (Hall et al. 1991; Lee et al. 2004). Reinfection with RSV occurs throughout life, however boosted serum antibody levels following natural infection are short-lived with a four-fold decrease after 1 year (Falsey, Singh, and Walsh 2006). Antibodies may also contribute to enhanced disease. Poorly neutralising antibodies can result in high levels of immune complexes, which have been shown to contribute to pathology in FI-RSV vaccinated mice (Polack et al. 2002). Further study has suggested that FI-RSV disease in infants may have been due to poor

stimulation of TLRs and lack of antibody affinity maturation (Delgado et al. 2009). Thus whilst antibodies are the most readily measurable correlate of protection from RSV, any potential vaccine must ensure to induce effective and long-lasting neutralising antibody levels.

1.2.5.3.2 Cytotoxic CD 8 T cells

Cell-mediated immunity is critical for the clearance of virally-infected cells and therefore recovery from infection. CD8 T cells control acute virus infection by direct lysis of infected cells via perforin and granzyme, induction of apoptosis via Fas ligand, and production of cytokines. The role of CD8 T cells in recovery from RSV is exemplified by children with defective T cell responses that exhibit prolonged viral shedding and increased disease (Hall et al. 1986). Pathological features of RSV bronchiolitis show CD8 T cells are the predominant infiltrating lymphocyte in the airways (Figure 1.5 and Johnson et al. 2007). Primary infection in BALB/c mice produces a strong CD8-mediated IFN-γ response and lymphocyte infiltration into the lungs; while CD_4 T cells are less frequent, Th_1 cells predominate even in Th_2-prone BALB/c mice (Openshaw 1989). CD8 T cells appear in the lungs at day 4, peak around day 6-14, and are critical for viral clearance but can also contribute to pathology (Cannon, Openshaw, and Askonas 1988) and their depletion in mice prior to RSV infection mitigates weight loss (Graham et al. 1991).

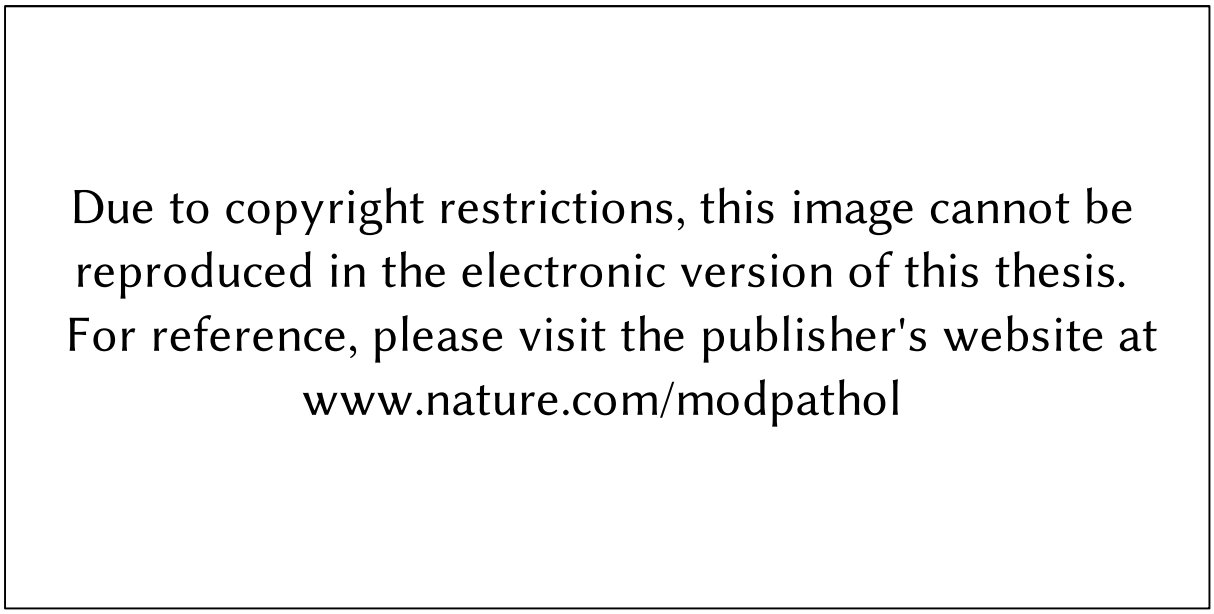


Figure 1.5 T-lymphocytes contribute to acute RSV-induced airway inflammation. (a-c) CD3+ cells (stained brown) infiltrate around small bronchioles (a, b) and alveolar interstitium (c). (d, e) CD8+ cells (stained brown) in the peribronchiolar region (d) and alveolar interstitium (e). (f) CD4+ cells (stained brown) are found rarely. Magnification: a and d = × 25, b = × 62.5, c, e, f = × 250. Reprinted with permission from Macmillian Publishers Ltd. Johnson *et al.* 2007. Modern Pathology 20:108-119. Copyright 2007 Nature Publishing Group.

1.2.5.3.3 T-helper cells

RSV-induced airway pathology is characterised by airway hyper-responsiveness and mucus production. The Th2 cytokines IL-4, IL-5, and IL-13 have been shown to play a role in these aspects of allergy and asthma in various experimental models (Holgate 2012), thus much work has been done to explore the role of these mediators in RSV. Reinfection of mice primed by intramuscular injection of FI-RSV results in enhanced disease, characterised by local eosinophilia and Th2 biased (IL-4, IL-5, IL-13 dominated) cytokine responses (Waris et al. 1996). In this case, disease is mediated primarily by CD_4 T cells (Connors et al. 1992; Connors et al. 1994). Priming with recombinant Vaccinia virus (rVV) expressing individual RSV proteins has proven to be a most valuable method for elucidating the contributions of various RSV proteins to protection and pathology. Cutaneous priming with rVV-F or rVV-G partially protects against RSV replication, but again results in enhanced disease. However rVV-G results in the Th2 biased eosinophilia, reminiscent of that seen in FI-RSV primed mice (Openshaw, Clarke, and Record 1992). The effects of RSV-G priming can be mitigated, either by simultaneous priming with M2 (which induces a strong CD8 T cell response and abundant IFN-γ) or by disruption of the dominant CD_4 T cell epitope of G with insertion of the

M2 T cell epitope (Hussell et al. 1997). The disease enhancing effects of specific protein priming can be largely reproduced by adoptive transfer of T cells from sensitised mice (Alwan, Record, and Openshaw 1993).

Evidence that Th2-biased responses are immunopathogenic in primary infantile bronchiolitis is not conclusive or wholly convincing (Bont et al. 1999), and in mice such responses are confined to secondary disease in sensitised animals. Indeed, several studies suggest that IFN-γ may be associated with disease severity (Bont et al. 2001; Chen et al. 2002), and there is even doubt that T cell mediated lung pathology plays an important part in the pathogenesis of bronchiolitis in infants undergoing primary infection (Heidema et al. 2007; Lukens et al. 2010; Welliver, Reed, and Welliver 2008).

In recent years, a distinct subset of CD4 T cells that secrete pro-inflammatory IL-17 family cytokines IL-17A and IL-17F have been identified as being responsible for autoimmune disease and defence against extracellular bacteria and fungi (Park et al. 2005; Annunziato et al. 2007). Elevated IL-17 levels have also been found in tracheal aspirates from infants with severe RSV bronchiolitis (Mukherjee et al. 2011). In mice, levels peak on day 8, the source appearing to be CD4 T cells. Treatment with anti-IL-17 antibody prior to and during RSV infection reduces neutrophilic lung inflammation and decreases viral load; knockout of IL-17 results in decreased lung mucus production. IL-17 blockade is associated with increased lung RSV-specific CD8 T cells, IFN-γ and granzyme B, suggesting an antagonism of Th1 and CTL responses. Possible mechanisms for IL-17 production in RSV-infected mice involve epithelial induced activation of complement with production of anaphylatoxin C3a and substance P (Bera et al. 2011). In contrast, a novel RSV vaccine strategy employing a live attenuated *Bordetella pertussis* strain (BPZE1) was shown to protect mice against subsequent RSV challenge by an IL-17-dependent mechanism (Schnoeller et al. 2014). Furthermore, it has been recently suggested that airway IL-17 may play a role in convalescence from bronchiolitis in human infants by facilitating antiviral responses (Faber et al. 2012), thus the exact role of Th17 cells and IL-17 in human RSV needs further study.

1.2.5.3.4 Regulatory T cells

Natural regulatory T cells (Treg) are a distinct subset of T cells that are critical for maintenance of self-tolerance and the prevention of autoimmune disease (Sakaguchi et al. 1995). The Treg population is generally found within the CD_4 T cell subset as cells that highly express the IL-2 receptor α-chain, CD25. The regulatory phenotype is dependent on expression of the forkhead box P_3 ($FOXP_3$) transcription factor, dysfunction of which results in scurfy mice and immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in humans (Lahl et al. 2007; Torgerson and Ochs 2007). In BALB/c mice undergoing primary RSV infection, depletion of Treg by anti-CD25 monoclonal antibody results in delayed recruitment of CD8 T cells to the lung, enhanced disease, and delayed viral clearance (Lee et al. 2010a). More specific Treg depletion of $FOXP_3$-expressing cells results in enhanced weight loss, greater lung inflammatory cell infiltration, and greater IFN-γ production by effector T cells in primary RSV infection (Fulton, Meyerholz, and Varga 2010). Subsequently, it has been shown that Treg in the lungs of RSV-infected mice actively resolve inflammation via inhibition of pro-inflammatory cytokines and chemokines, and reduce numbers of pro-inflammatory IFN-γ-secreting cells via granzyme B-mediated cytolysis (Loebbermann et al. 2012). Mice vaccinated with FI-RSV demonstrate a loss of Treg from the airways and restoration of local Treg numbers by delivery of Treg chemokine receptor ligands mitigates weight loss, suggesting that vaccine-enhanced disease may be due in part to a dysregulated immune response (Loebbermann et al. 2013). Thus, Treg appear to be the key resolvers of RSV-induced lung inflammation in mice, however their role in human RSV disease has not yet been investigated.

1.2.6 Treatment

As the majority of RSV infections are mild, treatment is supportive in all but the most severely-ill individuals. The antiviral drug ribavirin initially showed promise when given by aerosol in children (Hall et al. 1983a) and adults (Hall et al. 1983b), however it is expensive, difficult to administer, and evidence for its efficacy in infantile bronchiolitis is poor (Ventre and Randolph 2007). Palivizumab, a humanised mouse anti-RSV F-protein monoclonal IgG_1 antibody, is effective for prevention (The

IMpact-RSV Study Group 1998), however cost restricts its use in all but the most high-risk individuals. Furthermore, therapeutic trials of palivizumab and the more potent anti-RSV monoclonal, motavizumab, failed to show a benefit in established RSV disease (Malley et al. 1998; Ramilo et al. 2014). Novel therapies that have been evaluated in humans include RNA interference (RNAi) (DeVincenzo et al. 2010a) and ultra-potent nanobodies (Schepens et al. 2011). Most recently, GS-5806 (see Figure 1.6), a novel RSV fusion inhibitor based on a pyrazolo[1,5-a]pyrimidine scaffold that demonstrated potent antiviral activity against a broad panel of 75 clinical isolates of RSV A and B at nanomolar concentrations, was evaluated in an experimental adult human RSV challenge model (DeVincenzo et al. 2014). One hundred and forty participants were randomised to receive oral GS-5806 or placebo on the first occurrence of either the first day of detectable viral shedding in nasal lavage or day 5 post-inoculation. Participants were enrolled in seven sequential cohorts with the first four cohorts receiving 50 mg of GS-5806 or placebo, followed by four daily doses of 25 mg; subsequent cohorts received slightly different dosing regimens. The primary efficacy endpoint was area under the curve (AUC) for viral load from the time after the first dose and secondary endpoints included AUC for change in total symptom score and total mucus weight. Participants treated with GS-5806 had a lower mean AUC viral load (250.7 $\log_{10}$ PFU equivalent (PFUe) × hours per mL vs. 757.7 $\log_{10}$ PFUe × hours per mL), lower mean AUC change from baseline symptom score (-20.2 vs. 204.9), and lower total mucus weight (6.9 g vs. 15.1 g) than those treated with placebo. No serious adverse events were reported. Whilst this was only intended as a proof of concept study, GS-5806 represents a significant advance in the treatment of RSV and demonstrates the value of human experimental challenge in accelerating development of novel treatments for RSV infection.

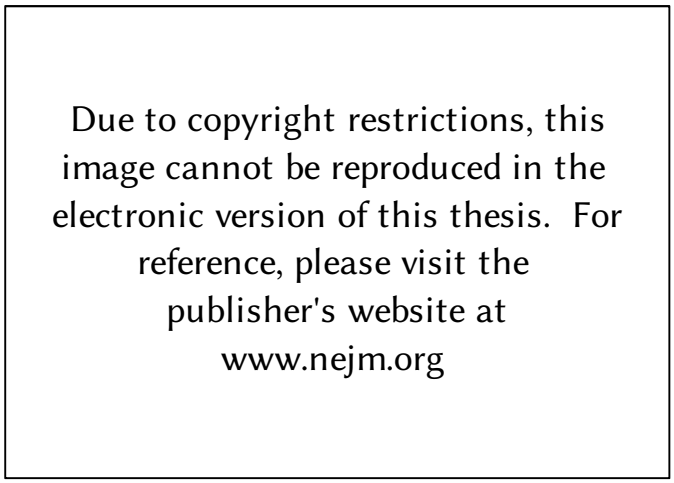


Figure 1.6 Chemical structure of GS-5806, an RSV fusion inhibitor. Reproduced with permission from DeVincenzo *et al.* NEJM 2014;371:711 doi: 10.1056/NEJMoa1401184. Copyright Massachusetts Medical Society.

1.2.7 Vaccination

There is currently no licensed human RSV vaccine and developments have been hampered following the disastrous results of trials of an RSV vaccine (Lot-100) conducted in North America during the 1960s (Fulginiti et al. 1969; Kim et al. 1969; Kapikian et al. 1969; Chin et al. 1969). Lot-100 was an alum precipitated 100× concentrated formalin-inactivated RSV (FI-RSV). Formalin inactivation theoretically prevents viral replication whilst preserving structure and hence immune recognition, and had at the time lead to a successful polio vaccine. Following receiving three doses of Lot-100, 43% of infant vaccinees displayed a four-fold or greater rise in serum neutralising antibody and 91% displayed a four-fold or greater rise in serum complement fixing antibody. However, following a natural RSV epidemic in the community, the rate of RSV infection in Lot-100 vaccinees was no different from children who had received a parainfluenza vaccine. Furthermore, 80% of Lot-100 vaccinees required hospitalisation compared with only 5% of children receiving the parainfluenza vaccine. Hospitalised children had pneumonia and bronchiolitis. Sadly, two Lot-100 vaccinees died. The mechanisms underlying this vaccine-enhanced disease were unclear; vaccinees showed serological responses and testing in guinea pigs, cynomologous monkeys, rabbits, and mice had demonstrated FI-RSV to be safe. However it has since been shown that prior exposure to FI-RSV causes similar disease enhancement on reinfection in mice (Waris et al. 1996), cotton rats (Connors et al. 1992), and non-human primates (Kakuk et al. 1993). Naïve mice vaccinated with FI-RSV develop pulmonary eosinophilia and Th2-biased responses characterised by IL-4, IL-5, and IL-13 on subsequent rechallenge with wild-type RSV. Post-mortem examination of the lungs of the two fatal

cases of FI-RSV vaccine-enhanced disease revealed peribronchiolar inflammation with exudates of epithelial cells, mononuclear cells, neutrophils, and eosinophils causing small airways obstruction (Kim et al. 1969), and immune complex deposition and complement activation (Polack et al. 2002). Subsequent serological testing revealed antibodies directed against F and G proteins in the sera of vaccinees to be poorly neutralising (Murphy and Walsh 1988), and that formalin-inactivation lead to the formation of carbonyl groups, which affected the quality of antibody and T cell responses (Moghaddam et al. 2006). The frequency of severe illness diminished in older children (Fulginiti et al. 1969), thus it seems plausible that early life priming with FI-RSV somehow alters the subsequent immune response on reencounter with the wild-type virus.

1.2.7.1 Live attenuated RSV vaccines

It should be noted that animals used to recapitulate the syndrome of FI-RSV enhanced disease are semi-permissive hosts thus it is possible that these models are imperfect and that animal models cannot be used to reliably exclude the possibility that a candidate vaccine will not cause enhanced disease in humans. However it has been shown that live attenuated RSV given to human infants does not result in enhanced disease (Wright et al. 2007), nor based on general observation does repeat natural infection, thus evaluation of live attenuated RSV vaccines in infants is considered to be safe. Live attenuated RSV vaccines can be administered intranasally, which aside from being needle-free, offers the advantages of permitting viral replication and thus immunogenicity in the face of pre-existing maternally-derived serum antibody, and inducing local mucosal immune responses. Important limitations of live attenuated RSV vaccines are the inherent instability of the virus itself, potentially complicating production and storage, and the fact that immunogenicity is inversely correlated with attenuation (Karron et al. 1997b; Karron et al. 2005; Wright et al. 2006). Live attenuated RSV vaccines based on biological and recombinant derivatives of wild-type RSV have been developed. A selection of live attenuated RSV vaccines is summarised in Table 1.2. However, with the possible exception of MEDI-559, a recombinant RSV A2 with both cold and temperature sensitive mutations plus deletion of the SH gene (Malkin et al. 2013), none has yet achieved the desired balance between attenuation and immunogenicity in infants.

Vaccine name and type	Population evaluated	Reference	Attenuation	Immunogenicity
***cp*RSV**, cold-passaged RSV	Adults	Friedewalde 1968	Virus passaged at 26 ℃ attenuated in adults, not evaluated in children	Poorly immunogenic in seropositive adults
RSV *ts*-1, RSV *ts*-2, temperature-sensitive mutant RSV	Adults and children	Wright 1971, Watt 1990, Pringle 1993	RSV *ts*-1 under attenuated (rhinorrhoea), possible reversion to virulence in excreted virus RSV *ts*-2 over attenuated and insufficiently infectious	Significant rise in serum nAb titre in RSV *ts*-1
***cpts*248/955, *cpts*530/1009**, cold-passaged, temperature sensitive RSV, with additional mutations	Adults and children including seronegative children	Karron 1997	Attenuated in adults, cpts248/955 insufficiently attenuated in seronegative children, *cpts*530/1009 attenuated, both strains transmitted from infants at frequency of 20-25%.	Significant rise in serum nAb titre
***cpts*248/404**, cold-passaged, temperature sensitive RSV, with additional mutations	Adults, children and young infants (1-2 months old)	Wright 2000	Non-infectious in seropositive children and adults, insufficiently attenuated in young infants (nasal congestion)	Significant rise in serum nAb titre except in infants where only rises in nasal antibody were seen
rA2cp248/404ΔSH, recombinant A2 with cold-passage and temperature sensitive mutations and additional deletion of SH gene	Adults and children	Karron 2005	As for *cpts*248/404	No significant rise in serum nAb titre
rA2cp248/404/1030ΔSH, recombinant A2 with cold-passage and further temperature sensitive mutations and additional deletion of SH gene	Adults, children, and infants (1-2 months old)	Karron 2005	Attenuated but evidence of mutation instability	Significant rise in serum nAb titre only seen in minority of seronegative infants
rA2cpΔNS2, recombinant A2 with cold passage mutations and deletion of NS2 gene	Adults and children	Wright 2006	Attenuated but viral replication too high to be evaluated in infants	No significant rise in serum nAb titre
rA2cp248/404ΔNS2, recombinant A2 with cold-passage and temperature sensitive mutations and additional deletion of NS2 gene	Adults and children including seronegative children	Wright 2006	Over attenuated	No significant rise in serum nAb titre in adults and seropositive children
rA2cp530/1009ΔNS2, recombinant A2 with cold-passage and temperature sensitive mutations and additional deletion of NS2 gene	Adults and children including seronegative children	Wright 2006	Over attenuated	No significant rise in serum nAb titre in adults and seropositive children
MEDI-559, recombinant A2 with cold-passage and alternate temperature sensitive mutations and additional deletion of SH gene	Seronegative children > 5 months old	Malkin 2013	Attenuated, although slightly increased frequency of lower RTI in vaccinees	Significant rise in serum nAb but dependent on pre-existing titre

Table 1.2 Summary of biologically-derived and recombinant live attenuated RSV vaccines (derived from Karron 2013).

The major protective antigens F and G protein of RSV have been expressed in viral vectors as potential live attenuated RSV vaccines. Two main vectors have been evaluated: human parainfluenza viruses (PIV) and Sendai virus (SeV), a murine parainfluenza virus. Human PIV-3 is a frequent cause of lower RTI in infants and thus is an appropriate choice for a bivalent RSV-PIV-3 vaccine. PIV vectors offer additional advantages over conventional live attenuated RSV vaccines, namely: 1) PIV replicate more efficiently *in vitro*, 2) PIV exists as spherical particles that are easier to process, and 3) PIV exhibits greater physical stability. MEDI-534, is a live attenuated chimeric bovine/human PIV-3 intranasal vaccine, genetically engineered to express human RSV F protein. In a study involving seronegative children aged 6-24 months, MEDI-534, was well-tolerated, and vaccine take (seroconversion or viral shedding) was dose-dependent, with 67% and 100% for RSV and PIV-3 respectively (Bernstein et al. 2012), however there have been concerns about genetic stability (Tang RS 2012, cited in (Karron, Buchholz, and Collins 2013)). SeV, modified to express RSV F protein, was immunogenic and protective against RSV challenge in African green monkeys (Jones et al. 2012), however safety of the SeV vector in young children has not yet been established.

1.2.7.2 Subunit vaccines

Whilst live attenuated virus vaccines are generally considered advantageous due to their ability to induce broad and lasting immunity, they are not suitable for all populations. Such vaccines can cause disease in immunocompromised persons and thus their use in this group is generally contraindicated. Also as discussed above, sufficiently immunogenic live attenuated RSV vaccines are frequently insufficiently attenuated to be safely used in young infants. Whilst, inactivated or subunit vaccines may not pose such an immediate risk, the experience following FI-RSV mandates extreme caution, and the absence of types of immune responses associated with enhanced disease must thus be a requirement for development of an inactivated or subunit RSV vaccine. The other challenge facing inactivated or subunit RSV vaccine development is induction of long-lasting immunity, given that even natural infection fails to achieve this. The F and G proteins are the known major protective antigens, however the latter exhibits significant variability between strains. This observation, and the knowledge of the efficacy of palivizumab for prevention, suggests that any

successful subunit vaccine must induce robust and effective responses directed against the well-conserved F protein.

With this in mind the first subunit vaccines to be evaluated were based on aluminium hydroxide or aluminium phosphate adjuvanted purified F protein. Three such vaccines (PFP-1, PFP-2, PFP-3) were sequentially produced with increasing purity, and evaluated in seropositive children (Belshe, Anderson, and Walsh 1993; Tristram et al. 1993; Piedra et al. 1996). Notably, four-fold or greater rises in serum neutralising antibody were seen with few adverse reactions, however all of the studies were small thus clear conclusions could not be drawn. A later meta-analysis of six double-blind randomised controlled trials of PFP in children reported a statistically significant reduction in RSV infection incidence but not serious lower RTI (Simoes et al. 2001). Additionally, the authors raised concerns about heterogeneity of included studies and publication bias. No studies of PFP in seronegative infants have been reported. A subunit vaccine containing F, G and M protein produced from RSV grown in Vero cells was evaluated in elderly people, (Falsey et al. 2008; Langley et al. 2009). These studies were not powered to assess efficacy, however, and no further development on this vaccine has occurred.

Subunit vaccines containing peptides from conserved regions of G protein have been developed. BBG2Na is a peptide spanning amino acids 130 – 230 of G protein fused to a streptococcal carrier protein. The peptide includes sequences completely conserved among all known human RSV isolates (Power et al. 2001). A vaccine based on alum adjuvanted BBG2Na was well-tolerated and immunogenic in healthy adults. The protein is produced in *Escherichia coli*, hence glycosylation is not required for immunogenicity (Power et al. 1997). A subsequent trial in elderly people demonstrated immunogenicity, however protection was short-lived and phase III trials were halted due to type III-type hypersensitivity reactions in a few individuals (Murata 2009; Nguyen et al. 2012).

1.2.7.3 Virus-like particle and virosome vaccines

Virus-like particles (VLP) are virus-sized particles containing repeating arrays of protein but no viral genome. Viral surface glycoproteins are embedded into the VLP membrane, mimicking infectious viruses, however replication is not possible. Thus VLP can contain important protective epitopes but do not need to be inactivated. VLP are potently immunogenic even without adjuvants and stimulate a broad range of B and T cell responses (reviewed in Jennings and Bachmann 2008). VLP expressing RSV structural proteins are not efficiently produced, however a chimeric Newcastle disease virus (NDV) expressing RSV F or G proteins fused to sequences encoding the transmembrane domains of NDV surface glycoproteins resulted in efficient expression of VLP incorporating the fused proteins (Murawski et al. 2010; McGinnes et al. 2011). Following a single intramuscular immunisation with VLP containing both glycoproteins, BALB/c mice demonstrated robust and durable neutralising antibody responses (Schmidt et al. 2012). VLP therefore are one approach by which the poor durability of RSV humoral responses may be overcome.

Virosomes are phospholipid vesicles containing viral glycoproteins. RSV virosome vaccines containing F and G protein induced potent neutralising antibody responses, however inclusion of adjuvants was required to avoid Th2-biased responses (Stegmann et al. 2010; Kamphuis et al. 2012). Vaccination with adjuvanted particles did not result in enhanced disease upon RSV challenge thus these vaccines show some promise. Safety and efficacy has not been demonstrated in humans, however phase I trials are ongoing.

1.3 Human infection challenge models

1.3.1 Background

The inoculation of James Phipps by Edward Jenner in 1796 might be regarded as the first documented deliberate human infection challenge. This experiment eventually would lead to vaccination and the eradication of smallpox nearly two centuries later, arguably one of the greatest triumphs of infectious disease science. Controlled human infection studies can provide great insight into pathogenesis, facilitating drug and vaccine development (Pollard et al. 2012). In

contrast to natural infection where similar disease may result from multiple different pathogens and is generally recognised only when symptoms occur, the precise nature and moment of infection in controlled studies is known. This allows detailed examination of pre-existing host and microbial factors that influence the course of disease.

1.3.2 Ethical issues

Deliberating infecting human beings, possibly with an aim to induce disease, clearly has ethical implications. Such experiments would not normally provide any meaningful benefit to the participants, so potential harms cannot be justified or offset, thus must be minimised. Whilst the fight against infection is relevant to all humankind, the interests of science or society must not override the interests of participants (Hope and McMillan 2004). Informed consent is especially central to the proper conduct of infection challenge studies so that potential participants are fully aware of the risks and possible harms to which they may be exposed. However, informed consent alone is not sufficient justification for research — there must be a limit to harms that potential participants are induced by researchers to expose themselves to, no matter what the perceived societal need. All research involving human beings in England is subject to review and approval by the National Research Ethics Service (NRES).

With respect to RSV, healthy adults would at most be expected to experience a common cold illness. Such illnesses are a part of normal life and as such, the risk and potential harms of acquiring one experimental cold is not considered significant. The research procedures themselves should be minimally invasive and comparable with what might be expected in a phase 1 pharmaceutical trial, e.g. monitoring of vital signs, blood tests, and collection of bodily fluids. More invasive sampling, e.g. a biopsy must be scientifically justified and its frequency the minimum necessary to acquire sufficient observations. Safety of participants is of primary concern at all times.

1.3.3 Historical RSV challenge studies

Kravetz first successfully infected twenty adult volunteers in 1961 with an RSV strain isolated in 1957 from a patient with bronchopneumonia (Kravetz et al. 1961). Since that time there have been

numerous challenge studies of human RSV involving over 300 subjects using both wild-type and attenuated strains without reported serious adverse events (Friedewald et al. 1968; Wright, Mills, and Chanock 1971; Watt et al. 1990; Pringle et al. 1993; Wright et al. 2000) and Table 1.3. Knowledge about the route of infection, incubation periods, durations and determinants of protective immunity, and duration of viral shedding has been gained from these studies, but much of what we have since learned from RSV in mice was not examined. Previous human RSV challenge studies have used virus strains (e.g. Long and A2) isolated by serial laboratory passage in multiple cell lines or live attenuated strains. Successful infection requires the use of high doses and is often minimally symptomatic, suggesting that these viral strains are extensively laboratory-adapted and likely attenuated (Cherrie 1992; Lee et al. 2004). These strains were isolated in the 1960s, so may differ somewhat to RSV strains in current circulation and it is difficult to ensure consistency between stocks in different laboratories. This poses a significant problem when trying to accurately reproduce and study natural infection, and casts some doubt over the relevance of the findings.

Characteristics and number of subjects	RSV strain used	Main observations	Year and reference
33 males, no history of cardiac, respiratory or allergic disease	RSV (Long) – wild-type strain isolated in 1957	High-dose (5 log TCID50) found to be effective. Titre of nasal wash antibody inversely correlated with susceptibility to infection.	Mills *et al* 1971
32 young adults, free of atopic disease	RSV (A2, wild-type strain isolated from Australia 1961)	5.2 log TCID50 found to be effective dose. Route of inoculation compared; eye and nose but not mouth found to be viable.	Hall *et al* 1981
105 healthy adults aged 18-50 years	RSS-2 (wild-type strain) (n=20) and 4 different ts-mutants (n=22 per group)	RSS-2 was virulent and induced serological response in 90% and clinical cold in 45%; ts-mutant strains induced much less illness and two strains produced serological response in 68%.	McKay *et al* 1988 and Watt *et al* 1990
102 healthy adults aged 18-55 years	RSS-2 (5 subjects challenged with saline)	Study designed to assess prophylactic and therapeutic effect of intranasal interferon α; benefit seen for prophylaxis but not as therapy.	Higgins *et al* 1990
15 young healthy adults with recent laboratory confirmed natural RSV infection	RSV A2	Study designed to determine duration of immunity to reinfection; immunity associated with serum neutralising antibody to F and G proteins, but protection short-lived.	Hall *et al* 1991
36 healthy adults aged 18-45 years	RSV A2 (8 subjects challenged with placebo)	Dose of 4.7 log TCID50 and 3.7 log TCID50 used; no difference in symptoms or infection rates with different doses; infection rate inversely correlated with serum neutralisation titre; virus appeared attenuated (mild symptoms only in all subjects).	Lee *et al* 2004

Table 1.3 Historical experimental human RSV challenge studies describing numbers of subjects and their characteristics, particular RSV strain used and main observations and conclusions.

1.3.4 Contemporary RSV challenge studies

More recently, a wild-type, low-passage strain of RSV A has been produced according to Good Manufacturing Practice methods in Vero cells. The progenitor virus, Memphis 37 (M37), was isolated from a child with bronchiolitis in 2006. RSV M37 has been grown to high titre and individual lots tested for the presence of adventitious agents. The first published study using this virus in humans was by Zaas *et al* in 2009 (Zaas et al. 2009). The purpose of the study was to identify a peripheral blood mRNA signature that could identify acute respiratory tract viral

infection. However only nine out of twenty subjects challenged became infected and the baseline characteristics of the subjects and course of the illness were not described in detail except that the symptoms peaked a median 141.5 hours post-inoculation. The following year DeVincenzo *et al* (DeVincenzo et al. 2010c) reported on a study involving thirty-five healthy adults challenged intranasally with doses of M37 from 3.0 – 5.4 $\log_{10}$ PFU mL^{-1}. Subjects enrolled had serum RSV neutralising antibody titres in the lower third of the population screened (mean titre of enrolled subjects 7.11 MU mL^{-1}). The key findings were an infection rate of 77% with no statistical differences in infection rate for different inoculating doses. The incubation period was 3.1 days and virus was shed for a mean of 7.4 days. Viral load and symptoms peaked around day 6 post-inoculation and a significant correlation was observed between viral load and respiratory tract symptom scores analysed as AUC. Additionally, nasal lavage concentrations of TNF, IL-6, IL-8, and MIP-1α were noted to be biomarkers of clinical disease and a significant positive correlation was observed between cumulative nasal IL-6 levels and AUC viral load. The authors concluded that viral load was the driver of clinical disease and thus antiviral therapy would likely be beneficial to reduce disease severity. A second lot of RSV M37 was acquired by Alnylam Pharmaceuticals, who developed ALN-RSV01, a small interfering RNA (siRNA) directed against the mRNA of RSV N protein. In a proof-of-concept study, eighty-eight subjects with low serum neutralising antibodies (mean titre 7.25 MU mL^{-1}) were challenged with ~5 $\log_{10}$ PFU mL^{-1} M37 and randomised 1:1 to receive ALN-RSV01 or placebo for two days before until three days after inoculation. Thirty-six (86%) of subjects in the placebo arm became infected (virus detected by PCR) compared with 29 (67%) in the ALN-RSV01 arm. ALN-RSV01 was well-tolerated and although a trend was seen in the ALN-RSV01-treated group toward a lower viral load, the study was not powered to detect a statistically significant difference.

These recent studies using RSV M37 involved inoculation of a total of 143 subjects with no serious adverse events described. The virus was demonstrated to be virulent in that a typical common cold illness was induced and virus shedding detected in approximately three-quarters of individuals. The

time course of disease and viral shedding has been described enabling further possibilities for controlled infection studies using this virus.

1.3.5 Summary of findings of existing human RSV challenge studies

Experimental infection of human volunteers with RSV has revealed much about routes of infection, infectious dose, incubation period, and duration of viral shedding, however study of the host immune response under these controlled conditions has been limited to the measurement of a few pro-inflammatory cytokines. The kinetics of the immune response has not been described. Whilst serum antibodies have been associated with protection from infection in the earlier studies, the viruses used were attenuated in humans. Studies using the recently developed M37 strain have pre-selected subjects based on especially low serum neutralising titres, therefore may have enrolled an unusually susceptible population limiting the generalisability of their conclusions.

1.4 Immune correlates of protection from RSV infection

As stated in 1.1.2, serum antibodies are the most readily measurable and definable correlate of protection against infectious disease. Serum neutralising antibody and binding antibody to RSV surface glycoproteins F and G have been associated with reduced risk of hospitalisation in infants (Piedra et al. 2003) and the elderly (Falsey and Walsh 1998) and protection from experimental infection of healthy adults (Lee et al. 2004). Protection is however incomplete, as it has been observed that subjects with high levels of serum antibodies can still be infected (Walsh and Falsey 2004) and that boosted levels of antibodies following natural infection decline to pre-infection levels within 6 – 12 months (Falsey, Singh, and Walsh 2006; Sande et al. 2013). As RSV replication is generally confined to the respiratory tract epithelium, serum antibody seems an unlikely direct mechanism of protection, at least in the upper respiratory tract where IgA is the dominant class of immunoglobulin (Brandtzaeg 2013). More likely, high serum antibody levels are a surrogate for generally robust RSV immune responses and may merely indicate more recent infection. Mucosal IgA specific to RSV F and G proteins has been demonstrated to be protective in adults (Walsh and Falsey 2004), and the appearance of RSV-specific IgA in nasal secretions following natural infection

of infants coincides with the disappearance of viral antigen (McIntosh et al. 1978; McIntosh, McQuillin, and Gardner 1979). However the mechanisms of nasal RSV-IgA generation and maintenance in humans have not been studied.

Cell-mediated correlates of protection from RSV infection in humans have not been directly studied, however RSV-specific CD8 T cells in the peripheral blood of elderly persons were observed to persist at lower frequencies than those specific for influenza (de Bree et al. 2005a). By contrast virus-specific differentiated CD8 T cells were found at higher frequencies in the human lung than in the circulation (de Bree et al. 2005b). A more recent study found frequencies of RSV F protein-specific IFN-γ-producing T cells to be lower in the blood of healthy elderly compared with younger subjects with no differences in neutralising antibody titres (Cherukuri et al. 2013). Whilst these observations suggest a role for T cells in protection from RSV infection, experimental studies will likely be needed to precisely define any cellular correlates of protection.

1.5 Mediators of human RSV disease

Although an association between viral load and respiratory tract symptoms and disease severity has been observed in children and adults (Buckingham, Bush, and DeVincenzo 2000; DeVincenzo et al. 2010c), RSV is not considered to be especially cytopathic (Collins and Crowe 2007). Neutrophils are the predominant leucocyte found in the BAL of bronchiolitis infants and their recruitment depends on IL-8 and other chemokines in the airway epithelium (McNamara et al. 2003; Smyth et al. 2002). Levels of pro-inflammatory cytokines and chemokines are also elevated in infant BAL (McNamara et al. 2004; McNamara et al. 2005; Hornsleth, Loland, and Larsen 2001). Studies of airway epithelial cells reveal their role in recruitment of inflammatory cells, however the precise nature of the human cell-mediated response against RSV is not fully characterised (Fonceca et al. 2012; McNamara et al. 2013). Recent evidence has suggested a role for Th17 cells (Faber et al. 2012), which promote neutrophilic inflammation and have been implicated in the pathogenesis of asthma (Newcomb and Peebles 2013). T cell immunity against RSV and enhanced disease have been well studied in mice but such mechanistic studies in humans are not possible. Difficulties extrapolating from animal

models have made studies of RSV in humans even more crucial. However, investigations of hospitalised children and frail adults are limited by difficulties with case definition and delayed diagnosis. Furthermore, immune measures are highly confounded by co-morbid conditions, medical interventions and generalised features associated with multi-organ failure that have resulted in a number of contradictory conclusions. In order to control for some of these factors and to study pre-existing and early pre-symptomatic immune responses, experimental human infection studies are an important complementary approach.

1.6 Key research questions

1) What are the kinetics of the host immune response in terms of local inflammatory mediators following RSV inoculation in unselected healthy adults?
2) What pre-existing correlates of natural resistance to virulent RSV challenge can be identified?
3) Which inflammatory mediators (and therefore which potential immune pathways) are measurable in local and systemic compartments during the pre-symptomatic, symptomatic, and recovery phases of infection?

