

**INVESTIGATING THE FUNCTION OF
NEUTROPHILS IN RESPIRATORY
SYNCYTIAL VIRUS BRONCHIOLITIS
USING A NOVEL *IN VITRO* MODEL OF
TRANSEPITHELIAL IMMUNE CELL
MIGRATION**

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Authors Declaration

I, Elisabeth Jane Robinson, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

This research is supported by the NIHR GOSH BRC. The views expressed are those of the author and not necessarily those of the NHS, the NIHR or the Department of Health.

Abstract

Background: Respiratory Syncytial Virus (RSV) infects almost all children before the age of 2. In most children, RSV infection causes a mild to moderate illness, but a small percentage of children develop severe RSV bronchiolitis and require hospitalisation. A significant proportion of these acutely unwell children were previously otherwise healthy. Clinical studies of infants with severe bronchiolitis have shown a massive infiltrate of neutrophils to the airway, comprising over 80% of all cells recovered. In this thesis I explore further whether the effect of neutrophil infiltration is necessary for viral clearance or detrimental to clinical disease, by exploring the interaction between neutrophils and RSV infected airway epithelial cells (AECs).

Aims: The aims of this thesis were to (i) describe neutrophil movement across RSV infected AECs, (ii) measure the effect of interaction with RSV infected AECs on neutrophil apoptosis, (iii) activation, then (iv) compare these effects between adult and cord blood neutrophils.

Methods: To examine the complex relationship between the RSV infected airway and neutrophil function, this study optimises a novel *in-vitro* transepithelial migration model of RSV infected AECs, comprising human neutrophils, ciliated epithelial cells cultured at air-liquid interface and RSV. This has allowed real-time video microscopy of neutrophil transepithelial migration, examination of dynamic neutrophil activation and assessment of neutrophil viability and apoptosis during infection.

Results and Conclusion: Three populations of neutrophils interacting with AECs were described, those basolateral to AECs, those apical and those adhered to AECs. Imaging neutrophil migration revealed adhered neutrophils appear to kill virally infected AECs during migration. Neutrophil apoptosis and activation were defined by their interaction with AECs and evidence presented in this thesis suggest physiological differences between adult and cord blood neutrophil response to RSV infected AECs may predispose to a more severe disease picture in neonates.

Impact Statement

The primary impact of this research will be through publications describing the interaction of neutrophils and RSV infected airway epithelial cells (AECs) in a sophisticated physiological *in vitro* model of transepithelial migration. The findings of my study would be of interest to academic researchers (immunologists, virologists, epithelial biologists), clinical researchers (paediatric intensivists, respiratory clinicians, general paediatricians and neonatologists), drug development companies, patients and their families.

In the first instance, the findings presented in this thesis demonstrate a novel contribution to the discipline, in particular the highlighted differences in adult and cord blood neutrophil response to RSV infected AECs. This in turn will enable future academic scholarship into the lung environment and dynamic immune response to RSV Bronchiolitis. This could take the form of additional research projects, PhD studentships and translational clinical studies.

Outside academia, these findings will also be of use to prescribers and pharmaceutical companies as these findings may inform how neonates are likely to respond to infection. My insight into neutrophil migration and apoptosis will also be of use in rational drug design and the potential development of neutrophil targeted therapies for RSV Bronchiolitis. The impact of this possibility is significant, as currently there are no licenced treatments for RSV Bronchiolitis. In the long term, children with RSV Bronchiolitis will benefit from the field's improved understanding of immune-mediated RSV pathophysiology and any novel therapies or management plans arising from it.

The methods described here will be of interest to academics across disciplines, especially the transepithelial migration model and adaptations for microscopy. This model is highly translational and could be adapted for use in immune cell migration research across tissues other than the lung. It will also be of benefit to pharmaceutical companies, as it provides an excellent system for testing novel therapeutics in a physiological manipulable model of human differentiated cells. This model will also enable future research into viral infection of the airway, and indeed, a collaborative study between this institute, Imperial College London and the Sanger institute has just been funded utilising this model to investigate the immune response to SARS-Cov2. Combining the knowledge and methodologies presented in this

thesis with specialist expertise in RNA SEQ and virology will enable powerful research which will ultimately benefit our understanding of infection and improve our ability to predict severe disease and treat it appropriately.

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Abbreviations

Abbreviation	Definition
AEC	Airway Epithelial Cell
ALP	Alkaline Phosphatase
APC	Allophycocyanin
APC-CY7	Allophycocyanin-Cyanine7
AU	Airy Unit
BAL	Bronchoalveolar Lavage
BD	Beckton Dickinson
BEGM	Basal Epithelial Growth Medium
BPI	Bacterial Permeability increasing protein
BS	Bovine Serum
CAD	Caspase Activated DNase
CARD	Caspase Recruitment Domain
CASPASE	Cysteine-Dependent Aspartate-Directed Proteases
CD	Cluster Differentiation
cDC	conventional Dendritic Cell
COPD	Chronic Obstructive Pulmonary Disease
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid

ELISA	Enzyme Linked Immunosorbent Assay
EPIC	Electronic Privacy Information Centre
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum
Fc	Fragment Crystallizable
FcR	Fragment Crystallizable Receptor
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
fMLP	n-formylmethionine-leucyl-phenylalanine
FSC	Forward Scatter
GCSF	Granulocyte Colony-Stimulating Factor
GFP	Green Fluorescent Protein
GMCSF	Granulocyte-Macrophage Colony-Stimulating Factor
GOSH	Great Ormond Street Hospital
HBSS	Hanks Balanced Salt Solution
HEP-2	Human Epithelial Type 2 cells
ICAM-1	Intercellular Adhesion Molecule 1
ICH	Institute of Child Health
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile Range
LD	Long Distance

LED	Light Emitting Diode
LFA-1	Lymphocyte Function-Associated Antigen 1
LTB4	Leukotriene B4
LWD	Long Working Distance
MEM	Minimum Essential Medium
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil Elastase
NETs	Neutrophil Extracellular Traps
NFkB	Nuclear Factor Kappa B
NK	Natural Killer
NPA	Nasopharyngeal Aspirate
p value	Probability Value
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCD	Primary Ciliary Dyskinesia
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein ligand 1
pDC	plasmacytoid Dendritic Cell

PE	Phycoerythrin
PE-CY7	Phycoerythrin-Cyanine7
PET	Polyethylene Terephthalate
PFA	Paraformaldehyde
PFU	Plaque Forming Units
PI	Propidium Iodide
PICU	Paediatric Intensive Care Unit
REDCAP	Research Electronic Data Capture
RIG-1	Retinoic Acid-Inducible Gene 1
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	revolutions per minute
RSV	Respiratory Syncytial Virus
SEM	Standard Error of the Mean
SSC	Side Scatter
TEER	Trans-epithelial Electrical Resistance
Th	T helper
TLR	Toll-Like Receptor
UCL	University College London
UCH	University College Hospital
VCAM-1	Vascular Cell Adhesion Molecule 1
WHO	World Health Organisation

Chapter 1 General Introduction

1.1 Respiratory Syncytial Virus (RSV)

Human Respiratory Syncytial Virus (RSV), first identified in 1956, is the leading cause of infant hospitalisation with lower respiratory tract infection and the leading cause of bronchiolitis worldwide(1). A ubiquitous virus, it is thought to infect almost all infants by the time they are two(2).

1.1.1 Virology

Human Respiratory Syncytial Virus (RSV) is an enveloped negative sense RNA virus, measuring 150-300nm in diameter, of the family *paramyxoviridae*. A pleomorphic virus, RSV virions can exist as either spherical or filamentous forms(3).

As shown in **Figure 1-1**, the RSV genome consists of 10 genes encoding for 11 proteins, 3 of which comprise the viral envelope – G glycoprotein (RSV G Protein), F glycoprotein (RSV F Protein) and small hydrophobic protein (RSV SH Protein). A nucleocapsid protein (RSV N Protein) encapsulates the single RNA stranded genome. RSV G protein is essential for attachment to cells, whereas F protein is essential for fusion of the viral envelope with the target cell membrane and also promoting syncytia formation(4). RSV has two antigenically separate subgroups separated mainly by structural differences in their RSV G protein, A and B(5).

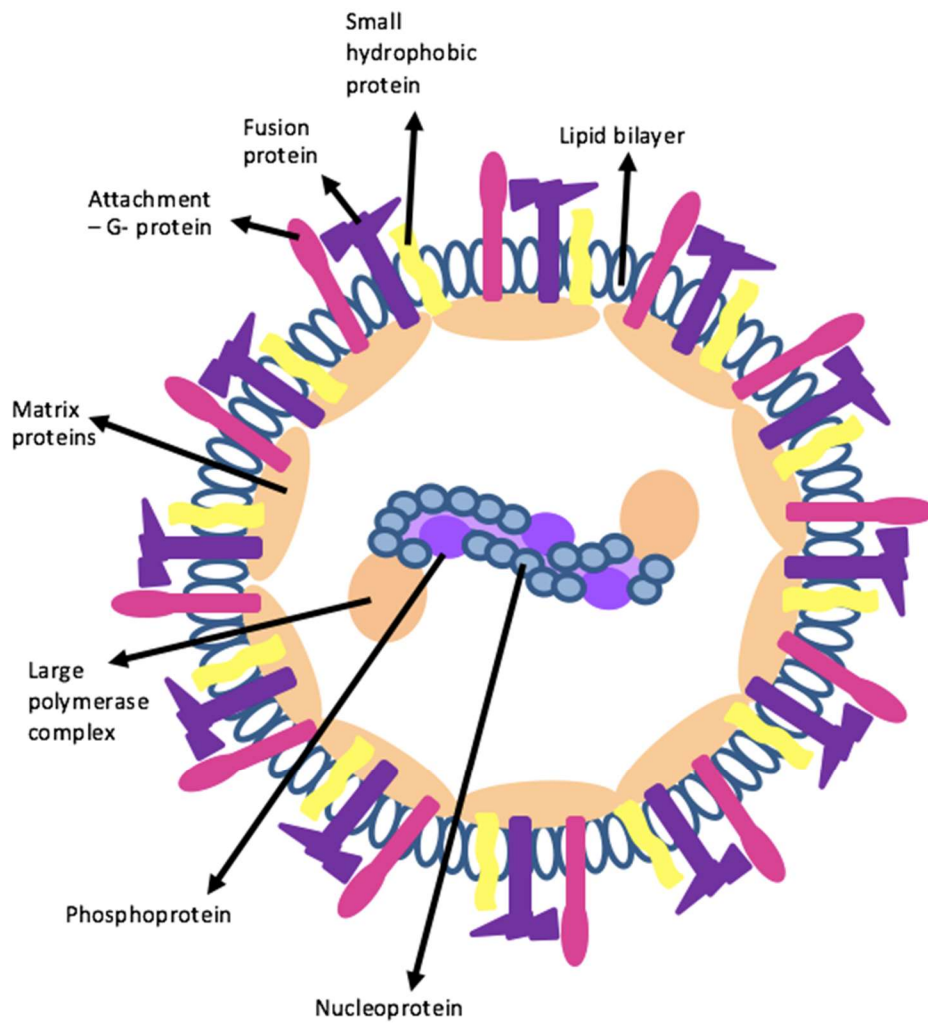


Figure 1-1 Schematic of Respiratory Syncytial Virus Virion, demonstrating its structural component proteins.

Adapted from Hall et al, 2001(5).

1.1.2 Life cycle

Much of our understanding of the life cycle of RSV comes from our study of other enveloped viruses such as influenza. RSV fuses with the target cell membrane, classically a respiratory epithelial cell, allowing its envelope content, including genome, to enter the cytoplasm and replicate. RSV protein transcripts can be detected 4 – 6 hours after initial infection, and reach peak concentration by 15 – 20 hours(6,7). New virus starts to be released from cells from 10 – 12 hours post infection and release continues until the cell dies(8). While it is well documented that RSV replicates within epithelial cells(9,10), there is conflicting evidence as to whether RSV can replicate within other cells, including those recruited to the respiratory tract such as the neutrophil.