1.7 Hypotheses

1) Adults (unselected for serum neutralising titres) will have a lower overall frequency of symptomatic infection than that previously observed following experimental challenge with virulent M37.
2) Serum virus neutralising antibody correlates with protection against infection and/or clinical disease following experimental RSV challenge in adults.
3) Nasal virus-specific IgA is more protective against infection and/or disease than serum antibody following experimental RSV challenge in adults.
4) Acute phase mediators (such as IL-1β, IL-6 and TNF) and Th1 pro-inflammatory mediators (such as IFN-γ) correlate with symptomatic disease severity.
5) The pattern of mediators in the pre-symptomatic, symptomatic, and recovery phases of infection can be used to characterise the evolving response to infection and thus explain the variability in responses to a standard controlled RSV challenge.

1.8 Aims of the project

The first objective of the project is to establish a safe and reproducible model of experimental RSV infection in healthy adults. The diverse responses of individuals to infection with RSV are well-suited to a prospective longitudinal study of local and systemic immune responses before and during the development and resolution of symptoms. The aim of the study is to achieve a

comprehensive understanding of the roles of antibody, soluble mediators and (in the future) T cells in the course of infection of adults challenged with RSV by intranasal infection, and thereby to guide the development of vaccines and other treatments for RSV disease.

2 Materials and methods

2.1 General methods

2.1.1 Buffers and solutions

Name	Usage or recipe	Supplier and product code
2-Mercapto-ethanol (βME) 14.3 M	Working solution of 50 mM: 10 µL βMe in 2850 µL pure water, aliquot and store at −80 ℃	Sigma M6250
3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate	Ready to use	Sigma T0440
3,3'-Diaminobenzidine (DAB) tablets	Dissolved in nanopure water according to instructions	Sigma D4293
3-Amino-9-ethylcarbazole (AEC) tablets	Dissolve 1 tablet in 2.5 mL of DMSO and add 47.5 mL 0.1M pH 5.0 sodium acetate buffer. Add 25 µL 30% hydrogen peroxide and filter through 0.45 µm PVDF membrane immediately before use.	Sigma A6926
Acetate buffer, pH 5.0	0.1M sodium acetate, 13.6 g sodium acetate trihydrate dissolved in 1000 mL deionised water, pH adjusted to 5.0 using glacial acetic acid	Sodium acetate trihydrate: Sigma S8625, acetic acid: Sigma 320099
Ampicillin (200mg, stock at 10 mg mL^{-1})	Reconstitute according to instructions	Invitrogen 11593-027
Avidin D-HRP	5 mg lyophilised, reconstitute with 1 mL pH 7.2 PBS	Vector Labs A2004
CpG ODN 2006 oligodeoxynucleotide [T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T] (* signifies phosphothionation)	Reconstitute at 1 mg mL^{-1} in nuclease-free water and store at −80℃	Eurofins MWG/Operon
Dimethylsulfoxide (DSMO)	Ready to use	Sigma 472301
Dulbecco's modified Eagle medium with high glucose and added pyruvate	Ready to use, supplement as required	Sigma
Dulbecco's modified Eagle medium with low pyruvate	Ready to use, supplement as required	Gibco 41965-039
Dulbecco's phosphate buffered saline, without $CaCl_2$ and $MgCl_2$	Ready to use, supplement as required	Sigma D5652
ELISA and ELISpot wash buffer	PBS + 0.05% Tween20 v/v	All from Sigma
ELISA stop solution	2N H_2SO_4, 196 mL concentrated H_2SO_4 added slowly to 500 mL deionised water then topped up to 1 L with deionised water	All from Sigma

Ethanol	Ready to use	Sigma E7023
Extravidin Peroxidase	Ready to use	Sigma E2886
Fetal calf serum	Heat-inactivate at 56 ℃ for 30 minutes, filter 0.2 µm, aliquot and store at −80 ℃	Source bioscience
Hemocyanin from *Megathura crenulata* (keyhole limpet)	Reconstitute according to instructions	Sigma H7017
Hydrogen peroxide (H_2O_2) 30% w/w	Ready to use	Sigma H1009
L-glutamine (200 mM)	5 mL is 500 mL of cell culture media	Invitrogen 25030-081
LB (Lysogeny broth) agar	Make up according to instructions, sterilise by autoclave 121 ℃, 1.03 bar for 15 minutes	Invitrogen 22700-025
LB (Lysogeny broth) media	10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L pure water	Invitrogen 12795-027
Lectin from *Phytolacca Americana* (Pokeweed mitogen)	Dilute 1:100 v/v in PBS + 1% FCS v/v and store at 4 ℃ for up to 6 months	Made at Emory University
Measles Virus antigen from Edmonston strain	Ready to use	Meridian Lifesciences 7604, UK distributer is AMSBio
Methanol	Ready to use	Sigma 322415
One Shot® TOP10 Chemically Competent *E. coli*	Ready to use	Invitrogen C4040-03
PBMC washing media	Sterile PBS + 2% FCS v/v	PBS: Sigma
PBS tablets	Dissolve according to instructions	Sigma P4417
Penicillin 10,000 U mL^{-1} + streptomycin 10,000 µg mL^{-1}	5 mL in 500 mL of cell culture media	Invitrogen 15140-122
Protein A from *Staphylococcus aureus* Cowan (SAC) strain, suspension, fixed	Resuspend thoroughly before use	Sigma P7155
Roswell Park Memorial Institute medium	Ready to use, supplement as required	Sigma
RSV stabilisation media	DMEM + 25% sucrose w/v + 1% pencillin-streptomycin v/v	All from Sigma
SAM elution buffer	PBS + 1% BSA w/v + 0.5 Triton-X v/v + 0.05% sodium azide w/v	All from Sigma
SOC (Super Optimal Catabolite) medium	Invitrogen (supplied with chemically-competent E. coli)	Invitrogen
Sodium azide (NaN_3)	Working solution is 0.1% w/v	Sigma S2002
TaqMan Universal Master Mix II, with UNG	Ready to use	Lifetechnologies 4440038

Trypan blue solution, 0.4%	Ready to use	Lifetechnologi es T10282
TWEEN-20 liquid	Ready to use	Sigma P1379

2.1.2 Antibodies for immunoassays

Name	Supplier
AffiniPure goat anti-human IgA+IgG+IgM	Jackson ImmunoR esearch 109-005-064 (UK distributer at time of writing is Stratech)
Biotin-SP-AffiniPure F(ab')2 fragment donkey anti-human IgG, Fcγ fragment specific	Jackson ImmunoResearch 709-066-098 (UK distributer at time of writing is Stratech)
Biotinylated goat anti-human IgA	Lifetechnologies 62 -7440
Biotinylated goat anti-human IgM	Lifetechnologies H 15015
Biotinylated polyclonal goat anti-RSV IgG	ABD Serote c 07950-0104
Peroxidase-conjugated goat anti-human IgA (α-chain specific)	Sigma A0295

2.1.3 Primers for molecular assays

Name	Sequence	Supplier
Random hexamer primers	N/A	Qiagen 79236
RSV A N-gene forward	5'-CATCCAGCAAATACACCATCCA-3'	Invit roen
RSV A N-gene reverse	5'-TTCTGCACATCATAATTAGGAGTATCAA- 3'	Invitrogen
RSV PAN A N-gene probe	5'-**FAM**-CGGAGCACAGGAGAT-**TAMRA**-3'	Eurofins MWG/Operon

2.1.4 Viruses

Type	Name or strain	Supplier
Challenge virus	RSV A Memphis 37	Purchased from Meridian Lifesciences
Laboratory-grade M37 stock	RSV M37 MH P3	Production described in section 2.1.4
	RSV M37 AJ P4	Production described in section 2.1.4
RSV quantitative standards	RSV A Long ATCC VR26	A gift from Prof. John DeVincenzo, University of Tennessee, US
UV-inactivated influenza	Influenza A/H1N1/England195	A gift from Prof. Wendy Barclay, Imperial College London, UK

2.1.5 Equipment

Name	Supplier
AID ELISpot reader	Oxford Biosystems
Conical tubes (15 mL and 50 mL, polypropylene, sterile)	BD Falcon
Countess Automated Cell Counter	Invitrogen
Cryotubes	Nunc
ELISA plate reader (SPECTRAMax PLUS S384)	Molecular Devices
Incubator (NU 5500 airjacketed automatic CO_2 incubator)	NuAire Inc
Inverted light microscope (Leica DME)	Leica Microsystems
Laboratory centrifuge (Heraeus 400R)	Heraeus
Laboratory centrifuge (Jouan CR422)	Jouan
Leukosorb, pre-cut strips for nasal epithelial lining fluid sampling	Pall Lifesciences (strips cut by Parafix)
Meso Scale Discovery SECTOR Imager 2400	Meso Scale Discovery
Microfuge (Eppendorf 5415D)	Eppendorf
Millex-HV 0.45 µm filter, nylon, 33 mm	Millipore SLHN033NS
Millex-HV 0.45 µm filter, PVDF, 33 mm	Millipore SLHV033NS
Non-sterile 96-well mixed nitrocellulose ester membrane plates (Multiscreen-HA)	Millipore MSHA NB450
Nuclease-free tubes	Invitrogen
PCR cycler (GeneAmp 9600)	Applied Biosystems
Real-time PCR cycler (ABI 7500 Fast)	Applied Biosystems

Refrigerated microfuge (Eppendorf 5415R)	Eppendorf
Rotary shaker	Jenway or equivalent
Screw-top cryotubes	Simport or Alpha Labs
Sonicator	Ultrawave Ltd.
Spin filter centrifuge tubes (Costar Spin-X, 0.22 µm pore size, cellulose acetate membrane, CLS8161)	Corning
Temperature-controlled water bath	Clifton or equivalent
Tissue culture flasks 225 cm^2 (T225)	Nunc

2.1.6 Computer software

Name	Supplier
Discovery Workbench	Meso Scale Discovery
R: A Language and Environment for Statistical Computing	R Foundation for Statistical Computing, www.r-project.org/
SoftMax Pro	Molecular Devices

2.1.7 Recombinant RSV fusion protein

RSV fusion (F) protein was a gift from Mucosis B. V. (Groningen, The Netherlands) and related production has been described elsewhere (Rigter et al. 2013). Briefly, F protein (GenBank accession AAS93654.1) was modified to lack the transmembrane domain and cytoplasmic tail and incorporate a Strep tag for purification purposes. Expression vectors containing this modified F protein sequence were transfected into HEK293T cells. Tissue culture supernatants were harvested at 5 days post transfection and F protein was purified using Strep-tactin Sepharose beads according to manufacturer's instructions (IBA, Germany). Total protein concentration was determined to be 0.6 mg mL^{-1}. Purity was estimated to be 70%.

2.1.8 Humanised murine monoclonal anti-RSV fusion protein antibody (Palivizumab)

Palivizumab is a humanised form of murine monoclonal anti-RSV F protein antibody 1129, described elsewhere (Beeler and Coelingh 1989; Johnson et al. 1997). A single 50 mg vial was obtained from Main Pharmacy, St. Mary's Hospital, Paddington, Imperial College Healthcare NHS Trust. Individual aliquots of 128 μg mL^{-1} in DMEM were made and frozen at −80 ℃.

2.1.9 Cell lines

HEp-2 cells (ATCC CCL-23) were a gift from Dr. Fiona Culley, Imperial College London. Cells were maintained in DMEM + 10% FCS v/v (D10) media at 37 ℃ in 5% CO_2 in 225 cm^2 tissue culture flasks (T225, Nunc). Flasks were observed daily for growth and microbial contamination using an inverted light microscope. Routine passage or harvesting of cells was undertaken when 50 – 70% confluence was observed. The procedure was as follows. Culture medium was removed and discarded followed by washing of the monolayer with 10 mL sterile D-PBS. Ten mL of 1× trypsin-EDTA was added and the flasks were incubated at 37 ℃ for approximately 5 minutes until cells were observed to be detached. Ten mL of D10 was added, the cell suspension was transferred to a 50 mL polypropylene tube (Falcon, BD) and cells were pelleted by centrifugation at 100 × *g* for 5 minutes at room temperature. The supernatant was discarded and the pellet resuspended in 20 mL of D10. Cells were split 1:4 to 1:8 depending on requirements, i.e. 2.5 – 5 mL of cell suspension was added to D10 to give a total volume of 25 mL in a T225.

2.1.10 Virus stocks

2.1.10.1 Production of RSV Memphis 37 challenge stock

RSV A Memphis 37 was purchased from Meridian Life Sciences (Memphis, Tennessee, US). A detailed description of its production is provided in section 3.4.1.

2.1.10.2 Production of laboratory virus stocks

A laboratory stock of non-GMP M37, designated RSV M37 MH P3, was prepared and expanded by two passages in HEp-2 cells according to standard methods. Briefly, one vial of M37 was thawed

and made up to a volume of 4 mL with serum-free DMEM. Two mL of this dilution was added to two of the flasks of 70% confluent HEp-2 cells; the third received 2 mL of serum-free DMEM alone as a control. All flasks were incubated for two hours at 37 ℃ in 5% CO_2 and the flasks were rotated 90° every 15 minutes to counteract any uneven surfaces. 13 mL of D10 was then added and the flasks were incubated for a further 24 hours before diluting the FCS to 2%. The flasks were returned to the incubator and examined twice daily for cytopathic effect (CPE). After 48 hours, 50% CPE was observed and the cells were harvested using a cell scraper, transferred to 50 mL polypropylene tubes, sonicated in an ice-water bath sonicator for 20 seconds, and centrifuged to remove debris. One mL aliquots were placed into cryotubes and snap-frozen in liquid nitrogen. Viral titre was determined by plaque assay in HEp-2 cells as described in section 2.1.10.3 to be 0.8×10^6 PFU mL^{-1}). A further passage in HEp-2 cells was performed by Dr. Agnieszka Jozwik to produce a higher titre (8×10^6 PFU mL^{-1}) of laboratory grade stock (designated RSV M37 AJ P4). This virus was used in neutralising antibody studies.

2.1.10.3 Plaque assay to determine live virus titre

Quantitative viral culture was performed in 96-well tissue culture plates as described (Martin J. 1987) with the following modifications. 100 µL of 2.5×10^5 mL^{-1} HEp-2 cells were added to each well and the plate was incubated overnight at 37 ℃. The plates were observed for ≥ 90% confluence before virus to be assayed was added in triplicate twofold dilutions across the long axis of the plate starting at 1 in 10. RSV quantitative standards (RSV A Long ATCC VR26, a gift from Prof. John DeVincenzo, University of Tennessee, US) were run in parallel as a positive control for every assay. The plate was incubated for two hours at 37 ℃ in 5% CO_2, and then 150 µL of DMEM with low pyruvate (LP) with 2% FCS was added to each well, followed by further incubation at 37 ℃ for 48 hours. Cells were then fixed for 20 minutes using 100 µL per well of methanol with 2% hydrogen peroxide. The plate was washed once in with 200 µL per well PBS before 200 µL per well of PBS 1% BSA 0.05% sodium azide (w/v) was added. The plates were then kept at 4 ℃ for at least 24 hours or up to one week prior to staining. Plates were washed once with PBS 1% BSA (w/v) before adding 100µL per well of 1 in 500 biotinylated polyclonal goat anti-RSV IgG antibody and incubated for two

hours at room temperature in darkness followed by four more washes with PBS 1% BSA (w/v). One hundred µL per well of 1 in 500 ExtrAvidin peroxidase was then added and the plates were incubated for a further two hours in darkness. Five further washes as above were performed before 50 µL per well of 3,3'-diaminobenzidine tetrahydrochloride substrate, prepared according to manufacturer's instructions, was added. Plates were observed for 5 – 10 minutes until clear plaques were visible in the control wells. The reaction is then stopped by washing once in PBS. Plates were then washed once with distilled water and blotted before being allowed to dry overnight in the dark at room temperature. Plaques were then counted using a microscope at 10× magnification. The mean of triplicate dilutions was calculated and the number of PFU per mL given by:

$$\frac{mean\ plaques \times fold\ dilution}{volume\ of\ inoculum\ (mL)}$$

2.1.11 Viral antigens

Measles antigen (Edmonston strain) was produced by Meridian Life Sciences and purchased from AMS Biotechnology. RSV M37 antigen (lysate) was produced by culture of laboratory-grade RSV M37 (see section 2.1.10.2) until 50% CPE was observed. Culture supernatant was then removed and 2 mL per flask of PBS 1% Triton-X v/v was added and the cells were scraped from the flask and transferred to a 15 mL polypropylene conical tube. The tube was then sonicated for 10 minutes and clarified by centrifugation at 400 × *g* for 10 minutes at room temperature. Fifty µL aliquots were stored at −80 ℃.

2.2 Laboratory techniques and assays

2.2.1 Serological assays

2.2.1.1 Internal controls for serological assays

RSV serological standards (Wyeth Lot 06937, 06938, 06939) were obtained through BEI Resources, NIAID, NIH (Panel of Human Antiserum and Immune Globulin to Respiratory Syncytial Virus, NR-32832, (Yang et al. 2007)) under a Material Transfer Agreement. The geometric mean ± standard

deviation neutralisation titres by plaque reduction in the absence of complement as stated in the certificate of analysis were: lot 06937 (high control) 1897 ± 864, lot 06938 (medium control) 255 ± 51, lot 06939 (low control) 310 ± 7. Additionally, a stock of serum was donated by the author for use as an internal positive RSV antibody control. Sera from four infants (kindly provided by Dr. Jethro Herberg, Imperial College London) under one year hospitalised but confirmed RSV PCR negative were determined to have RSV neutralising antibody titre below 3.3 $\log_2$ (lower limit of detection) and were used as a negative control.

2.2.1.2 Plaque reduction assay to determine serum neutralising antibody titre

Serum neutralising antibody titre was determined by plaque reduction assay. Plates were prepared as for plaque assay to determine virus titre (see section 2.1.10.3). Serum samples were thawed and incubated at 56 ℃ for 30 minutes in a water bath to inactivate complement. Eleven triplicate serial twofold dilutions beginning at 1 in 10 of serum to be assayed were made in DMEM in separate 96-well V-bottomed plates; the 12^{th} column received DMEM only. To ensure maximum comparability, pre- and post- infection samples from the same subject were run on the same plate, i.e. rows A – C contained a pre-infection serum and rows D – F contained a post-infection serum. Palivizumab was used as a positive neutralising control in row G, starting at 64 μg mL^{-1} with eleven serial twofold dilutions as for serum. Row H was a non-neutralised virus-only control. To these dilutions, 50 μL of 1 in 4000 RSV M37 AJ P_4 (= 100 PFU) was added and plates were incubated at 4 ℃ for one hour. Plates containing confluent HEp-2 cells were then removed from the incubator and washed once with 200 μL DMEM per well before 50 μL of the serum-virus mix from the V-bottomed plates was added to each corresponding well; thus each non-neutralised well would now have 50 PFU of virus. The cell plates were then incubated undisturbed at 37 ℃ in 5% CO_2 for two hours before adding 100 μL DMEM-LP + 2% FCS per well and incubating undisturbed for a further 48 hours. The plates were then fixed and plaques revealed by immunoperoxidase antibody staining and counted as in section 2.1.10.3. The serum neutralising titre was defined as the dilution that yielded a 50% reduction in plaques compared with the non-neutralised controls on the same plate. Numerical methods to calculate this are described in section 2.3.3. RSV serum standards and sera from

seronegative infants were tested with this assay to ensure accuracy and specificity (see section 2.2.1.1).

2.2.1.3 ELISA for nasal wash RSV-specific IgA

Nasal RSV-specific IgA was determined by endpoint titre ELISA in nasal lavage supernatant (see section 3.5.2.2). Optimal concentrations for coating antigens and detection antibodies were determined by prior titration. Ninety-six well ELISA plates were coated overnight at 4 ℃ with 50 µL per well of 1 in 1500 RSV lysate (see section 2.1.11) or 1 µg mL^{-1} recombinant F protein (see section 0) in PBS. Samples were thawed and 100 µL of lavage supernatant was added to an equal volume of PBS 1% BSA (w/v). This was done to maintain stability of proteins in nasal lavage after thawing. These diluted aliquots were stored at 4 ℃ for a maximum of two weeks. Plates were washed four times with 200 µL per well PBS-T and then blocked with 200 µL per well PBS 1% BSA for two hours at room temperature on a rotary shaker (300 – 500 rpm). Plates were washed four times as before and 50 µL each of six serial twofold dilutions starting at 1 in 16 of lavage supernatant (pre-diluted as above) was added per well; thus the effective actual range of dilutions was 1 in 32 to 1 in 1024. Pre- and post- infection samples were assayed concurrently as for serum neutralisation. A sample of serum from a bulk lot donated by the author that had been heat-treated (see section 2.2.1.2) was used as a positive control. This was diluted twofold from 1 in 10 to 1 in 2560 and 50 µL was added per well as above. The plate was laid out so that A1 to G12 contained samples, H1 to H8 contained positive control serum and H9 to H12 were plate blanks. The plates were incubated overnight at room temperature on a rotary shaker as before and then washed with 200 µL per well of PBS-T. Fifty µL per well of 1 in 4000 peroxidase-conjugated goat anti-human IgA was added and plates were incubated for two hours at room temperature on a rotary shaker as before. Plates were washed four times in PBS-T as before and 50 µL per well of substrate TMB was added. Plates were observed for colour development for approximately three to five minutes before the reaction was stopped by adding 50 µL per well of 2N H_2SO_4 and absorbance at 450nm (OD_{450}) was determined using a plate reader. The raw endpoint titre was defined as the last observed dilution in which the OD_{450} was greater than two times the mean plate background OD_{450}. Due to variable dilution of

epithelial lining fluid by nasal lavage, observed endpoint titre were corrected for dilution using the ratio of serum to nasal lavage urea before analysis as described (Rennard et al. 1986), except urea was measured using the Urea Assay Kit (Abcam ab83362)

2.2.2 Detection of RSV in volunteer samples

2.2.2.1 Nasal wash plaque assay

Live RSV excretion was detected by plaque assay of nasal lavage. Plates were prepared as in section 2.1.10.3. Eight duplicate serial two-fold dilutions of freshly collected nasal lavage in RSV stabilisation media were made from 1 in 2 to 1 in 256 and added to the plate. RSV M37 MH P3 was used as a positive control in two-fold dilutions from 1 in 100 to 1 in 12800. Uninfected wells were used as a negative control. The remainder of the protocol proceeded as for titration of live virus (see section 2.1.10.3).

2.2.3 RSV N gene qPCR

2.2.3.1 Nasal wash viral RNA extraction

Total RNA in nasal wash was extracted using the QIAamp Viral Mini kit (Qiagen) as per the manufacturer's instructions with the following modifications. Ten µL of carrier RNA was added to 1 mL of lysis buffer AVL per sample in a 15 mL polypropylene tube. Samples were thawed rapidly and 250 µL was added to the carrier RNA-AVL and the mixture was pulse vortexed for 15 seconds followed by incubation at room temperature for 10 minutes. One mL of 96% ethanol was added and the tubes were pulse vortexed for 15 seconds. The remainder of the procedure followed the kit instructions. Contaminating DNA was removed by an on-column DNAse digest using the Qiagen DNAse kit according to the manufacturer's instructions. Extracted RNA was eluted in 60 µL of nuclease-free water and stored in a screw-top cryotube at −80 ℃.

2.2.3.2 cDNA synthesis

Extracted RNA was converted to cDNA for use in PCR using the Omniscript RT kit (Qiagen) according to the manufacturer's instructions with the following modifications. Reverse

transcription was primed using random hexamer primers (Qiagen). RNAse inhibitor (Qiagen) was added to the reaction mix to prevent degradation of RNA as recommended by the manufacturers. Reaction conditions were 37 ℃ for 60 minutes for reverse transcription followed by 95 ℃ for 5 minutes to inactivate reverse transcriptase on the GeneAmp 9600 PCR cycler. Reaction volumes are shown in Table 2.1.

Reagent	Volume (µL) per reaction
10× buffer	2
dNTP	2
Primers	1
RNAse inhibitor	1
Omniscript RT	1
Extracted RNA	13
Total	20

Table 2.1 Reagent and sample volumes for cDNA synthesis using Omniscript RT kit

2.2.3.3 Real-time PCR

2.2.3.3.1 Primers and probe

Primers and TaqMan probe specific for sequences of all known strains of RSV A have been published (Perkins et al. 2005) and were used throughout. Optimum primer concentrations were determined by the method recommended by Applied Biosystems to be 900 nM forward and 900 nM reverse. Optimum probe concentration was determined by the same method to be 200 nM.

2.2.3.3.2 Production of N gene plasmid DNA standard

To permit approximate quantitation of N gene copy number, a plasmid DNA standard was made. RNA was extracted and cDNA produced from a mock infected nasal lavage, made from RSV M37 MH P3 diluted 1 in 10 in 0.9% sodium chloride, according to the procedures in sections 2.2.3.1 and 2.2.3.2 respectively. An N gene PCR product was produced by performing TaqMan real-time PCR as

in section 2.2.3.3.3. The N gene PCR product was purified from the PCR reaction using the QIAquick PCR Purification Kit according to the manufacturer's instructions.

2.2.3.3.2.1 Cloning of PCR product

The purified product was ligated into a plasmid vector (pCR 2.1-TOPO, Invitrogen) and transformed into chemically competent *Escherichia coli* (TOP10, Invitrogen) using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Briefly, for the ligation, 4 µL of purified PCR product was added to 1 µL of Salt Solution and 1 µL of TOPO vector and incubated at room temperature for 1 hour, then placed on ice. As a negative control, a further ligation reaction was performed without a PCR product (an empty vector). For the transformation, 2 µL of the cloning reaction was added to a tube of competent *E. coli* and incubated on ice for 5 minutes. The bacteria were heat shocked by rapid transfer to a 42 ℃ water bath for 30 seconds followed by placing back on ice. Two hundred and fifty µL of SOC medium was added and the tube incubated horizontally on a shaker at 200 rpm for 1 hour at 37 ℃. Two LB agar plates containing 50 µg mL^{-1} ampicillin were each spread with 50 and 150 µL of the *E. coli* culture to ensure one plate had a suitable density of growth to pick a single colony. One plate was spread with the control *E. coli* transformed with the empty vector. Plates were incubated overnight at 37 ℃. The pCR 2.1-TOPO vector contains an ampicillin resistance gene, hence transformants are able to grow on the plates but untransformed *E. coli* should not. As the vector is linearised, it is not efficiently expressed without a PCR product being incorporated, thus the control plates did not have any colonies. Thus colonies on the selective plates should be expressing the plasmid containing the N gene PCR product. Nine single colonies were picked from these plates and cultured for 16 hours in 15 mL tubes in LB broth with ampicillin at 37 ℃ with shaking prior to miniprep.

2.2.3.3.2.2 DNA miniprep of selected clones

Minipreps of the transformed clones were performed with the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. TaqMan real-time PCR was performed on all the miniprep clones using the N gene primers to detect expression of the N gene PCR sequence.

2.2.3.3.2.3 DNA maxiprep to produce plasmid stock

A single miniprep clone confirmed as containing the N gene PCR sequence by TaqMan was selected for production of the plasmid stock by maxiprep. One µL of miniprep DNA was used in the ligation reaction and transformed into *E. coli* as described in section 2.2.3.3.2.1. A single colony was picked and starter culture performed in 2 mL of LB broth with ampicillin for 8 hours at 37 ℃ with shaking. One hundred µL of this starter culture was grown in a vented flask containing 100 mL of LB broth with ampicillin overnight at 37 ℃ with shaking. The bacteria were pelleted by centrifugation at 3000 rpm for 30 minutes at 4 ℃ and the supernatant discarded. The remaining procedure was performed using the QIAfilter Plasmid Maxi Kit according to the manufacturer's instructions. The yield of N gene plasmid was determined by absorbance at 260 nm to be 537 µg. Assuming a molar mass per base pair (bp) of 662 g mol^{-1}, and a total plasmid size of 4,000 bp, using Avogadro's constant of 6.02×10^{23} molecules per mole, it was determined that the final prep had 3.06×10^{11} copies μL^{-1}. The presence of the N gene product was verified by TaqMan as before and the prep was diluted to a concentration of 10^{10} copies µL and stored at −80 ℃.

2.2.3.3.3 Semi-quantitative PCR (qPCR) using TaqMan and N gene plasmid DNA standard

Quantification of N gene was performed using real-time TaqMan PCR using the primers and probes described in section 2.2.3.3.1 as described in (Perkins et al. 2005) with the following modifications. A reaction volume of 12.5 µL was used containing 6.25 µL of TaqMan Universal Master Mix II, 1.125 µL each of forward and reverse primer, 0.25 µL of probe, 1.25 µL of nuclease-free water, and 2.5 µL of cDNA. Cycling conditions were as follows: 50 ℃ for 2 minutes (UNG incubation), 95 ℃ for 10 minutes (polymerase activation) followed by 40 cycles of 95 ℃ for 15 seconds (denaturing) and 60 ℃ for 1 minute (annealing and extension). A linear fit was performed on the standard curve and copy number was estimated using the model parameters. This quantification is only approximate as no allowance is made for efficiency of RNA extraction, cDNA synthesis or relative efficiencies of sample target cDNA amplification versus plasmid standard amplification. However providing one can accept the assumption that these factors are relatively constant across all samples tested, comparisons can be made.

2.2.4 Measurement of soluble mediators in nasal epithelial lining fluid

Soluble mediators were measured by a multiplex electrochemiluminescence method using custom kits purchased from and according to instructions provided by Meso Scale Discovery. Briefly, pre-coated plates were pre-wetted with 25 µL per well MSD Diluent 2 and incubated on a rotary shaker for 30 minutes at 750 rpm. Twenty-five µL of sample or standard (referred to as calibrator in MSD literature) was added per well and plates incubated for 2 hours on a rotary shaker as before. The plates were washed three times with 200 µL per well PBS-T followed by the addition of detection antibodies (MSD SULFO-TAG™ streptavidin-conjugated detection antibody blend) and incubation on a rotary shaker for 2 hours as before. The plates were then washed three times as before followed by the addition of 150 µL per well MSD 2X Read Buffer T using a reverse pipetting technique to avoid the creation of bubbles. Plates were then read on the MSD SECTOR Imager 2400 device. All incubations were at room temperature and plates were sealed using adhesive film during incubation. Concentration of each mediator was determined from the standard curve as in 2.3.4.

2.2.5 Measurement of peripheral blood acute antibody-secreting cell frequencies

2.2.5.1 B cell ELISpot

Antibody secreting cells (ASC) were detected in freshly isolated PBMC using enzyme-linked immunospot (ELISpot) as previously described (Saletti et al. 2013) with the following modifications. For acute plasmablasts, 96-well plates (MSHAN4B50 Millipore, UK) were coated 10 µg mL^{-1} anti-human IgG, IgA, IgM (Jackson ImmunoResearch), RSV antigen (1:1500 or 5 µg mL^{-1}), or 1 µg mL^{-1} purified F protein. Fifty-thousand PBMC were added to duplicate wells, serial three-fold dilutions were made in R10 and plates were incubated for 16 hours. For memory B cells, plates were additionally coated with 10 µg mL^{-1} measles antigen (Meridian Lifesciences), 1:10 UV-inactivated influenza virus A/H1N1/England/195 (for H1N1-specific MBC), and 5 µg mL^{-1} HEp-2 antigen, 2.5 µg mL^{-1} keyhole limpet haemocyanin (KLH, Sigma), and media as negative controls. Duplicate serial five-fold dilutions were made and cells were incubated for 4 hours at 37°C. ASC were detected using biotinylated anti-human IgG (Jackson ImmunoResearch), IgA, or IgM (both Life Technologies), horseradish peroxidase-avidin D (Vector Labs) and 3-amino-9-ethylcarbazole (Sigma). Spots were

counted on the AID iSpot Reader. Total ASC were expressed as spot-forming cells / 10^6 PBMC and antigen-specific ASCs as % of total Ig-secreting cells.

2.2.6 Measurement of memory B cell frequencies

2.2.6.1 Thawing of cryopreserved cells

All media was first warmed to 37 ℃. All other steps and centrifugation were performed at room temperature. Samples were removed from liquid nitrogen and placed onto dry ice. The samples were quickly thawed in a water bath at 37 ℃. The details of the sample were recorded and the cryotube cleaned with 70% ethanol. The contents of the vial were very carefully transferred to a 50 mL polypropylene tube using a Pasteur pipette. The cryotube was rinsed with 1 mL of warm PBMC washing media and this was added drop by drop to the 50 mL tube. The cells were then washed by adding 20 mL of warm PBMC washing media drop by drop to the tube before centrifugation at 250 × *g* for 10 minutes. The supernatant was discarded and a further wash with 20 mL PBMC washing media performed. The cells were resuspended in 1 mL RPMI + 10% FCS v/v (R10) and a count and viability was performed by Trypan blue dye exclusion. The cells were then resuspended at the desired concentration for use.

2.2.6.2 Polyclonal stimulation

PBMCs were cultured *in vitro* according to the method of Crotty *et al* (Crotty et al. 2004). Briefly, PBMCs were resuspended in R10 + 50 μM β-mercaptoethanol (βME) and incubated for 5 days with a mitogen mix containing, pokeweed mitogen extract (kindly donated by Prof. Rafi Ahmed, Emory Vaccine Center, US), CpG ODN 2006 and *S. aureus* Cowan protein A. Antibody secreting cells were detected by B cell ELISpot as in 2.2.5.1. Subjects exhibiting a total Ig^+ cell responses the below the 10^{th} centile in either the day 0 or day 28 sample for either IgG or IgA were excluded (N = 9) as inadequate responders to stimulation. As the MBC assay involves polyclonal stimulation that may produce high non-specific background activity, to estimate true spot count, the following procedure was observed. Spot counts in the negative control wells were low overall and could be shown to approximate a negative binomial distribution. The mean and variance of the spot count in the

negative control wells for each sample was determined and therefore a theoretical negative binomial distribution and associated 95th centile cutoff count for each sample could be calculated. This cutoff was subtracted from spot counts in the antigen-coated wells. Values now below this cutoff were then assigned a value of the geometric mean of the negative control cutoffs for MBC of that Ig class (i.e. IgG or IgA), giving the limit of detection for MBC of that class in the assay (2.0 spots and 2.2 spots respectively).

2.3 Statistical analysis

2.3.1 General

All data analyses and graphs were produced using the software R (R Core Team 2013). Trellis plots were produced using package 'lattice' (Sarkar 2008). Results are expressed as median and interquartile range unless otherwise indicated. Non-parametric data was compared using one-sample (indicated by *V*) or two-sample (indicated by *W*) Mann-Whitney-Wilcoxon tests with Holm's correction for multiple comparisons where appropriate. Comparisons between more than two groups were by Kruskal-Wallis χ^2. A p-value of < 0.05 was taken to indicate statistical significance.

2.3.2 Estimation of cumulative responses by area under the curve

For cumulative data, area under the curve (AUC) by the trapezium rule was used. This is a standard technique for approximating the definite integral. Let y be a function at time x, then for a measured response of y (e.g. symptom score) at point x (day post-inoculation) over an interval X (e.g. days 0 to +14 post-inoculation), assuming a straight line between discrete observations of y, the area can be calculated by the following formula:

$$\sum_{k=1}^{X} \frac{x_{k+1} - x_k}{2} (y(x_{k+1}) + y(x_k))$$

In other words, the area is approximated by adding together the areas of multiple trapezia spanning each day interval. All AUC calculations were performed on data on the native scale, i.e. log

transformations were performed *after* the AUC calculation. Values are then expressed as log (values × days).

2.3.3 Estimation of serum neutralising antibody midpoint titres (ED50)

Weighted (1/y) three-parameter logistic models were used with the upper limit parameter (A) fixed at the mean plaque count of the non-neutralised control wells. These models were fitted to the plaque counts and the 50% neutralising titre (ED50) was derived from the midpoint parameter (E) of the curve using package 'drc' (Ritz and Streibig 2005).

2.3.4 Estimation of nasal soluble mediator concentration from standard curves

The supplied software for the MSD SECTOR Imager 2400 (Discovery Workbench) is able to fit a four-parameter logistic model to the standard curve data and thereby provide a calculated concentration for the samples. However, the software available at the time of writing is not able to apply limits of detection to the data or use alternate standard curves in the event of a failure. The software R was used with package 'drc' (Ritz and Streibig 2005) to perform the curve fitting (weighted $1/y^2$ as per MSD recommendations) and standard curves for each assay were visualised together. A lower limit of detection for each mediator for each run date was chosen to exclude the lower portion where the curve is flat (where concentration cannot be accurately quantified). The geometric mean of this lower limit of detection for each mediator was calculated and any calculated concentrations below this value were assigned this value.

2.3.5 Logistic regression models

Binary response variables (infected vs. uninfected) were related to continuous explanatory variables ($\log_2$ antibody titre) by a generalised linear model (GLM) using a binomial (logit) link function (logistic regression). Odds ratios (OR) and 95% confidence intervals (CI) of the OR for the explanatory variables were calculated. The GLM was used to predict the probability of protection (i.e. being uninfected) for a given antibody titre. A grid of 120 points of antibody titre across the range of theoretical values from lowest to highest was used to produce the prediction curves. Point-

wise 95% confidence intervals for the predicted response curves were derived by a bootstrap method (see 2.3.6).

2.3.6 Bootstrap method to derive 95% confidence intervals

The percentile method of Efron was used (Michael R Chernick 2008). Briefly, 1,000 bootstrap replicates of the data were generated by random sampling with replacement, a GLM was fitted to each replicate, and the predicted response values for the 120-point antibody titre grid derived, producing a 120 by 1,000 matrix of predicted response values. The 2.5^{th} and 97.5^{th} centiles for each row of the matrix were extracted yielding the lower and upper bound of the estimated 95% CI for the predicted response at that level of antibody titre.

2.3.7 Loess smoothing

Loess regression is a generalisation of locally-weighted scatterplot smoothing (LOWESS) techniques. The term 'loess' may be taken to mean 'LOcal regrESSion'. The details of how the technique works are beyond the scope of this thesis, however the specific application to the nasal mediator data is described in section 6.2.1.2. Functions to perform loess regression and obtain predicted values are part of the standard distribution of R. The technique was used to fit smoothed lines of best fit to scatterplot data to illustrate trends between pairs of continuous variables.

3 Method of experimental RSV infection, containment and sampling of adult volunteers

3.1 Introduction

Experimental infection of humans has been performed with numerous infectious agents including bacteria, viruses, and parasites. Kravetz and colleagues first successfully infected 20 adult volunteers in 1961 with an RSV strain (Long) isolated in 1957 from a patient with bronchopneumonia (Kravetz et al. 1961). Since that time there have been many RSV challenge studies involving a total of over 400 subjects, using both wild-type and attenuated viruses without reported serious adverse events (McKay et al. 1988; Watt et al. 1990; Higgins et al. 1990; Lee et al. 2004; Zaas et al. 2009; DeVincenzo et al. 2010c). Valuable knowledge about the route of infection, incubation periods, and duration of viral shedding has been gained, but the role of diverse immune responses in protection and disease pathogenesis were not studied in great depth. In addition, virus strains used in some studies were attenuated (Lee et al. 2004) or poorly characterised (McKay et al. 1988; Watt et al. 1990).

At present, the only other facility performing live experimental RSV human challenge is a commercial organisation with extensive purpose-built facilities including consulting rooms, a level 2 quarantine suite with individual rooms and onsite laboratories. Such facilities offer great control, but are arguably unnecessary for RSV challenge. There is no UK regulation that mandates their use, and the costs of such facilities are prohibitive for academic studies on a normal research budget.

3.2 Study design

3.2.1 Ethical issues

Research involving humans is subject to approval by Research Ethics Committees (REC). At the time of writing in the England, this oversight is provided by the National Research Ethics Service (NRES). As experimental infection is not for therapeutic benefit, viral challenge agents are not

classed as Investigative Medical Products or 'new drugs', thus these studies fall outside the requirements of the European Union Clinical Trials Directive (EUCTD, 2004), the Medicines and Healthcare Products Regulatory Agency (UK MHRA), and European Medicines Evaluation Agency (EMEA). In particular live viral inocula do not currently have a legal requirement for Good Manufacturing Practice (GMP). However, the tenets of Good Clinical Practice (GCP) apply with key principles being ensuring the safety of and minimisation of harm to research subjects, and ensuring that all subjects provide full written informed consent. This study was approved by the West London REC 2 on 7th February 2011, reference number 10/H0711/94 with the registered title "The Development of a Human Model of Respiratory Syncytial Virus Infection".

3.2.2 Safety issues

RSV is a Hazard Group 2 biological agent as described within the Control of Substances Hazardous to Health Regulations (COSHH) Approved list of Biological Agents (Advisory Committee on Dangerous Pathogens 2014). Published studies suggest that subjects experimentally infected with RSV excrete virus for up to ten days following the viral challenge. (DeVincenzo et al. 2010b; Kravetz et al. 1961; Hall et al. 1981; Lee et al. 2004) Therefore, in this study, the hazard (i.e. RSV) cannot be entirely contained, thus COSHH regulations can only apply to the safety of staff and laboratory workers when the virus is handled in the clinical and laboratory setting and do not apply to the subjects once they are infected. The Academy of Medical Sciences (UK) has issued a guidance document entitled: "Microbial Challenge Studies of Human Volunteers" (Academy of Medical Sciences 2005). Whilst not intended to be a regulatory document, this guidance provided a working framework for research within which to ensure safety of subjects deliberately infected, those conducting the research, and third parties not directly involved in the research.

3.2.2.1 Safety monitoring of study subjects

To the author's knowledge, there are no reported instances of serious adverse events following experimental RSV challenge of healthy volunteers. Since safety is of paramount importance, all subjects were screened (see section 3.3.2.1) prior to enrolment by the study doctor (Maximillian

Habibi) to ensure they were at minimal risk of developing severe disease following RSV inoculation. All live RSV inoculations took place in the Imperial Clinical Respiratory Research Unit (ICRRU), part of Imperial College Healthcare NHS Trust (ICHNT), where emergency facilities were immediately available, and where subjects remained for a period of one hour following inoculation. Since inoculation procedures occurred in NHS facilities the study was also subject to scrutiny by the Trust Safety Committee in addition to Imperial College's safety advisors.

Following inoculation, subjects were seen and examined daily by the study doctor and research nurse (Allan Paras). Assessments included observation of vital signs and measurement of lung function (see section 3.5.1.4). The research protocol included detailed guidance on safety monitoring and adverse events. Whilst one aim of the study was to induce common colds, in the event of any concerns, subjects could be withdrawn from the study and necessary treatment provided by St. Mary's Hospital, Paddington, ICHNT.

3.2.2.2 Protection of staff

All staff working in the study (clinical and laboratory) underwent biological safety health screening by Imperial College and specific training in the handling of respiratory viruses and material likely to be infectious to humans. Risk assessments for all procedures were approved by the College safety department. All live RSV inoculation procedures (see section 3.4) were carried out by the study doctor in the negative pressure procedure room in the ICCRU. Personal protective equipment (PPE) comprising of visor, face mask (FFP2), gloves, and liquid-impervious gown were used during inoculation. Following inoculation, subjects were instructed to wear a standard surgical face mask and wash their hands to minimise the risk of contamination of the immediate environment with infected respiratory secretions. During all study visits and sampling procedures for 10 days following inoculation, the same PPE was used by staff as for inoculation.

3.2.2.3 Protection of the community

Subjects deliberately infected with RSV would be expected to develop colds and shed infectious virus, thus be contagious. Since it was especially important that the risk of someone not directly

involved in the research becoming infected should be minimised, all subjects were quarantined from the day before to the tenth day after viral inoculation. Previous rhinovirus challenges have been performed on ambulant out-patients (Message et al. 2008), and RSV challenge studies carried out in the early 1990s in the department of Respiratory Medicine at St. Mary's Campus were on staff who continued to work and live at home (Cherrie 1992).

More recent studies have used private residences, hotels or dedicated quarantine facilities. In this study, a 5-bedroom self-contained flat in Imperial College student residences (Wilson House, Paddington, London) was used. Subjects were housed in groups of four and had their own bedroom, but a kitchen, bathroom, and recreation room shared with the other study subjects. There was to be no routine contact with resident staff or other residents although other residents were informed that the study was taking place. A domestic support worker provided all necessary cleaning duties during the stay and assisted with delivery of food and drink from local caterers. This worker was also trained to use appropriate PPE (see section 3.2.2.2).

It should be noted that such confinement offers only partial containment, and is only justified if environmental release of the pathogen is unlikely to cause serious harm. Subjects could (but did not) withdraw from the study at any time and no legal power existed to prevent them leaving the facility. In such an eventuality, the possibility of onward sustained community transmission was deemed unlikely due to the following additional measures:

- The study was conducted in the summer months, outside usual RSV season.
- Subjects had no regular contact with persons vulnerable to severe RSV disease.
- After the period of confinement, subjects were instructed to avoid ALL close contact with elderly persons, children under 12 months, those with chronic lung disease, or the immunocompromised, until the end of the study (day 28 post viral challenge).

- In the event of a subject withdrawing from the study during the confinement period, or becoming symptomatic late into the confinement period (i.e. possibly being secondarily infected from another subject), a supply of face masks and alcohol gel would be issued and strict advice given regarding isolation at home until the tenth day post viral inoculation or symptoms subside, as appropriate.

These measures were agreed by the study investigators and the regulatory committees to sufficiently minimise the potential risk of transmission to others.

3.2.3 Sampling protocol

Subjects attended for a baseline 'healthy' visit one to fourteen days (nominally day −14) prior to planned inoculation (day 0). Subjects then entered quarantine the day before inoculation where they remained for eleven nights (day 0 to +10). During this period they were visited daily by the study doctor and nurse who undertook daily sampling from the subjects' respiratory tracts and less frequent sampling of the peripheral blood. On the tenth day (day +10), after sampling was complete subjects were allowed to leave quarantine but were advised to self-isolate at home until the study finished. Subjects returned at day +14 and +28 for further sampling of the respiratory tract and blood. Further details of the sampling protocol are shown in Figure 3.1.

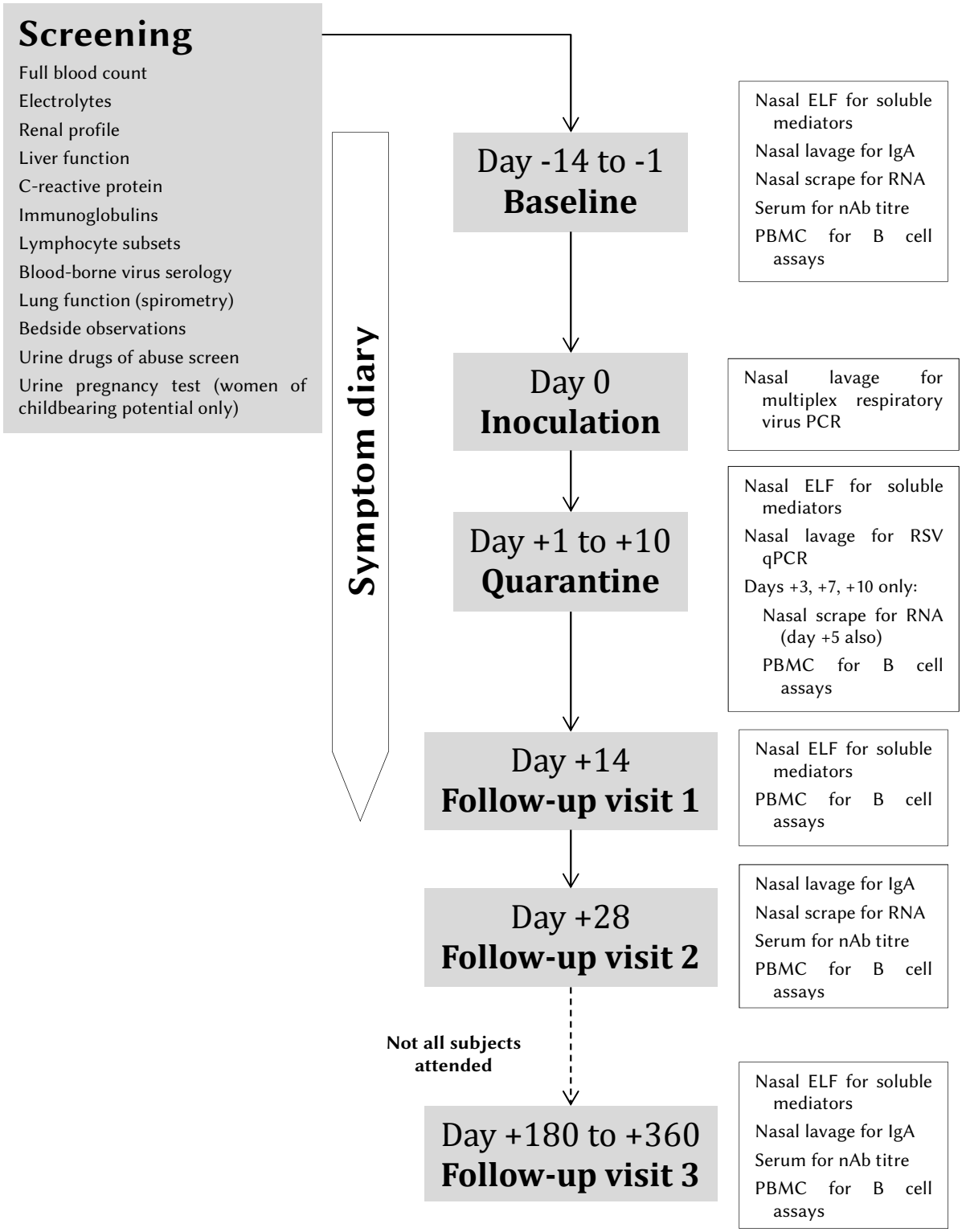


Figure 3.1 Study design, subject visits, quarantine and timing of samples. ELF = epithelial lining fluid, nAb = neutralising antibody.

3.3 Study enrolment

Logistical factors (availability of quarantine flat, numbers of staff, safety) dictated that the challenge studies be conducted during the period June – September in 2011 and 2012. Twelve subjects were enrolled in 2011 and twenty-eight subjects in 2012. Subjects were in groups of four to optimise use of the quarantine flat. Twelve subjects in 2012 were part of an RSV challenge study supervised by Dr. Christopher Chiu (subject numbers beginning 'RF'), with which the author of this thesis assisted with inoculation, sampling and assays. The study protocol for these subjects was identical except that they also underwent bronchoscopy (after nasal sampling) on days -28, +7, and +28.

3.3.1 Recruitment

Subjects were recruited by direct email from previous lists of research study participants, advertisement of ICHNT website, registration on clinicaltrials.gov website (indexed by specialist research study search engines), and by word of mouth from those already screened. Interested subjects were given the study participant information sheet and details of the remuneration for participation (£1000). Such methods proved highly effective and straightforward to obtain suitable volunteers. Newspaper advertisements, although prepared and approved, were not required.

3.3.2 Screening

Subjects attended a screening visit at the ICCRU where they were seen by the study doctor and research nurse for a brief interview and medical examination to determine their suitability for enrolment. Following an opportunity to discuss the study and address any questions they may have had, written informed consent was obtained. Questions were asked about their current and previous health, lifestyle, and social and domestic circumstances. A physical exam and bedside observations (temperature, pulse, blood pressure, and oxygen saturation) were performed. Lung function tests (spirometry) were obtained and blood for full blood count, electrolytes, renal function, liver function, coagulation, C-reactive protein, immunoglobulin subsets, lymphocyte subsets, and blood borne virus serology (HIV, hepatitis B, hepatitis C) was taken. A urine test for drugs of abuse was performed for all subjects and women of childbearing potential underwent a pregnancy test.

3.3.2.1 Inclusion criteria

Healthy persons aged 18 to 55 years, able to give informed consent, were eligible for the study.

3.3.2.2 Exclusion criteria

Any significant medical or psychological condition or medication deemed by the study doctor to make the participant unsuitable resulted in exclusion. Specifically, volunteers with any of these conditions were excluded:

- Chronic respiratory disease (asthma, COPD, rhinitis, sinusitis) in adulthood
- Inhaled bronchodilator or steroid use within the last 12 months
- Use of any medication or other product (prescription or over-the-counter) for symptoms of rhinitis or nasal congestion within the last 6 months
- Acute upper respiratory infection (URTI or sinusitis) in the past 6 weeks
- Smoking in the past 6 months OR > 5 pack-year lifetime history
- Subjects with allergic symptoms present at baseline
- Those in close domestic contact (i.e. sharing a household with, caring for, or daily face to face contact) with children under 3 years, the elderly (> 65 years), immunosuppressed persons, or those with chronic respiratory disease
- Subjects with known or suspected immune deficiency
- Receipt of systemic glucocorticoids (in a dose ≥ 5 mg prednisone daily or equivalent) within one month, or any other cytotoxic or immunosuppressive drug within 6 months prior to challenge
- Known IgA deficiency, immotile cilia syndrome, or Kartagener's syndrome
- History of frequent nose bleeds
- Pregnant or breastfeeding women
- Positive urine drug screen

3.3.2.3 Screening case report form

To ensure consistency and accurate record-keeping, screening visits were conducted using a pre-printed case report form (see Appendix).

3.3.2.4 Outcome from screening

The target for enrolment was twelve subjects in 2011 and twenty-eight subjects in 2012. To achieve this, twenty-three subjects were required to be screened in 2011 and sixty-three in 2012. The outcome from screening and reasons for exclusion are shown in Figure 3.2.

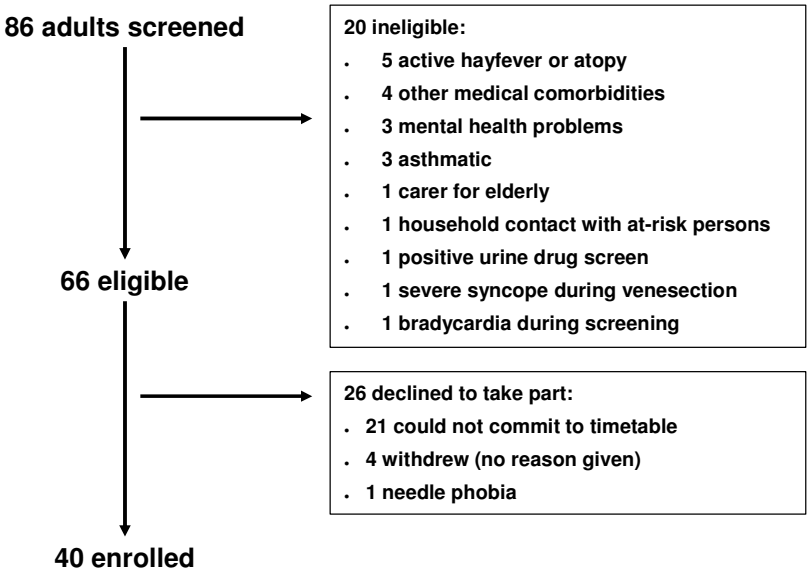


Figure 3.2 Outcome from screening and reasons for exclusion

3.3.3 Characteristics of study subjects

Forty subjects were enrolled, twenty-five were male, median age was 21 (range 18 – 50) years.

3.4 Virus inoculation

3.4.1 Viral inoculum

The RSV stock used for inoculation was designated RSV A Memphis 37 and was purchased from Meridian Life Sciences (Memphis, TN, US). The virus was isolated from a child with severe RSV

infection in the US in 2007 and manufactured according to current Good Manufacturing Practice (cGMP) by expanding the plaque purified virus through 5 passages in clinical grade qualified Vero cells (WHO 10-87) in Meridian Life Science's cGMP biomanufacturing suite. This process was conducted according to cGMP as defined in Title 21 of the United States Code of Federal Regulations, Parts 210 and 211, as applicable to the manufacture of Phase I clinical trial material. The material was then tested according to the appropriate US Food and Drug Administration (FDA) guidance documents for the production of a human clinical vaccine product. Briefly, from the investigator's brochure, the identity of the inoculating virus (RSV) was confirmed by an immunofluorescent antibody assay, electron microscopy, and N-gene sequencing. It was determined at several steps in the selection and manufacturing process to be free of adventitial agents and human pathogens by four methodologies:

- Twenty-eight day culturing in 5 indicator cell lines (MRC-5, Vero, MDBK, HeLa, MEF) while under specific RSV neutralisation conditions (high concentrations of RSV-specific monoclonal antibody, palivizumab. Routine cytopathic effect observations, haemabsorption and haemagluttination at 14 days were performed, followed by blind passage and identical repeat evaluations after an additional 14 days (Bioreliance Inc. Rockville MD, USA).
- Product-enhanced reverse transcriptase assay for the general detection of retrovirus reverse transcriptase (Bioreliance Inc. Rockville MD, USA)
- An expansive series of individual PCR assays designed to detect human pathogens
- Electron microscopy

The M37 virus inoculum was presented in 1 mL vials diluted in DMEM with 25% w/v sucrose. The titre of the virus was reported to be 3×10^{6} PFU mL^{-1} by plaque assay in Vero cells by the manufacturers.

3.4.2 Preparation of inoculum

Prior to commencement of challenge studies in June 2011, the titre of the M37 stock held in −80 ℃ freezers at Imperial College was determined to be 3.9 $\log_{10}$ (approximately 8000) PFU mL^{-1} by plaque assay in HEp-2 cells (see section 2.1.10.3). The volume of M37 used for inoculation was chosen to be 1 mL (thus approximately 3.9 $\log_{10}$ PFU) for convenience and based on a published consistent infection rate of 75-80% using doses ranging from 3 – 6 $\log_{10}$ PFU mL^{-1} (DeVincenzo et al. 2010b). On the day of inoculation, the required number of vials of M37 were removed from −80 ℃ freezers and placed on dry ice for transport to the ICRRU. The vials were placed inside a class 2 microbiological safety cabinet (MSC) in the ICCRU laboratory and thawed on wet ice. In order to minimise any vial to vial variation, the contents of all vials were combined in a 5 mL tube using a 1 mL pipette and the required number of 1 mL aliquots made in individual 1.7 mL microfuge tubes and placed on wet ice until inoculation. To maximise viability of the inocula, the time from thawing to completion of inoculation was restricted to less than thirty minutes.

3.4.3 Inoculation technique

Subjects were asked if they were feeling fit and well and briefly examined by the study doctor prior to inoculation. A single nasal lavage sample was taken to be used for respiratory virus multiplex PCR to exclude coincident infection (performed by Department of Infection and Immunity, Charing Cross Hospital, Imperial College Healthcare NHS Trust). Subjects were then positioned semi-recumbent on an examination couch with the back rest angled at approximately 45°. The viral inoculum was administered drop by drop directly into alternate nostrils using a sterile 1 mL pipette over a period of two minutes. Subjects were instructed to sniff gently at regular intervals during inoculation.

3.4.4 Titration of inoculum by plaque assay

To determine and ensure consistency of the dose used for inoculation, the combined inocula were titrated by plaque assay in HEp-2 cells on each inoculation occasion. The day prior to inoculation, confluent HEp-2 monolayers in 96-well tissue culture plates were prepared according to

section 2.1.10.3. Prior to making individual aliquots but after mixing of virus vials, 200 µL of viral inoculum was transferred to a 1.7 mL microfuge tube and placed on wet ice. This aliquot was immediately transported back to the main laboratory and inoculated into the HEp-2 cells within thirty minutes of original thawing and the procedure for plaque assay was followed (see section 2.1.10.3). The results of the titration for each inoculation day are shown in Figure 3.3. The mean titre over all inoculation procedures was 3.8 $\log_{10}$ PFU mL^{-1}.

3.4.5 Inoculation case report form

Details of the inoculation procedure were recorded on an inoculation case report form (see Appendix).

3.5 Sampling methods and processing

Prior to commencement of challenge studies in 2011, methods for sampling and sample processing were recorded in a Standard Operating Procedure document, distributed to all staff involved in the study. Techniques were practiced on willing volunteers from the laboratory including (primarily) the author.

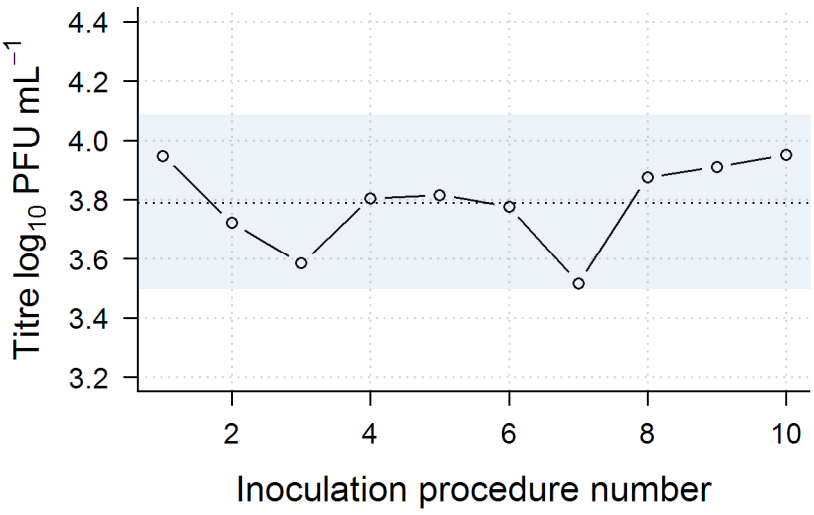


Figure 3.3 Consistency of inoculum titre between inoculation days. Subjects were inoculated in groups of 4 requiring 10 occasions in total. An aliquot of the pooled inocula was titrated by plaque assay on each occasion. Dotted line is the geometric mean titre of all the pooled inocula, shaded region represents ± 2 standard deviations.

3.5.1 Clinical data collection

All clinical data was recorded on the study case report form (see Appendix).

3.5.1.1 Daily symptom score

Subjects recorded daily upper respiratory tract symptom scores according to a modified Jackson score (Jackson et al. 1958; Jackson, Dowling, and Muldoon 1962) into a pre-printed symptom diary issued to them at their first study visit (day −14). Symptoms were recorded at day −14 and days +0 to +14 inclusive. Eight symptoms were assessed: sneezing, headache, fatigue, fever, nasal discharge, nasal obstruction, sore throat, and cough. Symptoms were graded 0 – 3: 0 = absent; 1 = mild – present, but does not affect normal daily activities; 2 = moderate – some interference with or limitation of normal daily activities; 3 = severe – prevents some normal daily activities. Each subject was advised to record symptoms at the same time towards the end of the day to reflect how they felt on that particular whole day. An example symptom diary can be found in the Appendix.

3.5.1.2 Infection definition

Infection was pre-defined as RSV detectable in the nasal lavage on two consecutive days between day +2 and day +10 by either PCR or culture. The inoculating virus could theoretically be detected by PCR on day +1, thus given the known incubation period of approximately three days, it was decided that detection on day +1 did not count toward the definition of infection.

3.5.1.3 Clinical cold

Subjects were asked each day if they thought that they had a cold. A clinical cold was defined as 2 out of 3 of: a cumulative symptom score over the 14 day period of ≥ 14; nasal discharge present for ≥ 3 days; a subjective feeling of a cold. These criteria are based on the findings of Jackson *et al*, with modification to take into account the longer incubation period and less abrupt onset of symptoms following RSV infection compared with rhinovirus. Subjects were deemed only to have a cold if they were subsequently confirmed as RSV infected.

3.5.1.4 Daily observations

At each study visit and for 10 days following viral inoculation, observation of vital signs (temperature, pulse, blood pressure, oxygen saturation) and measurement of lung function (peak expiratory flow rate and forced expiratory volume in 1 second) were performed. Results were recorded on pre-printed laminated forms in the subjects' rooms and later transcribed into the case report form.

3.5.2 Nasal samples

All nasal sampling was documented on the pre-printed laminated daily sample case report form in the subjects' rooms. Pre-made pre-labelled sampling and processing packs were produced with all the required materials to streamline the process and minimise errors. The order in which nasal samples were collected is presented here and is critical as it is theoretically possible for one sampling technique to interfere with the performance of another. For example, performing a nasal lavage prior to nasal epithelial lining fluid sampling may result in an overly dilute epithelial lining fluid sample.

3.5.2.1 Nasal epithelial lining fluid collection

Pre-cut strips of Leukosorb (Pall Life Sciences, Portsmouth, UK) were adapted by Dr. Trevor Hansel, Imperial College London, to obtain samples of epithelial lining fluid from the nasal mucosa and other sites for the analysis of soluble mediators of inflammation (Chawes et al. 2010; Nicholson et al. 2011). The following procedure was used. A refrigerated microcentrifuge (Eppendorf 5415R) was pre-cooled to 4 ℃. Centrifuge tubes with filter cups (Costar Spin-X, 0.22 μm pore size, cellulose acetate membrane, CLS8161) were labelled with study ID and date. The filter cup was removed and the weight of empty tube recorded (E). The filter cup was replaced and 280 μL of elution buffer was added to the cup. For practical purposes, the procedure thus far was carried out in the ICCRU laboratory. The subject was placed in a sitting position with the neck extended. The Leukosorb strip was inserted into the nostril using sterile blunt forceps and placed on the lateral wall of the nasal cavity to overly the inferior nasal turbinate. A nasal clip or finger pressure was applied for two minutes. The now moist strip was removed and placed into the filter cup containing elution buffer. The tube was capped and placed on ice. On return to the laboratory, tube was centrifuged at 16,000 × *g* for ten minutes at 4 ℃. The filter cup and dry strip was discarded, filtered eluate retained in the tube, and the combined weight recorded (F). The volume of recovered fluid was estimated by subtracting E from F (assuming a density of 1 kg m^{-3}). Aliquots of 130 μL were made and frozen at −80 ℃.

3.5.2.2 Nasal lavage

Samples of nasal lavage were obtained for determination of RSV infection by culture and quantitative PCR (qPCR) and for nasal antibody measurement. A sterile 10 mL syringe was filled with 5 mL sterile 0.9% sodium chloride and fitted with a 20 mm metal nasal olive (Clinical Engineering, Royal Brompton Hospital). The subject was seated with the chin tucked into the chest and asked to hold the syringe and olive firmly in one nostril whilst occluding the other. The syringe was flushed gently ten times over one minute. Aliquots (1 mL) of lavage were made for RSV culture and qPCR. Aliquots for culture were transported immediately to the laboratory on wet ice and inoculated into confluent HEp-2 monolayers as in section 2.1.10.3. Aliquots for qPCR were

immediately frozen on dry ice and subsequently stored at −80 ℃. On day 0 and +7, a 0,5 mL aliquot was taken for multiplex PCR (performed by Department of Infection and Immunity, Charing Cross Hospital, Imperial College Healthcare NHS Trust). The remaining lavage was transferred to a 15 mL polypropylene tube and placed on wet ice before transport to the laboratory. The tube was centrifuged at 400 × *g* for ten minutes at 4 ℃. Aliquots of 500 µL of the supernatant were made and frozen at −80 ℃ for subsequent nasal antibody measurement.

3.5.2.3 Nasal epithelial scrape for RNA

Single nasal epithelial scrape samples were taken on day 0 and days +3, +5, +7, +10, and +28 post-inoculation using the ASI-Rhinopro nasal curette (Arlington Scientific). Samples were immediately placed into 800 µL TRIZol reagent (Life technologies) for lysis and vortexed thoroughly before storage at −80 ℃. Analysis of nasal RNA is not discussed in this thesis.

3.5.2.4 Nasal sample processing case report form

Details of each nasal sample processed were recorded on a case report form. An example can be found in the Appendix.

3.5.3 Peripheral blood samples

All peripheral blood sampling was documented on the pre-printed laminated daily sample case report form in the subjects' rooms. Pre-made processing packs were produced with all the required materials to streamline the process and minimise errors. One plain 6 mL tube with clot activator (BD Vacutainer 367837) and variable numbers according to requirements of glass 10 mL sodium heparin (NH) tubes (BD Vacutainer 368480) were taken each day.

3.5.3.1 Serum

Serum was obtained from whole blood via clotting at room temperature for thirty minutes followed by centrifugation at 400 × *g* for ten minutes at room temperature. Aliquots of 1 mL were made and stored at −80 ℃.

3.5.3.2 Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were obtained from heparinised whole blood within four hours of collection using Ficoll PAQUE PLUS (GE Healthcare) by the following method optimised for large volumes of blood and rapid processing. Prior to sample collection, one sterile 50 mL polypropylene tube for every two 10 mL NH tubes was labelled and 20 mL of sterile D-PBS was added to each. Two sterile 50 mL polypropylene tubes for every three 10 mL NH tubes were labelled and 15 mL of ficoll was added to each tube. Using aseptic technique, 20 mL of heparinised blood was transferred to each tube containing D-PBS and mixed thoroughly using a pipette. The blood–D-PBS mixture was then carefully layered on top of the ficoll using an automatic pipette under gravity. This was repeated for all blood samples and then the ficoll tubes were centrifuged at 400 × *g* for thirty minutes at room temperature with the brake off to produce a separation gradient.

The middle white 'buffy coat' was carefully removed using a 3 mL Pasteur pipette and transferred to a new 50 mL polypropylene tube containing 10 mL of sterile PBS + 2% FCS (P2). Each Pasteur pipette was only used once – reuse of pipettes was found to produce unacceptable degrees of platelet contamination of PBMC. After removal of all buffy coats, the tubes were topped up to 50 mL with P2, ensuring a minimum 1:1 ratio of P2:ficoll-buffy coat mix. Insufficient dilution of ficoll resulted in poor yields of PBMC as they did not efficiently pellet in high concentrations of ficoll at the *g*-force used. The tubes were then centrifuged at 150 × *g* for ten minutes at room temperature and the ficoll-rich supernatant discarded. The pellets were resuspended in 30 mL P2 and centrifuged as before. This was repeated thrice more for a total of four washes to remove serum contamination. Cell count and viability was determined by Trypan blue dye exclusion using the Countess device (Life Technologies). Fresh PBMC were then used as per requirements (see section 2.2.5). Remaining PBMC were centrifuged as before, resuspended in 90% FCS / 10% DMSO at a concentration of $10 - 15 \times 10^6$ PBMC mL^{-1}, and 1 mL aliquots were made in labelled cryovials. These were transferred to a freezing container (Mr. Frosty, Nalgene) and kept overnight at –80 ℃ before transfer to liquid nitrogen for long term storage.

3.5.3.3 Peripheral blood RNA

Two Tempus RNA tubes (Life technologies) were filled with blood to indicated capacity on day 0 and days +3, +7, +10, and +28 post-inoculation and frozen at -80 ℃. Analysis of blood RNA is not discussed in this thesis.

3.5.3.4 Peripheral blood case report form

Details of each peripheral blood sample processed, including numbers and viability of stored PBMC, were recorded on a case report form. An example can be found in the Appendix.

3.6 Adverse events

No serious adverse reactions to the challenge virus or study procedures occurred. One unexpected adverse reaction occurred: one subject developed a generalised non-itchy papular rash the day following viral inoculation but was otherwise completely well. The rash resolved over five days without complications. Subjects experienced varying degrees of upper respiratory tract illness following inoculation (discussed in Chapter 4), none unusually severe and none requiring any specific treatment or necessitating withdrawal from the study. All subjects tolerated quarantine and the study procedures. No subject withdrew from the study after enrolment. No subjects were withdrawn from the study by the study doctor for any reason. No staff member experienced symptomatic upper respiratory tract illness during the study. To the author's knowledge, there was no breach of quarantine or containment of the study virus and no secondary infections occurred.

3.7 Discussion

Recruitment of healthy adult volunteers for a study involving an eleven night stay in quarantine was surprisingly straightforward, the primary reason for refusal being unable to commit the time. The motives for volunteering for paid medical research are diverse, but studies of this sort inevitably attract those whose financial need exceeds their concern for their health and wellbeing. The exclusion criteria and demands of the study dictated that mainly young people with no conflicting

work or family commitments were recruited, and the majority of those enrolled were students. Any persons who were deemed as possible to suffer harm from RSV infection were specifically excluded.

The stock of M37 used had a consistent titre, albeit much lower than that reported by the manufacturers. It seems likely that either the virus had degraded since manufacture, or the methods used for determination of viral titre perform differently. Meridian Life Sciences reported on a 36 month stability study during the period in which this research project was carried out. Titre of aliquots from the same stock as used at Imperial College (lot 070504, manufacturing date 12 June 2007) declined from 2.9×10^6 PFU mL^{-1} to 1.09×10^6 PFU mL^{-1} by 6 July 2010 despite being stored unopened continuously below −65 ℃. The decline in titre over time would be expected to follow an exponential decay, i.e. the rate of decay will be proportional to the titre and thus slow down with time, so this does not fully explain the discrepancy in titre.

The titre determined by plaque assay is a function of the absolute number of live virus particles present, the relative infectivity of those particles to the cells used, and the permissibility of those cells to the particular virus. RSV M37 is produced in Vero cells. It is possible that the determined titre in Vero cells, within which RSV M37 would be expected to replicate most efficiently, is higher than that determined in HEp-2 cells. One group has reported that RSV A2 grown in Vero cells has a truncated G protein, rendering viral G and host glycosaminoglycan interaction (which may be important for HEp-2 infectivity) inefficient (Kwilas et al. 2009). In support of this, Prof. DeVincenzo of University of Tennessee, has stated that the titre of RSV M37 in HEp-2 cells in his laboratory was ~16000 (4.2 $\log_{10}$) PFU mL^{-1} (personal communication), although whether RSV M37 actually has a truncated G protein has not been determined. Whilst it is reassuring that another group has independently found a similar titre for identical lots of RSV M37, the stability of GMP-manufactured RSV is concerning. The costs of producing these agents are considerable and the potential commercial market small, thus the time to recoup the cost of development will be long. A shelf life of a few years may make development of future agents and thus subsequent challenge studies prohibitive.

In summary, healthy adults can readily be recruited for viral challenge studies, even when they are required to be resident. The stability of GMP viral challenge agents is vital and short shelf lives may erode the advantages of their use. Experimental challenge of healthy adults with RSV M37 is safe and can be reasonably carried out without the use of purpose-built quarantine facilities.

4 Clinical and virological responses of inoculated volunteers

4.1 Introduction

Previous experimental human RSV infection studies have used strains isolated in the 1960s e.g. Long and A2. Whilst both of these strains have been well-characterised at the genetic and protein level and A2 has been extensively studied in animal models, both have been passaged in laboratory cell culture therefore lots may be heterogeneous and have lost virulence (Lee et al. 2004). RSV A Memphis 37 (M37) was used in this project as it exists as a near-identical stock derived from a single plaque (clone) of virus produced according to Good Manufacturing Practice (see section 3.4.1). A prior published human challenge study using an identical lot of this exact virus demonstrated it to be infectious and capable of causing typical upper respiratory tract illness in approximately three-quarters of those inoculated (DeVincenzo et al. 2010b). However, this study pre-selected the subjects based on low serum RSV neutralising antibody titres. Those persons with especially low serum RSV neutralising antibody titres have been previously demonstrated to be at higher risk of hospitalisation from RSV infection (Falsey et al. 2005). Furthermore, low serum RSV neutralising antibody in otherwise healthy adults may be indicative of a more generalised RSV-specific immunological naivety or deficiency. Thus the infection rates and induced disease in this especially susceptible population may not be representative of an unselected population in whom RSV-specific immune responses would be expected to be generally more vigorous. In order therefore, to better understand the human immune response to RSV, it was necessary to conduct the experimental infection in an immunologically unselected population.

4.2 Outcome from challenge

Forty subjects were enrolled and inoculated intranasally with an equivalent dose (8000 PFU) of RSV M37. All subjects completed the study as planned and no serious adverse reactions occurred.

4.2.1 Infection rates and colds

Twenty-four (60%) subjects were confirmed infected by qPCR of nasal lavage. Of these, seventeen (71%) developed clinical colds as previously defined (see section 3.5.1.3). Seven (29%) subjects therefore were confirmed infected but did not develop the typical symptoms of a cold. Furthermore, sixteen (40%) subjects received the same dose of live RSV yet did not become infected. Thus three outcomes following virus inoculation are defined: infected, clinical cold; infected, asymptomatic; and uninfected. There were no significant differences in infection rates or colds between subjects enrolled in 2011 and 2012. Subject characteristics age, gender, and ethnicity had no observed effect on infection rates or colds. These results are summarised in tables Table 4.1 and Table 4.2.