1.2 RSV Bronchiolitis

Bronchiolitis is the most common cause of hospitalisation in infants under the age of one and around 80% of cases are caused by RSV(11). Approximately 2-3% of infants will develop bronchiolitis severe enough to warrant hospital admission before the age of one (12).

1.2.1 Epidemiology

RSV infections occur in seasonal epidemics during the winter months and it's predicted almost every child will have been infected at least once by the time they are two years old(2). Despite causing a mild, upper respiratory tract illness in most children, RSV can cause severe respiratory illness and around 1-3% of infected children develop a more severe bronchiolitis illness and require hospital admission or intensive care facilities (11,12). RSV is also of importance in the elderly with some estimating that a significant proportion of apparent influenza cases may actually be caused by RSV (1). In spite of an increase in research investigating RSV, there is still no vaccine licenced in 2020 and a high number of winter admissions to hospital of infants with acute bronchiolitis continue.

Extensive modelling studies have found significant variation of incidence and mortality, year on year, within any given population. Shi et al described that in 2015, in children under 5 years there were an estimated 33.1 million (uncertainty range (UR) 21.6–50.3) cases of RSV acute lower respiratory tract infections which resulted in 3.2 million (2.7–3.8) hospital admissions, and 59,600 (48,000–74,500) hospital deaths(13). The same study estimated the overall RSV acute lower respiratory infection deaths in 2015 as 118,200 (Uncertainty range 94600–149400). RSV mortality occurs almost entirely in the developing world (14).

In England during 2018/19, there were 7,860 recorded admissions where acute bronchiolitis due to RSV was the primary diagnosis, and, in addition, there were 40,505 admissions recorded as acute bronchiolitis (unspecified cause), a large proportion of which are likely attributable to RSV infection(15).

1.2.2 Disease presentation

Despite being widespread in the winter season, there is no universally recognised clinical definition of bronchiolitis. However, it is widely accepted as an acute respiratory illness

affecting infants, including coryza and low grade fever which progresses over a few days to a cough, tachypnoea, hyperinflation, chest retraction and widespread crackles and wheeze(16).

1.2.3 Risk factors

Risk factors which correlate with more severe RSV infection include prematurity, immunocompromise, passive smoke exposure, pre-existing lung pathologies, congenital heart disease and low birth weight, however 50% of children who develop severe RSV bronchiolitis have no identifiable risk factors for disease(11,17).

1.2.4 Transmission

RSV is highly infectious and disseminates from host to host, borne in aerosolised droplets and secretions(18). The incubation period for RSV infection has been estimated as a median of 4.4 days but can be up to eight days(19). RSV is accepted as one of the most contagious infections in humans; sero-epidemiological studies have shown that almost all infants have been infected by the time they have reached the age of two years(2).

1.2.5 Management

Management of infants with RSV bronchiolitis is limited to supportive care, including nasogastric feeding, fluids for rehydration, management of fever, mechanical ventilation if in respiratory failure and supplementary oxygen if hypoxia is present(20). No conventional drugs and therapies to date have passed phase three trials in the treatment of RSV bronchiolitis despite research, including salbutamol, montelukast, antibiotics, ipratropium bromide or inhaled corticosteroids(21).

Recent developments into the use of macrolide antibiotics have proved promising. Azithromycin has been shown to be safe at randomised phase two trial at a low and high dose, and at high dose was shown to reduce MMP-9 levels detectable in patients ETT secretions(22). More and more studies have investigated azithromycin in treating RSV bronchiolitis with various clinical end points investigated. It was found that low dose azithromycin treatment did not improve disease course in infants with RSV bronchiolitis, when the outcome measure was duration of hospital stay(23). However more recent studies have shown that azithromycin treatment during RSV bronchiolitis reduced upper airway levels of IL-8, and in terms of clinical

benefit prolonged the time to third wheezing episode and reduced overall respiratory morbidity over the subsequent year of the study(24,25). These studies alongside other laboratory research support azithromycin as a candidate for a multicentre randomised trial, with outcome measures yet to be defined.

1.2.6 Prophylaxis

First described in 1997, Palivizumab is a humanised IgG1 monoclonal antibody which targets the 'A' antigenic site of RSV F glycoprotein, which mediates fusion of the virus with the host cell membrane(26). Synagis® solution is the only licenced formulary of Palivizumab prescribed in the UK as prophylaxis against RSV infection, and is indicated for use in certain at-risk groups of children:

- 'Children born at 35 weeks or less of gestation and under 6 months of age at the onset of the RSV season
- Children under two years of age and requiring treatment for bronchopulmonary dysplasia (chronic lung disease) within the previous six months
- Children under two years of age and with significant congenital heart disease
- Children under two years of age with severe combined immunodeficiency syndrome (SCID)
- Infants on long term ventilation for certain disease states(27).'

In these high-risk groups of children, mortality from RSV infection is estimated at 3%.(28)

1.2.7 New therapies and vaccines

Developing an effective vaccine or drug for RSV has challenged scientists for nearly 60 years. The first vaccine, devised in the 1960s, of formalin-inactivated RSV, not only proved to be ineffective in providing protective immunity to RSV, but in fact enhanced severity of disease in those vaccinated(29). Further efforts to formulate a vaccine were marred by the potential risks of causing more severe disease to vaccinated children and development of RSV vaccines slowed. Recent advances in structural biology have kick-started vaccine and therapeutic development once more with several vaccine candidates in development and some trials have started in children (see **Figure 1-2**)(30). Candidate vaccines rely on various approaches to induce immunity, including viral vectors, live attenuated viruses and protein subunit vaccines,

but still the only licensed preventative intervention for RSV is Palivizumab(31). Of those in later development and licensed use, targeting and promoting neutralisation of the RSV F (fusion) glycoprotein is a common target of vaccines(32). RSV F protein occurs in two conformations, 'pre fusion' and 'post fusion', both of which have been targeted in vaccine development, including phase III clinical trials.

RSV Vaccine and mAb Snapshot

TARGET INDICATION: P = PEDIATRIC M = MATERNAL E = ELDERLY

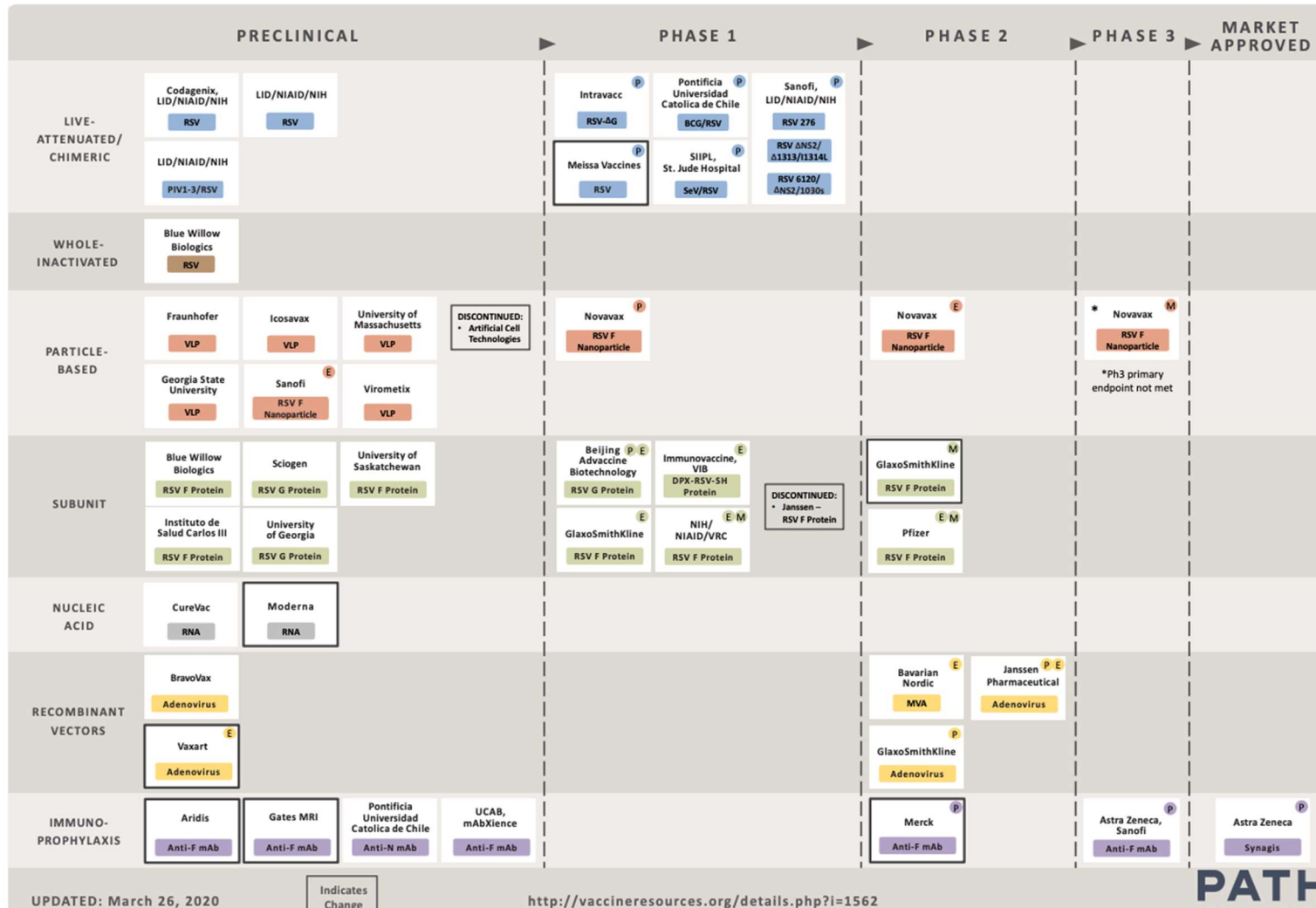


Figure 1-2 Table of novel vaccines and therapies in development or trials at present collated by PATH.

Downloaded on 25/07/2020 from URL: <https://www.path.org/resources/rsv-vaccine-and-mab-snapshot/>

1.2.8 Long term effects of RSV Bronchiolitis

It has been well evidenced over the past 40 years that children who develop severe RSV bronchiolitis as infants are more likely to wheeze in the first year of life(33). A longitudinal study following a large cohort of American infants from their birth in the 1980s into adulthood, showed that infants which develop RSV infection are more likely to develop pre-school wheeze and asthma in childhood but this effect disappears in their teenage years(34). Furthermore, in smaller studies, RSV bronchiolitis in infancy has been correlated with poor lung function and the development of wheeze and asthma in adults (35–37). One study demonstrated that 50% of children hospitalised with severe RSV bronchiolitis subsequently received an asthma diagnosis, and 13% of cases of asthma diagnosed in a cohort were considered to be attributable to RSV. However these studies did not continue into adulthood, unlike the Tucson study(35)(38). Sigurs et al followed-up children admitted to hospital with RSV bronchiolitis in their first 12 months of life, comparing them to matched infants of the same age who did not experience respiratory disease as an infant. This study revealed that at 18 years, children who had had bronchiolitis showed an increased prevalence of asthma (39% versus 9%), clinical allergy (43% versus 17%), and atopic sensitization (41% versus 14%) compared with controls, leading to the conclusion that the risk of asthma increases with the severity of infant bronchiolitis(39).

Bronchiolitis not requiring hospitalisation has also been implicated in delayed respiratory sequelae. In a retrospective birth cohort study of 90,341 children born between 1995 and 2000, episodes of bronchiolitis and subsequent asthma diagnoses were identified alongside the severity of bronchiolitis, which was graded based on the highest level of healthcare provider they accessed. 18% of the cohort experienced bronchiolitis needing medical attention as an infant and these children contributed to 31% of asthma diagnoses between four and five-and-a-half years of age(40). This study not only showed a link between bronchiolitis in infancy and later asthma diagnosis, it also suggested a 'dose response' relationship between bronchiolitis severity and the odds of asthma and resultant morbidity.