	2011		2012		Total		W | χ^2	p
	N		N		N			
Number of subjects enrolled (% of total for both studies)	12	30%	28	70%	40	100%	5.6	0.02
Infected (% of subjects for that year)	8	75%	16	57%	24	60%	0.04	ns
Clinical colds (% of subjects for that year)	4	33%	13	46%	17	43%	0.18	ns
As proportion (%) of infected	4/8	50%	13/16	81%	17/24	71%	1.24	ns
Asymptomatic infection (% of subjects for that year)	4	33%	3	11%	7	18%	1.62	ns

Table 4.1 Infection rates and outcome for the 2011 and 2012 challenge studies. Mann-Whitney-Wilcoxon test (W) or Chi-squared (χ^2) test statistics and p-values for 2011 vs. 2012 are shown as appropriate (ns = $p > 0.05$)

	Uninfected		Asymptomatic, infected		Clinical cold		W | χ^2	p
Number of subjects (% of total)	16	40%	7	17.5%	17	42.5%	4.55†	ns
Median age (range)	22	19–39	20	18–27	20	18–50	1.46	ns
Gender (M : F)		10 : 6		4 : 3		11 : 6	0.12	ns
Ethnicity (% with each outcome)							7.24⁋	ns
White	14	40%	7	20%	14	40%		
Black	1	100%	0	0%	0	0%		
Asian	0	0%	0	0%	1	100%		
Mixed	1	100%	0	0%	0	0%		
Other‡	0	0%	0	0%	2	100%		

Table 4.2 Subject demographical characteristics and outcome of challenge. Mann-Whitney-Wilcoxon test (W), Kruskal-Wallis (χ^2) test or Chi-squared (χ^2) test statistics and p-values for differences in frequencies of the three outcomes for each characteristic are shown as appropriate (ns = $p > 0.05$). †: χ^2 for probability (asymptomatic = clinical cold = 0.5) = 4.2, $p = 0.04$. ⁋: Chi-squared (χ^2) test of 5 × 3 contingency table of ethnicity and outcome. ‡: Other included two subjects identifying themselves as Chinese.

4.2.2 Upper respiratory tract symptoms

Subjects completed an upper respiratory tract symptom diary on day −14 and days 0 to +14 post-inoculation. Graphical depictions of symptom diaries from subjects demonstrating the three different outcomes are shown in Figure 4.1. The magnitude of symptoms reported, both in terms of peak score recorded and cumulative score over the 14-day period, varied considerably between all subjects. For subjects experiencing clinical colds, the peak of symptoms occurred at a median 7 (IQR 6 – 7) days post-inoculation. Cumulative symptom scores (as AUC) for the 14-day period post-inoculation for each outcome are shown in Figure 4.2. Those subjects experiencing asymptomatic infections did not record significantly different cumulative 14-day symptom scores from uninfected subjects. Cumulative symptom scores of subjects with clinical colds were significantly different from both other outcomes, however this is an artefact resulting from the definition of clinical cold depending on reporting sufficient symptoms. There was considerable variation of the cumulative symptom scores for subjects experiencing clinical colds.

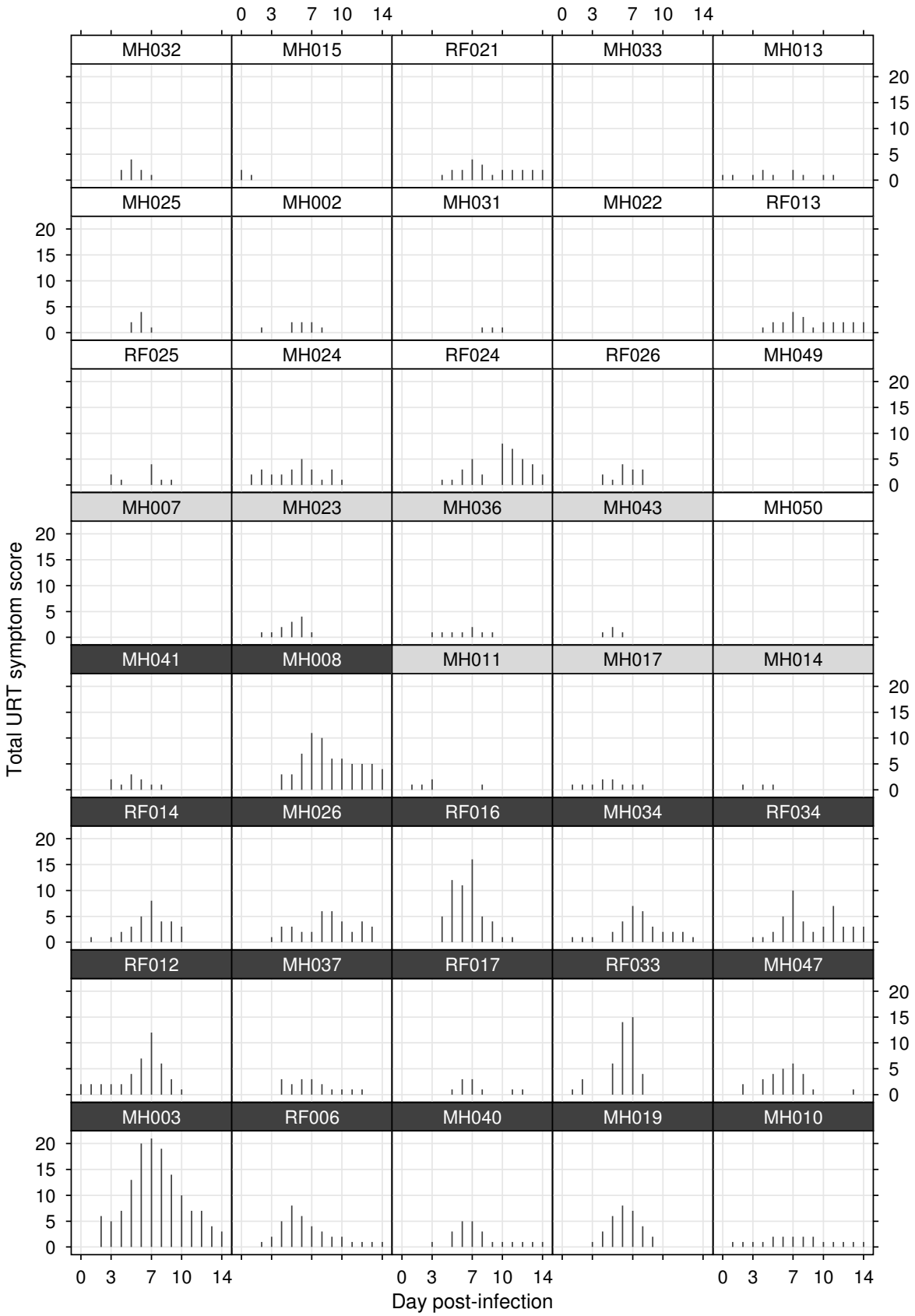


Figure 4.1 Daily total symptom scores for all subjects. Horizontal strips indicate outcome from infection. White: uninfected, light grey: asymptomatic, dark grey: clinical cold.

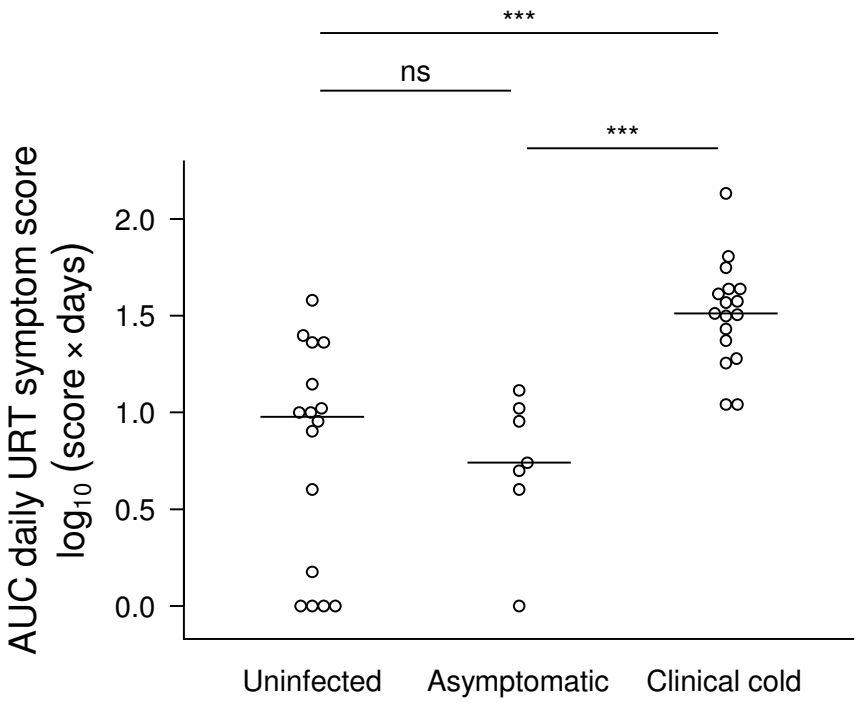


Figure 4.2 Cumulative (trapezoidal area under curve) upper respiratory tract symptom scores for all subjects by outcome of challenge. Symptom scores of 0 were assigned a value of 1 to permit plotting on log scale. Mann-Whitney-Wilcoxon test *p*-values are shown ** $p < 0.01$, * $p < 0.05$, ns $p > 0.05$.

4.2.3 Viral load

Nasal lavage was performed on days −14, +1 to +10 and +14. Viral load was assessed by culture of nasal lavage in HEp-2 cells and by qPCR as described in sections 4.2.3.1 and 4.2.3.2 respectively.

4.2.3.1 Virus detected by culture

It was not possible to perform virus culture on all study days due to resource constraints. During 2011, culture was performed on days +3, +6, and +8. During 2012, additional effort was made and culture was performed for all subjects on days +2 to +8 inclusive. Results are summarised in Table 4.3. Overall, fourteen (35%) subjects had virus detected by culture on at least one day; all were classed as infected and also met the criteria for clinical colds. The sensitivity of culture for infection and clinical cold was therefore 58% and 82% respectively.

	2011		2012		Total		χ^2	p
	N		N		N			
Number of subjects enrolled	12	30%	28	70%	40	100%	5.6	0.02
Days culture performed (total number of days)	+3, +6, +8	3	+2 to +8	7	NA	NA	NA	NA
Culture positive								
As proportion of number performed	3/36	8%	23/196	12%	26/232	11%	0.09	ns
Number of subjects	3	25%	11	39%	14	35%	0.18	ns
As proportion of infected	3/8	38%	11/16	69%	14/24	58%	1.05	ns
As proportion of clinical colds	3/4	75%	11/13	85%	14/17	82%	0	ns
As proportion of asymptomatic infections	0/4	0%	0/3	0%	0/7	0%	NA	NA

Table 4.3 Virus detected by culture. Chi-squared (χ^2) test statistics and p-values for differences between 2011 and 2012 are shown as appropriate (ns = $p > 0.05$).

Although a much greater number of viral cultures were performed in 2012 (196 vs. 36), there were no significant differences in the proportion of positive cultures, the numbers of subjects found to be culture positive, or the sensitivity of culture for the outcome of clinical cold. Although the sensitivity of culture for infection in 2012 was greater at 69% vs. 38% in 2011, this difference was also not significant. No subject experiencing asymptomatic infection had virus detectable by culture. The proportion of positive cultures by day post-inoculation is shown in Figure 4.3.

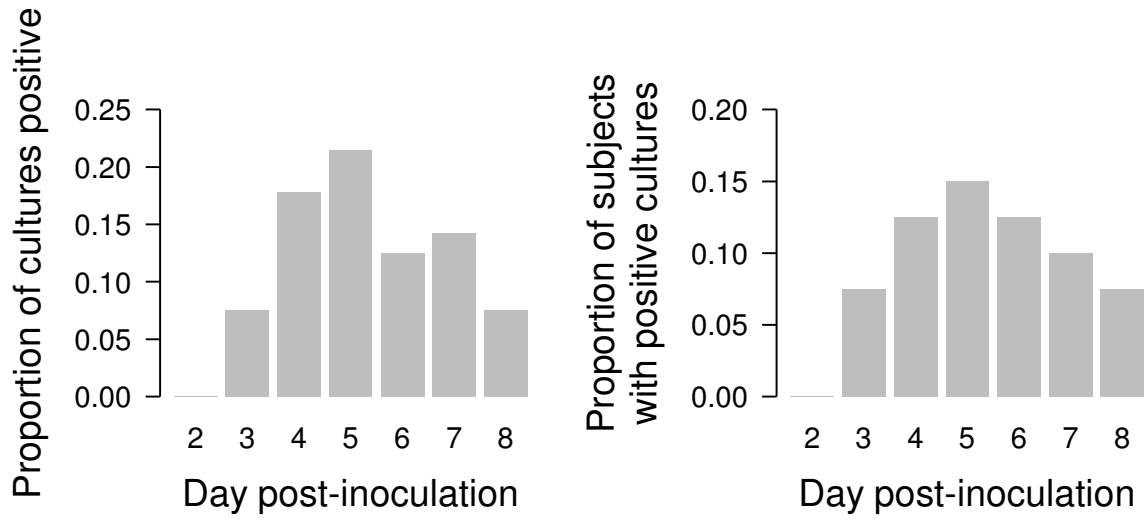


Figure 4.3 Proportion positive of total cultures taken (left) and proportion of subject with positive cultures (right) by day post-inoculation.

Taking cultures on day +5 post-inoculation yielded the greatest proportion of positives (21%) and the greatest proportion of subjects (15%) had positive cultures on this day followed by days +4 and +6 (12.5% each). An attempt was made to quantify viral load by culture as has been done in other published studies however this was not possible in the majority of cases thus aggregate analysis was not performed.

4.2.3.2 Virus detected by qPCR

Virus detection by qPCR for RSV N gene was performed on days 0 to +10 and +14. Graphical depiction of daily nasal viral load is shown in Figure 4.4. Summary statistics are presented in Table 4.4. The median onset of viral shedding as detected by PCR was day 4 (IQR 4 – 5.25) post-inoculation. The peak of nasal viral load occurred at a median 7 (IQR 6 – 8) days post-inoculation. Subjects shed virus as detected by PCR for a median of 5 (IQR 3.75 – 7) days. Subjects experiencing clinical colds shed virus beginning a median one day earlier and for three days longer than those experiencing asymptomatic infections, although the former difference was not significant. Quantity of viral shedding, both in terms of peak and cumulative viral load, were significantly greater in those experiencing clinical colds.

	Asymptomatic infection		Clinical cold		*W*	*p*
	Median	**IQR**	**Median**	**IQR**		
Onset (day post-inoculation)	5	4–6	4	4–5	75.5	ns
Duration of shedding (days)	3	3–4	6	5–7	13	0.003
Peak viral load (log_{10} copies mL^{-1})	2.84	1.49–3.15	3.94	3.34–4.86	25	0.028
Cumulative viral load (AUC log_{10} (copies mL^{-1} × days))	2.99	1.66–3.44	4.10	3.5–4.9	24	0.024

Table 4.4 Nasal lavage viral load by qPCR for asymptomatic-infected subjects and subjects with colds. Mann-Whitney-Wilcoxon test (*W*) statistic and *p*-values are shown (ns = $p > 0.05$).

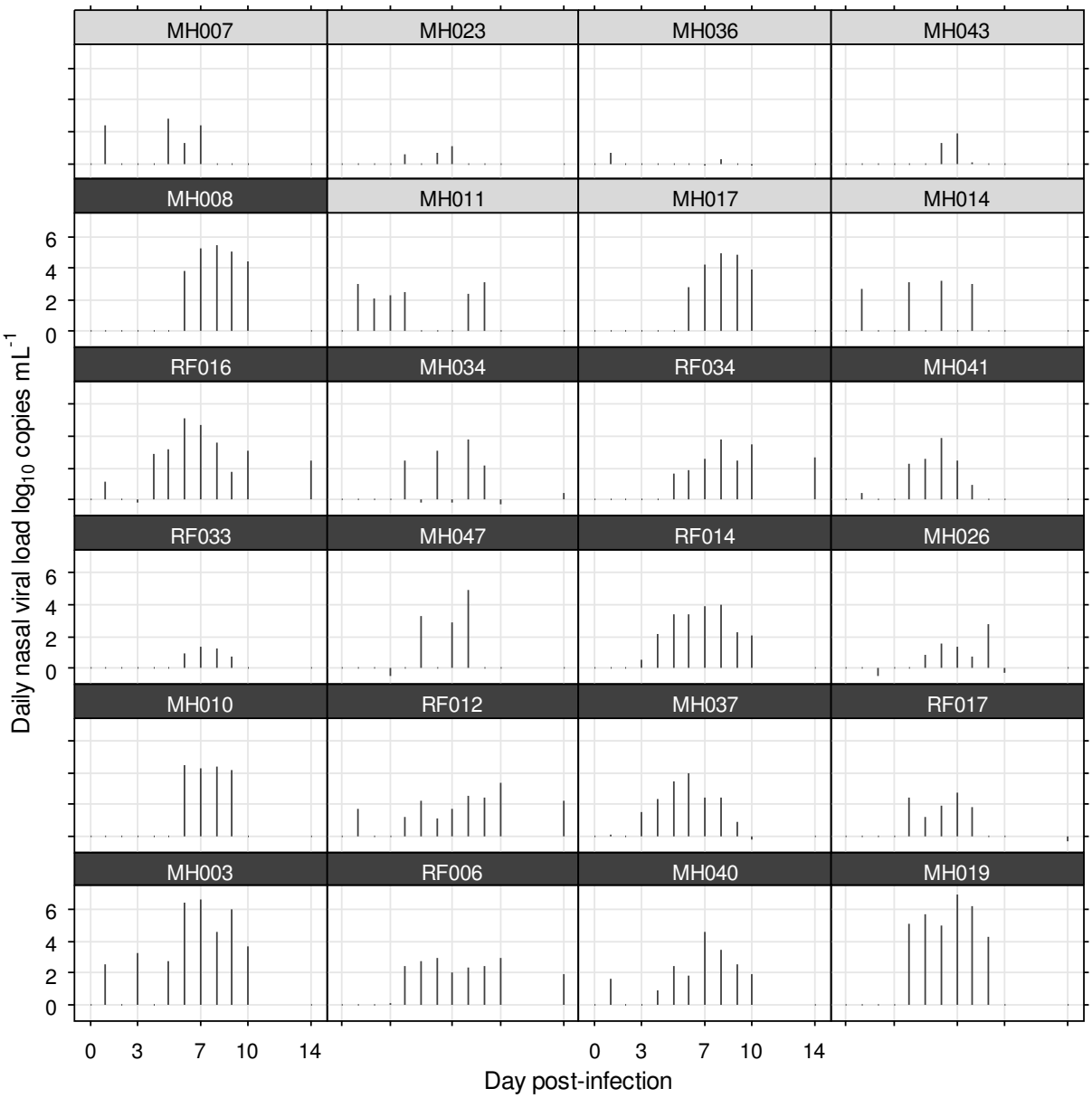


Figure 4.4 Daily viral load for all infected subjects. Horizontal strips indicate outcome, dark grey = clinical cold, light grey = asymptomatic infection.

4.2.4 Dynamics of symptoms and viral load

The mechanisms of RSV-induced airway inflammation in humans are not known. It has been suggested that clinical disease in terms of symptoms are quantitatively related to viral load although this does not indicate causality (Buckingham, Bush, and DeVincenzo 2000; DeVincenzo et al. 2010b). Furthermore, the only experimental infection study to examine this relationship (DeVincenzo et al. 2010b) was conducted in adults selected to have especially low serum neutralising antibody titres in whom symptoms might be unusually strong.

4.2.4.1 Correlation

Figure 4.5 shows the positive correlation between cumulative symptom score and cumulative viral load (both as $\log_{10}$ AUC) for infected subjects. Using any summary statistics like AUC reduces the detail of the data, potentially masking important temporal and between-subject differences in the variables.

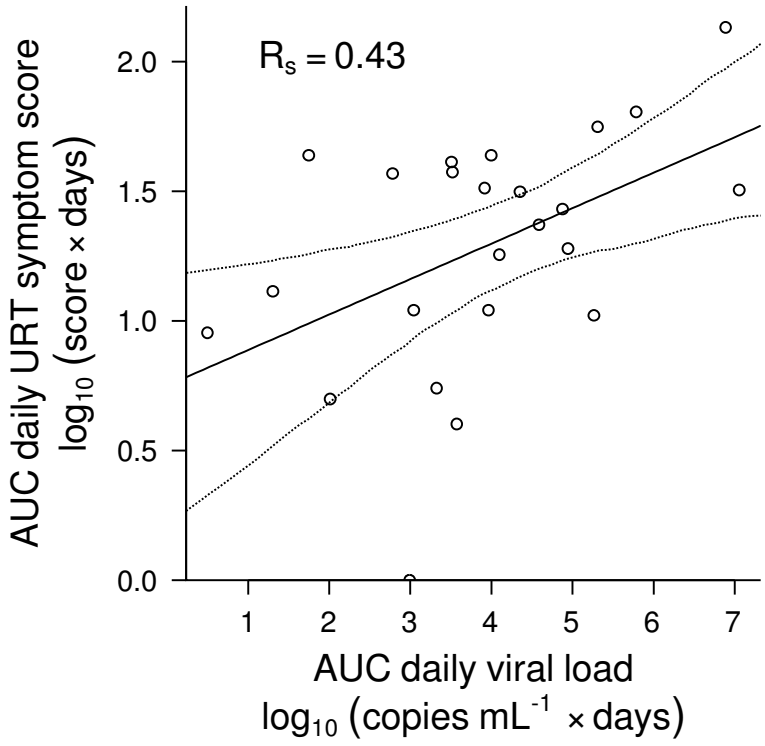


Figure 4.5 Correlation of cumulative daily upper respiratory tract score and cumulative viral load (both log10 area under curve) for infected subjects. Solid line is predicted response from linear model, dotted lines are point-wise 95% confidence intervals. Spearman's rank correlation coefficient (R_s) is shown.

Figure 4.6 shows correlations of daily symptom scores and viral load for each subject. Statistically significant correlation was shown for 15 out of 17 subjects with colds compared with only one asymptomatic subject (MH023).

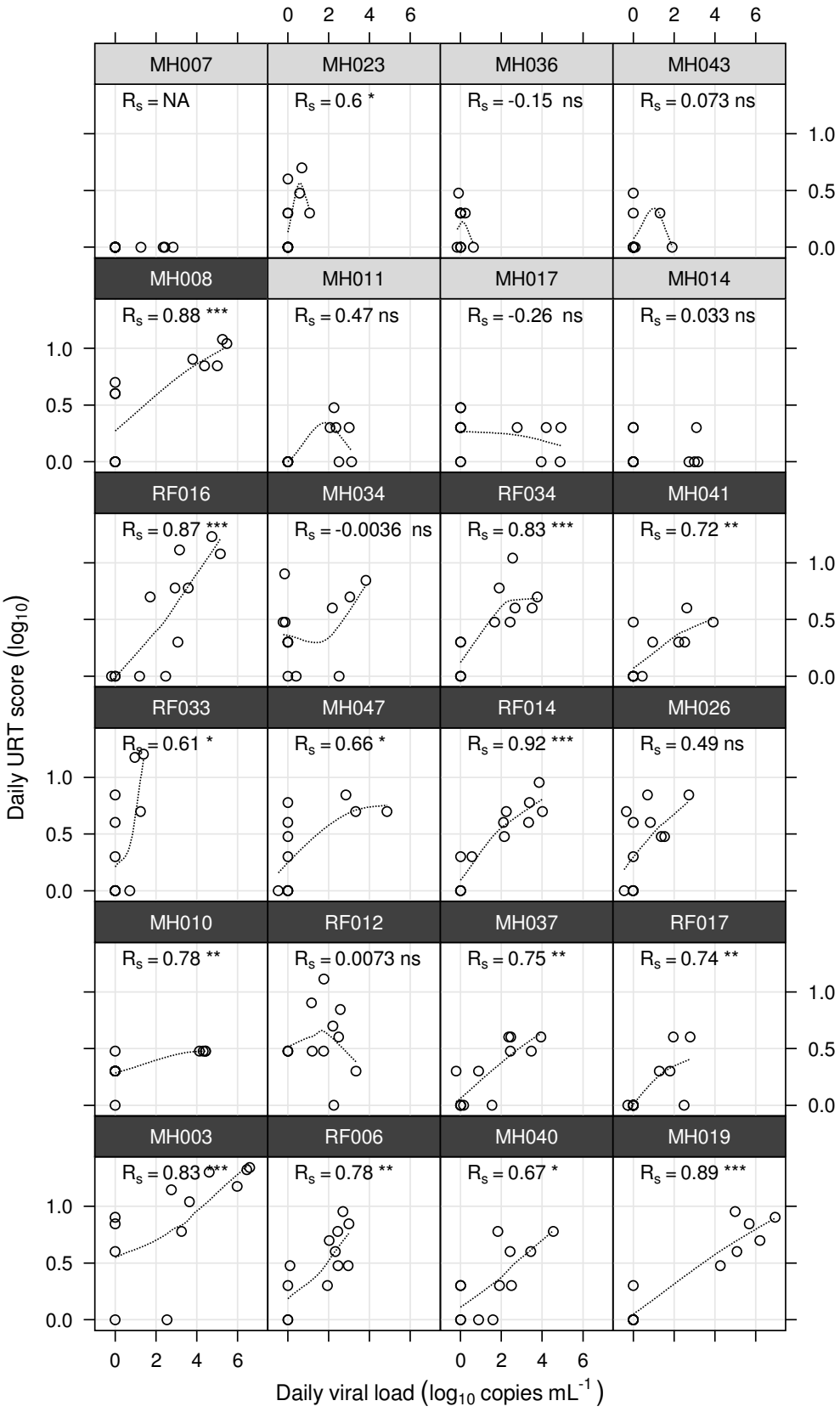


Figure 4.6 Individual correlation plots of daily upper respiratory tract score and viral load (both $\log_{10}$) for each infected subject. Dotted line is loess smooth to aid visual interpretation only. Horizontal strips indicate outcome, dark grey = clinical cold, light grey = asymptomatic infection. Spearman's rank correlation coefficient (R_s) is shown. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns $p > 0.05$.

Each post-inoculation day is considered separately in Figure 4.7. Correlation was significant on days 3, 5, 7, 8, and 9 only and strongest on days 8 and 9, thus later than the median peak of viral load.

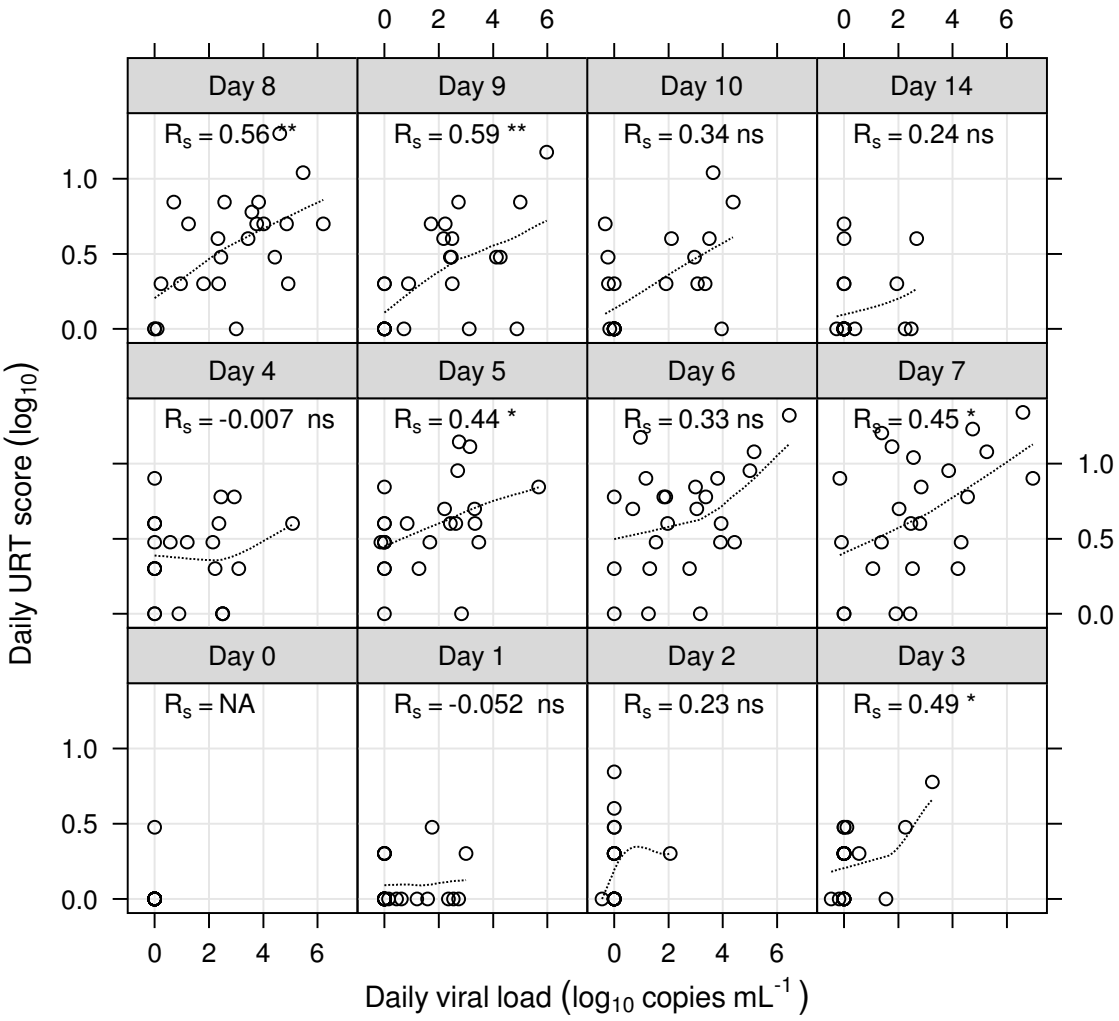


Figure 4.7 Individual correlation plots of daily upper respiratory tract score and viral load (both log_{10}) each day post-inoculation. Dotted line is loess smooth to aid visual interpretation only. Spearman's rank correlation coefficient (R_s) is shown. *** p < 0.001, ** p < 0.01, * p < 0.05, ns p > 0.05.

Figure 4.8 relates the magnitude of the correlation coefficient between cumulative symptoms and viral load with the magnitude of the cumulative symptom score (left) and the cumulative viral load (right). Subjects with greater cumulative symptom scores or viral loads also had the strongest correlation. Thus the observed correlation between symptoms and viral load holds for subjects with

clinical colds only, is confined to a few days post-inoculation only, and is itself dependent on the magnitude of the two variables.

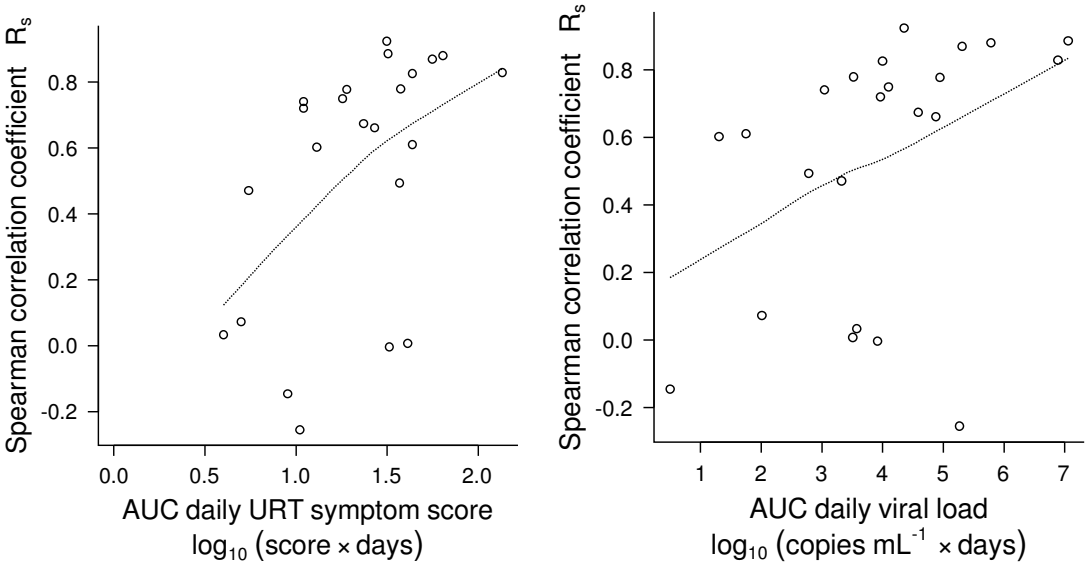


Figure 4.8 Relationship of correlation coefficient to magnitude of symptoms (left) and viral load (right). Dotted line is loess smooth to aid visual interpretation only.

4.3 Discussion

Infection rates in this unselected population were lower than previously published studies involving adults with especially low serum neutralising antibody titres, however were in line with expectations. The majority of infected subjects experienced a typical cold, however a small proportion of individuals became infected but did not experience typical symptoms. These asymptomatic 'subclinical' infections have not been reported following experimental challenge with RSV M37. Notably, none of these subjects had virus identified by culture. As culture was not performed on all days for all subjects, it is not possible to exclude the possibility that some asymptomatic subjects were shedding culturable virus. Alternatively, nasal lavage may not be an efficient method to recover virus from subjects not experiencing a typical cold.

The mechanisms of RSV-induced airway inflammation in humans are not known. It has been suggested that clinical disease in terms of symptoms and length of hospital stay are quantitatively

related to viral load although this does not indicate causality (Buckingham, Bush, and DeVincenzo 2000; DeVincenzo et al. 2010b). Furthermore, the only experimental infection study to examine this relationship was conducted in adults selected to have especially low serum neutralising antibody titres. If viral load were the cause of symptoms, it would be expected that the emergence of virus in nasal secretions would precede the start of symptoms. Whilst the former time point is straightforward to identify, it is not similarly straightforward to define the start of symptoms. The symptom scoring system used was not designed to identify the day when symptoms began or the start of a cold and it is probable that most subjects would not be able to define this exactly. Symptoms by their nature are subjective and imprecise. A statistically significant correlation between cumulative symptoms and viral load was observed and the median peaks of symptoms and viral load by qPCR did coincide. However when subjects and days were examined individually, it became clear that this relationship was driven by those subjects most symptomatic. Given that the recovery of virus depends on shedding in nasal secretions it is not surprising that subjects with the most nasal symptoms, on which symptom scores are heavily weighted, have the greatest viral loads. Thus symptom scores and nasal viral load may in fact be measuring the same process therefore correlation is expected.

How can the fact that some subjects have detectable virus in their nasal lavage by PCR yet experience no apparent symptoms be explained? It may be that some subjects simply do not report symptoms because they did not comply with the study protocol. If this were the case, a significant difference in cumulative viral load between the asymptomatic subjects and those with colds would not be expected. Alternatively, some subjects may genuinely not experience symptoms of a cold but still be infected with RSV. Given that these subjects demonstrated lower overall viral loads it seems plausible that they are experiencing an atypical infection. Possible explanations would be that the inoculation virus was attenuated, failure of inoculation technique leading to insufficient dosing, or that these subjects are able to better control viral replication, limiting subsequent epithelial damage and host inflammation. Given that subjects were inoculated in batches and that inoculating virus

was titrated by plaque assay from the pooled inoculum on each occasion, the former two explanations cannot explain this phenomenon.

Over a third of subjects who were inoculated did not become infected at all, despite receiving a dose ten times higher than the minimum reported infectious dose (Hall et al. 1981) via the nasal route directly into their noses. For reasons stated above, inoculation inconsistency cannot adequately explain this variability. A significant proportion of adults enrolled were completely resistant to experimental RSV infection.

In summary, a spectrum of outcomes occurred following experimental challenge with RSV M37 ranging from uninfected to heavy cold. How some individuals are able to control their virus infection and limit replication and others are apparently completely resistant offers promise for the development of live attenuated RSV vaccines. The mechanisms underlying this resistance to infection and disease will be explored in subsequent chapters.

5 Correlates of protection in inoculated subjects

5.1 Introduction

Humans remain susceptible to reinfection with similar or even identical strains of RSV throughout life (Hall et al. 1991). However, in this study 16/40 (40%) of the inoculated subjects did not become infected. The inoculating virus had a consistent titre and on no occasion did a batch of inoculations fail to induce at least one infection. Thus just under half of subjects were apparently completely resistant to RSV challenge.

The observed protective effect of maternal antibody and the efficacy of palivizumab suggest that serum antibodies can protect against severe lower respiratory tract disease. IgA is generally the most abundant class of immunoglobulin at mucosal surfaces but IgG may be more abundant in the lower respiratory tract (Berneman et al. 1998; Brandtzaeg et al. 1999). Thus serum antibody and IgG may protect the lower respiratory tract but leave the upper respiratory tract vulnerable, hence prevent severe disease (LRTI) but not nasal infection. In support of this, observations of natural RSV infections have found a relationship between serum neutralising antibody and risk of hospitalisation (Piedra et al. 2003; Falsey and Walsh 1998), but there is no defined level of antibody in the serum above which protection from infection is consistent and the correlation between serum neutralising antibody and protection from RSV infection is partial at best (Walsh and Falsey 2004).

Boosted antibody levels in the serum following natural RSV infection decline to pre-infection levels within 6 – 12 months. The reasons for this poor durability are unknown and the mechanisms of RSV antibody generation and maintenance have not been examined in humans under controlled conditions. Antibody levels in serum are thought to be maintained by a population of long-lived plasma cells derived from post-germinal centre (GC) plasma cells that persist in specially adapted survival niches (Manz, Thiel, and Radbruch 1997; Slifka et al. 1998; Manz et al. 2005). Maintenance of locally-produced mucosal antibody is less well-understood, but would require the persistence of plasma cells in subepithelial layers. These could potentially be long-lived cells analogous to bone

marrow plasma cells or plasma cells arising from differentiation of naïve or memory B cells (MBC) via persistent antigenic stimulation. Such lymphoid tissue is not readily available for sampling in RSV-infected humans, so these plasma cells cannot be directly studied. Memory B cells represent a population of long-lived circulating B cells that produce affinity-matured, class-switched antibody, thus MBC and long-lived plasma cells arise via a common parent pathway. Following polyclonal activation with mitogen, MBC with specificities to antigens encountered in the distant past can be readily detected, allowing the B cell memory repertoire of an individual to be studied (Crotty et al. 2003; Crotty et al. 2004). Unlike plasma cells, which must migrate to survival niches, MBC exist in the circulation; thus the peripheral blood represents an easily accessible compartment to study B cell memory in humans.