Although these epidemiological associations are clear, we are still left with the question as to whether the children who will go on to develop wheezing and asthma in later childhood are also more likely to develop bronchiolitis with RSV, or if RSV bronchiolitis itself causes long

term effect on the airways and or immune system leading directly to asthma and recurrent wheeze.

Impetus for further RSV research is driven by the assertion that, if the initial infection is better understood and successfully prevented or treated it may save these children from long term health issues(41).

1.3 Host response to RSV infection

Examining the host's response to RSV is also important. Multiple studies of paediatric and elderly patients have shown a multi-factorial and varied response to RSV infection in the airways, including multiple different cell types and cytokine profiles. These studies however raise key questions. Is it better to have a strong inflammatory response to raise a strong front against the viral infection or will this inflammatory response contribute to greater lung damage with systemic illness and long-term sequelae?

Another key question is, why are the very young and the very old more vulnerable? And are they vulnerable for the same reasons or are there different age-related variations to how the body responds to RSV? This study focusses on the immune response of the very young, but comparative studies examining the response of those at extremes of age, both the elderly and neonates has shown interesting parallels(42). In innate cells, neutrophilic migration into the airways has been shown to be reduced in both healthy infant and older adult studies, as well as reduced neutrophil mediated pathogen killing and reduced NK cell cytotoxicity(43–47). Adaptive immune parallels have also been implicated with both neonates and the elderly being suggested to have blunted humoral responses to exogenous antigen(44,48).

The mechanism behind these age related differences in immunity is particularly important because, as we continue to develop our understanding of these immune responses, it may come to be that when designing a vaccine, different modalities of vaccine target and delivery may confer immunity better in different age groups(49).

1.3.1 The airway during RSV infection

RSV is thought to enter the respiratory tract via inhaled droplets, where it infects the ciliated epithelium of the nasopharynx and replicates, eventually descending to the lower airways, potentially aided by aspirated mucus or nasopharyngeal secretions(50). Previous studies have demonstrated it is the ciliated cells of the small airways and type 1 alveolar pneumocytes which are infected by RSV with basal progenitor cells being largely spared(51). Established RSV infection of the epithelium causes syncytia formation between neighbouring cells, a process considered characteristic of the virus(52).

In fatal cases of RSV bronchiolitis, histopathology of the airways has shown necrosis of the airway epithelium, preferential destruction of ciliated epithelial cells and peri-bronchiolar infiltration of immune cells, specifically neutrophil infiltration to the airway walls and the bronchiolar lumen(53,54). *In vivo*, histological hallmarks of RSV disease include epithelial cell sloughing, oedema, syncytia formation and increased mucus production(54,55).

1.3.1.1 Structure of the bronchioles in health

Bronchiolitis is an inflammatory disease of the small airways, bronchioles, which sprout from the bronchi and communicate air to the gas exchange surface in the alveoli(56). There are three classifications of bronchiole, determined by their size. Lobular bronchioles, the largest passages which first enter each lobe of the lung; terminal bronchioles, smaller passages which connect lobular bronchioles to the respiratory bronchioles, of which there are 50-80 in each lobe and respiratory bronchioles which branch from the end of terminal bronchioles and lead to alveolar ducts(57).

The bronchioles are the end of the conducting airway and the entrance to the respiratory portion of the lung. Conducting airways, such as the lobular and terminal bronchioles, warm, humidify and remove particles from air which enters the lung to ensure efficient gas exchange and these functions are enabled by specialised cells which form the respiratory epithelium. Five cell types reside in this region, Ciliated cells, Club cells, Basal Cells, Brush cells and Neuroendocrine cells. Ciliated cells are the most abundant cell type and are key for the function of the mucociliary escalator which allows the removal of particulates and debris from the airways as described later in the section.

1.3.1.2 Mucus, surfactant and physical innate immunity

The role of mucus in the airway is to entrap inhaled particles, which may be damaging or infectious, neutralise them and facilitate their movement out of the airway. This is a fine balance and excessive mucus, during infection or disease process, may cause 'plugging' especially in smaller airways. RSV stimulates mucin production via its F protein, which enhances epidermal growth factor receptor (EGFR) phosphorylation(58). RSV can also cause ciliary dyskinesia which, together with loss of ciliated cells, may result in impaired airway clearance and mucus obstruction(59).

The importance of innate antimicrobial peptides secreted in response to RSV infection, has also been investigated. Cathelicin (LL-37) is a conserved peptide which inhibits epithelial cell infection by RSV *in vitro* and in murine models. Baseline higher levels of LL-37 detected by nasosorption are also correlated with protection following experimental challenge in human studies of RSV infection(60).

In addition, surfactant proteins, present on the epithelial surface, have been shown to bind directly to RSV F protein. This is interesting as prematurity correlates with reduced lung levels of surfactant, and indeed Kerr et al showed that infants suffering more severe RSV bronchiolitis had reduced levels of detectable surfactant in the airways however we cannot say whether this is due to cause or effect(61,62). Further to this, a number of polymorphisms in surfactant-encoding genes have been associated with worsened or reduced disease severity(63,64).

1.3.1.3 Ciliated epithelial cells in RSV infection

Ciliated cells are the main cell type infected by RSV in culture, with the virus binding directly to cilia as a first point of contact with the epithelium, by a mechanism, which is as yet unclear (65,66). *In vitro* models of ciliated epithelium have demonstrated virus antigen is present not only in ciliated cells, but on the surface of cilia themselves(59,66). Early RSV infection *in vitro* is characterised by ciliary dyskinesia, which if mirrored *in vivo* potentially contributes to viral spread and hindering viral clearance. Furthermore, late infection *in vitro* is characterised by ciliary loss, which, if reflected *in vivo*, could contribute to mucus plug formation and mechanical blocking of the airway(59).

1.3.1.4 Alveolar Macrophages in RSV infection

Alveolar macrophages (AMs) may play an important part in initiation of responses in the distal airways. AMs are important for clearing debris and for lung homeostasis and are ideally placed to sense viruses by the same mechanisms described above(49).

1.3.1.5 Recognition of RSV by host cells

Interaction of virus particles with host epithelial cells and immune cells leads to recognition of the virus by means of pattern recognition receptors (PRRs). These can detect both intracellular and extracellular virus by distinct cytoplasmic and membrane bound receptors.

Intracellular virus detection

Cytoplasmic receptors such as retinoic acid-inducible gene I-like receptors (RIG-1), an RNA helicase, which binds intracellular double stranded RNA and triggering NFkB and IRF3 cascade signalling(67,68).

Extracellular virus detection

Membrane bound receptors such as toll like receptors (TLRs) detect viral particles and proteins coming into contact with the membrane of the cell(69,70). TLR 2 has also been shown to impact on RSV replicative ability, modulation of cytokine production by leucocytes and subsequent migration of neutrophils into the lung lumen(71). Especially important in RSV detection signalling are TLR 3 and 4 leading to an inflammatory response by activation of NFkB(72,73). RSV infection can modulate the differential expression of TLRs, with an upregulation of TLR 4 seen on epithelial cells when infected, increasing their ability to detect other pathogen associated molecular patterns (PAMPS) such as lipopolysaccharide(74). Interestingly, a study examining neutrophils isolated from BAL of children with RSV bronchiolitis showed a downregulation of neutrophil TLR 4 expression compared to controls, and further to this the neutrophils of pre-term babies with severe RSV expressed more TLR 4 than those born at term(75). It has previously been shown that expression of TLRs increases with gestational age, and so this observation of lower TLR 4 in term infants with severe RSV than preterm may suggest that term infants with severe RSV may have an abnormality with TLR 4 expression(75,76).

1.3.1.6 Inflammatory response to RSV infection

Detection of RSV PAMPs by PRRs enables the removal of I κ B (inhibitor of kB) from NFkB which translocates to the nucleus and ultimately leads to the production and secretion of inflammatory chemokines and cytokines, notably neutrophil chemoattractants IL-8, IP-10, MCP-2 and IL-9(77–79). NFkB signalling also leads to the production of type I interferons, which once released, bind to recognition receptors on nearby epithelial cells and induce a signalling cascade within to prime these cells for viral attack. These secreted factors not only act on nearby epithelium, but also recruit immune cells such as neutrophils, dendritic cells and lymphocytes.

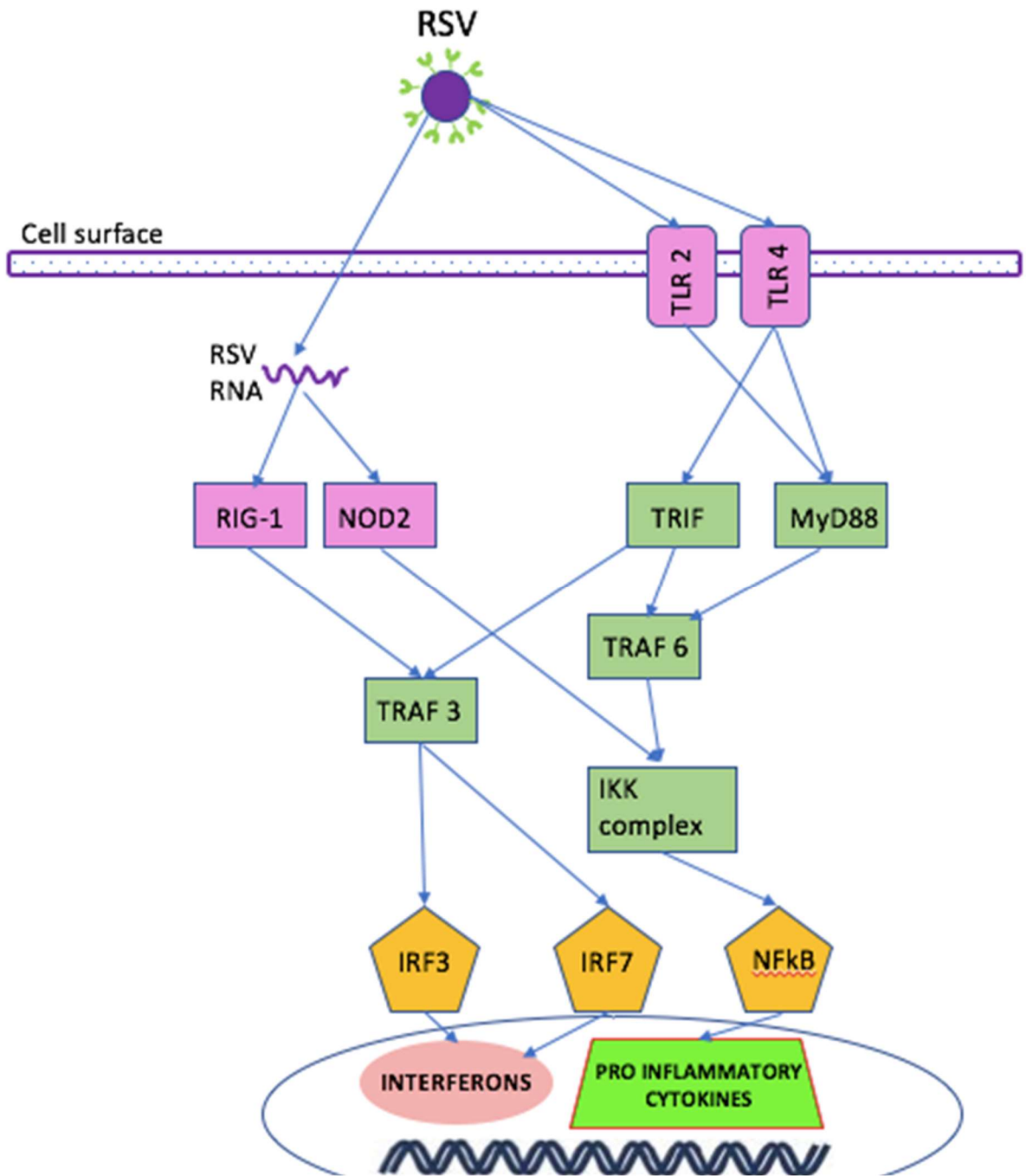


Figure 1-3 illustration of the detection of RSV by PRRs and signalling pathways induced within host cells.

Diagram adapted from Brealey et al 2009(80).

1.3.2 Cellular immune response to RSV infection

Biopsy samples from young children with RSV bronchiolitis demonstrated that large areas of epithelium infected with RSV remained intact, indicating that other host factors may contribute to localised epithelial damage and disease severity(53). Examining the cells found in the lungs of children with RSV bronchiolitis, it was found that a greater number of cells is found in the lungs of children with severe RSV bronchiolitis in comparison to age matched uninfected control infants intubated for routine surgery reasons(81). The proportions of different cell types recovered from BAL of children with severe RSV disease by McNamara is described in **Table 1 – 1** below (81).

Table 1-1 Cellularity of bronchoalveolar lavage samples from Term and Preterm infants intubated with RSV bronchiolitis and age matched control infants intubated for routine surgery.

Data displayed as Median (IQR). Adapted from McNamara et Al Arch Dis Child 2003(82).

	Term	Preterm	Control
Number of samples	47	73	10
% Live cells	77.8 (17.2)	80.0 (17.0)	68.8 (18.7)
Neutrophils	85.3 (12.7)	78.0 (16.0)	52.3 (27.7)
Alveolar macrophages	8.0 (6.3)	12.0 (17.0)	32.7 (23.3)
Lymphocytes	6.0 (4.3)	8.0 (7.0)	11.0 (13.0)

1.3.2.1 Macrophages

Macrophages destroy invading pathogens by phagocytosis and then act as professional antigen presenting cells and assist in control of viral infection through direct interaction with CD4+ and CD8+ T-cells and through secretion of type one interferons (Type 1 IFN) in response to RSV infection(83). In addition to type 1 IFN, Macrophages secrete a cocktail of cytokines which in turn recruit and prime neutrophils, NK cells and T-cells to the site of infection(84,85).

1.3.2.2 The Neutrophil during RSV infection

Lung neutrophilia has become a hallmark of RSV disease and McNamara et al demonstrated that the bulk of cells recovered from the broncho-alveolar lavage (BAL) samples of children with severe RSV bronchiolitis are neutrophils(82). The same group also demonstrated that in

infants with severe bronchiolitis, concentration of neutrophils recovered from the lungs decreased after intubation in infants born at term which correlated with disease resolution(81).

First, neutrophils migrate to the site of infection down a concentration gradient of secreted chemoattractant, leaving the bloodstream and entering tissues. Here, neutrophils can perform their primary role of pathogen destruction. Neutrophils can cause pathogen destruction in three ways, phagocytosis of the pathogen, secretion of destructive factors such as Reactive Oxygen Species (ROS) granule contents and 'Neutrophil Extracellular Traps' (NETs). As the infection resolves, neutrophils die and are removed by tissue resident macrophages and degraded, contributing to the dampening of inflammation(86,87). This is arguably the most interesting step of all, as due to the non-specific nature of neutrophil secreted effectors such as defensins and reactive oxygen species (ROS), prolonging their presence at the site of infection can cause unnecessary damage to host tissue (88,89).

1.3.2.3 Natural Killer cells

NK cells have an important antiviral role during RSV infection. By producing IFN- γ they promote Th1 responses and kill infected cells(90). The recruitment of NK cells is enhanced by AMs(91). In mouse models, it has been suggested that NKT cells may contribute to IL-4 production(92).

1.3.2.4 Dendritic cells

Both conventional and plasmacytoid dendritic cells (cDCs and pDCs) are trafficked to the nasal mucosa of infants infected with RSV and their population remained raised for weeks after initial infection(93). pDCs have been shown to be protective against pathological change during RSV infection of a mouse model, which also suggested their activation is mediated by epithelial cell interaction(94–96). In humans, activation of DCs during RSV infection is dependent on autophagy in part and is modulated by differential gene transcription mechanisms(97–100). Conversely, inflammatory DCs interaction with PD-1 on T-cells by upregulating their own PD-L1 expression has appeared to limit immune mediated damage(101).

1.3.2.5 Adaptive immune response to RSV bronchiolitis

During viral infection, virus is taken up by professional antigen presenting cells (APCs) which then process and present viral antigen to adaptive immune cells using MHC class one for intracellular detected antigen and MHC class two for extracellular(49).

RSV is an interesting disease for adaptive immunity as natural infection does not confer long lasting protection and symptomatic infections reoccur throughout life.

Humoral response

Protective antibody to RSV is stimulated only against RSV F and G protein, the two major surface glycoproteins, but protective antibody responses although varied are short lived(102,103). Local protection in the upper airway is mediated by secretory IgA, however its effects are not long lasting. RSV-specific IgG accesses the lower airway mucosa much more readily than that of the upper airways where passive transudation occurs(50,104). During acute infection, IgM is present within days of infection and is sustained for around 2 weeks. IgG is detectable in serum after 1 week of infection, rises to peak at about 1 month then begins to decline(103).

In neonates, RSV neutralising antibody is usually present due to passive transfer of maternal antibody via the placenta(102). Correlation has been observed between low RSV antibody titre in cord blood and earlier or more severe subsequent RSV disease, which implies some degree of protection(102). In addition, the only licensed prophylactic for RSV infection is passive transfer of RSV neutralising antibody as described previously (105,106), however levels of antibody facilitated by treatment with palivizumab are rarely achieved by natural infection(105).

The recent discovery that IgA+ B cell generation is impaired following experimental RSV infection in adults suggests RSV may modulate the generation of long-lived memory B-cells which would confer sustained levels of antibody to protect against reinfection (90)

T-cell response

CD4+ and CD8+ T-cells are present in the lung during RSV infection and play vital roles in resolving RSV infection and have also been implicated in determining infectious shedding of virus. Humans with compromised T-cell responses shed live RSV for months after initial

infection and in mouse models where CD4+ and CD8+ T-cells were depleted, prolonged shedding of RSV was also detected(107–109). In these same studies, depleting T-cells also resulted in reduced lung pathology(108). However, children with T-cell immunodeficiency display prolonged and persistent RSV infection which further demonstrates their critical role in terminating disease(49).

1.3.2.6 Primary immunodeficiencies and RSV infection

Children with primary immunodeficiencies provide insight into the cell types involved in the immune response to RSV infection, and what role they may play. Infants with reduced or absent T and B lymphocytes (humoral response), such as severe combined immunodeficiency (SCID) and athymic Di George syndrome, have been shown to be at increased risk of significant pathology from RSV disease and other respiratory viruses (110,111). In contrast, children with congenital absence of neutrophils (neutropenia) and those with defects in neutrophil function such as in chronic granulomatous disease are at much greater risk of fungal and intracellular bacterial infection and less susceptible to viral infections such as RSV(112,113). This implies that in health it is the humoral response which is necessary for clearing the virus and that neutrophils contribution is more likely to be an inflammatory response to infection.

1.3.3 Prematurity and the immune response

It is clear from the evidence that infants born preterm are more at risk of experiencing a severe episode of RSV bronchiolitis, but exactly the mechanism behind this remains to be explained (11). Preterm infants are often excluded from clinical studies of RSV bronchiolitis, despite this group suffering a greater incidence of respiratory failure, airway obstruction and overall morbidity than term infants(11). We know from studies of ventilated children with RSV bronchiolitis that infants born at preterm require ventilation for a longer period of time than those born at term, and that a lower concentration of cells are recoverable from the airways of preterm infants than those born at term(81). This would immediately suggest that there is a lesser cellular response in preterm infants which may contribute to prolonged disease. However, the differential cellularity, and in particular, differing neutrophil concentrations recoverable (see **Table 1-1**) suggests abnormal cell recruitment to the lung potentially due to underlying pathological changes to lung caused by prematurity impeding

normal cell migration(114). Premature babies also have smaller and less compliant airways and so the amount of inflammation required to obstruct the airway is lower, meaning a smaller cellular influx could cause critical obstruction(115). This would help explain why a higher proportion of premature infants, compared to those born at term, require ventilation during an episode of RSV Bronchiolitis.

Total cell concentration recoverable from the airways did not decrease over subsequent days of intubation during RSV infection in preterm infants which was the case in those born at term(81). This is probably due to the relatively low levels of cells present on day one recovered from the preterm lung compared to the term children, and indicates preterm infants likely have a lower physiological reserve. Cell concentration and in particular neutrophil concentration decreasing over time in term infants is concordant with resolution of infection and reducing inflammation caused or aided by neutrophils themselves. The fact this was not found in preterm infants, where the cellular infiltrate was relatively constant, is further indication that clinical course is determined by pathological changes of the lung rather than the inflammatory response.

1.4 The Neutrophil

First described by Paul Ehrlich in 1886 as 'polynuclear cells', neutrophils are now known to be polymorphonuclear granulocytes and are regarded as the 'foot soldiers' of the immune response, being the first cell type recruited to a site of infection(116). Their role in combatting bacterial and fungal infections is well documented in literature, but their role in viral infection remained unclear, despite it being well recognised that large numbers of neutrophils are recruited to the site of many viral infections.

1.4.1 Structure

On average 10 – 15 μm in diameter, neutrophils consist of 63% cytoplasm, 21% nucleus, 15.4% granules and 0.6% mitochondria(117). Neutrophil granules can be classified into three types according to their contents; Azurophilic (primary) granules containing myeloperoxidase (MPO), BPI, Neutrophil elastase, cathepsin G and Defensins; Specific (secondary) granules containing alkaline phosphatase (ALP), NADPH oxidase, lactoferrin, lysozyme, collagenase and cathelicidin; Tertiary granules containing cathepsin and gelatinase. Each neutrophil is thought to contain 200 granules of all three types(118). Granule contents can be released intracellularly on fusion with phagosomes or extracellularly when stimulated to, via ligand binding to FcR receptors on the neutrophil surface(119).

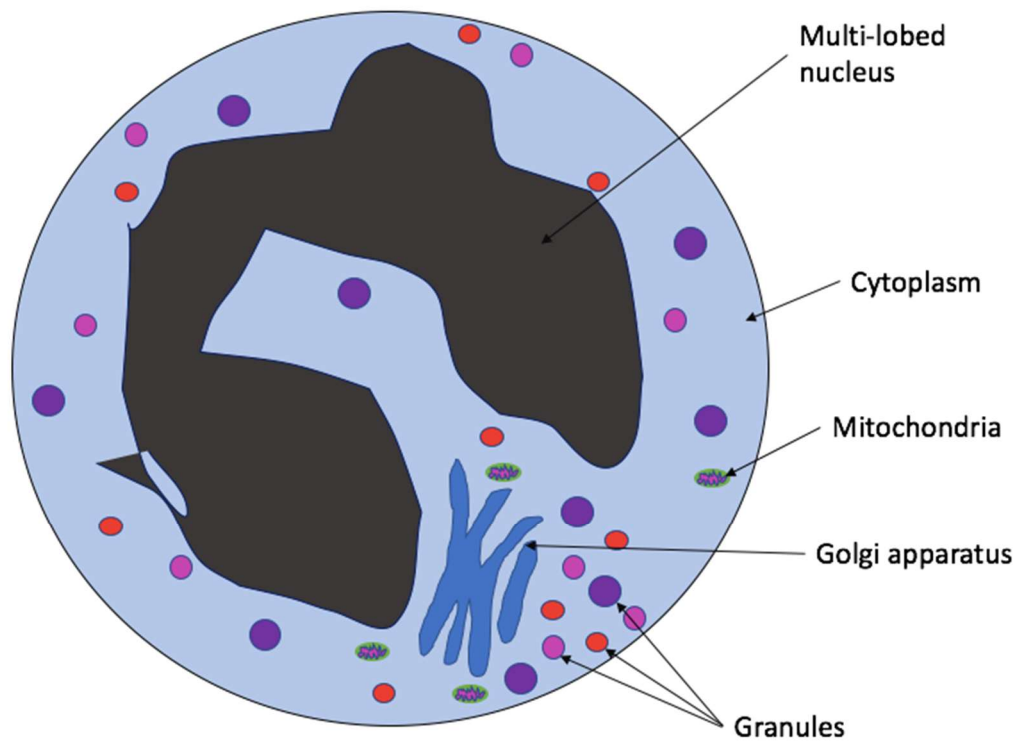


Figure 1-4 Schematic of neutrophil ultrastructure and its component multilobed nucleus, multiple granules, mitochondria and Golgi apparatus.