Previous experimental studies that examined the relationship between antibodies and protection used attenuated virus strains (Lee et al. 2004), and contemporary studies using RSV M37 have pre-selected their subjects for especially low serum neutralising antibody titres (DeVincenzo et al. 2010c). Thus the ability of antibodies to protect from virulent RSV has not been fully examined and the protective effect of mucosal IgA has not been reported in a controlled infection study. As subjects enrolled in this study were not selected on the basis of low antibody levels, an evaluation of the protective effects of humoral immunity on infection rate and clinical disease was possible.

5.2 Protection from infection by antibody

The author had the opportunity to collaborate with Dr. Christopher Chiu on another RSV infection challenge using the same stock of M37 virus in 2013 where a further twenty subjects were inoculated. The inoculation and experimental procedures were identical to those reported in Chapter 3. Ten (50%) were infected, six of these (60%) experienced a clinical cold. There were no statistically significant differences in infection rate or colds between these additional twenty subjects and the original forty (Chi-squared test, 10/20 and 24/40, χ^2 = 0.43, d.f. = 1, p = 0.51 for infection rate; 6/10 and 17/24, χ^2 = 1.2, d.f. = 2, p = 0.55 for colds). There were no statistically significant differences in cumulative (as AUC) symptom scores or viral load between these

additional twenty subjects and the original forty (Mann-Whitney-Wilcoxon test, median = 12.5 and 17.0 score × days, $W = 452$, $p = 0.6$ for symptoms; median = 2.5 and 0 $\log_{10}$ copies mL^{-1} × days, $W = 372.5$, $p = 0.5$ for viral load). Therefore all sixty subjects (infected = 34, uninfected = 26) were pooled for the following analyses, except where indicated when samples were limited.

5.2.1 Serum neutralising antibody

Serum antibody was determined by plaque reduction neutralisation titre (see section 2.2.1.2) at baseline and day +28. Somewhat in contrast to what has been previously published, although infected subjects had a lower median pre-existing serum neutralising titre compared with uninfected subjects, the difference was not significant (median 7.8 and 7.6 $\log_2$ titre, $W = 337$, $p = 0.12$, Figure 5.1).

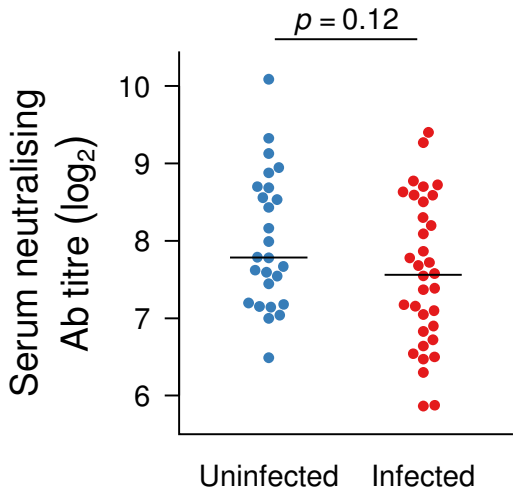


Figure 5.1 Protective effect of baseline serum neutralising antibody. Serum neutralising antibody was determined by plaque reduction neutralisation titre from serum samples collected at the day -14 visit. Horizontal bars indicate median for uninfected ● and infected ● subjects, *p*-value for Mann-Whitney-Wilcoxon test is shown.

5.2.2 Nasal virus-specific IgA

Serum antibody may effectively protect against LRTI but not URTI, where IgA may predominate. Therefore, IgA titres to whole RSV and F protein were determined by ELISA binding in nasal lavage (Figure 5.2, top panel). Uninfected subjects had significantly greater median pre-existing nasal IgA

titres for both whole RSV and F protein than did infected subjects (anti-RSV-IgA, 10.2 and 9.4 $\log_2$ titre, $W = 266$, $p = 0.0081$; anti-F-IgA, 10.5 and 9.6 $\log_2$ titre, $W = 278$, $p = 0.014$). A significant effect of nasal IgA against whole RSV and F protein on probability of protection from infection was also found using logistic regression (anti-RSV-IgA, OR = 1.9, 95% confidence interval 1.2 – 3.4, $p = 0.015$; anti-F-IgA, OR = 1.7, 95% confidence interval 1.1 – 2.9, $p = 0.016$, Figure 5.2, bottom panel). Hence in experimentally challenged adults, for whom URTI is the outcome of a failure of pre-existing immunity, nasal IgA appeared to be a superior correlate of protection to serum neutralising antibody.

5.3 Induction of humoral responses

5.3.1 Convalescent antibody

Antibody levels induced following RSV challenge were determined in the serum and nasal compartments (Figure 5.3, left-hand column). Following RSV challenge, infected subjects demonstrated a significant median 3.4-fold, IQR 2.3 – 4.6 , increase in serum neutralising antibody (one-sample Wilcoxon signed rank test $V = 589$, $p < 0.001$). Similar increments were seen in nasal anti-RSV IgA (2.5-fold, IQR 2.0 – 5.4, $V = 592$, $p < 0.001$) and nasal anti-F-protein IgA (3.0-fold, IQR 1.4 – 4.4, $V = 563$, $p < 0.001$). By contrast, individuals who were inoculated but remained uninfected showed much lower rises in serum neutralisation titre (1.4-fold, IQR 1.0 – 2.0, vs. infected, $V = 757$, $p < 0.001$), nasal anti-RSV IgA (0.9-fold, IQR 0.6 – 2.5, vs. infected, $V = 675$, $p = 0.0014$), or nasal anti-F-protein IgA (0.8-fold, IQR 0.6 – 1.6, vs. infected, $V = 705$, $p < 0.001$).

5.3.2 Durability of the RSV-induced antibody response

Previous studies have demonstrated that antibodies induced in response to RSV infection are poorly maintained in both adults (Falsey, Singh, and Walsh 2006) and children (Sande et al. 2013). All infected subjects were therefore asked to return for further blood and nasal sampling approximately 6 – 12 months post-inoculation (nominally day +180). In those subjects that attended this follow-up visit (N = 12 provided serum, N = 11 provided nasal lavage), the boosted antibody levels were not maintained and levels had declined to those observed pre-infection (Figure 5.3, right-hand column).

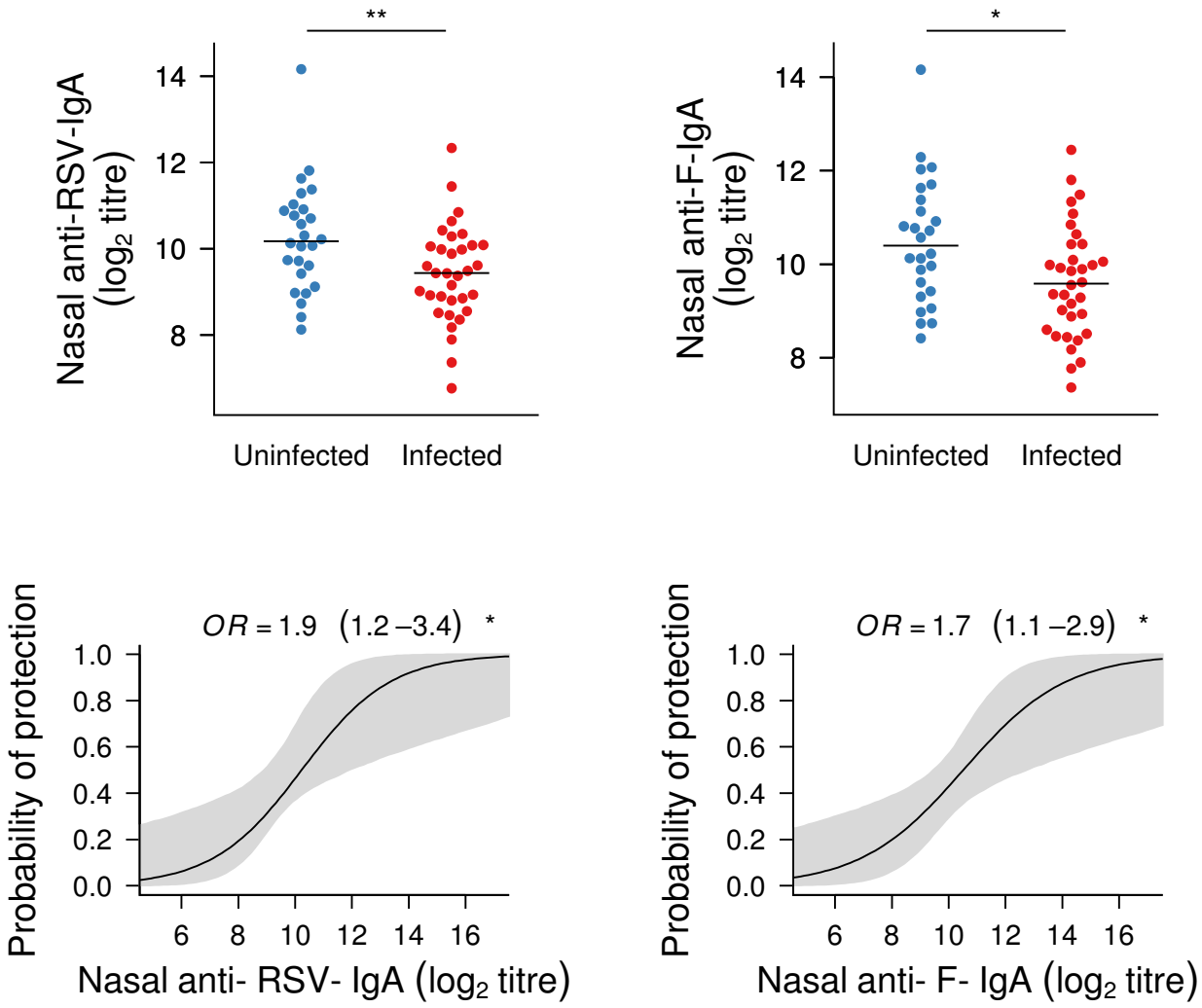


Figure 5.2 Protective effect of baseline nasal IgA. Top: nasal IgA titre was determined by ELISA binding to whole RSV and F protein from nasal lavage samples collected at the day -14 visit. Bottom: logistic regression showing predictive effect of nasal IgA on protection from experimental infection. Smooth curve is predicted probability, shaded area is 95% confidence interval for the prediction. OR = odds ratio, horizontal bars indicate median titre for uninfected ● and infected ● subjects, stars indicated *p*-value for Mann-Whitney-Wilcoxon test or *z*-test as appropriate (= $p < 0.01$, * = $p < 0.05$).**

5.3.3 Circulating antibody secreting cells during experimental infection

In order to understand how RSV-specific antibody is induced, acutely generated whole-RSV- and F-protein- specific antibody-secreting cells (ASC) were assayed from fresh PBMC on days 0, +7, +10, +14, and +28 by ELISpot (Figure 5.4, top panel). Twenty-eight subjects (sixteen infected, twelve uninfected) had sufficient cells available for this analysis. RSV- and F-protein- specific ASC were absent on day 0 and were not detectable until day +10 when maximum response (hereon referred to

as burst) in the majority of subjects was observed (Figure 5.4, middle panel). IgM^+ ASC were not significantly induced. By day +14 frequencies of virus-specific ASC had declined almost to zero and were completely absent by day +28. Neither the median peak frequencies (ASC / 10^6 PBMC) nor the percentages of total Ig^+ ASC differed significantly between virus-specific IgG^+ and IgA^+ ASC (Figure 5.4, bottom panel). Pre-existing serum neutralising antibody, but not nasal IgA, was negatively correlated with ASC burst (Figure 5.5, top panel). ASC burst was positively correlated with fold-change in serum neutralising antibody and nasal IgA (Figure 5.5, bottom panel).

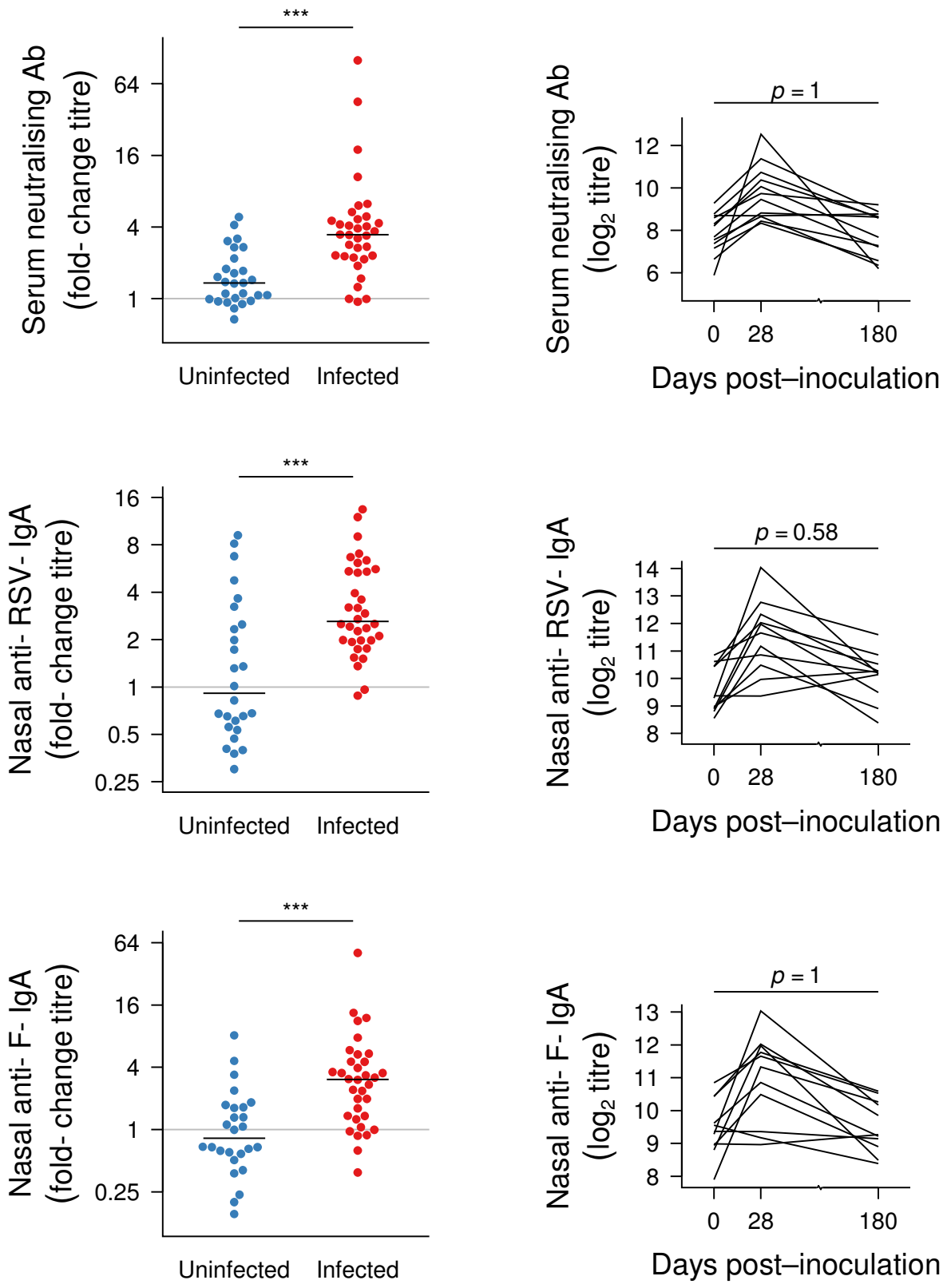


Figure 5.3 Fold-change and durability of induced antibody response. Left-hand column: fold-change at day +28 over baseline of serum neutralising antibody (top), nasal anti-RSV-IgA (middle), and nasal anti-F-protein-IgA (bottom) for uninfected ● and infected ● subjects. Right-hand column: durability of serum neutralising antibody (top), nasal anti-RSV-IgA (middle), and nasal anti-F-protein-IgA (bottom) for selected subjects that returned for further blood and nasal sampling approximately 6 – 12 months (nominally day +180) after initial inoculation. Stars and *p*-values indicate significance of Mann-Whitney-Wilcoxon test. *** = $p < 0.001$.

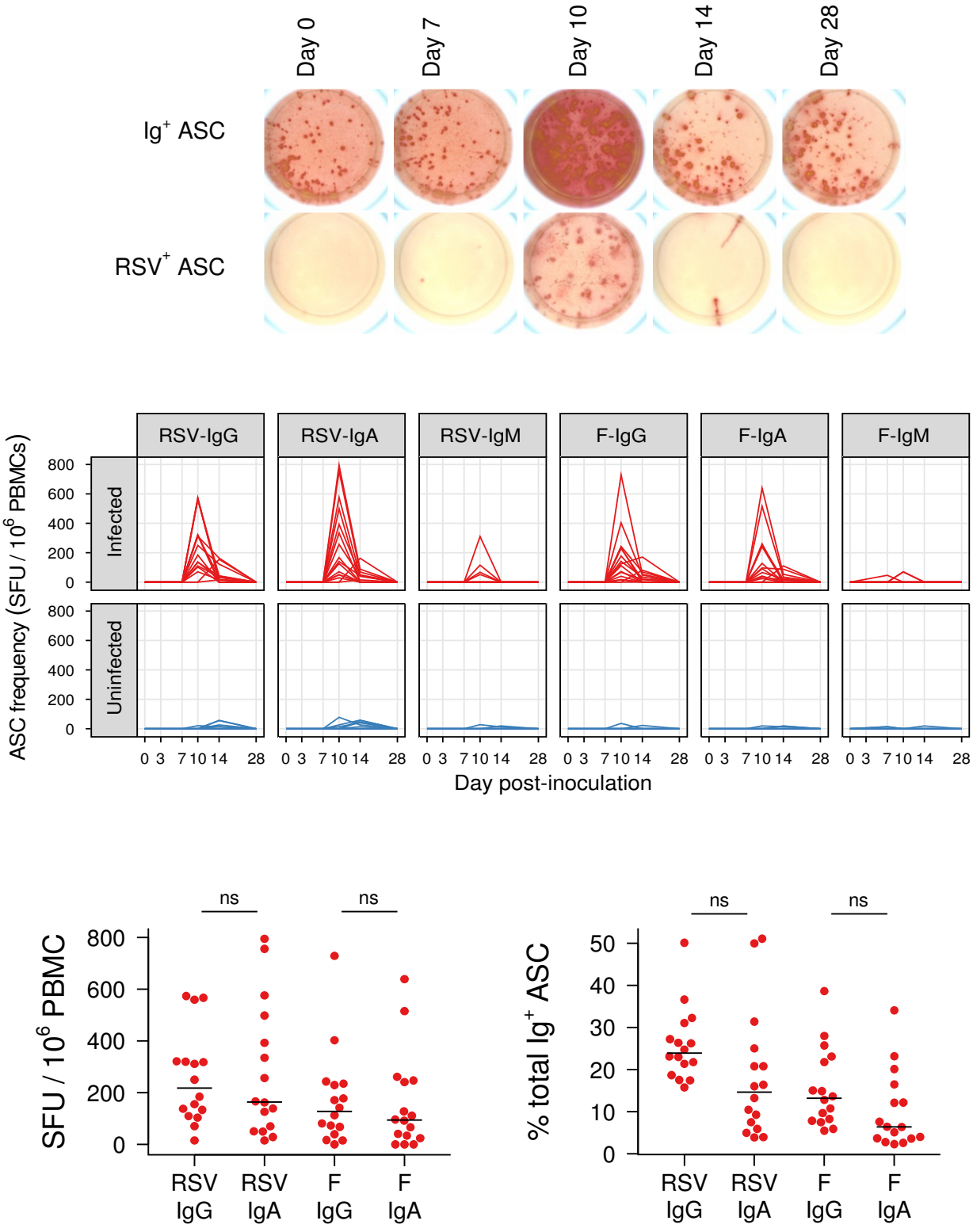


Figure 5.4 Measurement of RSV-specific ASC in fresh PBMC by ELISpot for uninfected (N = 12) and infected (N = 16) subjects where sufficient cells were available. Top and middle: RSV-specific ASCs peak at day 10 post-inoculation. Bottom: infected subjects only, peak whole RSV- and F protein-specific ASC frequency (SFU / 10^6 PBMC) and percentage total Ig^+ ASC. All comparisons shown non-significant by Mann-Whitney-Wilcoxon test.

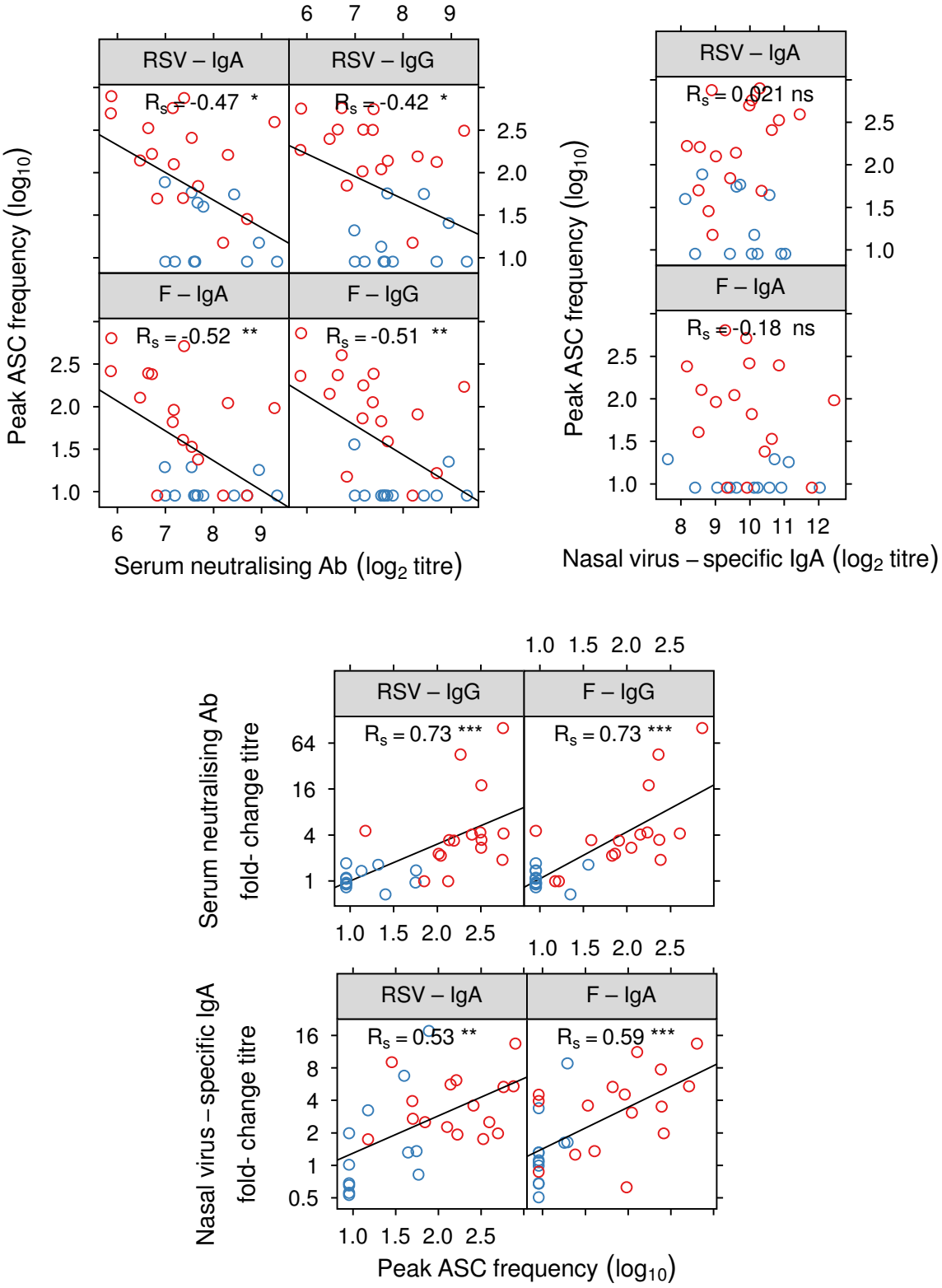


Figure 5.5 Correlation of ASC burst with pre-existing serum neutralising antibody (top panel, left), nasal IgA (top panel, right), fold-change in serum neutralising antibody (bottom panel, upper), and fold-change in nasal IgA (bottom panel, lower). Horizontal strips indicate specificity of ASC and virus-specific IgA where appropriate. Uninfected subjects indicated by ○ infected subjects indicated by ○ however all subjects have been analysed together for the statistics. Spearman's rank correlation coefficient (R_S) is shown, stars indicate significance of correlation coefficient (ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Where correlation is significant, least-squares linear best fit line is shown.

5.3.4 RSV-specific memory B cells

Circulating memory B cells (MBC) rapidly differentiate into ASC and plasma cells on re-encounter with cognate antigen and are essential for rapid humoral response to recurrent infection. MBC can be activated from fresh or frozen PBMC using a cocktail of mitogens and a TLR9 agonist (cytosine—phosphate—guanine (CpG) oligodeoxynucleotide (ODN) 2006, see section 2.2.6 and (Crotty et al. 2004)). Frozen PBMC from baseline and day +28 were stimulated *in vitro* for five days using the method of Crotty *et al* and antigen-specific ASC were identified by B cell ELISpot. Subjects with an inadequate polyclonal response to stimulation were excluded (see section 2.2.6.2) leaving twenty-six subjects (seventeen (65%) infected, nine (35%) uninfected) remaining for the analysis. Frequencies of RSV-specific MBC (as % total Ig^+ cells) were compared with those against measles virus (MV), a related pathogen to which protection following natural infection or live attenuated vaccination is known to be long-lasting (Amanna, Carlson, and Slifka 2007).

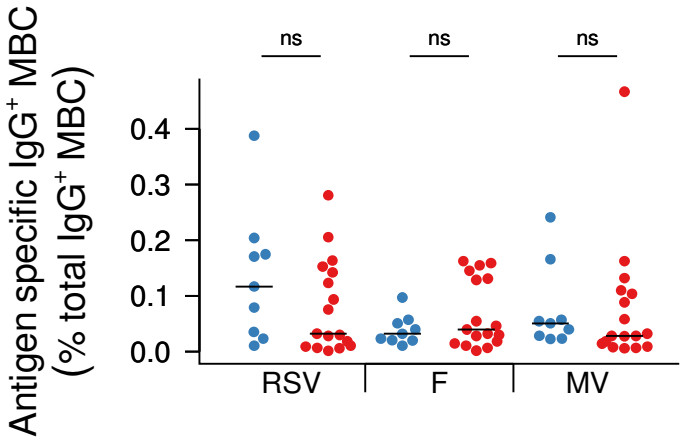


Figure 5.6 Pre-infection IgG^+ MBC frequencies with specificities for whole RSV, F-protein (F), and whole measles virus (MV). MBC frequency is expressed as % total Ig^+ cells. Horizontal bar indicates median for uninfected ● and infected ● subjects. All comparisons shown were non-significant by Mann-Whitney-Wilcoxon test.

5.3.5 IgG^+ memory B cells

There were no significant differences in IgG^+ MBC frequencies against whole RSV, F-protein or MV prior to RSV challenge (whole RSV, median frequency 0.08%, IQR 0.02 – 0.16%; F-protein, median frequency 0.04%, IQR 0.02 – 0.1 ; MV, median frequency 0.04%, IQR 0.02 – 0.09; Kruskal–Wallis

χ^2 = 1.2, d.f. = 2, p = 0.5). There was no significant difference between the frequencies of virus-specific MBC in infected or uninfected subjects (Figure 5.6).

Following infection, subjects demonstrated a median increase in IgG$^+$ MBC frequencies of 0.4% (IQR 0.2 – 0.5) against whole RSV and 0.1% (IQR 0 – 0.2) against F-protein (Figure 5.7, top panel). These equated to a median 3.8 (IQR 3.1 – 5.1) –fold increase in RSV-specific MBC (V = 153, p < 0.001) and 1.8 (IQR 1.3 – 4.4) –fold increase in F-specific MBC (V = 139, p = 0.0017) compared with pre-infection frequencies. By contrast, MV-specific IgG$^+$ MBC frequencies remained unchanged (median fold-change 0.8, IQR 0.6 – 1.3, V = 59, p = 0.43). Comparison of fold-change in RSV- and F-protein-specific and MV-specific IgG$^+$ MBC is shown in Figure 5.7 (bottom panel, left). The fold-change in both whole RSV- and F-protein specific IgG$^+$ MBC correlated with the size of ASC burst specific for the homologous antigens, suggesting that these populations were generated by associated mechanisms (Figure 5.7, bottom panel, right).

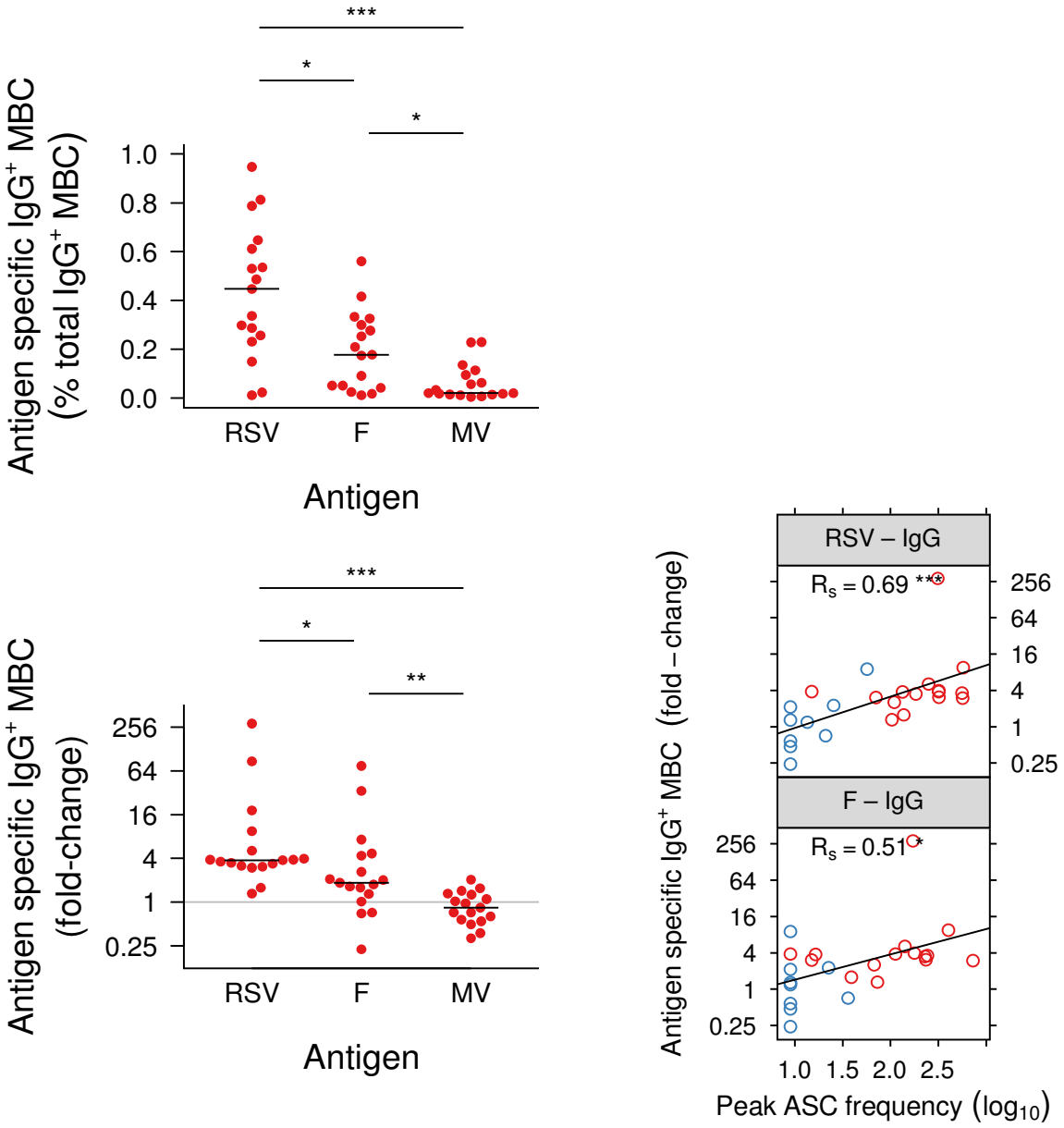


Figure 5.7 IgG⁺ MBC frequency and fold-change at day +28. Top panel: day +28 frequencies for whole RSV-, F-protein (F)-, and whole measles virus (MV)- specific IgG⁺ MBC for infected subjects only. Bottom panel: fold-change at day +28 over baseline for infected subjects only (left) and correlation and linear line of best fit for day +28 MBC frequency and ASC burst for all subjects where data on both variables available (right, N = 24, uninfected ○ = 9, infected ○ = 15) . Black horizontal bar indicates median (top and bottom left). Horizontal strips indicate specificity of IgG⁺ MBC and acute ASC (bottom right).

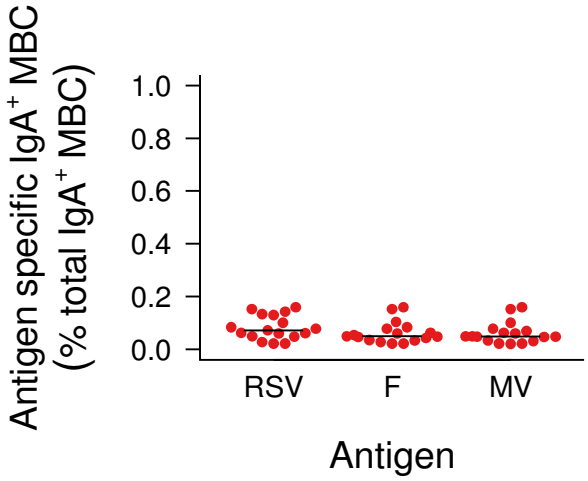


Figure 5.8 Day +28 IgA⁺ MBC frequencies with specificities for whole RSV, F-protein (F), and whole measles virus (MV) for infected subjects only. MBC frequency is expressed as % total Ig⁺ cells. Horizontal bar indicates median.

5.3.6 IgA⁺ memory B cells

In contrast to IgG⁺ MBC, IgA-producing MBC could not be detected at baseline. Furthermore, even after RSV infection, RSV- and F- specific IgA⁺ MBC frequencies could not be quantified for most subjects. Figure 5.8 shows the frequencies of IgA⁺ MBC at day +28. After subtraction of background from the negative control, spot counts below the geometric mean spot count of the negative control (2.2 spots for IgA) were assigned that value (RSV: 15 subjects, F-protein: 23 subjects, MV: 20 subjects; see section 2.2.6.2). Hence the majority of data in Figure 5.8 represents values below the limit of quantification.

The subjects enrolled would have received routine childhood intramuscular measles vaccination, thus it might be expected that observed MBC specific for MV are IgG⁺ as antigen exposure was not via the mucosal route. To determine whether there was a particular deficiency in RSV-specific IgA⁺ MBC generation and not just a failure to detect IgA⁺ MBC in general, comparison with a condition where IgA⁺ MBC are known to be generated was required. Following intranasal vaccination with live attenuated influenza (LAIV), influenza-specific IgA⁺ and IgG⁺ MBC in the blood are present at similar frequencies (Sasaki et al. 2008). The author of this thesis was fortunate to obtain frozen convalescent PBMC from 8 patients hospitalised with virologically-confirmed influenza H1N1

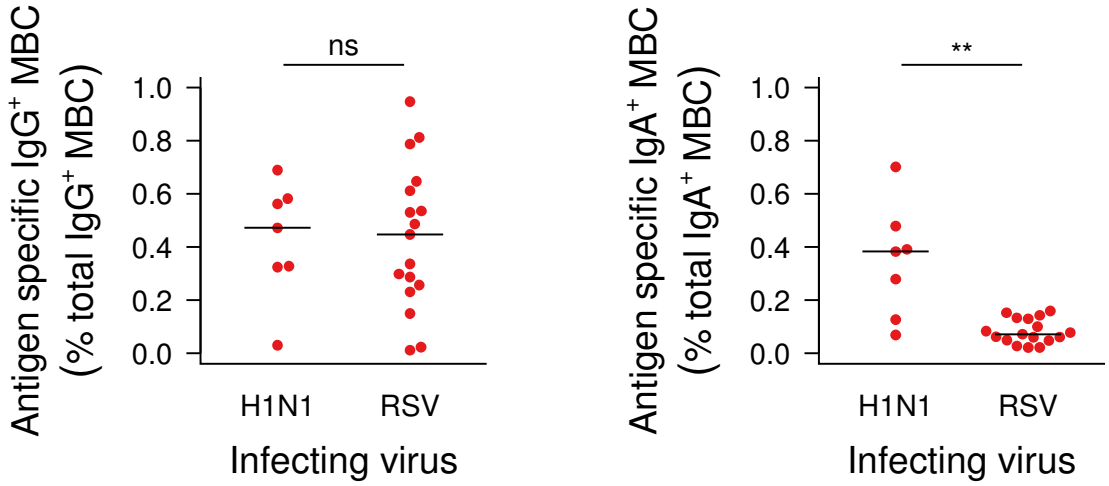


Figure 5.9 Convalescent MBC frequencies in patients recovering from experimental RSV infection (day +28 post-inoculation) and hospitalised with natural H1N1 influenza infection (day +42 post-admission). Left: IgG⁺ MBC frequencies. Right: IgA⁺ MBC frequencies. MBC frequency is expressed as % total Ig⁺ cells. Horizontal bar indicates median. Stars indicate significance of Mann-Whitney-Wilcoxon test. ns = $p > 0.05$, ** = $p < 0.01$.

infection during the 2009/10 and 2010/11 influenza seasons (a gift from the Mechanisms of Severe Acute Influenza Consortium, MOSAIC). The median number of days post-admission when the samples were taken was 42, at which point all patients were deemed to have fully recovered by the treating physician. All patients were previously healthy with no known immunosuppressive disease. PBMC were polyclonally stimulated under identical conditions to those used for the RSV-specific MBC assays and antigen-specific MBC identified by B cell ELISpot. As expected, virus-specific IgG⁺ MBC following both H1N1 and RSV were found at similar frequencies (Figure 5.9, left). However, PBMC from patients naturally infected with influenza showed significantly higher numbers of virus-specific IgA⁺ MBC than did those from subjects infected with RSV (Figure 5.9, right). In influenza, virus-specific IgA⁺ and IgG⁺ MBC number were therefore comparable and IgA⁺ MBC were generally abundant in those who had recovered from infection, contrasting with the absence of IgA⁺ MBC after RSV infection. This implies that a specific defect exists in the ability to induce IgA memory for RSV in humans.