1.4.2 Origins

All cellular components of blood originate from pluripotent haematopoietic stem cells or hemocytoblast, resident in the bone marrow, and neutrophils are no exception(120). This hemocytoblast then develops into either a common myeloid progenitor or a common lymphoid progenitor cell – neutrophils deriving downstream from the former. The common myeloid progenitor differentiates further into myeloblast and then again into a neutrophil promyelocyte where granules first start to be apparent. The neutrophil promyelocyte then differentiates more rapidly, into a neutrophil myelocyte, neutrophil metamyelocyte and finally an N band – where the beginnings of the characteristic polymorphic nucleus is discernible. Neutrophils are then stimulated for maturation and release into the bloodstream where they become the mature neutrophil. This process is regulated by granulocyte colony stimulating factor (G-CSF), a cytokine, and results in the continual release of neutrophils into the circulation at an estimated rate of 10^{11} neutrophils daily(121).

1.4.3 Neutrophil function in health

1.4.3.1 Activation

Neutrophil activation can be considered a two stage process, with an initial priming step followed later by a second ‘activation’ step allowing release of granule products and generation of reactive oxygen species (122). The ‘priming’ step is postulated as a protective mechanism against inappropriate neutrophil activation necessary to prevent damage to the host. *In vitro*, neutrophil activation-inducing agents can only effect degranulation or ROS generation after neutrophils have first encountered a ‘priming’ stimuli such as contact with activated endothelium, encountering foreign antigen or foreign secretagogues such as LPS(123). After priming, neutrophils exhibit an adherent state and altered NADPH organisation, an important enzyme for production of superoxide in response to invading pathogens(124). This reorganisation, depending on mechanism of priming stimuli, allows translocation of NADPH subunits from the neutrophil cytosol to the plasma or granular membrane, but does not allow complete assembly required for superoxide generation which occurs later during neutrophil activation(124,125). The priming step also inhibits chemotaxis and apoptosis which may be a mechanism to hold neutrophils at the site of infection and enhance their longevity of antimicrobial properties (122). In some systemic inflammatory

disease states, such as vasculitis it has been demonstrated that neutrophils primed in the circulation enter pulmonary microcirculation and remain there until they are stimulated to 'activate', a mechanism which has been implicated as dysfunctional in adult respiratory distress syndromes(122,126,127). Once stimulated to activate by a secondary stimulus, granules present in the neutrophil fuse with the cell membrane, releasing their contents extracellularly and presenting extracellular surface receptors on the external membrane which facilitate adhesion to other cells and indeed activation potential (128,129). Completed neutrophil activation can be defined as 'complete assembly of the membrane-linked and cytosolic NADPH oxidase components on a neutrophil granule or plasma membrane'(124). Specific contents of neutrophilic granules and their functions are discussed in earlier in this chapter and again later in **Chapter 5**.

Measuring neutrophil activation is important because, the more activated neutrophils are the more able they are to mount a response to the exogenous RSV, but also the more likely collateral tissue damage may be caused to healthy cells surrounding. There are many recognised correlates of neutrophil activation we can measure, including surface bound proteins or receptors or secretory products stored in granules for eventual release.

1.4.3.2 Chemotaxis

Chemotaxis can be defined as the directed migration of a cell type to a distant chemoattractant (130). Chemotaxis by neutrophils at the time of infection and inflammation is one of the most critical arms of the innate immune response and is heavily relied upon to clear pathogens especially in the immature immune systems of the young. Effective neutrophil chemotaxis is critically effected by endogenous chemokines including IL-8 (CXCL8), a potent known neutrophil chemoattractant (131). Disordered chemotaxis and other directional movement of neutrophils has been observed in chronic inflammatory disorders such as COPD (132). On a signalling level, there is recent evidence that phosphoinositide 3-kinase (PI3-K) signalling governs the process of chemotaxis, influencing cell polarisation, F-actin cytoskeleton remodelling and myosin contraction (133). Neutrophils in particular have been shown to organise their chemotaxis and form 'swarms' in response to stimulus, notably of their own cell death, which has interestingly been shown to be leukotriene mediated in a murine model(134).

1.4.3.3 Migration into the airway

Once activated, neutrophils from general circulation arrive at the infection site, migration first through endothelium then through epithelium into the lung lumen can be described in four stages: capture and rolling, firm adhesion, crawling, and transmigration(135). During capture and rolling, the first contact between the neutrophil and endothelium is initiated, mediated by L-selectin molecules on the neutrophil and P or E-selectin on the endothelium, and neutrophils roll along the endothelium in the direction of blood flow (136). Tethering the two cells in close proximity allows further binding between chemokine receptors on the endothelium and neutrophil. This triggers a conformational change in the neutrophil, precipitating the shedding of L-selectin and the initiation of β_2 integrin expression including lymphocyte function associated antigen 1 (LFA-1) and Complement Receptor 3 (CR3)(137). The additional β_2 integrins interact with adhesion molecules on the endothelium such as intercellular adhesion molecules (ICAM) or vascular cell adhesion molecule 1 (VCAM-1)(138). These new bindings connecting the neutrophil and the endothelium leads to firm adhesion of the neutrophil. Neutrophils then traverse the endothelium, 70-90% via paracellular migration at the borders of endothelial cells and the rest transcellularly (139,140). Neutrophils then bind to the basolateral aspect of airway epithelial cells (AECs) mediated by CD11b/CD18 and traverse the epithelium via paracellular migration at the borders of epithelial cells(137).

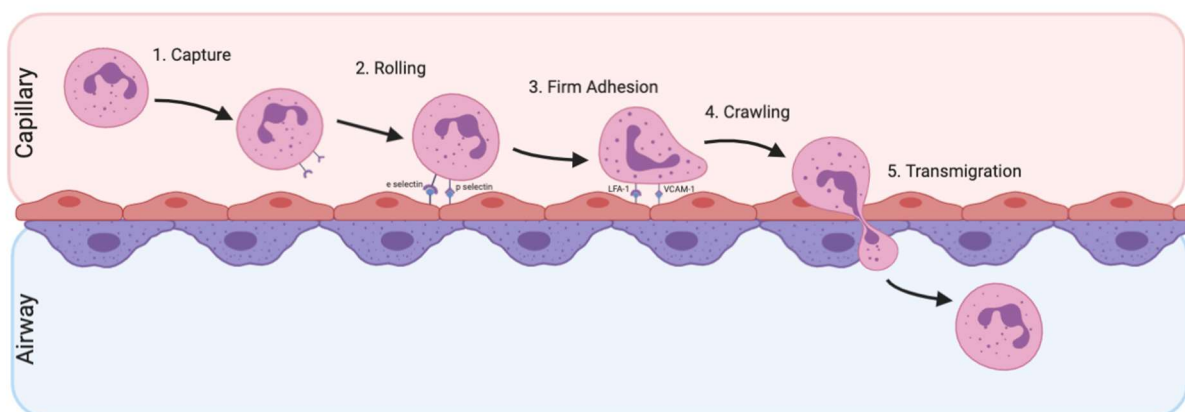


Figure 1-5 Schematic demonstrating extravasation of neutrophils from the vasculature into the airway lumen

Individual steps as detailed in **Section 1.4.3.3**. Drawn using biorender.com with reference to (120).

1.4.3.4 Phagocytosis

As neutrophils are often the first cells to arrive at infection site, their primary role, for which they are well known, is that of phagocytosis of pathogens, be that bacteria, fungi or viruses. Often when encountering foreign material, it is already opsonised by complement or immunoglobulin. Neutrophils, post-migration, demonstrate upregulated Fc receptors and TLRs and so are well equipped to recognise the opsonin and pathogen(141). By means of these receptors inducing intracellular changes, the neutrophil is able to remodel its cytoskeleton and engulf the pathogen in an envelope of its cell membrane known as the phagosome. Classically, it is thought that mitochondria in the neutrophil cytoplasm move to the phagosome membrane and generate superoxide anions (ROS) such as $H_2O_2^-$ which are released into the phagosome and cause fatal damage to the engulfed pathogen(142). However, more recent studies have demonstrated it is more likely that ROS generation leads to the excretion of protons into the cell cytoplasm, and as a result the ion fluxes produced create an environment within the phagosome that is suited to the neutral proteases (elastase and cathepsins) to digest the phagosome contents(143). In addition, neutrophils' azurophilic granules, containing MPO and other destructive enzymes, fuse with the phagosome, releasing their destructive contents and degrading the engulfed pathogen into absorbable amino acids(144).

1.4.3.5 Reactive oxygen species

The generation of superoxide anions or reactive oxygen species (ROS) is dependent on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, as is the generation of NETs described later in this section(122). Intracellular killing of fungi and bacteria are dependent on the production of superoxide anions as evidenced by recurrent severe infections by these pathogens seen in patients with chronic granulomatous disease, a disease characterised by inability of this mechanism(145). This process has been shown to be Pi3 kinase signalling dependent in mouse models(146).

1.4.3.6 Neutrophil Extracellular Traps (NETS)

Definitively characterised in 2004, NETs were first recognised as a suicidal mechanism of neutrophils, by which a net of chromatin embossed with serine proteases to attach and 'trap'

extracellular pathogens, marking them for destruction by the immune system (147). Their formation is known as NETosis and can occur on activation of neutrophils (147).

Large quantities of DNA are recoverable from mucus plugging the airways of infants with RSV bronchiolitis, and it is hypothesised that neutrophils undergo NETosis in response to RSV (148). The presence of DNA in mucus acts as a thickener and, as such, NETosis may contribute to airway obstruction in RSV disease (149). In a bovine model of RSV disease, it was demonstrated that NETs trapped viral particles preventing them reaching their target epithelium in the lung, but whilst doing so also contributed to the plugging of small airways (150). RSV F glycoprotein induces NETosis in neutrophils by interacting with TLR4 (151).

1.4.3.7 Cell death, apoptosis and necrosis

Spontaneous apoptosis of neutrophils is a protective mechanism which is key for the proper resolution of inflammation and infection, whereby obsolete or cells containing pathogen are essentially 'packaged' for removal, preventing excess collateral damage to tissue by non-specific neutrophil cytotoxicity and preventing escape or replication of any engulfed pathogen (86). This is an energy dependent process distinct from necrosis, an unregulated 'accidental' cell death characterised by loss of membrane integrity, leakage of intracellular contents and resultant inflammatory consequences. While necrosis can occur due to damage or exhaustion of energy supplies for apoptosis, it is now understood that apoptosis and necrosis are not mutually exclusive and a spectrum of semi-regulated forms of necrosis exist which incorporate some features of both(152).

Apoptosis can be described as 'regulated or programmed cell death involving a controlled disassembly of cellular contents without disruption to the plasma membrane and minimal inflammation'(153). Apoptosis can be split into two phases, the latent phase and the execution phase. During the latent phase, apoptosis inducing signalling pathways have been activated but cells appear morphologically the same. During the execution phase we first see plasma membrane asymmetry as a result of phosphatidylserine appearing on the outer leaflet, chromatin condensation and the fragmentation of DNA (153). At this point the membrane blebs and the entire cell is fragmented into membrane enclosed apoptotic bodies which are then removed from tissues by phagocytic tissue resident macrophages (154).

Apoptosis is executed by a cascade of proteolytic enzymes, the cysteine-dependent aspartate-directed proteases (CASPASEs), zymogens present in the cell cytoplasm as pro-CASPASEs, which once activated dismantle the cell as described above. All pro-CASPASEs contain a large and small protein subunit, and once pro-CASPASEs are cleaved, two large subunits and 2 small subunits combine to form an 'active tetramer' – which is the CASPASE. CASPASEs can be initiated by extrinsic factors, such as the binding of helper T cells mediated by FAS receptors and FASL ligand, or intrinsic factors such as loss of mitochondrial membrane integrity and leakage of cytochrome C into the cytoplasm (155–157). CASPASEs are subdivided into initiator CASPASEs, which initiate the apoptosis cascade by means of extra binding domains either (CASPASE Recruitment Domain) CARD or (Death Effector Domain) DED present in their structure which assist in the cleavage of nearby pro-caspases, and effector CASPASEs which facilitate the breakdown of the cell (158). Effector CASPASEs, once activated, cleave and inactivate cellular proteins and complexes, such as nuclear laminins leading to the breakdown of the nucleus, and activate further destructive enzymes such as CASPASE Activated DNAase (CAD), a protein kinase which breaks down chromatin (155).

1.4.4 Neutrophils in neonates

Like in adults, neutrophils are the first cell to be recruited to combat pathogen insult. Foetuses develop in a site of immune privilege, and have not yet encountered natural antigen in the environment to form their own antigen specific immunity, relying on persisting maternal antibody from placental transfer(159). Mature neutrophils are detectable in foetal blood by the end of the first trimester and rapidly expand in number immediately prior to birth, stimulated by granulocyte-colony stimulating factor (GCSF)(44). In the days after birth, concentration of neutrophils return to a stable level(160). In addition to this, neonates have fewer neutrophil progenitor cells resident in the bone marrow, so in times of stress and infection, when cytokines stimulate new neutrophils to mature and be released, neonates are especially vulnerable to a quantitative defect(161).

However, it is widely accepted that neonatal neutrophils show some impairment or variation in function, the severity of which has been shown to inversely correlated to gestational age (162). An example of this is that neonatal neutrophils produce different cytokine profiles in response to ligands on the same TLRs, notably increased IL-8 production, a potent neutrophil

chemoattractant, on the stimulation of TLR 1 and 2 in comparison to healthy adult neutrophils(163). It has been suggested that these differences are attributable to the immaturity of neonatal neutrophils, and by extension other differences in bactericidal mechanisms despite the mechanisms being poorly understood. Another variation is the differential protein expression profile of neonatal neutrophils and healthy adults, including many integral proteins involved in NETosis(164). It has also been demonstrated that preterm neonatal neutrophils show considerable impairments in the production of bactericidal and viricidal secreted products including bactericidal/permeability- increasing protein (BPI), elastase and lactoferrin(159,165). Neonatal neutrophils have also been demonstrated to show impaired phagocytosis of several pathogens(166).

The most consistently observed functional abnormality of neonatal neutrophils is depressed chemotaxis, first described in an animal model by Christensen and Rothstein irrespective of gestational age(167,168). As surface marker expression has been investigated and shown to be comparable on neutrophils from neonates and healthy adults, this depression of chemotaxis is historically attributed to reduction in intracellular calcium mobilisation, impacting the cytoskeleton rearrangement induced by chemoattractant mediated signalling(169,170). Neutrophil migration via chemotaxis, assessed by *in vitro* assays, is abnormal at birth in both term and pre-term infants, with term infants rapidly gaining normal chemotactic function, but preterm infants showing delayed development of normal chemotactic function post birth(161).

Apoptosis is also delayed in neonatal neutrophils in comparison to adult neutrophils, which could be partially explained by a relative lack of caspase 3(171,172). These neonatal neutrophils exhibiting prolonged survival may secrete mediators promoting chronic inflammation(173).

1.5 Neutrophils and their role during RSV infection

Historically, neutrophils have been thought to combat bacterial infection and their antiviral roles have remained poorly defined. Extensive clinical research over the last 20 years has investigated the impact of the neutrophil in infants with RSV bronchiolitis and we now have a better picture of what contributions these cells make. We know infants infected with RSV bronchiolitis exhibit an enhanced inflammatory state not only in the lungs but in the peripheries, with evidence of immune cell activation detectable in peripheral blood(75,174,175).

This inflammatory state in the lungs of infants with RSV bronchiolitis may be, in part, driven by neutrophils as 'CXC' neutrophil chemoattractant chemokines dominate the inflammatory milieu, in particular CXCL-8 (IL-8)(33,79). This is supported by the observation that neutrophils are the dominant cell type recoverable from the airways of ventilated infants with severe RSV Bronchiolitis, although whether this infiltrate is as a result of initial inflammation or a causative enhancer remains to be fully explained (81). In these infants, high levels of CXCL-8 in the lungs and the blood have been correlated with increased severity of RSV Bronchiolitis, a first indicator that neutrophil action may contribute to host damage during infection(176). *In vitro* studies of RSV infection of epithelial cell lines have shown that CXCL-8 first peaks two hours after infection, showing that established viral infection is not required for this inflammatory response(177). A second peak in CXCL-8 is seen after 24 hours, and this is reliant on active replication(177). BAL samples taken from children intubated with severe RSV have shown vast quantities of IL-9, a type 2 cytokine, both secreted into the lungs and actively produced in the neutrophil which, may contribute to pathogenesis of severe RSV disease(78). Furthermore, concentrations of IL-9 decreased over the course of infection in infants with RSV (78).

Neutrophils have been found to represent a median of between 76% and 84% in the lower airway counted from BAL and 93% of cells in the upper airway collected by nasopharyngeal aspiration (81,178). Examining the neutrophils themselves recovered from BAL of ventilated RSV infected neonates, has shown an increase in the integrin CD11b, an important activation marker and MPO, a key secreted neutrophil effector enzyme present in granules in comparison to those found in the bloodstream and those recovered from uninfected control

children (75). Further to this, Halfhide et al demonstrated that neutrophil TLR4 expression, a key PRR implicated specifically in RSV detection, is low both in the blood and in the airways of children with severe RSV, but TLR4 mRNA within the neutrophil is within normal levels. This raises the hypothesis a constitutive neutrophil TLR4 deficiency may contribute to severe RSV bronchiolitis(75).

It could be argued, the recruitment of neutrophils must be physiologically beneficial to counteract pathogens, and the differential neutrophil recruitment seen in premature infants with RSV may be testament to their immature immune system or more likely to pathological changes to the lung associated with prematurity. Studies of neutrophil depletion using mouse models have shown us, mice infected with RSV were given a monoclonal neutrophil depleting antibody – These mice lost weight and suffered greater lung pathology compared to controls but had no change in viral load(179). Albeit murine, this suggest neutrophil presence is beneficial to the resolution of disease but no evidence that they contribute to removal of virus itself.

In clinical studies of infants with RSV bronchiolitis, we have found evidence that RSV enters the neutrophil and undergoes transcription of its genome, which indicates that neutrophils do take up RSV but whether this is an active process is unclear(180,181). It is also unclear whether transcription of RSV within the neutrophil leads to the production of new virions.

On the other hand, neutrophils are innate immune cells and provide fast responses to infection which are non-specific and if excessive or aberrant may cause damage to the host epithelium. Our group have shown using *in vitro* models of the airway, that RSV infection increases neutrophil adherence to epithelial cells, in part mediated by LFA-1 and ICAM-1 binding which is linked to epithelial damage and detachment (182–185). There are many reports suggesting damage inflicted by neutrophils to the epithelium, but few describing the differences in their function which may explain or abrogate this (186).

1.5.1 Modelling RSV infection of the airways

A number of limitations to observational studies of infants with RSV complicate progression of research in this area. In studies of BAL cellularity, it is not possible to compare with infants with milder or moderate disease as they are not intubated and may not present to healthcare professionals at all(81). In the same way, it is difficult to recruit appropriate age matched

controls who do not already have underlying pathology. As RSV bronchiolitis is most often diagnosed in infants under 6 months there are also ethical and technical limitations to consenting and sample collection(187).

1.5.1.1 Animal models of RSV infection

A number of animal models have been employed to investigate RSV disease to varying success including chimpanzees, cotton rats, mice ferrets, hamsters and neonatal lambs. A limiting factor to these models is, with the exception of chimpanzees and the cotton rat, these animals are only semi permissive to RSV infection and require large non-physiological inocula to become infected(188). Few animals infected with RSV show the same clinical symptoms as humans and none have demonstrated recurrent infection with RSV which is a characteristic of human infection. Although mouse and cotton rat models were widely used to great success in development of palivizumab and immune mechanism studies, interpretation of in particular vaccine studies in these animals has been confounded by sensitization to non-viral antigen found in virus preparation and vaccine (105,189). In some animals, grossly different pathology is induced to what is observed in humans and although infection occurs it is not hugely comparable or helpful in mechanistic studies(190).

1.5.1.2 Human challenge models of RSV infection

Of course, the ultimate host to investigate RSV infection is humans themselves. Clinical studies of infants and adults admitted to hospital with severe RSV allow us to observe and sample the disease course in the population which can benefit most from advancing research. However, it is impossible to study these infections in their entirety as it is often a few days before patients present to hospital due to the long incubation period of RSV infection. It is also true that while clinical trials in this population are possible, a multitude of confounding factors limit their validity. Mutually decided end points for clinical trials are yet to be clearly defined, which limits the design and efficacy of such studies to extremely large sample sizes. Experimental infection of healthy volunteers, therefore, allows controlled dose of inocula and longitudinal sampling of participants from even before infection has taken place. RSV infection rarely causes severe symptoms in otherwise healthy young adults and so this group present a good population for challenge studies(191). Human challenge models also present difficulties and risks, clinical trials and experimental infections are of course more expensive

than animal experiments and indeed, as humans are a natural host of RSV, there is a risk of transmission of RSV through droplets and fomites to attending research staff and the volunteers' families. In addition, deliberately infecting otherwise healthy humans carries ethical implications. Although discovering potentially lifesaving treatments or vaccines will benefit countless individuals in the future, this does not override the interests of individual participants – and so informed consent of all volunteers is key(192). Infecting healthy adults is also a compromise as children and the elderly are the age demographics at greatest risk of RSV pathology, this still applies in a challenge model and infecting already vulnerable groups who cannot give informed consent is not justifiable(193).

Human challenge models of RSV have been used previously by several groups to explore the mechanisms of RSV infection and compare RSV induced disease to that of other respiratory viruses(188). The first human experimental infection with RSV, isolated from a patient with bronchopneumonia, was performed by Kravetz et al in 1961(194). Since this point, there have been many human challenge studies of RSV with few reported adverse effects which have showed insight into protective immunity, route of infection and incubation periods of RSV disease(195–200). Human challenge models are a fantastic utility for future vaccine development but require much more nuanced interpretation when it comes to molecular and mechanistic studies where a less complex more manipulable simple *in vitro* model may be preferable.

1.5.1.3 In-vitro modelling of RSV infection

To further interrogate the complicated relationship between the neutrophil, RSV and the airway epithelium during acute infection, it was necessary to develop a sophisticated *in vitro* model of the airway epithelium. A fairly inexpensive *in-vitro* model which enables experimental manipulation of viral infection, epithelial barrier, and neutrophil in a well-controlled, highly reproducible system was required. The first proof of concept study was based on air-liquid interface cultures documented previously but using Human type-2 alveolar basal epithelial cells (A549) (ATCC CCL-185), a highly reproducible cell line, and inverted to allow transepithelial migration of neutrophils(201). This model was then further adapted to include ciliated primary human airway epithelial cells, which allowed neutrophils, potentially from a matched donor to the epithelium, to migrate across the epithelium in a physiological basolateral to apical direction in response to RSV infection(185).