5.4 Pre-existing humoral immunity and measures of disease

The protective effect of antibodies from acquiring infection has been demonstrated, however whether pre-existing antibody can influence the course of disease once infection is established is not known. Therapeutic trials of monoclonal antibodies in bronchiolitis (Malley et al. 1998; Ramilo et al. 2014) were disappointing, however it may be too late to supplement antibody when infants are already hospitalised. The theoretical capacity of antibody to limit extracellular spread of virus coupled with the observation that severe bronchiolitis is rare in healthy term infants under one month in whom transplacentally-acquired maternal antibody is present at the highest levels would suggest that antibody present in early in the course of an established infection can still be beneficial.

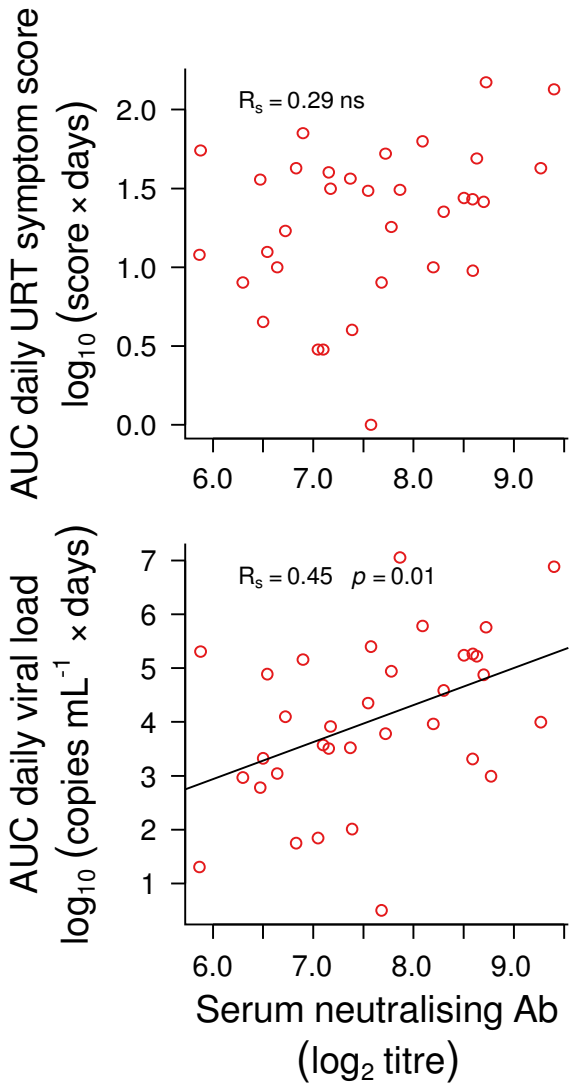


Figure 5.10 Correlation between serum neutralising antibody and measures of disease in infected subjects (N = 34). Top: serum neutralising antibody and AUC daily URT symptom score. Bottom: serum neutralising antibody and AUC daily viral load. Spearman's rank correlation coefficient (R_S) is shown, *p*-values indicate significance of correlation coefficient (ns = $p > 0.05$). Where correlation is significant, least-squares linear best fit line is shown.

The relationship between pre-existing humoral immunity (antibody and MBC) was therefore explored for subjects who became infected (all 34 infected subjects included for antibody analyses, 17 of these had sufficient cells remaining for MBC analyses). Surprisingly, pre-existing serum neutralising antibody had a weak positive correlation with symptoms, as measured by AUC (Figure 5.10, top panel), and a moderate and significant positive correlation with viral load (also AUC, Figure 5.10, bottom panel). By contrast, nasal RSV-IgA had no correlation with symptoms and a weak negative correlation with viral load (Figure 5.11, left column). Nasal F-IgA had a moderate but

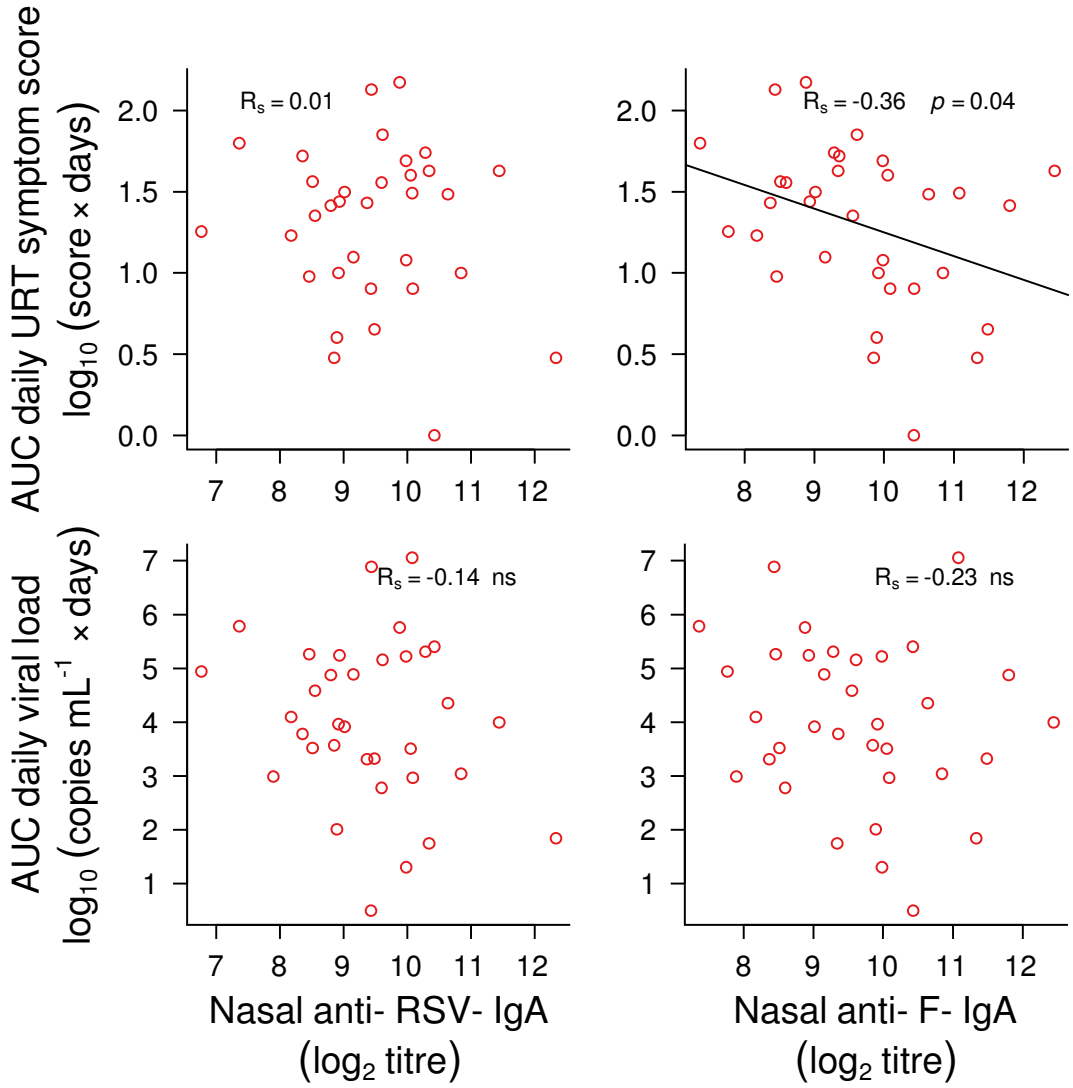


Figure 5.11 Correlation between nasal virus-specific IgA and measures of disease in infected subjects (N = 34). Top: nasal RSV- (left) and F-protein- (right) IgA and AUC daily URT symptom symptom score. Bottom: nasal RSV- (left) and F-protein- (right) IgA and AUC daily viral load. Spearman's rank correlation coefficient (R_S) is shown, *p*-values indicate significance of correlation coefficient (ns = $p > 0.05$). Where correlation is significant, least-squares linear best fit line is shown.

significant negative correlation with symptoms, and a weak negative correlation with viral load (Figure 5.11, right column).

As memory B cells can rapidly differentiate into antibody-secreting plasma cells, they may contribute to early viral control by rapidly boosting levels of antibodies. Seventeen infected subjects had sufficient stored PBMC for the MBC analysis. Weak, non-significant, negative correlations were observed between pre-existing RSV- and F- specific IgG^+ MBC in the blood and

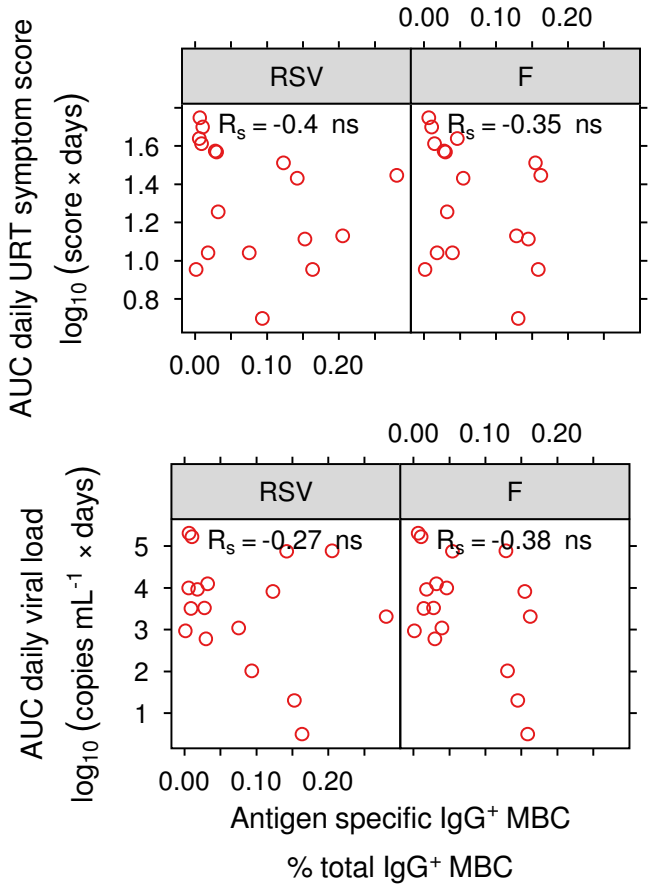


Figure 5.12 Correlation between pre-existing circulating IgG⁺ MBC frequency and measures of disease in infected subjects (N = 17). Top: IgG⁺ MBC frequency and AUC daily URT symptom score. Bottom: IgG⁺ MBC frequency and AUC daily viral load. Spearman's rank correlation coefficient (R_S) is shown, *p*-values indicate significance of correlation coefficient (ns = $p > 0.05$).

symptoms (Figure 5.12, top panel). Similar weak negative correlations were observed between MBC and viral load (Figure 5.12, bottom panel).

5.5 Discussion

In contrast to published studies, serum neutralising antibody was not found to be a reliable correlate of protection from infection. From a mechanistic point of view, this is not entirely surprising. RSV replication in the immunocompetent host is confined to the respiratory tract and epithelium. Access of serum antibody to the respiratory tract is by passive diffusion and locally-produced IgA is thought to be the dominant form of antibody in most mucosal sites. Whilst serum IgG may diffuse adequately to protect the lung (especially if inflamed), the presumed site of entry in natural and experimental infection is via the upper respiratory tract. Thus local IgA in the nose has the greatest

opportunity to protect from infection. It is thought that local infection in the nasopharynx proceeds to lower respiratory tract infection by aspiration of secretions (Openshaw and Tregoning 2005). Hence immunocompetent adults, in whom serum neutralising antibody is readily detectable, are protected from clinically significant LRTI, but remain susceptible to URTI (i.e. RSV colds) unless local RSV-specific IgA levels are sufficient. This is supported by the data obtained that showed a robust association between virus-specific nasal IgA and protection from infection.

Analysis of circulating acute ASC showed robust responses of both IgG^+ and IgA^+ ASC that correlated with convalescent antibody levels and peaked on day +10 post-inoculation. This was in contrast to what was expected from published studies of acute ASC generation following trivalent intramuscular influenza vaccination (Halliley et al. 2010), where the peak is around day +7 post-vaccination, and natural RSV infection in the elderly, where ASC were detectable for several weeks after the onset of illness (Lee et al. 2010b). The kinetics of the humoral response following an intramuscular dose of a relatively large amount of non-replicating antigen will likely differ from those following an experimental live RSV challenge, where the maximum viral load is not achieved until approximately seven days have elapsed. Similarly, viral replication in elderly persons, and thus the signals for humoral activation, may be prolonged. The interesting observation that ASC burst correlated negatively with pre-existing serum but not nasal antibody and positively with convalescent antibody levels suggests that low antibody levels may be effectively boosted by intranasal vaccination despite pre-existing nasal antibody from prior or recent RSV infection.

All subjects in this study received an antigen load, and some uninfected subjects manifest a low-level virus-specific ASC response and an increment of antibody titres (Figure 5.3 and Figure 5.4). However, following successful infection, subjects' exhibit much stronger ASC and antibody responses, indicating that local replication enhanced subsequent humoral responses. Induced antibody did not persist and titre waned back to pre-infection levels within 6 – 12 months in nearly all subjects who were followed up for this length of time. These 'basal' levels of antibody imply the existence of RSV-specific long-lived plasma cells in survival niches such as the bone marrow, but

the inability of RSV infection to produce a sustained increment in serum antibody suggests that the factors promoting the persistence of this compartment of long-lived plasma cells have reached their maximum effect in adults. Examination of RSV-specific IgG^+ MBC in the peripheral blood revealed frequencies pre-infection comparable to those with specificities to measles virus and an expansion post-infection that correlated with the acute ASC burst, implying that the IgG^+ MBC compartment is responding appropriately. RSV-specific IgA^+ MBC were absent pre-infection and remained undetectable even at day +28. By contrast, following natural H1N1 influenza infection, virus-specific IgG^+ and IgA^+ MBC were found at comparable levels, suggesting that a specific impairment exists in RSV-specific IgA B cell memory.

Several possible explanations might account for this impairment. It is possible that antigen-specific IgA^+ MBC are so infrequent that the assays used cannot reliably detect them. This is unlikely as influenza-specific IgA^+ MBC were readily identified post-H1N1 infection. It could be argued that immune responses to influenza or novel epitopes in the pandemic virus might be more vigorous, hence virus-specific MBC might be generated at higher frequencies. However, there was no difference in frequencies of IgG^+ MBC between the two infections. It is also possible that since IgA^+ MBC derive from mucosal GC reactions e.g. in MALT, they preferentially migrate to and reside in mucosal sites and are not present in the circulation. However, if this were the case, influenza-specific IgA^+ MBC would not be expected to be detected in convalescent PBMC. In addition, studies of oral cholera vaccination show that vaccine antigen-specific IgA^+ MBC appear in the circulation of vaccinees (Harris et al. 2009), suggesting that B cells activated in the mucosa spend at least some time in the circulation. Therefore it is hypothesised that RSV infection may be unusual in failing to induce mucosal GC activation and thus produces an impaired B cell memory. If true, this could explain why the defect is specifically in IgA^+ MBC and does not affect IgG^+ MBC.

While antibodies have a decisive role in prevention of pathogen entry into cells, virally-infected cells must also be eliminated to ensure recovery from infection. Antibody bound to viral antigens expressed on the surface of virally-infected cells may facilitate cytotoxicity by NK cells via the

receptor FcγRIII (antibody-dependent cell-mediated cytotoxicity, ADCC). The positive correlations between pre-existing serum neutralising antibody and AUC symptoms and AUC viral load were thus unexpected. Binding of viral particles by antibody can facilitate infection of macrophages via Fc receptors. Such antibody dependent enhancement (ADE) is well-described for flaviviral disease, however there are also reports that dilute human neutralising sera can increase the yield of RSV from infected macrophage cell lines by 50-fold (Gimenez, Keir, and Cash 1989; Krilov et al. 1989). It has been speculated that waning levels of maternal antibody may contribute to the observed peak incidence of severe bronchiolitis around 3 – 6 months of age (Glezen et al. 1986), and that ADE may have been involved in the FI-RSV enhanced disease (Murphy and Walsh 1988). The major hypothesised mechanism of ADE involves incomplete neutralisation and promotion of virus uptake via Fc receptors however, thus it would not be expected that the high neutralising titres observed in the adults enrolled in this study would result in ADE. It may be that serum antibody is somehow interfering with viral clearance by cytotoxic T cells and NK cells, however this is speculation. The relevance of the observed association of higher serum neutralising titres and higher viral loads in this study is thus unclear.

In contrast to serum antibody, nasal IgA was not associated with increased viral loads or symptoms, however the negative correlation of nasal anti-F-protein IgA was similarly unclear. Studies of ADE have used sera, which is richer in IgG, to produce enhancement, so it is possible that the phenomenon is limited to IgG. IgA is a comparatively weak activator of complement, and thus is perceived as less inflammatory than IgG. All of the correlation coefficients and the statistical significance between antibody and measures of disease were moderate at best, suggesting much uncertainty about the true relationship. At the least, pre-existing serum or mucosal antibodies do not markedly influence the course of disease once infection is established. Similarly, pre-existing MBC frequencies neither correlated with later symptom score nor viral load in infected subjects. Taken together, these observations suggest that pre-existing RSV-specific humoral immunity does not contribute substantially to control of disease or viral replication once infection is established in healthy adults.

In summary, nasal RSV- and F-protein- specific IgA were the only reliable observed correlates of protection from infection, however most adults do not have sufficient levels to prevent infection. This, and the poor durability of induced nasal IgA, probably explains the continued susceptibility of adults to recurrent RSV infection. A deficiency of RSV-specific humoral memory was suggested and the absence of RSV-specific IgA^+ MBC, but not influenza-specific IgA^+ MBC (even shortly after infection), suggesting that RSV-specific B cell memory is selectively impaired. Putative RSV vaccines will need to overcome this to ensure long-lasting protective immunity. Humoral immunity did not appear to influence the course of disease once infection was established; much of the variation in symptoms and viral load among infected subjects remains unexplained.

The magnitude and antiviral efficiency of the ensuing host inflammatory response to viral replication likely impacts RSV-induced disease. This will be explored in the next chapter.

6 Mediators of disease in infected volunteers

6.1 Introduction

Thus far, the protective role of pre-existing antibody in prevention of experimental infection has been demonstrated. However, once infection was established, pre-existing antibodies in the serum or nasal fluid neither influenced the course of symptoms nor the quantity or duration of viral shedding. Antibody therefore appears to provide an effective barrier to infection if levels are sufficient and it is in the appropriate site, but does not limit viral replication or disease if this barrier is overcome.

The exact cause of RSV-induced airway inflammation is not known. Whilst viral load in nasal fluid and symptoms correlate, this relationship is driven by subjects exhibiting higher symptom scores and viral loads and limited to a few post-inoculation days only. As previously stated, the recovery of virus by nasal lavage may itself depend on having typical cold symptoms (e.g. sufficient nasal mucus), thus any observed correlation may be artefactual. How then can the observed variability in symptoms and viral load in infected subjects be explained? Given that the inoculations were performed from a pooled lot of virus vials in an identical manner by the same operator, and that titration of inoculating virus by plaque assay showed the dose to be consistent throughout the study, inoculation variability is unlikely to be the explanation. Rather, the variation in host immune response may determine the extent of viral replication, subsequent host epithelial injury, and thus symptoms experienced.

In order to investigate this, levels of soluble inflammatory mediators in nasal lining fluid were measured. Whilst there exist published reports of levels of soluble inflammatory mediators in airway fluid in both natural (McNamara et al. 2004) and experimental subjects (Noah and Becker 2000), these studies have been restricted to one or a few discontinuous time points or summary statistics like area under curve (AUC) to represent an 'average' response from serial repeated measures. Which particular mediators participate at which times during infection has not been

defined. More importantly, early pre-symptomatic responses that may influence the subsequent course of events have not been studied.

6.2 Nasal soluble mediators

Samples of nasal epithelial lining fluid were obtained pre-infection, on days +1 to +10, and day +14 as described in section 3.5.2.1. Levels of soluble inflammatory mediators were determined by multiplex electrochemiluminescence as describe in section 2.2.4. Due to sample limitation, analysis of soluble mediators was only possible for 37 out of 40 subjects inoculated. Of the three excluded subjects, one had an asymptomatic infection (MH011), and two were uninfected (MH022 and MH015).

6.2.1 Exploration of mediator data

Simple plots of mediator concentration over time for each mediator for all subjects demonstrating the three outcomes (uninfected, asymptomatic–infected, and clinical cold) were therefore produced. Due to the sheer number of plots, it is not possible to show all mediators assayed so representative plots demonstrating the different patterns are shown in Figure 6.1. Due to the large variance of the data, log transformation was required. Despite the variability and noise in the data, two patterns were apparent. Some mediators, such as IFN-γ shown here, were not detectable on most days post-inoculation except during the peak of viral shedding in subjects exhibiting colds, where strong induction was seen. Conversely, mediators such as chemokines IP-10 and IL-6 were detectable to some extent throughout and induction during a cold was less immediately apparent. In order to better define the trends in mediators over time, methods for plotting an average response for groups of subjects exhibiting the three different outcomes were tried.

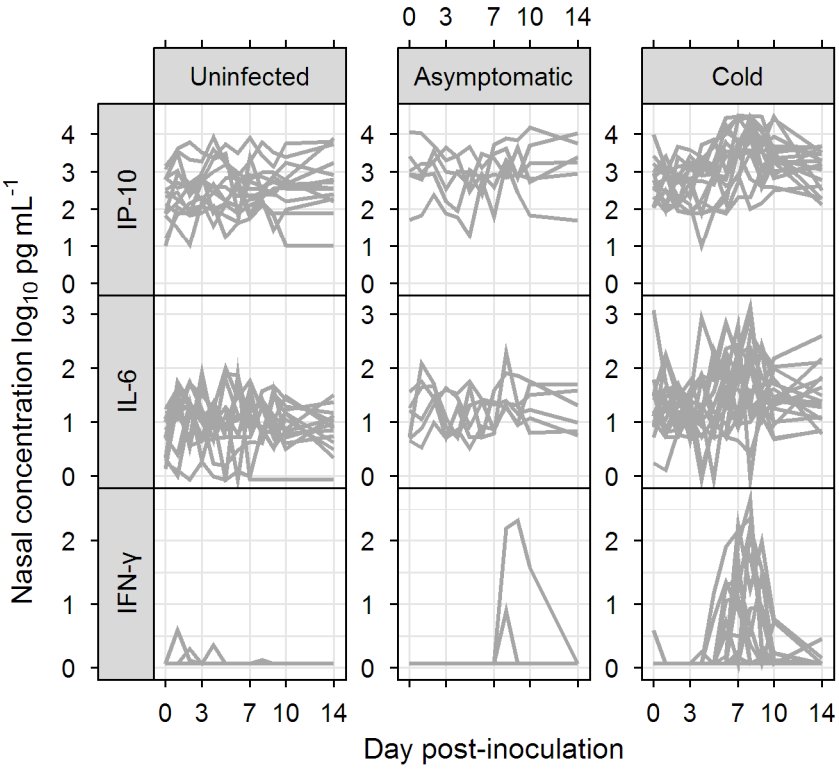


Figure 6.1 Nasal IFN-γ, IL-6, and IP-10 (log_{10} pg mL^{-1}) for all 37 included subjects demonstrating the three different outcomes to experimental challenge. Horizontal strips indicate outcome and vertical strips indicate mediator being assayed.

6.2.1.1 Standard measures of central location

The data in Figure 6.1 was replotted. Figure 6.2 shows the daily mean and median response overlaid respectively. Both measures are susceptible to baseline signal noise however trends began to emerge. It was clear that inflammatory mediators such as IFN-γ were not detectable in the absence of viral shedding and induction was strongly associated with cold symptoms. Asymptomatic-infected subjects showed little or no induction of IFN-γ. For IP-10, there was some suggestion of induction in subjects experiencing a cold. No clear differences were discernable between uninfected subjects and those who were infected but remained asymptomatic. There was no clear superiority of mean or median in terms summarising the main features of the data or susceptibility to noise, however it must be conceded that such judgements are subjective.

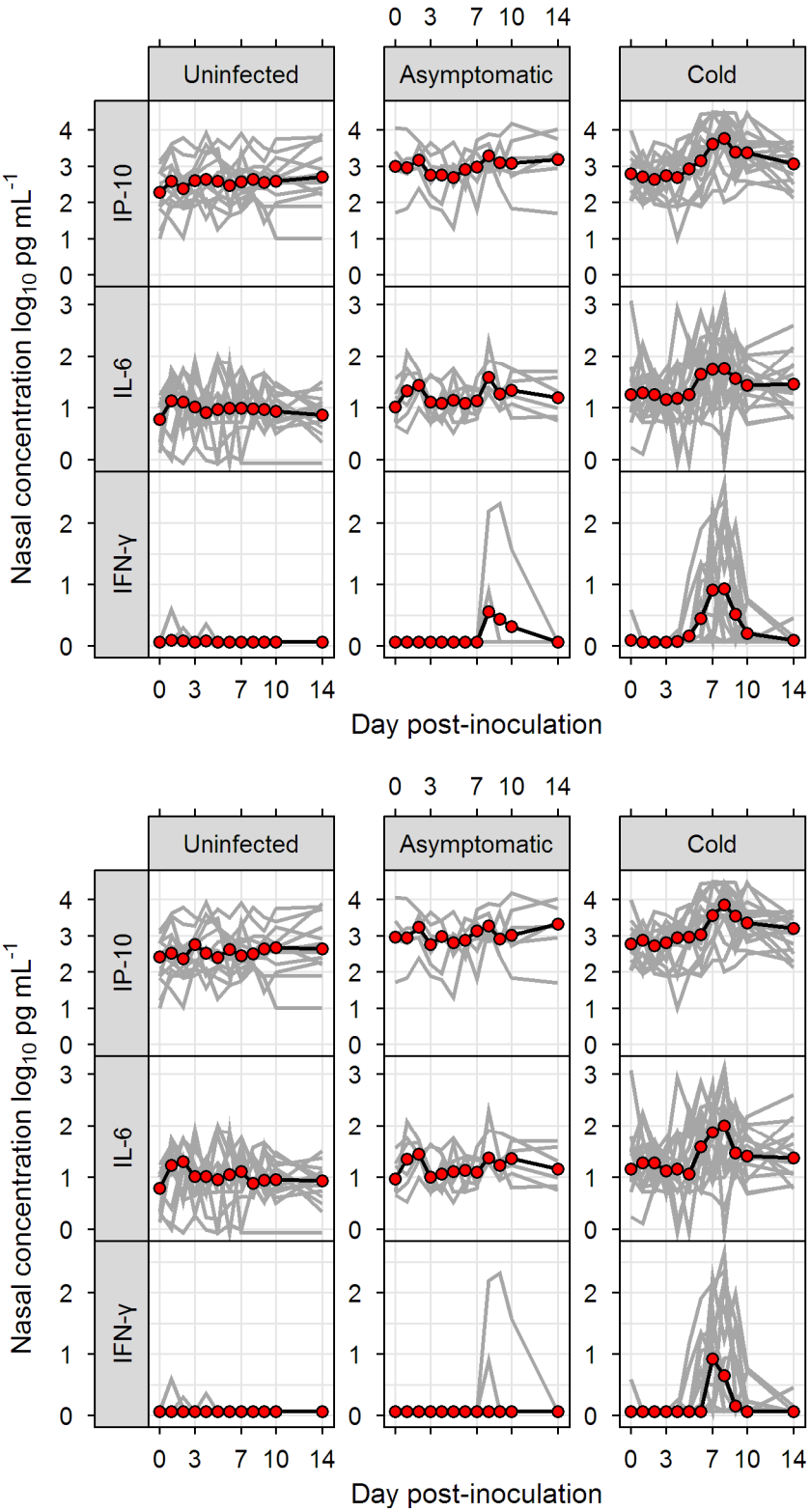


Figure 6.2 Nasal IFN-γ, IL-6, and IP-10 (log_{10} pg mL^{-1}) for all 37 included subjects demonstrating the three different outcomes to experimental challenge. Thick black line and filled red circles indicate daily mean (top panel) or median (bottom panel) of the log mediator concentration. Horizontal strips indicate outcome and vertical strips indicate mediator being assayed.

Whilst summary measures such as mean and median can be used here to capture gross features of the response, they cannot be made free of baseline noise, which could be mistaken for subtle features of the response. A method for smoothing the mediator concentrations was therefore required.

6.2.1.2 Loess smoothing of mediator data

Loess regression is a non-parametric regression method, first described by William Cleveland in 1979. The technical details will not be described. The aim of the method is to fit low-order polynomial functions to a localised subset of data to build a function to describe the variation in the data. Localised functions are fit using weighted least squares, i.e. more weight is given to points near the point whose response is being estimated. The chief advantage over conventional non-linear regression techniques is that no functional form of the data need be provided or even known. Furthermore, confidence intervals for the fitted curve can readily be derived. Loess regression can easily be performed with modern computers and was the method chosen for smoothing based on simplicity and the author's familiarity with the technique. The degree of polynomial used is conventionally limited to two. The fraction of the data (known as span or bandwidth) used for calculation of the fit can be specified. Use of a large span results in a smoother curve, however small features might be lost. Conversely, use of a small span may result in over fitting with the function capturing random noise in the data. Typical values might be 0.5 – 0.75, however in this case, the choice of span was determined empirically, with the objective that the fitted function captured the important features of the data without over fitting random noise. A representative example of differing spans for the mediators IFN-γ and IP-10 is shown in Figure 6.3.

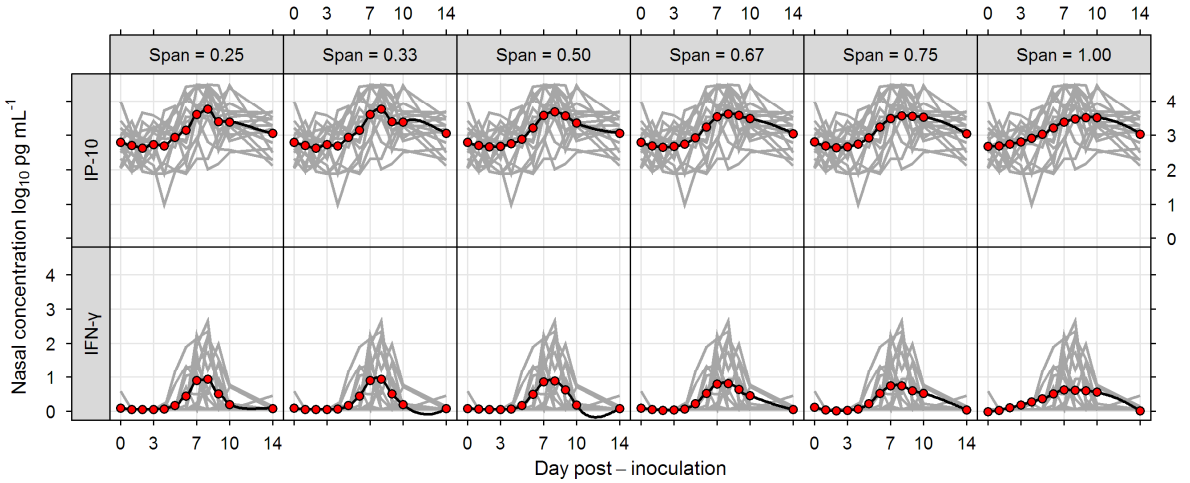


Figure 6.3 Different parameters for span for loess smooth of log-transformed nasal IFN-γ and IP-10 concentration (as indicated by vertical strips) for subjects exhibiting colds. Raw data is plotted as grey lines, loess smooth for indicated span (horizontal strips) is overplotted as thick black line with filled red circles.

The chosen parameter for span for IP-10 in this case was 0.75 and for IFN-γ, 0.67. The procedure was repeated for all mediators measured. Chosen span parameters for each mediator are shown in Table 6.1.

Mediator name	Choice of span parameter
MIG	0.75
IFN-α	0.5
IFN-γ	0.67
IL-15	0.67
IL-6	0.67
TNF	0.67
IP-10	0.75
IL-1β	0.75
IFN-β	0.67

Table 6.1 Span parameters for individual mediators

Example plots showing the raw data and loess fit with 95% confidence intervals for the predicted smooth response for each mediator for the three different outcomes are shown in the Appendix.

6.2.2 Dynamics of symptoms, viral load, and inflammatory mediators

The loess smoothing was applied to all mediators measured and the dynamics of symptoms, viral load and nasal inflammation for subjects exhibiting the three different outcomes was explored.

6.2.2.1 Mediators expressed during an RSV cold

It was clear from the preliminary analyses presented above that IFN-γ was strongly induced in subjects experiencing a cold. Figure 6.4 shows the temporal relationship between symptoms, viral load, and IFN-γ concentration. In order to produce this plot, the IFN-γ concentration has been scaled. The smoothed concentration was divided by the maximum observed smoothed IFN-γ concentration to produce unitless values between 0 and 1 representing the proportion of maximum response. This proportion was then scaled to fit the same range as that observed for symptoms and viral load (both plotted as geometric mean for each day) and expressed as percentage of maximum measured concentration. This was done to permit plotting of these otherwise unrelated quantities on the same scale but retain relative differences in IFN-γ response between subjects.

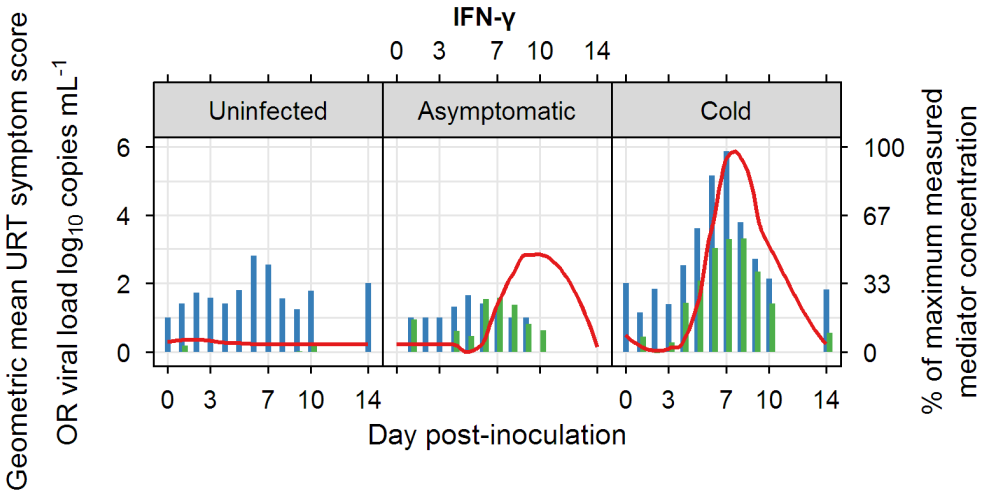


Figure 6.4 Dynamics of symptoms (blue bars), viral load (green bars) and smoothed IFN-γ response (red line) for subjects demonstrating the three different outcomes (uninfected, n = 14, asymptomatic, n = 6, cold, n = 17). Mediator levels expressed as % of maximum concentration.

Uninfected subjects demonstrated no detectable IFN-γ response. Subjects experiencing a cold however showed strong induction that coincided with the start of (approximately day +3), peaked with (approximately day +7), and decayed with detectable viral replication. Asymptomatic-infected subjects also show induction, but to a lesser extent, in parallel with the lower overall viral load in these subjects. IFN-γ is a key mediator of Th1 responses and promotes the elimination of intracellular pathogens such as viruses. As IFN-γ acts on monocytes and endothelial cells to promote secretion of chemokines IP-10 and MIG, it was expected that a similar pattern of responses of these mediators would be observed. The dynamics of these chemokines is shown in Figure 6.5.