This model sourced primary human airway epithelial cells from the nasal respiratory tract collected by nasal brushing, the procedure for which is described in **Chapter 2**. It is possible to obtain AECs for research both from the nasal and lower respiratory tract by bronchial brushing, bronchial biopsy or isolation from resected lung tissue. Sampling method is an important consideration, especially when performing research using 'healthy' donor samples as ease of sample collection and minimising intervention to otherwise healthy individuals are key to reproducibility and study design. Bronchial AEC sampling techniques require the use of an endotracheal tube or bronchoscopy, both considerable interventions to an otherwise healthy individual whereas a simple nasal brushing is easier and more acceptable to healthy volunteers. Lung resection is rarely performed on healthy respiratory tissue. It is also an option to purchase human bronchial epithelial cells from commercial suppliers, however this introduces further variability into the experimental design as techniques used for isolation purification and processing by the company are proprietary.

Comparative studies of AEC samples collected from nasal and bronchial airways of healthy volunteers showed similarities in ciliary beat frequency, *in vitro* growth rate, cell morphology and expression of cellular markers including ICAM-1, integrins and CD44(202,203). McDougall et al compared nasal AECs with bronchial AECs derived from the same donors and found nasal AECs produce constitutively higher levels of inflammatory markers than bronchial AECs(203). However, when challenged with TNF- α and interleukin-1 β , similar levels of receptor expression upregulation occurred on nasal and bronchial AECs(203).

Another study using single cell RNA sequencing of nasal and bronchial brushing samples showed that same types of cells are present in both, including basal, ciliated and goblet cells, however that the proportions of these cells differed between the two(204). Specifically, there were a greater proportion of goblet cells, lesser proportion of ciliated and a very small proportion of basal cells found in nasal brushing samples compared to bronchial brushings, where ciliated cells were predominant (204). This study also found that nasal brushings lacked presence of club cells which were present in bronchial samples (204).

These studies demonstrate there are similarities between nasal and bronchial brushing samples from healthy donors which support the use of nasal AECs as a correlate of bronchial AECs in research due to the ease of their availability. However, some differences between nasal and bronchial samples have been shown, further studies to accurately define these

characteristics are required to fully validate in vitro experimentation. This knowledge would better inform the tradeoff between ease of sample collection and relevance to pathology under investigation when using nasal AECs in place of harder to sample bronchial AECs.

1.6 Aims and Objectives

The overall aim of this study is to explore the interaction between neutrophils and RSV infected airway epithelial cells (AECs) and the effect of this interaction on neutrophil function.

Specifically, I aim to:

1. Explore the profile of neutrophil movement across RSV infected AECs.
2. Examine the effect of RSV infection of AECs on neutrophil apoptosis
3. Examine the effect of RSV infection of AECs on neutrophil activation.
4. Compare the difference in function of neutrophils from adults and neonates during RSV infection.

Hypotheses derived from these distinct aims are described in the corresponding thesis chapters.

Chapter 2 Experimental methods

In order to investigate the aims outlined in **Chapter 1**, a model of neutrophil transepithelial migration needed to be established and adapted for the study of neutrophil function. **Section 2.1 – 2.6** describe materials, methods and preparations undertaken to optimise an *in vitro* model of transepithelial migration assay. **Sections 2.7 – 2.10** describe the standard procedures for experiments presented in **Chapters 3 – 6**.

2.1 Chemicals, solvents and media

All chemicals used in this thesis were of analytical grade and were purchased from Sigma Aldrich, unless otherwise stated. Distilled and deionised water (ddH₂O) was obtained from a Millipore Q Plus water purification system (Merck KGaA, Darmstadt, Germany) and was used to prepare all media where mentioned.

All sterile culture media, sterile tissue culture grade trypsin/EDTA, tissue culture antibiotics and FBS were purchased from Thermo Fisher unless otherwise stated. Sterile tissue culture flasks and plates were purchased from Nunc.

Several different culture media were used in this thesis and their composition are detailed in **Appendix 1**. All media were stored at 4°C.

2.2 RSV propagation

Recombinant GFP tagged RSV A2 strain was kindly provided by Professor Jean-François Eléouët (Unité de Virologie Immunologie Moléculaires, France). Viral propagation was performed in HEp-2 cells grown to 80% confluency on the bottom of a T175 tissue culture flask. Cells were infected for 1 hour with RSV (MOI 0.1) in 25ml Opti-MEM (Gibco) with 1mM L-glutamine supplement and 2% Foetal Calf Serum (FCS) (Gibco). After incubation for 3-5 days – level of infection and syncytia formation was assessed by fluorescence microscopy. When at peak of infection, characterised by first sign of cytopathic effect such as dead cells floating, infected cells were scraped from the bottom of the flask using a cell scraper. Scraped cell solution was placed in a universal container then lysed in a sonicating water bath for 15 minutes, which was then centrifuged at 1200rpm for 5 minutes. Supernatant was collected and frozen at -80 until purification. Virus was concentrated and purified by placing the thawed supernatant in a vivaspin column (MW 1,000,000, Sigma) then centrifugation for 4 hours at °C and 15000rpm. Virus was collected in HBSS + (Gibco) and stored in 100µl aliquots at -80°C for up to 3 months.

2.2.1 Viral quantification

Viruses were quantified by plaque assay in Hep-2 cells. 2.5×10^4 HEp-2 cells were seeded into each well of a flat bottomed 96 well plate (Corning) and cultured overnight in Opti-MEM + 10% FCS at 37°C 5% CO₂. The following day cells were washed in PBS and serial tenfold dilutions of viral stock added to triplicate wells (50µl) for 2 hours at 37°C 5% CO₂. After this, the viral solutions were replaced with 200µl of Opti-MEM + 10% FCS and the plate returned to the incubator for 1 to 2 days at 37°C and 5% CO₂. Each well was then imaged were using a Nikon Eclipse Ti-E microscope equipped with Hamamatsu ORCA 4.0 camera in the FITC channel. Visible viral plaques from each well were counted manually and pfu/ml was calculated by multiplying the plaques/well by dilution factor and x10.

2.2.2 Viral proteins

RSV G protein was produced at the Oxford Protein Purification Facility, (Diamond light source, Didcot, UK) by Professor Raymond Owens and Dr Maud Dumoux (University of Oxford, UK).

RSV F protein was purchased from Sino Biological (11049-V08B). Both RSV F and G protein were kept in high concentration aliquots at -80 degrees.

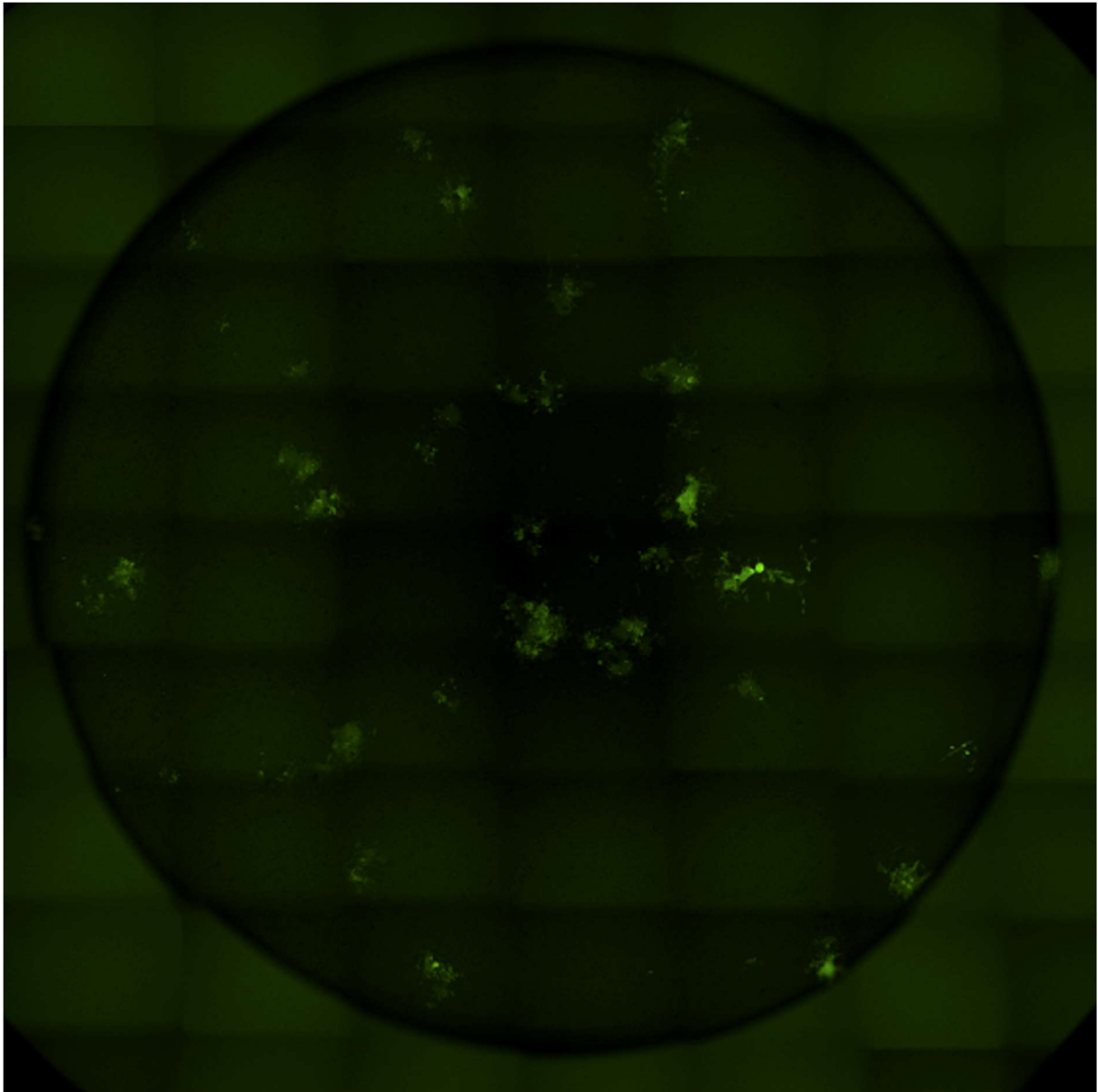


Figure 2-1 Representative image of visible viral plaques during plaque assay to quantify virus

Image captured as described in **Section 2.2.1**. Green corresponds to GFP tagged RSV. Viral plaques were counted, and viral concentration calculated as described above.

2.3 Donor recruitment

This study recruited two groups of participants to donate samples;

- 1) healthy adult volunteers for venous blood and brushings of the nasal epithelium;
- 2) infants born at term for cord blood

2.3.1 Ethical approval

Healthy volunteer donors – Ethical approval for the collection venous blood and nasal brushings from healthy volunteers within UCL for the study of respiratory virus infections was obtained from the UCL Research Ethics Committee (Project ID Number: 4735/002). A maximum volume of 50ml of venous blood in a 28-day period was permitted to be collected from each donor. A copy of the information sheet and consent form is available in **Appendix 2**.

Cord blood – Ethical approval for the collection of blood from placental umbilical vein at University College London Hospital (Grafton Place, London) was obtained from the Bloomsbury Research Ethics committee (Project ID Number: 14/LO/0863). Blood samples were obtained from the placenta and umbilical cord after birth and not the baby. A copy of the information sheet and consent form is available in **Appendix 3**.