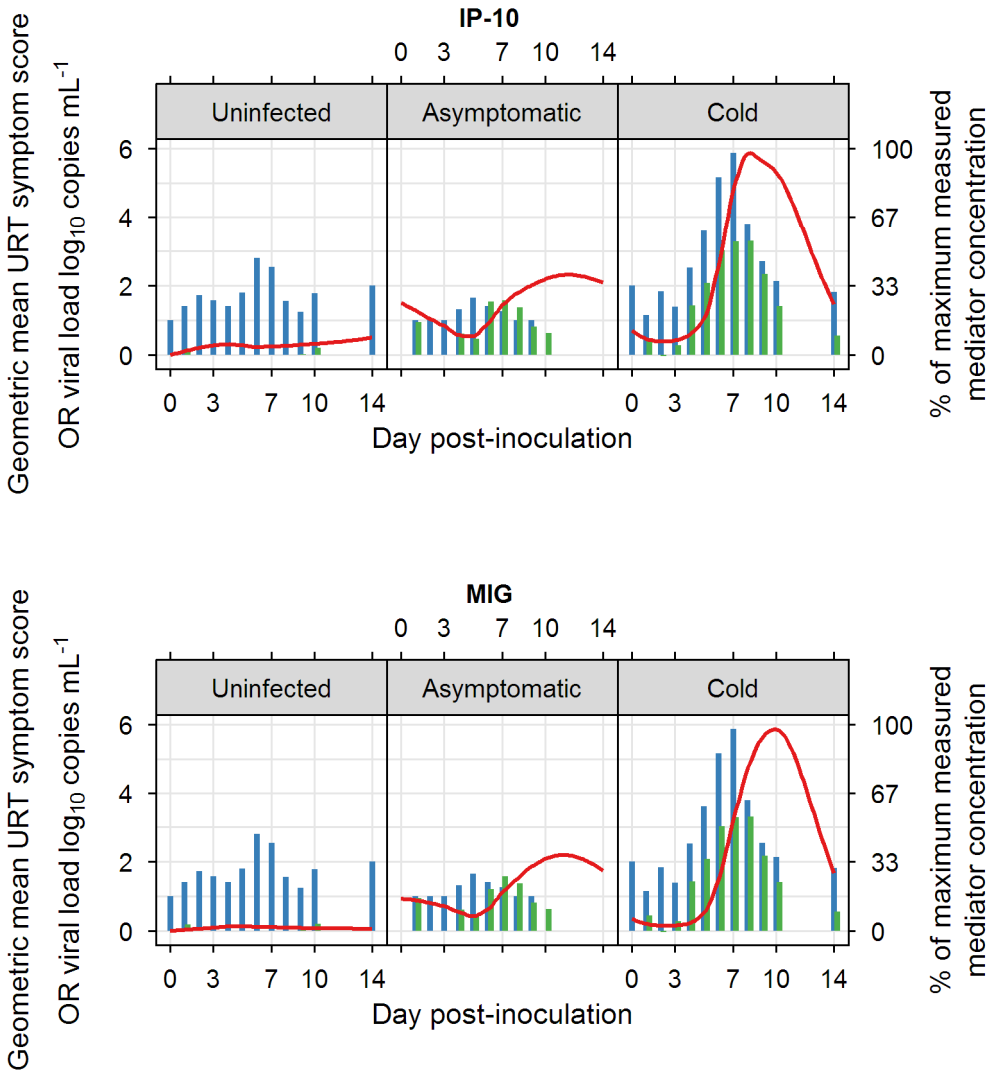


Figure 6.5 Dynamics of symptoms (blue bars), viral load (green bars) and smoothed IP-10 (top) and MIG (bottom) response (red line) for subjects demonstrating the three different outcomes (uninfected, n = 14, asymptomatic, n = 6, cold, n = 17). Mediator levels expressed as % of maximum concentration.

Both of these chemokines were strongly induced in subjects experiencing a cold and to a lesser extent in asymptomatic-infected subjects. However, the initiation and peak were slightly later (day +8 to +10, especially for MIG) than for IFN-γ and thus viral load, supporting the idea that these mediators act downstream from IFN-γ.

The acute-phase response to infection is initiated by TNF, IL-1β, and IL-6, thus it was expected that these mediators would show similar patterns of induction during colds (Figure 6.6). The appearances of IL-1β were surprising in that the subjects exhibiting colds showed higher baseline

levels than did uninfected and asymptomatic infected subjects. Examining the observed IL-1β concentrations rather than the values from the smoothed curves revealed that there was a true, statistically significant, difference between baseline IL-1β levels in subjects experiencing a cold and those subjects who were uninfected (Figure 6.7). All three mediators of the acute-phase response were induced in subjects experiencing a cold, closely tracking the trend of symptoms and detectable viral replication. Surprisingly, there was also some earlier induction in asymptomatic subjects prior to the period of detectable viral replication (day +1 to day +3).

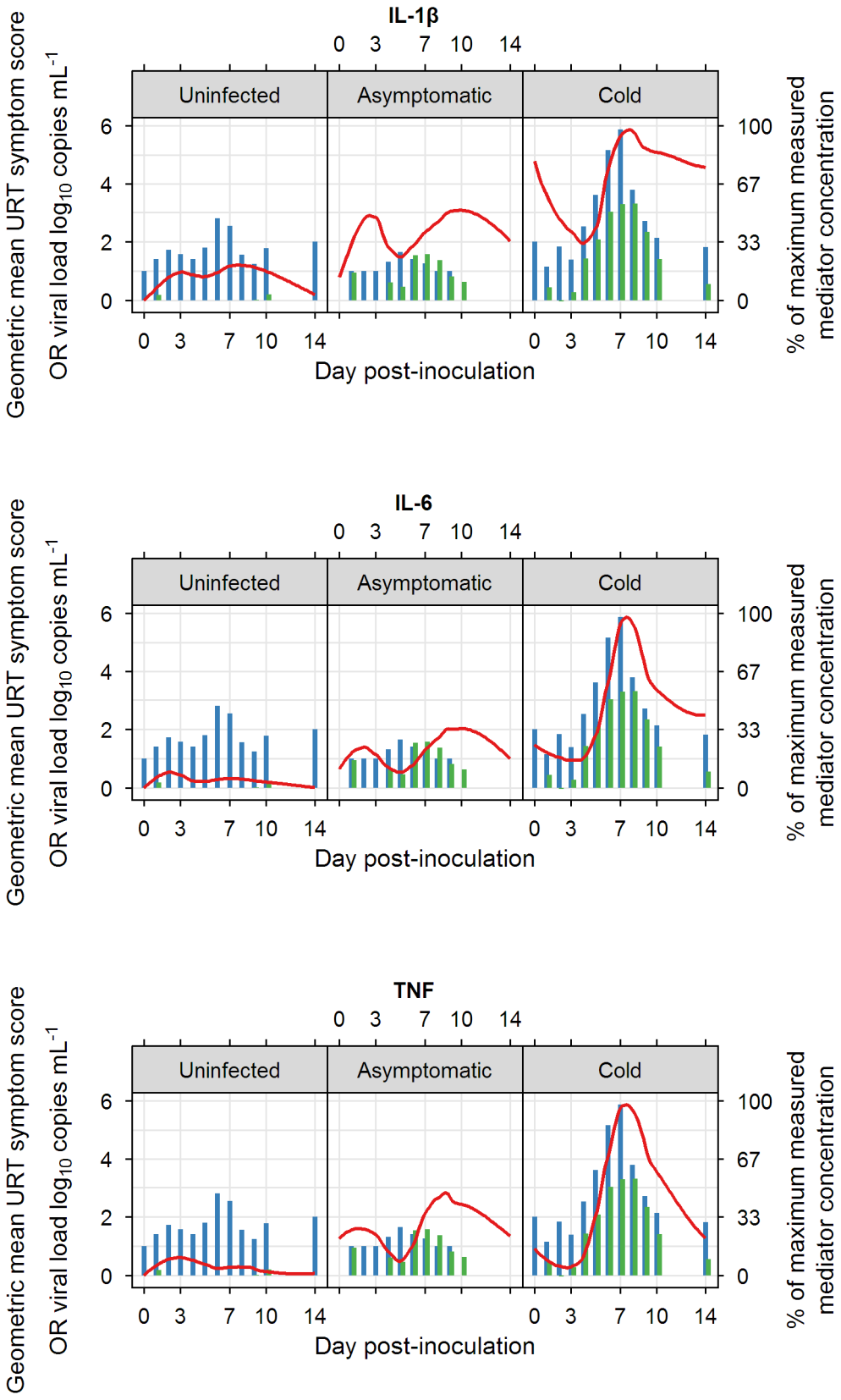


Figure 6.6 Dynamics of symptoms (blue bars), viral load (green bars) and smoothed IL-1β (top), IL-6 (middle) and TNF (bottom) response (red line) for subjects demonstrating the three different outcomes (uninfected, n = 14, asymptomatic, n = 6, cold, n = 17). Mediator levels expressed as % of maximum concentration.

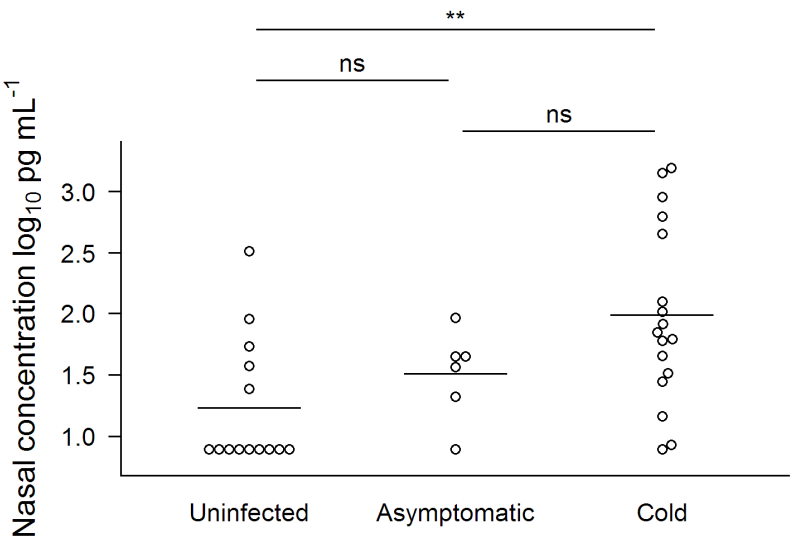


Figure 6.7 Nasal IL1-β concentration (log10 pg mL-1) at day 0 for subjects exhibiting the three outcomes. Horizontal bar indicates median. Kruskal-Wallis Chi-squared = 8.5, df = 2, p-value = 0.014. Star indicates significance for Mann-Whitney-Wilcoxon test (= p < 0.01, * = p < 0.05, ns = p > 0.05).**

6.2.2.2 Antiviral responses

The temporal relationship between detectable viral replication and a host inflammatory response apparently initiated by IFN-γ and induced chemokines IP-10 and MIG, combined with the acute phase response involving IL-1β, IL-6, and TNF has been observed for subjects experiencing a cold. There has also been a suggestion that subjects who ultimately remain asymptomatic despite being infected may display early pre-symptomatic acute phase responses perhaps indicating earlier viral recognition. Type I interferons (IFN-α and IFN-β) are made by host cells following viral recognition by pattern recognition receptors such as RIG-I and TLR-3. They are able to induce an antiviral state in neighbouring cells by promoting the destruction of mRNA and inhibition of translation. They are also potent activators of NK cells, enhancing cytotoxicity. IL-15 is important for T cell and NK cell survival and has recently been shown to enhance the CD8 T cell and NK responses in rhinovirus infection (Jayaraman et al. 2014). Figure 6.8 shows the dynamics of IFN-α and IL-15 in subjects experimentally challenged with RSV. Induction of both mediators closely followed the trend of detectable viral replication in subjects experiencing a cold, supporting the idea that secretion of these mediators is driven by detection of virus. Most striking was the observation of early induction of both mediators in subjects infected but remaining asymptomatic. IFN-β appeared to be detectable with a pattern of suppression during the quarantine period noted in all three groups.

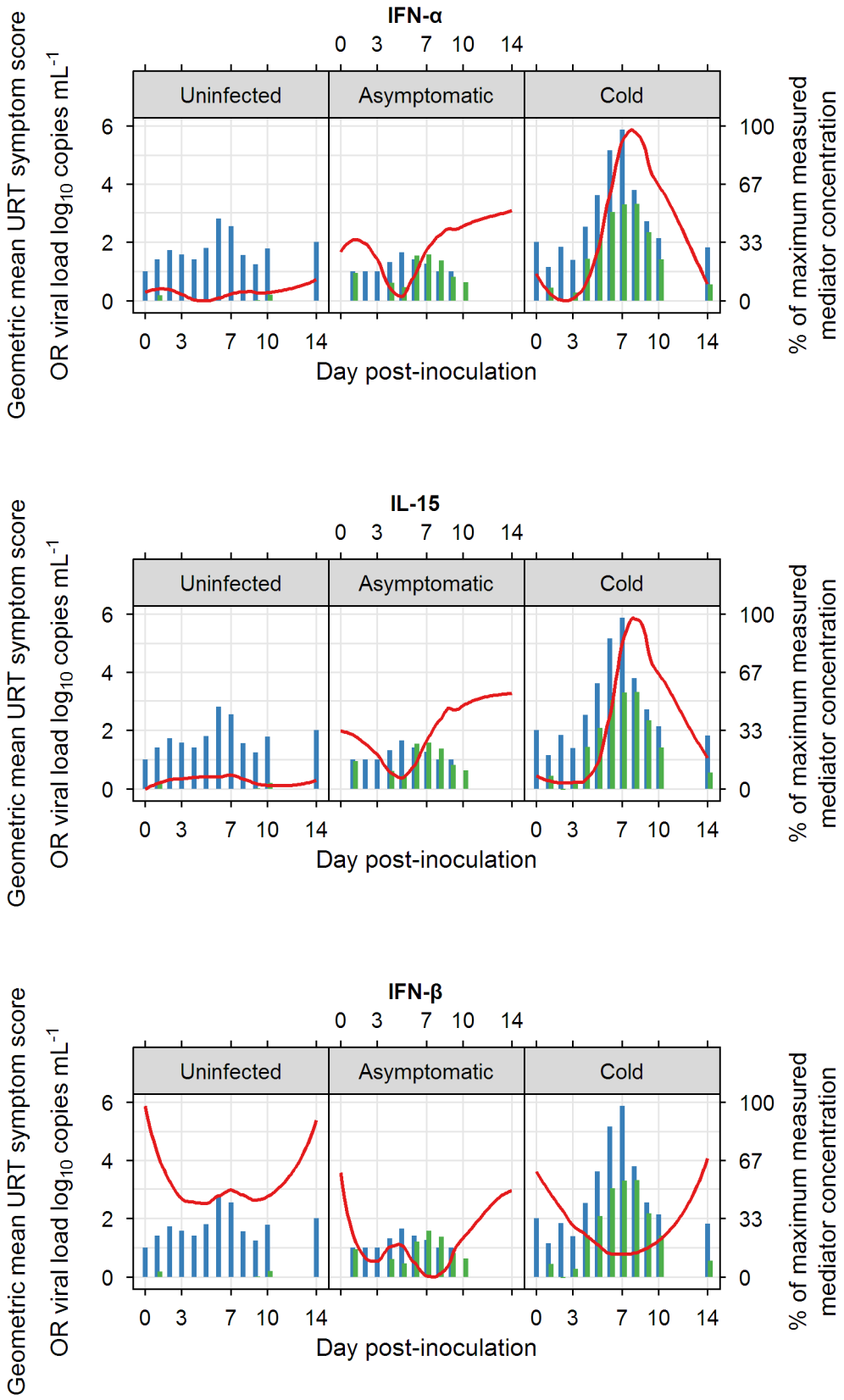


Figure 6.8 Dynamics of symptoms (blue bars), viral load (green bars) and smoothed IFN-α (top), IL-15 (middle) and IFN-β (bottom) response (red line) for subjects demonstrating the three different outcomes (uninfected, n = 14, asymptomatic, n = 6, cold, n = 17). Mediator levels expressed as % of maximum concentration.

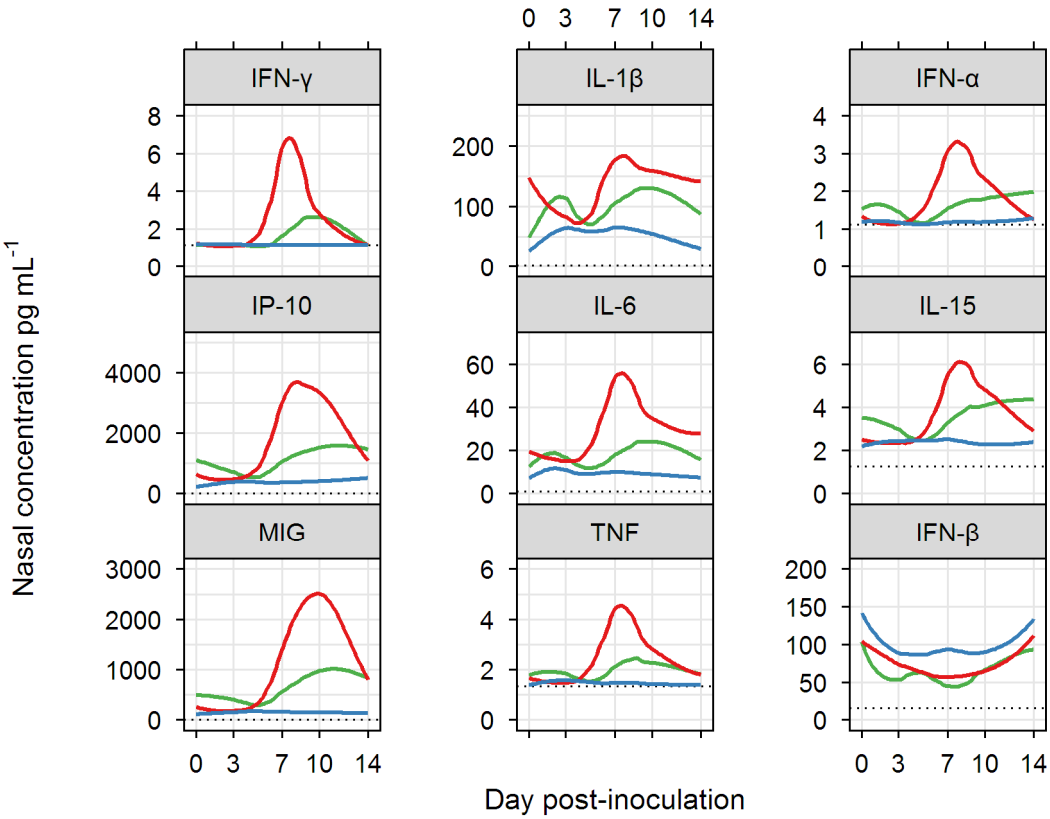


Figure 6.9 Comparison of smoothed mediator response in subjects exhibiting the three different outcomes (uninfected, n = 14, asymptomatic, n = 6, cold, n = 17). Mediator concentrations are expressed as pg mL^{-1}. Horizontal strips indicate mediator being assayed. Dotted lines indicate the lower limit of detection for the assay.

6.2.2.3 Summary of mediator dynamics

The dynamics of mediators expressed as pg mL^{-1} for subjects exhibiting the three different outcomes is summarised in Figure 6.9. Infected subjects show dynamic changes in nasal mediator levels during periods of viral shedding. By contrast, uninfected subjects show little changes in nasal mediator levels during the study period (except for IFN-β where changes occurred in all three groups). Uninfected subjects had lower IL-1β concentrations at baseline than did those who developed a cold. The acute phase mediators IL-1β, IL-6, and TNF were strongly induced during the period of symptoms and viral shedding in subjects with colds. These subjects also showed strong induction of IFN-γ during the period of viral shedding with slightly later induction of induced chemokines IP-10 and MIG. The kinetics of IFN-γ follow those of viral shedding almost exactly (Figure 6.4). Asymptomatic-infected subjects also demonstrated induction of these six mediators

during the period of viral shedding, however levels were proportionally lower except for TNF. Notably, asymptomatic-infected subjects showed some early induction of IL-15, and IFN-α prior to the onset of symptoms and viral shedding, with lower levels of induction during the period when symptoms and viral shedding were present. The trend of IFN-β was surprising. This mediator appeared to be expressed constitutively in the nose pre-infection with levels declining during the quarantine period (day +1 to day +10) before returning to near baseline. The nadir appeared around day +7 to day +8, consistent with the peak of viral shedding in infected subjects. This effect might be attributed to RSV-mediated IFN suppression by NS1 and NS2 if the same trend were not also observed in uninfected subjects. The trend was for a lower nadir of IFN-β in infected subjects; however, the comparison was not significant (Figure 6.10).

6.2.3 Correlations of mediators with measures of disease

A measure of RSV disease has yet to be defined. In this study, infection with RSV was defined by virus detectable in the nasal secretions on two consecutive occasions. The amount of viral shedding could therefore be used as one measure of disease. An RSV infection is expected to cause some respiratory tract symptoms and correlations between viral shedding and symptoms have been explored in Chapter 4. Thus symptoms reported by infected subjects, irrespective of whether criteria for a cold are met, may also be used as a measure of disease. So far, the dynamics of nasal mediators, viral shedding, and symptoms have been explored to investigate which particular mediators may be participating in which phases of the host response to RSV infection. To support the hypothesis that certain mediators are associated with measures of disease, correlation between nasal mediator levels and levels of symptoms and viral shedding were explored.

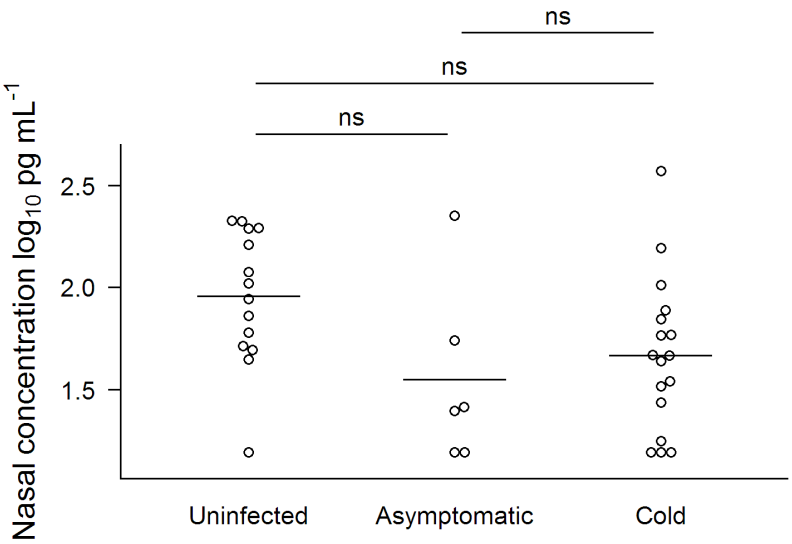


Figure 6.10 Nasal IFN-β concentration (log_{10} pg mL^{-1}) at day +7 for subjects exhibiting the three outcomes. Horizontal bar indicates median. Kruskal-Wallis χ^2 = 6.8, df = 2, p-value = 0.034, however subsequent pairwise comparisons by Mann-Whitney-Wilcoxon test all non-significant.

6.2.3.1 Correlations with symptoms

The relationship between the magnitude of nasal mediator concentration and symptom score was investigated by first calculating the AUC of each variable using the trapezium rule (see section 2.3.2). Whilst the point that such summary statistics might mask some details of the data has already been made, the intention here was to investigate the relative overall magnitudes (rather than trends over time) of two variables that have been measured serially on a group of subjects. Alternatively, a single arbitrary time point could have been used or the peak value. Both of these would be more prone to bias than the AUC which uses all the observations. The author is aware of statistical methods (e.g. mixed effects models) to analyse data with repeated measures, however such methods are complex and thus the AUC was chosen as a compromise.

The hypothesis was that symptoms might result from host inflammation as measured by nasal mediators. Figure 6.11 shows a scatterplot of AUC upper respiratory tract symptom scores on the y-axis and nasal mediator concentration on the x-axis for the nine mediators described in section 6.2.2.3. A loess smooth has been added to aid visual interpretation of the trend, but no claim is made about causality. Surprisingly, weak positive correlations were observed for IFN-γ, IL-1β, and

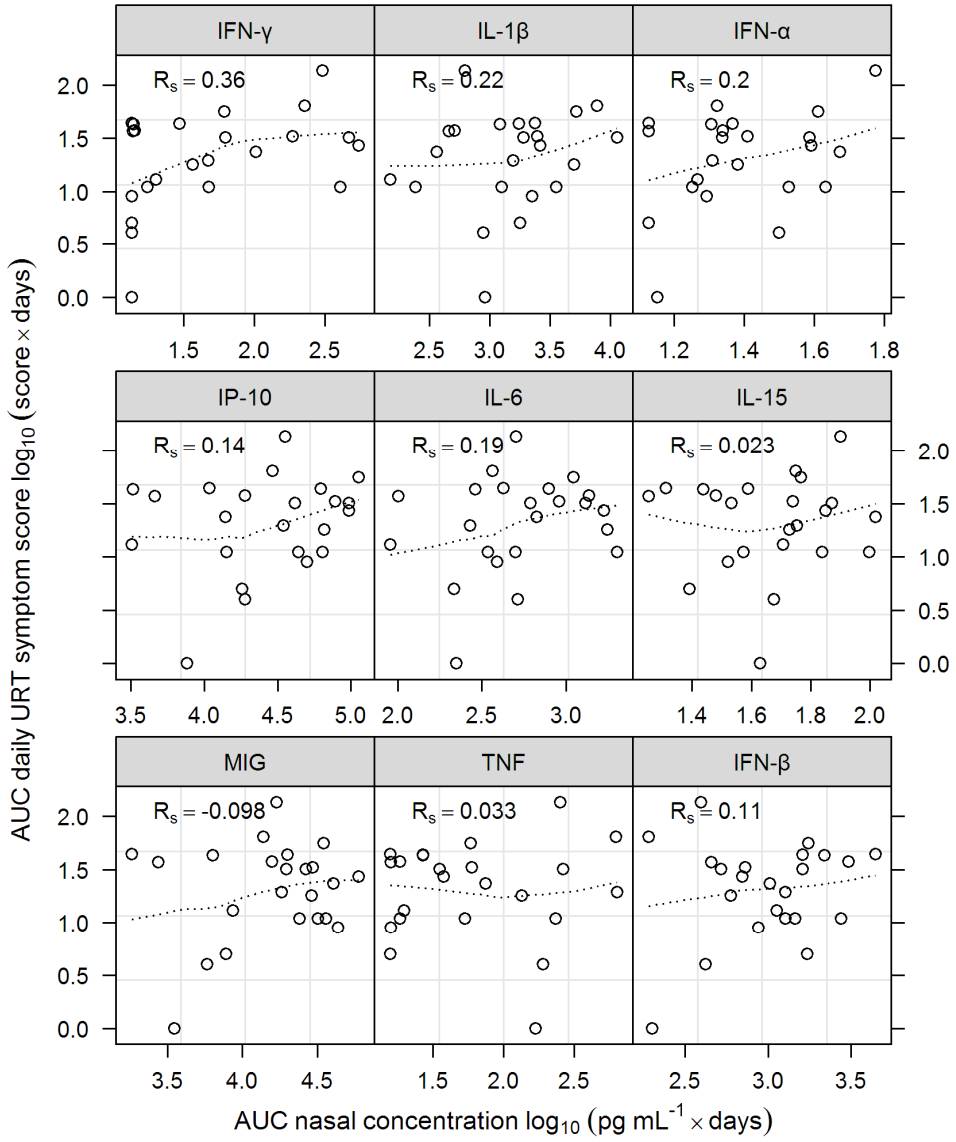


Figure 6.11 Scatterplot of upper respiratory tract symptoms scores and nasal mediator levels (both as AUC for the total period measured). Dotted line is loess smooth to aid visual interpretation of trend. Horizontal strips indicate mediator. Spearman's rank correlation coefficient (Rs) is shown, all non-significant.

perhaps IFN-α and IL-6 only; none reached statistical significance (closest was for IFN-γ, p = 0.09). No relationship was observed for the remaining mediators.

6.2.3.2 Correlations with viral load

The hypothesis was that nasal mediators are produced as a response to viral replication. Figure 6.12 shows a scatterplot of AUC nasal lavage viral load on the x-axis and nasal mediator concentration on the y-axis for the nine mediators described in section 6.2.2.3. A loess smooth has been added to

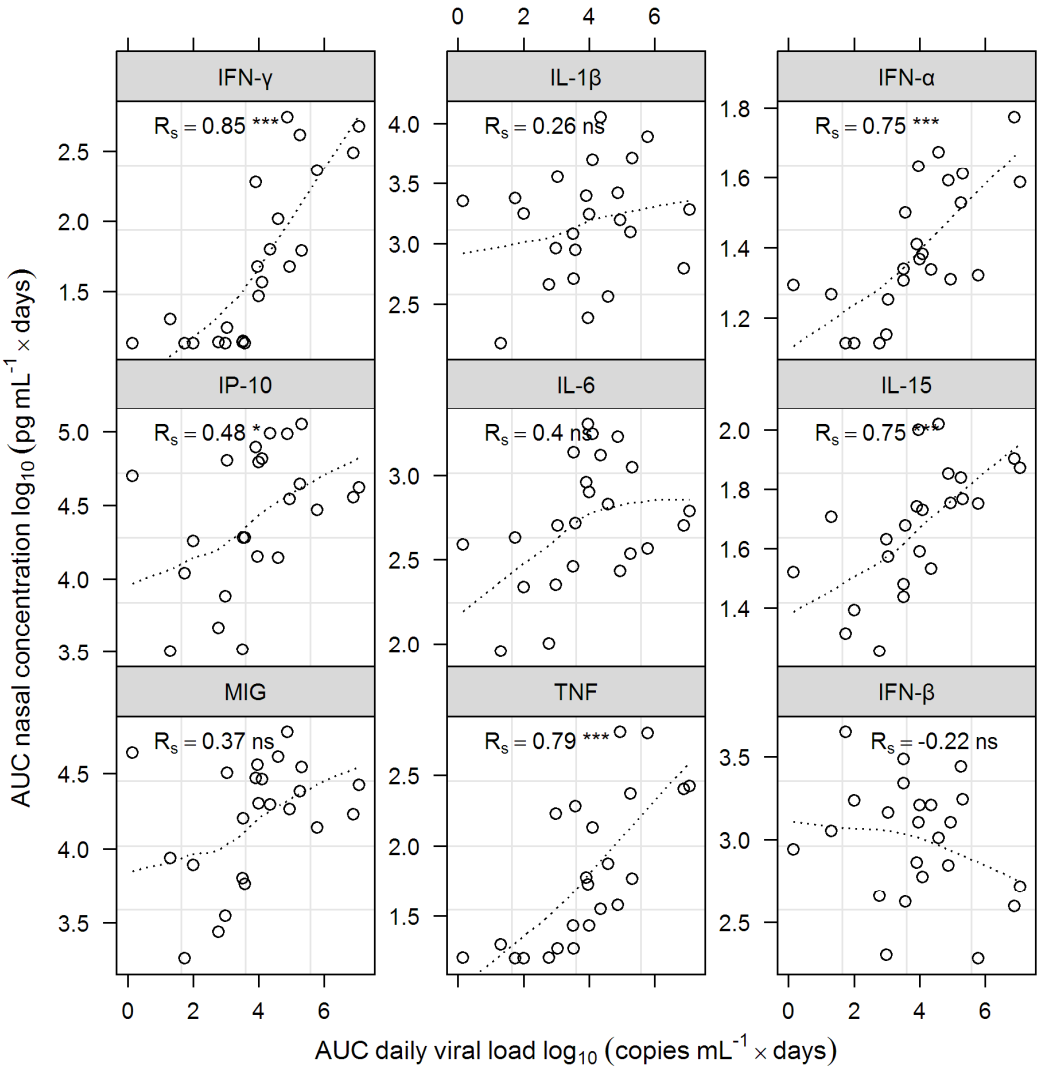


Figure 6.12 Scatterplot of nasal lavage viral load and nasal mediator levels (both as AUC for the total period measured). Dotted line is loess smooth to aid visual interpretation of trend. Horizontal strips indicate mediator. Spearman's rank correlation coefficient (Rs) is shown. Stars indicate significance of the correlation coefficient: ns p > 0.05, * = p < 0.05, ** = p <0.01, *** = p < 0.001.

aid visual interpretation as before. Strong correlations were observed for IFN-γ, IFN-α, IP-10, IL-15, and TNF, suggesting that these mediators are expressed in proportion to the degree of viral shedding. A weak negative correlation was observed for viral load and IFN-β, suggesting again that this mediator might be inhibited by viral replication, however the correlation coefficient was not significant.

6.2.3.3 Summary of correlations of mediators with measures of disease

No correlation using AUC could be demonstrated for symptoms and mediators. By contrast a strong correlation was observed for viral load and several pro-inflammatory (e.g. IFN-γ and TNF) and antiviral (e.g. IFN-α) mediators. This suggests that the host mediator response is proportional to the degree of viral replication thus viral replication may cause host inflammation and lead to epithelial injury.

6.3 Discussion

Analysis of serial mediator measurements proved extremely challenging due to the variation between subjects and baseline noise. Optimisation of nasal epithelial lining fluid sampling and elution may well be required however the application of loess smoothing allowed trends to be observed. Subjects experiencing a cold showed strong induction of antiviral mediators IFN-α and IL-15, Th1 mediator IFN-γ and associated chemokines IP-10 and MIG, and the acute phase response mediators IL-1β, IL-6, and TNF. IFN-α induced by virally infected cells limits spread of virus and enhances the adaptive immune response. IFN-γ is critical for Th1-mediated responses involved in elimination of virally infected cells and appears to initiate a proportionate response to viral replication. The chemokines IP-10 and MIG are both ligands for the receptor CXCR3, which is present on lymphocytes, especially T cells and NK cells. Their secretion may therefore act to draw in cytotoxic cells to facilitate clearance of virally-infected epithelial cells. As well as participating in the acute phase response, TNF is produced by CD_4 T cells during Th1-mediated responses and activates macrophages to kill intracellular pathogens. Cumulative viral load correlated very strongly with cumulative nasal TNF, IFN-α, IFN-γ and associated chemokine levels. Taken together, these observations support the hypothesis that a host inflammatory response to viral replication is driven by TNF, IFN-α and IFN-γ.

Seven subjects (6 included in the analysis of mediators) became infected but did not develop typical common cold symptoms. Antibodies have been demonstrated to correlate with protection but not influence the course of disease once infection is established. Viral load and symptoms correlate and

it is hypothesised that the degree of viral-induced epithelial injury leads to a proportionate inflammatory response that is manifested by the host as a cold. It follows that early and effective antiviral responses manifested by IFN-α and IL-15 may limit viral replication and therefore host disease. The numbers of subjects exhibiting this response are few so it is difficult to draw firm conclusions. Furthermore, the mechanisms of the observed early antiviral responses are unknown. It is possible that these subjects were recovering from another recent virus infection that was not detected by the multiplex respiratory virus PCR. Alternatively they may have been responding to something in the local environment that was somehow able to trigger PRR and hence induce IFN. The most interesting, albeit speculative, hypothesis is that these subjects are somehow better equipped to recognise RSV early and mount efficient and rapid antiviral responses. Interferon-mediated viral restriction via recognition of cytoplasmic dsRNA from replicating virus by RLH is an important mechanism of innate immunity to RSV (Liu et al. 2007; Ling, Tran, and Teng 2009). Although no associations between SNP of RIG-I and RSV severity in children have so far been demonstrated, it remains possible that more complex variations in RIG-I response or that of other PRR may contribute to susceptibility to RSV disease in adults.

The behaviour of IFN-β was in contrast to most other mediators measured. This mediator was constitutively detectable in all groups and levels appeared to decline during the period of quarantine, perhaps more so in infected subjects but this difference was not significant. There is no clear explanation for this phenomenon. It has been suggested that interferons can be expressed in the absence of viral infection and that this is important for the rapid response to infection (Taniguchi and Takaoka 2001). Such 'tonic' secretion is thought to be driven by as yet unidentified stimuli, however it is possible that in the nose, stimulation comes from inhaled antigen. The very act of isolating subjects from the outside world may therefore alter the innate mucosal immune response. This remains speculation, however if true, it would have significant implications for experimental challenge studies of respiratory viruses and for natural colds. Moreover, it may indicate ways in which to non-specifically boost nasal resistance to a range of common cold viruses, and in biodefence applications against unknown novel agents.

The absence of a relationship between cumulative nasal inflammatory mediators and cumulative symptoms was unexpected. It may be that the subjective nature and variable reporting of symptoms by subjects leads to dilution of any real relationship. Symptoms therefore may not be a reliable measure of local disease. Daily mucus weight has been previously used as a disease measure in studies of URTI, however the infection precautions required in this study meant performing this was not feasible. Alternatively, the pathways of the pro-inflammatory mediators measured may be modulated downstream leading to a more attenuated overall host response, thus epithelial injury and clinical disease is reduced. It is also possible that the mediators measured in this project do not fully reflect all the possible disease pathways and that additional mediators remain to be discovered that will better reflect disease.

Whilst serial measurement of nasal mediators in this way can offer great insights into the dynamics of the host response, definite conclusions about the underlying mechanisms are not possible. The cellular source of mediators can only be speculated. Many mediators are produced by numerous cells and act on more than one receptor. Some mediators cannot be readily measured as secreted proteins in nasal fluid. The great between- and within- subject variability of nasal mediator levels poses a challenge for analysis. Optimisation of the timing, frequency, and technique of nasal lining fluid sampling and mediator measurement is necessary. Prior to the analysis of the data, it was thought that the induced illness would be resolved by day +10, hence subjects left quarantine and did not return for further sampling until day +14, meaning no data on mediators or viral shedding was collected on days +11 to +13. The rapid dynamics of nasal mediators was not anticipated. It may be informative to take samples more frequently, especially in the early pre-symptomatic stage where subtle responses may be important. Finally, the measurement of host airway mRNA expression by microarray may reveal some of the cellular pathways involved and identify a signature associated with development or protection from symptomatic disease.

In summary, RSV colds were associated with nasal inflammation driven by TNF, IFN-α, and IFN-γ. Viral load was the disease measure found to correlate best with host pro-inflammatory mediator

response. Early pre-symptomatic antiviral responses manifested by IFN-α and IL-15 appeared protective against symptomatic disease.

7 Discussion of the project

7.1 Summary and implications of main findings

For this project, 40 healthy volunteers were enrolled and challenged without pre-selection according to virus-specific antibody levels. Of the 40, 24 (60%) became infected according to the pre-specified criteria of RSV detection in nasal secretions on at least two consecutive occasions from day +2 to day +10 post-inoculation. Of the 24 infected individuals, 17 (71%) developed common colds, and 7 (29%) did not develop significant symptoms. Experimental adult human challenge with a controlled and standardised inoculum of RSV M37 thus produced a spectrum of outcomes from no infection, to asymptomatic infection, to typical colds. Given that the inoculation and experimental conditions were as consistent as possible, variations in host immunity can be assumed to determine the variable outcome of challenge.

7.1.1 Correlates of protection from infection

Mucosal virus-specific IgA was found to be the most predictive correlate of protection from infection. However, sufficiently protective levels were not found in the majority of adults and durability of antibody in all compartments was found to be poor. The acute ASC response in RSV-infected subjects was dominated by virus-specific IgA^+ ASC consistent with the mucosal site of induction, and both IgG^+ and IgA^+ ASC frequencies correlated with respective convalescent antibody levels in serum and nasal lavage. Thus the mechanisms of acute antibody generation appeared intact. However, a specific deficiency in the induction of IgA^+ MBC was found that may indicate an impairment of humoral memory to RSV. This novel discovery may be of considerable significance.

The observed defect in RSV-specific IgA^+ MBC and poor durability of RSV-specific antibody suggests that RSV plasma cell memory may also be impaired. Serum IgG antibody levels are thought to be maintained by a population of long-lived plasma cells (LLPC) derived from mature acute ASC that migrate to survival niches in the bone marrow (Slifka et al. 1998; Manz et al. 2005).

Bone marrow stromal cells express IL-6, an important factor for B cell survival, and the chemokine stromal cell derived factor (SDF)-1, whose receptor (CXCR4) is present on mature plasma cells (Manz et al. 1998; Cassese et al. 2003), facilitating their migration to and retention in this site. More recently, tumour necrosis factor superfamily members B cell activation factor (BAFF, TNFSF13B) and A proliferation-inducing ligand (APRIL, TNFSF13), have also been shown to be critical for bone marrow plasma cell survival (Belnoue et al. 2008; Benson et al. 2008). The receptor for BAFF and APRIL on naïve and memory B cells is BAFF receptor (BAFF-R also known as TNFSFR13C), however plasma cells downregulate BAFF-R and upregulate a second receptor, B cell maturation antigen (BCMA, Rickert, Jellusova, and Miletic 2011). This receptor can bind both BAFF and APRIL, however the latter interaction with BCMA is of greater affinity (Day et al. 2005). The observed plateau of serum RSV antibody levels following repeated reinfection (Hall et al. 1991) suggests that in adults under natural conditions, RSV-specific plasma cells induced following infection are not able to develop into LLPC. Potential explanations could be a failure to migrate to or remain in the bone marrow or a failure of signalling via BCMA. Immunoglobulin G antibodies may be able to diffuse into and protect the lower airway, but leave the upper airway vulnerable. Immunoglobulin A predominates in most mucosal secretions and exists as glycoprotein-linked multimers known as secretory IgA (sIgA). Production is by plasma cells that have undergone class switch recombination (CSR) and reside in the subepithelial layers such as the intestinal lamina propria. Both T cell-dependent (TD) and T cell-independent (TI) pathways to IgA CSR exist (Mora and von Andrian 2008). The former requires cognate interaction with follicular T-helper (Tfh) cells via CD40-CD40L binding and cytokines including transforming growth factor (TGF)-β1 (Cazac and Roes 2000; Borsutzky et al. 2004) and is thought to be especially important in response to pathogens. The latter involves epithelial and DC recognition of commensal microbial associated molecular patterns via PRR and secretion of IL-10, BAFF, and APRIL (Mora et al. 2006; He et al. 2007; Cerutti, Chen, and Chorny 2011). This mechanism is thought to be primarily involved in immune exclusion of commensals under homeostasis, however it might be much more general, as APRIL-deficient mice fail to make IgA following mucosal immunisation (Castigli et al. 2004).

Maintenance of mucosal antibody is less understood (Hiepe and Radbruch 2006). It has been suggested that it is derived in part from a population of long-lived IgA^+ plasma cells in subepithelial layers or mucosa-associated lymphoid tissue (MALT), analogous to bone marrow LLPC. In support of this hypothesis, it has been shown that human mucosal plasma cells can survive for several weeks, at least *in vitro*, when tissues are removed and cultured intact. In these studies, survival depends on intact tissue architecture as well as IL-6 and ARPIL signalling (van Laar et al. 2007; Mesin et al. 2011). Whether these cells are truly LLPC with a lifespan of many years *in vivo* is unclear.

Alternatively or in addition, IgA may be derived from nasal-associated lymphoid tissue (NALT). Following influenza infection, a population of virus-specific ASC is found in the nasal-associated lymphoid tissue (NALT) of mice that persists for the life-time of the animal (Liang, Hyland, and Hou 2001). By contrast, RSV infection of mice produces only short lived virus-specific plasma cells in the NALT even after repeat challenge, and animals are not protected despite significant levels of serum antibody (Singleton et al. 2003). This observation bears a striking resemblance to the life-long susceptibility of humans to RSV. Further work by the same group showed that the poor durability of RSV-specific mucosal ASC and antibody responses could be improved by use of bacteria-derived adjuvants, demonstrating that nature's 'best effort' can be bettered (Etchart et al. 2006). However, it is not clear from this later study whether the mucosal ASC are truly long-lived mucosal plasma cells or short-lived plasma cells continuously generated by an ongoing immune stimulation. The requirement for a potent adjuvant favours the latter.

More recently it has been shown that APRIL-rich plasma cell survival niches may exist in the human mucosa (Huard et al. 2008). In this study, neutrophils infiltrating the human tonsillar epithelium produced APRIL that was retained by binding to subepithelial heparan sulfate proteoglycans. In the absence of neutrophil inflammation, production was by tonsillar keratinocytes. Both wild-type and APRIL-deficient mice were equally able to mount specific-Ig to ovalbumin. However after three weeks, production continued and plasma cells persisted in wild-

types but ceased in APRIL-deficient mice. Thus neutrophil- and epithelial- derived APRIL might promote mucosal antibody longevity. Human infants hospitalised with bronchiolitis were observed to have readily detectable levels of BAFF, but not APRIL in airway lavage fluid (McNamara et al. 2013). Furthermore, RSV infection of human primary airway epithelial cultures failed to induce APRIL secretion in the same study. This lack of APRIL induction in the airway epithelium following RSV infection may explain why RSV-specific IgA shows such poor longevity, however such a hypothesis would need experimental confirmation. If true however, improving epithelial APRIL induction might be a promising strategy to include in a potential RSV vaccine and APRIL induction might be predictive of long-lasting protection.

7.1.2 Determinants of disease

In these studies, the protective effect of humoral immunity on RSV infection was clearly present, but antibody had no clear protective effect against illness once infection was established. Innate recognition of virus by PRR of epithelial cells and DC results in an acute phase response, and an antiviral state mediated by interferon, limiting spread to uninfected cells and activation of immune effectors (such as NK cells). However, virally-infected cells are eliminated by NK and CD8 T cells, and free virus and cellular debris must be removed by phagocytes to facilitate recovery and tissue healing. Data from mouse models suggest that NK cells peak around day 3 and are an important early source of IFN-γ that is vital for later recruitment of CD8 T cells (Anderson et al. 1989; Hussell and Openshaw 1998). The numbers of CD8 T cells peak in the airway after day 6 post-infection when they contribute to viral clearance. It is therefore hypothesised that the early type I IFN-induced activation of NK cells results in IFN-γ-driven endothelial secretion of chemokines such as IP-10 and MIG. These chemokines are ligands for CXCR3 present on activated T lymphocytes, thus production leads to recruitment of these effectors. TNF secreted by CD_4 T cells activates macrophages to kill ingested viral particles, and cytotoxic CD8 T cells then kill remaining virally-infected cells leading to recovery from infection. A diagram showing this theoretical scheme of the host response to RSV infection in the upper airway is shown in Figure 7.1. A recent study using IFN-α receptor knockout mice illustrated the central role of IFN-α in induction of the host antiviral

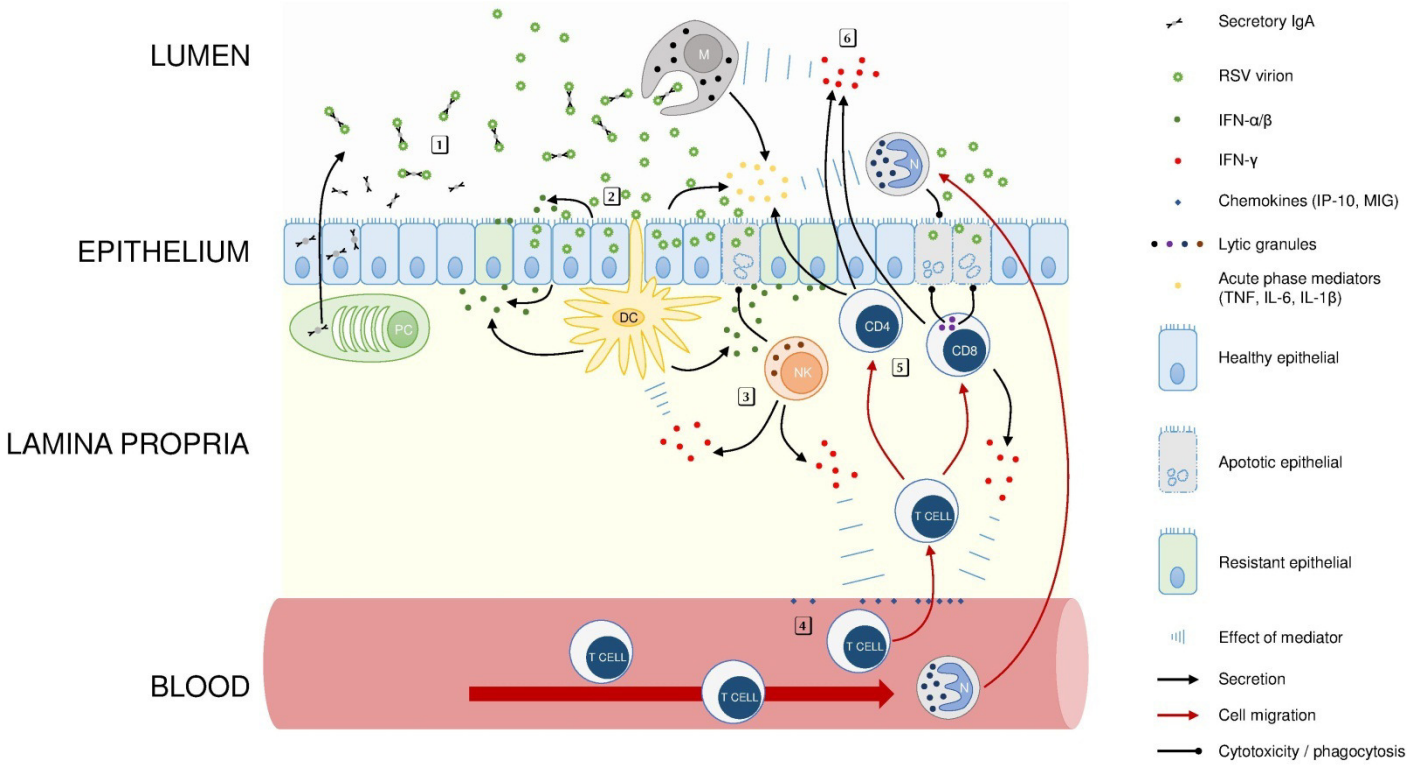


Figure 7.1 Theoretical diagram of host response to RSV infection in the nasal airway. (1) Secretory IgA (sIgA) produced by resident plasma cells (PC) is actively transported across the epithelium and can bind and neutralise virus. (2) Protective sIgA is overwhelmed in most adults, leading to infection of epithelial cells. Recognition of viral proteins by Toll-like receptors results in production of acute phase mediators and recognition of viral RNA by cytosolic RIG-I result in IFN-α and IFN-β production by epithelial cells, fibroblasts (not shown), and dendritic cells (DC). Neighbouring epithelial cells rendered resistant to infection by IFN. (3) Natural killer (NK) cell activity is enhanced by IFN-α leading to killing of virally-infected cells and early IFN-γ production. Additional DC subsets are activated by IFN-γ to migrate to lymph nodes and prime T cells (not shown). (4) IFN-γ induces endothelial chemokine secretion and chemoattraction of activated lymphocytes. (5) Activated CD_4 T cells secrete IFN-γ and TNF. Cytotoxic CD8 T cells kill virally-infected cells, removing source of virus and halting further replication. (6) Macrophages (M) are activated by IFN-γ to engulf and destroy viral particles. Neutrophils (N) are attracted into the lamina propria and lumen by chemokines and contribute to restoration and healing.

response (Goritzka et al. 2014). Deficient mice exhibited greater viral loads and weight loss although still recovered from RSV infection. Induction of type I IFN, IFN-γ and IP-10 was markedly impaired.

The data presented in Chapter 6 demonstrates the robust induction of IFN-α, TNF, and IFN-γ and induced chemokines during the period of maximum viral shedding and symptoms among subjects with colds, supporting the hypothesis that this acts to draw in and activate effector cells to

ultimately clear the virus. However, it must be appreciated that the observations from mice mostly relate to primary infection, and that human NK and T cells have not been directly studied in this project. Thus it is possible that other mechanisms (including local resident mucosal T cells) play a greater part in protection and viral clearance during reinfection in man.

The host inflammatory response is likely to be a major determinant of the variable outcome of established infection. The observed correlations between various mediators and viral load support the concept that some types of inflammation occur in proportion to viral load and thus virus-induced cell injury, thus reflecting both viral replication and symptoms. Observations of hospitalised infants have demonstrated high nasal viral loads and elevated levels of certain inflammatory mediators in those with the most severe illness (Buckingham, Bush, and DeVincenzo 2000; McNamara et al. 2004). Therapeutic antivirals can reduce viral load and resulting disease when given early (DeVincenzo et al. 2010a; DeVincenzo et al. 2014). Therefore it is also hypothesised that early host antiviral responses will limit subsequent viral replication and thus host inflammation, leading to reduced symptoms. This is supported by the observation of early induction of IL-15 and IFN-α, and subsequent lower AUC viral loads and proportionate inflammatory response in subjects infected but remaining asymptomatic.

Deficient IFN production has been found in airway epithelial cells of asthmatics and is thought to play a role in virus-induced exacerbation (Wark et al. 2005; Cakebread et al. 2011). In a recent placebo-controlled therapeutic trial in moderate to severe asthmatics, early augmentation with IFN-β increased recovery and reduced need for additional treatment (Djukanović et al. 2014). Leucocytes from children experiencing recurrent symptomatic respiratory viral infections have been observed to have deficient IFN-α responses to stimulation with respiratory viruses including RSV, rhinovirus and influenza *in vitro* (Isaacs et al. 1981; Pitkäranta, Karma, and Hovi 1993; Pitkäranta et al. 1999). Taken together, these observations and findings in this project support the contention that early IFN production is associated with protection from symptomatic disease.

7.1.3 Implications

These findings emphasise the importance of even greater focus on mucosal vaccination. Measurement of nasal virus-specific IgA is to be preferred to serum in predicting the effect of putative vaccines. However, the unique absence of IgA^+ MBC generation following RSV infection requires further consideration of which types of intranasal vaccines to develop. Furthermore, the lack of pre-existing IgA^+ MBC may necessitate prime-boost strategies rather than being able to rely on boosting existing memory. Sufficiently attenuated intranasal live RSV vaccines have so far been relatively poorly immunogenic (Karron, Buchholz, and Collins 2013; Karron et al. 2013). Strategies to boost the host IFN response by deleting the non-structural proteins of RSV resulted in over attenuation (Wright et al. 2006). However a more creative strategy such as bacteria-like particles or novel adjuvants to modulate host innate immunity may result in a vaccine that can both induce robust mucosal antibody (e.g. by inducing epithelial APRIL production), yet be free of undesired symptomatic effects (e.g. by inducing an early but moderate type I IFN response). This approach has been well-studied in influenza and in animal models of RSV but not in humans (Wijesundara, Xi, and Ranasinghe 2014).

7.2 Future directions

Whilst antibody, acute plasma cell, and MBC specific against RSV have been examined in this project, it was not possible to directly sample secondary lymphoid compartments. Although a plausible chain of events leading to poor IgA-mediated protection has been provided, the mechanism behind the poor durability of RSV-specific antibody needs to be further unravelled. A detailed study on the phenotype and function of acute ASC during RSV infection may provide many insights. For example, a failure of acute ASC to upregulate $CXCR_4$ or BCMA during RSV infection may result in impaired plasma cell survival hence short-lived antibody responses.

However, an analysis of the cellular subsets directly responsible for antibody memory is also required. The bone marrow of adults can be sampled relatively easily and may permit the relative frequencies of RSV-specific LLPC to be measured and compared with those of related viruses such

as measles. The immunoglobulin gene repertoire of mature RSV-specific B cells may also reveal much about the specificity of protective antibodies. Acute ASC underwent single cell sorting during this project. Cells were immediately treated with lysis buffer to permit future studies of rearranged immunoglobulin gene sequences (Wrammert et al. 2008; Smith et al. 2009). The superior protective effect of mucosal IgA demonstrated in this project however mandates a study of mucosal plasma cells. This would require invasive sampling of the respiratory tract by nasal mucosal or endobronchial biopsy, however such procedures in human respiratory and allergy research are well established (Nouri-Aria et al. 2004; Nouri-Aria et al. 2005; Message et al. 2008; Mallia et al. 2011).

The importance of innate immunity in determining outcome to RSV challenge was highlighted by the early antiviral responses seen in some subjects in this project who remained minimally symptomatic despite being infected. An experimental study of prior innate immunity and subsequent experimental infection challenge may allow these responses to be characterised. For example, the response to intranasal TLR agonists such as LPS and poly I:C followed by experimental RSV challenge after an appropriate washout period. The analysis of airway epithelial mRNA by microarray has already been mentioned; nasal scrape samples from subjects enrolled in this project were taken and analysis is planned for a future project.

Finally, the role of T cells as mediators of severe pathology in RSV using sensitised mice and the potential for protection from enhanced disease by Treg needs to be properly explored in a controlled human study. There can be little doubt that T cell responses are crucial for viral clearance and are likely critical for effective and durable antibody responses. However the ability of T cells to cause pathology in human RSV is disputed (Welliver et al. 2007). Ongoing work at Imperial College by Dr. Christopher Chiu and Dr. Agnieszka Jozwik identifying RSV-specific CD8 T cells in the blood and lungs of experimentally infected humans using peptide-MHC multimers is beginning to shed light on the role of these cells in protection and disease. Parallel work by Alex Guvenel in collaboration with GlaxoSmithKline is aiming to elucidate the role of Tfh in the induction of RSV-specific antibody responses.

7.3 Conclusions

a. A specific impairment in the induction of memory B cell responses against RSV was found that could explain the poor durability and thus continued susceptibility of adults to upper respiratory tract infection.
b. Early local antiviral responses were observed in some infected subjects that resulted in markedly reduced viral shedding and upper respiratory tract symptoms.
c. Experimental RSV challenge can safely and feasibly be carried out without the need for purpose-built quarantine facilities. This promotes involvement of research groups without industry funding and widens the possibilities of studies.
d. The utility of the human experimental infection challenge was again demonstrated, permitting controlled and detailed study determinants of infection and disease.

Future studies involving experimental human RSV challenge in more diverse populations e.g. the healthy elderly, atopics, or asthmatics, may provide even greater insight into the mechanisms of this globally important disease.

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9 Appendix

9.1 Main Case Report Form (screening, inoculation, and study visits)

The Development of a Human Model of Respiratory Syncytial Virus Infection — Subject ID no: _____

Case Report Form, Page 1 of 12 — **CONFIDENTIAL** — Subject Initials: _____

SUBJECT DETAILS

Hospital: St. Mary's Hospital, Imperial College Healthcare NHS Trust
Principal Investigator: Prof Peter Openshaw

☐ **Male** ☐ **Female** **Date of Birth** ☐☐ (dd) ☐☐ (mm) ☐☐☐☐ (yyyy)

Subject Name: _____

Address: _____

_____

Contact numbers: _____

Email: _____

GP Name: _____

GP Address: _____

_____

BASELINE INFORMATION

☐ **Informed Consent** **Date Consented** ☐☐ (dd) ☐☐ (mm) ☐☐☐☐ (yyyy)

ELIGIBILITY CRITERIA (tick to confirm)

☐ **Healthy and aged 18-55**

☐ **No** history of Chronic Respiratory Disease (Asthma, COPD, Rhinitis, Sinusitis) in adulthood

☐ **No** known use of inhaled bronchodilator or steroids within the last 12 months

☐ **No** known use of any medications for rhinitis or nasal congestion within the last 6 months

☐ **No** history of Acute Upper Respiratory Infection in the past 6 weeks

☐ **No** history of smoking in the past 6 months or >5 pack-year lifetime

Completed by: _____ Date form completed ☐☐ (dd) ☐☐ (mm) ☐☐☐☐ (yyyy)

Version 1, 22nd February 2011 — Please return to: Dr. Maximillian Habibi, Imperial College London

SUBJECT DETAILS

☐ **No** allergic symptoms present at baseline

☐ **No** close domestic contact (i.e. sharing a household with, caring for, or daily face to face contact) with children under 3 years, the elderly (>65 years), immunosuppressed persons, or those with chronic respiratory disease

☐ **No** known or suspected immune deficiency

☐ **No** previous use of systemic glucocorticoids within one month, or any other cytotoxic or immunosuppressive drug with 6 months prior to challenge

☐ **No** Known IgA deficiency, immotile cilia syndrome, or kartagener's syndrome

☐ **No** history of frequent nose bleeds

☐ (women of childbearing age only) **Negative** pregnancy test or **NOT** breastfeeding

☐ **No** significant medical condition or prescribed drugs deemed by the study doctor to make the participant unsuitable for the study

Completed by:_____ Date form completed ☐☐ ☐☐ ☐☐☐☐
dd mm yyyy

SCREENING VISIT

Visit date: _____

PHYSICAL EXAMINATION					
BP:	**mmHg**	**Temp:**	**°C**	**PR:**	**bpm**
O2 sats:	**%**	**Ht:**	**cm**	**Wt:**	**kg**

System	Normal	Specify abnormalities
General		
Head Ears/Nose/Throat		
Chest		
Cardiovascular		
Gastrointestinal		
Other:		

Allergies: **Alcohol intake:**

Document any relevant medical history briefly below:

Completed by:_____ Date form completed ☐☐ ☐☐ ☐☐☐☐ (dd mm yyyy)

SCREENING VISIT

BLOOD TESTS			
Date:		**Time:**	
Tube	**Taken?**	**Specify reason if not taken**	**Results**
1x 4.5mL purple EDTA (FBC)			
1x 6mL yellow gel (U&E, LFT, CRP)			
1x 4mL blue citrate (clotting)			
1x lymphocyte subsets			
2x 6mL red plain (serology including Hep B/C, HIV)			
2x 10mL green (PBMCs)			

Urine drug screen: ☐ **Negative** ☐ **Positive – specify, inform Dr**

HIV test explained? ☐ **Yes** **Consent for HIV test given?** ☐ **Yes**

LUNG FUNCTION TEST				
	FEV_1 (L)	**FVC (L)**	**PEFR (L/min)**	**FEV_1/FVC**
Performed? (If not, specify reason)				
Actual				
% predicted				

Completed by:_____ Date form completed ☐☐ ☐☐ ☐☐☐☐
dd mm yyyy

BASELINE (-14 DAYS PRIOR TO INOCULATION)

Visit date: _____

PHYSICAL EXAMINATION					
BP:	**mmHg**	**Temp:**	**°C**	**PR:**	**bpm**
O2 sats:	**%**				

System	No change	Specify abnormalities
General		
Head Ears/Nose/Throat		
Chest		
Cardiovascular		
Gastrointestinal		
Other:		

Pregnancy test: ☐ **Negative** ☐ **Positive – inform Dr and exclude**

☐ **Symptom diary and instructions for completion given**

Completed by:_____ Date form completed ☐☐ ☐☐ ☐☐☐☐ (dd mm yyyy)

BASELINE (-14 DAYS PRIOR TO INOCULATION)

BLOOD TESTS		
Date:		**Time:**
Tube	**Taken?**	**Specify reason if not taken**
1x 6mL red plain (RSV serology)		
2x 10mL green (PBMCs)		

LUNG FUNCTION TEST				
	FEV_1 (L)	**FVC (L)**	**PEFR (L/min)**	**FEV_1/FVC**
Performed? (If not, specify reason)				
Actual				
% predicted				

RESPIRATORY TRACT SAMPLES		
Date:	**Time:**	
Sample	**Taken? (specify site R or L)**	**Specify reason if not taken**
x2 SAM (single side)		
1x Nasal lavage (specify volume recovered)		
1-2x Rhinoprobe (single side only)		
1x Bacteriology throat swab (blue with gel)		
1x 16S throat swab (white)		

Completed by:_____ Date form completed ☐☐ ☐☐ ☐☐☐☐ (dd mm yyyy)

DAY 0 (VIRAL INOCULATION)

Visit date: _____

PHYSICAL EXAMINATION					
BP:	**mmHg**	**Temp:**	**°C**	**PR:**	**bpm**
O2 sats:	**%**				

System	No change	Specify abnormalities
General		
Head Ears/Nose/Throat		
Chest		
Cardiovascular		
Gastrointestinal		
Other:		

RESPIRATORY SYNCYTIAL VIRUS INOCULATION			
Batch no:	Dose:	Time given:	Signature:

☐ **Isolation advice given** ☐ **Symptom diary checked**

☐ **Alcohol gel given** ☐ **Masks given**

Completed by:_____ Date form completed ☐☐ ☐☐ ☐☐☐☐
dd mm yyyy

TEN DAILY POST INOCULATION VISITS										
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**
Date										
Attended?										
Serum										
Blood for PBMCs (x2)										
Blood in Tempus tube (x2)										
x1 Bacteriology throat swab										
x1 16S PCR throat swab										
x1-2 SAM (single side, opposite from last rhinoprobe)										
x1 Nasal lavage (specify side and volume)										
x1 Rhinoprobe (single side, alternate between visits)										
FEV_1 (L) / %										
FVC (L) / %										
PEFR (L/min) / %										
Symptom diary checked										

If any samples are not taken, specify reason

Completed by:_____

Date form completed ☐☐ ☐☐ ☐☐☐☐ (dd mm yyyy)

DAY 14 POST VIRAL INOCULATION VISIT

Visit date: _____

PHYSICAL EXAMINATION					
BP:	**mmHg**	**Temp:**	**°C**	**PR:**	**bpm**
O2 sats:	**%**				

System	No change	Specify abnormalities
General		
Head Ears/Nose/Throat		
Chest		
Cardiovascular		
Gastrointestinal		
Other:		

☐ **Symptom diary collected and filed with CRF**

Completed by:_____ Date form completed ☐☐ ☐☐ ☐☐☐☐ (dd mm yyyy)

DAY 14 POST VIRAL INOCULATION VISIT

RESPIRATORY TRACT SAMPLES		
Date:	**Time:**	
Sample	**Taken? (specify site R or L)**	**Specify reason if not taken**
x2 SAM (single side)		
1x Nasal lavage (specify volume recovered)		
1-2x Rhinoprobe (single side only, opposite from D10 sample)		
1x Bacteriology throat swab (blue with gel)		
1x 16S throat swab (white)		

Completed by:_____ Date form completed ☐☐ ☐☐ ☐☐☐☐ dd mm yyyy

FINAL VISIT

Visit date: _____

PHYSICAL EXAMINATION					
BP:	**mmHg**	**Temp:**	**°C**	**PR:**	**bpm**
O2 sats:	**%**				

System	No change	Specify abnormalities
General		
Head Ears/Nose/Throat		
Chest		
Cardiovascular		
Gastrointestinal		
Other:		

BLOOD TESTS		
Date:		**Time:**
Tube	**Taken?**	**Specify reason if not taken**
1x 6mL red plain (RSV serology)		
2x 10mL green (PBMCs)		

Completed by:_____ Date form completed ☐☐ ☐☐ ☐☐☐☐ (dd mm yyyy)

FINAL VISIT

LUNG FUNCTION TEST				
	FEV_1 (L)	FVC (L)	PEFR (L/min)	FEV_1/FVC
Performed? (If not, specify reason)				
Actual				
% predicted				

RESPIRATORY TRACT SAMPLES		
Date:	**Time:**	
Sample	**Taken? (specify site R or L)**	**Specify reason if not taken**
x2 SAM (single side, record weight of dry SAM and post-sampling)		
1x Nasal lavage (specify volume recovered)		
1-2x Rhinoprobe (single side only)		
1x Bacteriology throat swab (blue with gel)		
1x 16S throat swab (white)		

☐ **Explained that study has ended and care transferred to primary care provider**

☐ **Evaluation form given**

Completed by:_____ Date form completed ☐☐ ☐☐ ☐☐☐☐ dd mm yyyy

The Development of a Human Model of Respiratory Syncytial Virus Infection

Subject ID no: _____

Symptom diary, Page 1 of 1

CONFIDENTIAL

Subject Initials: _____

PLEASE COMPLETE ON THE DAYS INDICATED

Upper respiratory tract symptoms assessment & scoring																
Symptoms	**D-14**	**D0**	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**	**D11**	**D12**	**D13**	**D14**
Sneezing																
Headache																
Malaise/fatigue																
Fever/chills																
Nasal discharge																
Nasal obstruction																
Sore Throat																
Cough																
Total Score																

Please grade the above 8 symptoms using a score of 0-3 as follows:
0: absent
1: mild – present, but does not affect normal daily activities
2: moderate – some interference with or limitation of normal daily activities
3: severe – prevents some normal daily activities

PLEASE BRING THIS DIARY CARD WITH YOU ON EACH VISIT

Version 1, 7th January 2011

9.3 Daily vital signs and sampling CRFs (during quarantine period)

The Development of a Human Model of Respiratory Syncytial Virus Infection

Subject ID no: _____

Sampling Record

CONFIDENTIAL

Subject initials: _____

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Date										
Serum										
Blood for PBMCs (x2)										
Blood in Tempus tube (x2)										
x1 Bacteriology throat swab										
x1 16S PCR throat swab										
x1-2 SAM (single side, opposite from last rhinoprobe)										
x1 Nasal lavage (specify side and volume)										
x1 Rhinoprobe (single side, alternate between visits)										
FEV_1 (L) / %			X				X			X
FVC (L) / %			X				X			X
PEFR (L/min) / %			X				X			X
Symptom diary checked										

If any samples are not taken, specify reason
X = mandatory lung function measurement

Version 1, 7th July 2011

If found please return to: Dr. Max Habibi, Imperial College London, Respiratory Medicine, London W2 1PG

9.4 Blood and nasal sample processing CRFs

The Development of a Human Model of Respiratory Syncytial Virus Infection Subject ID no: _____

Case Report Form, Page 1 of 1 **CONFIDENTIAL** Subject Initials: _____

BLOOD SAMPLE PROCESSING CASE REPORT FORM

Visit No:	Date: / /	Specimen Identification No:	
Time collected:	Processor's initials:	Time peripheral blood processing commenced:	

PBMCs (green tube – mix thoroughly)

Number of tubes collected (N)	
Volume (mL) used for cell count (A) (suggested volume: N x 6)	
PBMC count (cells x 10^6/mL) (B)	
Cell viability (%) (C)	
Total PBMCs isolated (D = A x B x (C/100))	
Concentration (x10^6/mL) required for fresh cell assay (E)	
Volume to resuspend PBMC pellet (V = D/E)	
Volume needed for fresh cell assays (F)	
Fresh cells used (G = F x E)	
Remaining cells (H = (V−F) x E)	
Volume of freeze down media used (mL) (K)	
Frozen cell count (10-25x10^6/mL) (H/K) (cells/mL)	
Number of vials (2-5)	
Time of entry into -80°C freezer (use Mr. Frosty container)	
Date and time placed into liquid nitrogen	
Rack number and box in liquid nitrogen	
Date of thawing (if appropriate)	
Thawed cell viability (%)	

Serum (red tube – keep upright at room temp)

Volume of blood collected (mL)	
Number of 0.5mL serum aliquots	
Time of entry into -80°C freezer	

Tempus® tubes (gene expression – mix thoroughly)

Number collected	
Time of entry into -80°C freezer	

Other notes and calculations

Completed by:_____

Date form completed ☐☐ ☐☐ ☐☐☐☐ dd mm yyyy

Version 3, 10th August 2012

Please return to: Dr. Maximillian Habibi, Imperial College London

DAILY NASAL PROCESSING CASE REPORT FORM

Visit No:	Date: / /	Specimen Identification No:	
Time collected:	Processor's initials:	Time filter paper processing commenced:	Time nasal lavage processing commenced:

Nasal SAM

Number of SAM samples performed (1-2)	
Specify side (R, L, or both)	
Wt of labelled empty Eppendorf (without spin filter)	E
Wt of Eppendorf post spin (recovered sample in buffer) (g)	F
Whole sample wt (g)	F-E

No. of aliquots (130 µL) in -80°C freezer	

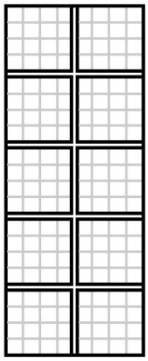
Nasal Lavage

Specify side (R or L)		Aliquot for viral qPCR (unspun, 500µL, freeze at -80°C)	
Post lavage volume recovered (mL)		Aliquot for viral culture in transport media on ice (unspun, 0.5mL in 0.5mL media (1:1))	
		Time inoculated into HEp2 cells:	
Volume of lavage remaining (mL)	L	Aliquot for multiplex PCR (600µL DAY 0 and DAY 7 only)	

Lavage cell counts: FastRead 102 disposable counting slides
10 chambers per slide, 5x2 grids per chamber, each grid has a volume of 100nL
Total count = mean number of cells per grid x 10000 x dilution factor

Resuspend cell pellet in 210µL of PBS, then dilute 10µL into a known volume of Trypan Blue
e.g. 10µL cells + 10µL Trypan Blue for a 1 in 2 dilution *(i.e. dilution factor = 2)*
ONLY leukocytes to be counted; ignore epithelials and RBCs

Indicate cell number in each grid counted (minimum 4 grids)



Dilution factor	a
	Leukocytes
Number of grids counted	b
Total number of cells	c
Mean per grid	d = c / b

Cell count comments (debris, RBCs, epithelials)

Leukocytes / mL of suspension	$c = a \times d \times 10^4$

Lavage cytospin

Dilution (1 in *x*) required to prepare cytospin (if <1, use neat)	c / 100,000
100µL of cells at $1x10^5$ leukocytes/mL in PBS per cytospin block; if fewer than 2 cells per grid, use 200µL neat	
	Satisfactory (Yes/No)
Quality of stained cytospin	

Cytospin differential counts for <400 leukocytes

	Eos	Neuts	Mac	Lymph	Total Leuk
Absolute					
% of leukocytes	%	%	%	%	%

Lavage supernatant

No. of 0.5mL aliquots stored in -80 °C freezer	

Completed by:_____

Date form completed ☐☐ ☐☐ ☐☐☐☐ (dd mm yyyy)

Version 3, 14th June 2012

Please return to: Dr. Maximillian Habibi, Imperial College London

9.5 Loess smoothing of all nine mediators

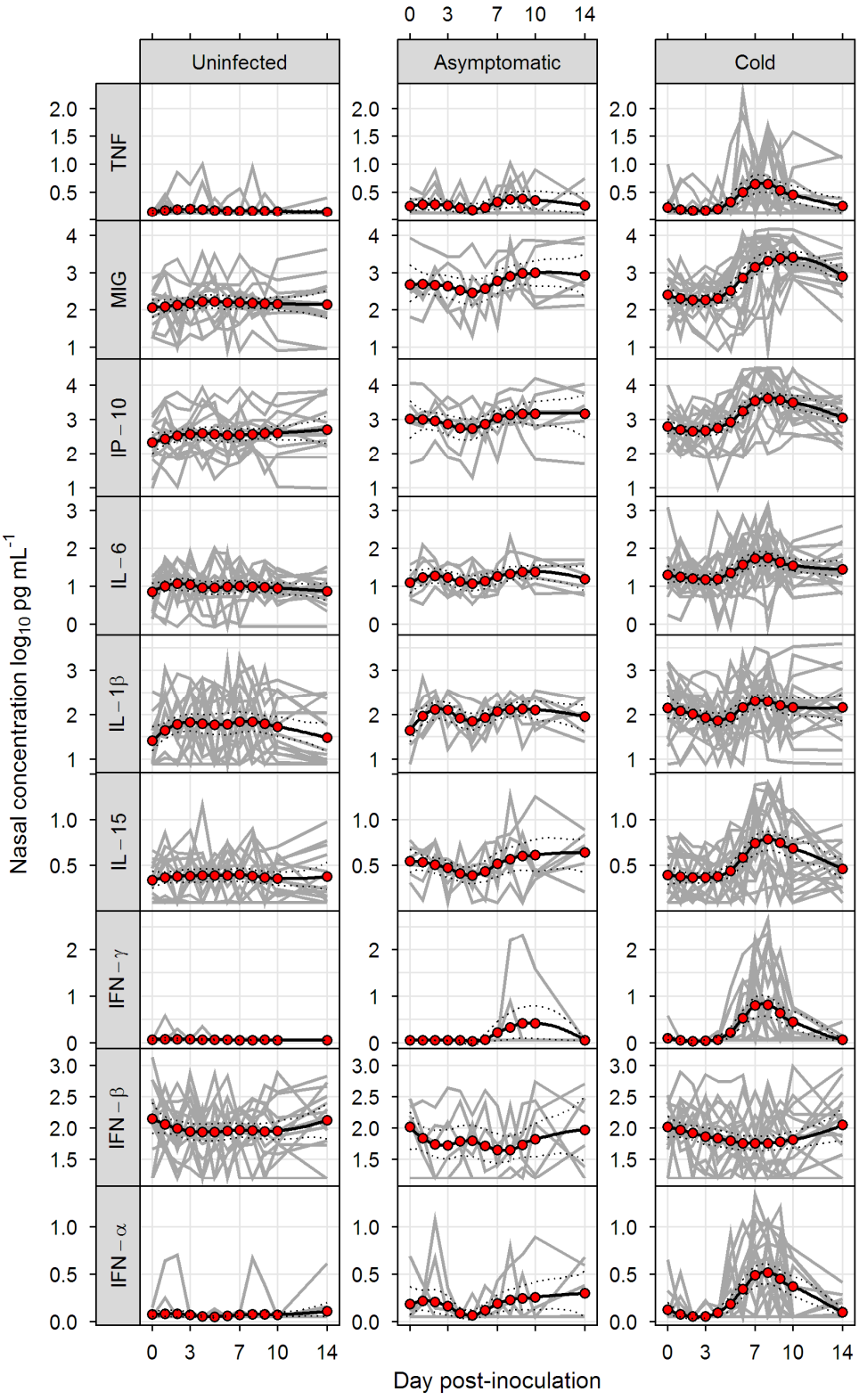


Figure 9.1 Loess technique applied to all mediators measured for the three different outcomes. Grey lines are the raw log-transformed data, thick black line with filled red circles represent the predicted response from the loess model, dotted lines are the point-wise 95% confidence intervals for the prediction. Horizontal strips indicate outcome from challenge, vertical strips show the mediator being assayed.

9.6 Permissions for reuse of figures (in order of use)