2.3.2 Healthy adult recruitment

Healthy adults studying or working within UCL were recruited to donate peripheral venous blood and nasal brushing samples as part of a study to investigate *in vitro* the role of the neutrophil in airway infections such as RSV. All volunteers received a patient information sheet about the study (**Appendix 2**) and gave written informed consent prior to any sample collection. After collection, donor consent forms were inputted into REDCAP, a secure database managed by UCL, and a unique donor number assigned. From this point on, blood samples were labelled with this unique donor number.

2.3.2.1 Exclusion Criteria

Volunteers were excluded if they were under 18, pregnant, had had a respiratory infection in the previous 3 weeks or had given blood in the last month for another purpose. Nasal brushing donors were excluded from the study if they were smokers, had known respiratory disease

(i.e. Asthma, COPD, PCD), had a current respiratory infection, hay fever or known anatomical abnormality of the nasal passages.

2.3.2.2 Healthy Volunteer venous blood collection

Venous blood samples were obtained immediately before experiments by venepuncture of the volunteer's median cubital vein using a 21G butterfly needle (Greiner) and vacutainer (Greiner). Blood was collected directly into K₂ EDTA (Greiner) blood tubes. No more than 50ml was taken from any one donor in a 1-month period.

2.3.3 Cord blood donor recruitment

Pregnant women attending labour ward at University College Hospital (UCH) were recruited to donate a sample of their umbilical cord blood post-delivery as part of a study to investigate the role of stem cells in diseases of the newborn, including in airway infections such as RSV. Written informed consent was obtained from all participants prior to their enrolment in the study. Demographic data was also collected including the babies' sex and gestational age. Parents received an approved patient information sheet on the study (**Appendix 3**) and gave written informed consent prior to any data or sample collection. After collection, donor consent forms were inputted into EPIC, the clinical data management system at UCH, and recorded into the study database where a unique donor number assigned. From this point on, cord blood samples were labelled with this unique donor number.

2.3.3.1 Exclusion Criteria

Participants were excluded if they, or their newborn had concurrent infection, had an existing medical condition which would affect their immune system, were prescribed steroids, were prior to 37 weeks gestation or had consented to donate cord blood for another study. Eligible patients were identified from the elective caesarean section list on the morning of collection or identified by hospital staff.

2.3.3.2 Cord blood collection

Post-partum, the placenta and umbilical cord were checked by the midwife before being given to the researcher. Venous blood was obtained from the umbilical cord vein using a 21G needle and a 20ml syringe (BD Biosciences) and then decanted into K₂ EDTA blood tubes (Greiner).

2.4 Neutrophil Purification

Neutrophil purification was performed using a direct whole blood neutrophil isolation kit (Stem Cell Technologies #19666). The mean number of neutrophils isolated from 10ml of blood was 1.5×10^7 cells with a percentage purity for adult blood neutrophils after purification of 99.8-99.9% as confirmed by flow cytometry and microscopy (See **Figure 2-2**). Purification was performed according to the manufacturers' instructions using a 50ml magnetic collection column (New England Biosciences). The isolated neutrophil mixture was then centrifuged at 1200rpm for 5 minutes, cell pellet suspended in 1ml Robosep buffer and then placed on a 1.5ml magnetic column (New England Biosciences) to ensure maximum purity. Isolated neutrophils were counted using a standard manual Neubauer haemocytometer. Cell population purity was assessed using flow cytometry for markers CD66c and CD11b (Both Biolegend) as previously described, and visually with DiffQuik™ staining and light microscopy. It is not possible to discuss the demographics of adult blood donors as ethical approval for this study did not include collecting this information.

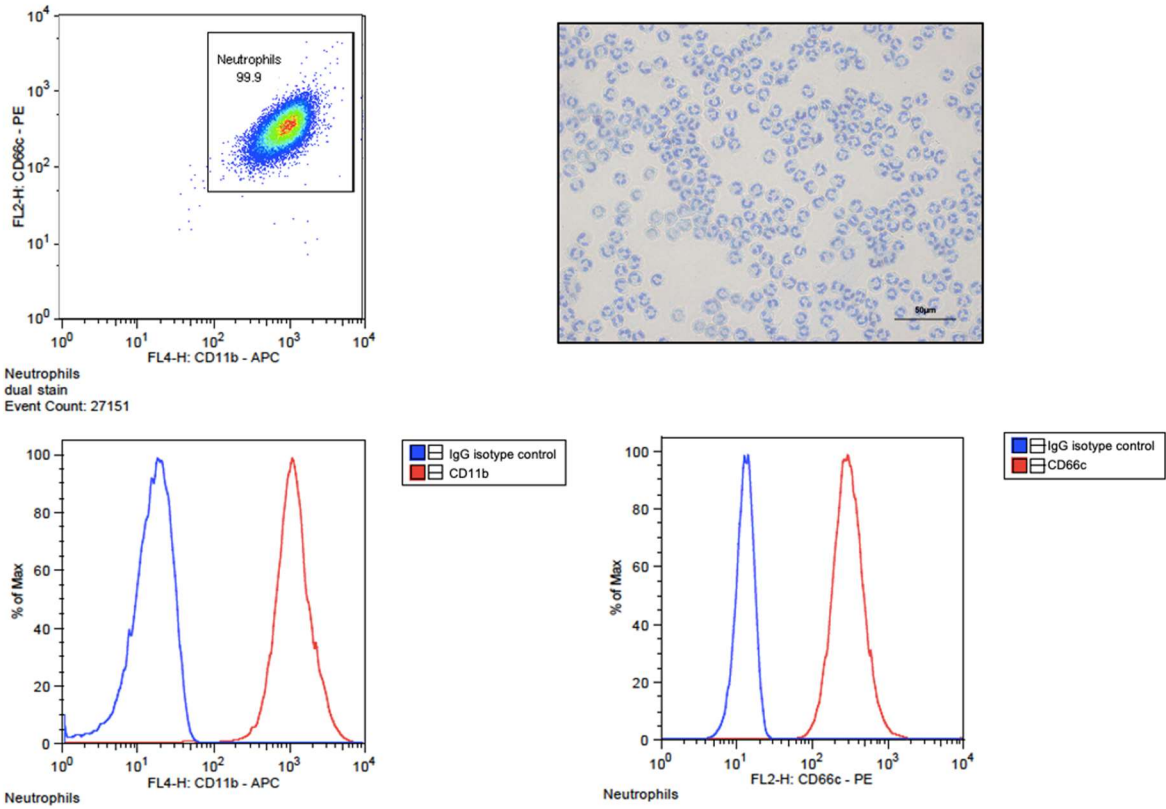


Figure 2-2 Neutrophils isolated using negative immunoselection to 99.9% purity.

(A) Naïve neutrophils isolated using the direct whole blood isolation kit (Stem Cell technologies) showing 99.9% purity by proportion of cells which are both CD66c and CD11b positive; (B) Representative image of a cytospun neutrophil sample with DiffQuik™ staining demonstrating 99.8% purity of neutrophil preparation.

2.5 Respiratory epithelial culture

Primary human airway epithelial cells were either isolated from healthy donors by means of nasal brushing (procedure described in **Section 2.5.1.1**) or purchased from Epithelix Sarl (Switzerland). It is not possible to discuss the demographics of adult airway donors as ethical approval for this study did not include collecting this information. The number of respiratory epithelial cells obtained from each brushing was between 10^3 and 10^6 cells as counted using a manual Neubauer haemocytometer. Respiratory cells obtained were then expanded in co-culture with mitotically inactivated 3T3-J2 'feeder' cells (J2F cells – a mouse fibroblast cell line, herein referred to as 'J2F cells') and Rho-associated protein kinase (ROCK) inhibitors as described by Butler et al(205). This method, previously used in epidermal cell expansion, allows culture of large enough numbers of multipotent stable airway cells to seed multiple permeable membrane inserts, which are capable of differentiating. This method is not only more efficient than culture based methods using BEGM in rapidly expanding respiratory cells from minimal patient samples, it is also more effective in producing multipotent cells which can differentiate to form the pseudostratified epithelium and cilia (205,206).

2.5.1.1 Nasal epithelial brushing processing

A sample of the respiratory epithelial cells was collected by passing a 2mm sterile cytology brush (Keymed, Southend-on-Sea, UK) along the epithelium inside the nose. Cytology brushes collected were then immersed in 2ml collection media {20mM HEPES buffered M199 media (Gibco) supplemented with 1mM Penicillin/Streptomycin (Gibco) and 1mM Amphotericin B (Thermofisher)}. Samples were then placed in the fridge overnight, and the next morning centrifuged for 5 minutes at 1200rpm. The cell pellet was suspended in 1ml F-Media and added to a tissue culture flask containing pre-prepared J2F cells prepared the previous day (see **Section 2.5.1.2**). Tissue culture flasks were placed in a humidified incubator at 37°C 5% CO₂, humidity was provided with sterile water containers inside the incubator. Cultured cells were fed cells with F-media alternate days until 80-100% confluent. At this point basal cells were collected by a two-step trypsinisation protocol. The media was removed from flasks, cells washed with 5ml PBS and then 2ml Trypsin-EDTA (Gibco) added and the flasks were briefly returned to the incubator. Detachment of fibroblast feeder but not basal cell colonies was confirmed with light microscopy. The trypsin solution was removed and the cells rinsed

with 5ml PBS, then 2ml of TrypLE (Gibco) was added and flasks again returned to the incubator for 5-10minutes until all remaining cells had dissociated from the flask. F-media was then added to rinse out and inactivate TrypLE then entire cell suspension placed in a 15ml Falcon tube, which was then centrifuged for 5 minutes at 1200rpm prior to manual cell counting.

2.5.1.2 Preparation of J2F fibroblast feeder flasks

J2F cells were kindly provided by Professor Samuel Janes (University College London Hospital, Grafton Place, London). 1.4×10^6 J2F cells were seeded in a T75 tissue culture flask with 10ml J2F growth medium. Cells were passaged to feeder layer density (20,000 cells/cm²) then rinsed once with PBS before addition of 1ml Trypsin-EDTA (Gibco). After 2-3 minutes at 37°C, trypsin was neutralised by the addition of 3ml J2F growth medium. Cell suspension was centrifuged at 1200rpm for 5 minutes, and the cell pellet suspended in 2ml J2F growth medium. To generate feeder layers, J2F cells were mitotically inactivated by treatment with 4µg/ml Mitomycin C (Sigma) in growth medium. Cells were then counted by trypan blue exclusion using a standard manual neubauer haemocytometer and then seeded in a new T75 flask at feeder layer density of 20,000cells/cm² in growth medium.

2.5.1.3 Expansion of primary epithelial basal cells in co-culture

J2F 'feeder' cells were prepared as described above. An aliquot of primary epithelial cells, 4×10^6 cells in 400µl medium, was defrosted quickly in a 37°C water bath and added to the flask containing J2F cells with 10ml of F-media.

The flasks were then incubated at 37° 5% CO₂ until the cells were completely confluent, with F-media replaced with fresh every 2 days. Confluency was usually achieved within 3-5 days. Basal epithelial cells were harvested by the two step trypsinisation method described in **Section 2.5.1.1**. The cell pellet was suspended in 1ml of F-media and counted at 1 in 20 dilution in trypan blue using a manual haemocytometer and a 10x objective on inverted bright field microscope (Olympus). Cells were prepared for freezing by combining 4×10^6 cells in 200µl of media with 200µl of freezing solution containing 85% ProFreeze™ (Lonza) and 15% DMSO (Sigma) v/v, in a 1ml cryogenic vial which was then stored at -150° until required.

2.6 An *in vitro* model of neutrophil transepithelial migration

To investigate the interactions of the neutrophil with the airway during RSV bronchiolitis, an *in-vitro* model of primary airway epithelial cells grown at air-liquid interface was developed; this was first established and published using a human alveolar cell line (A549) and then further adapted using primary human nasal or bronchial epithelial cells(185,201). This system presents an opportunity to study neutrophil transepithelial migration in a physiological basolateral to apical direction through differentiated airway epithelial cells (AECs).

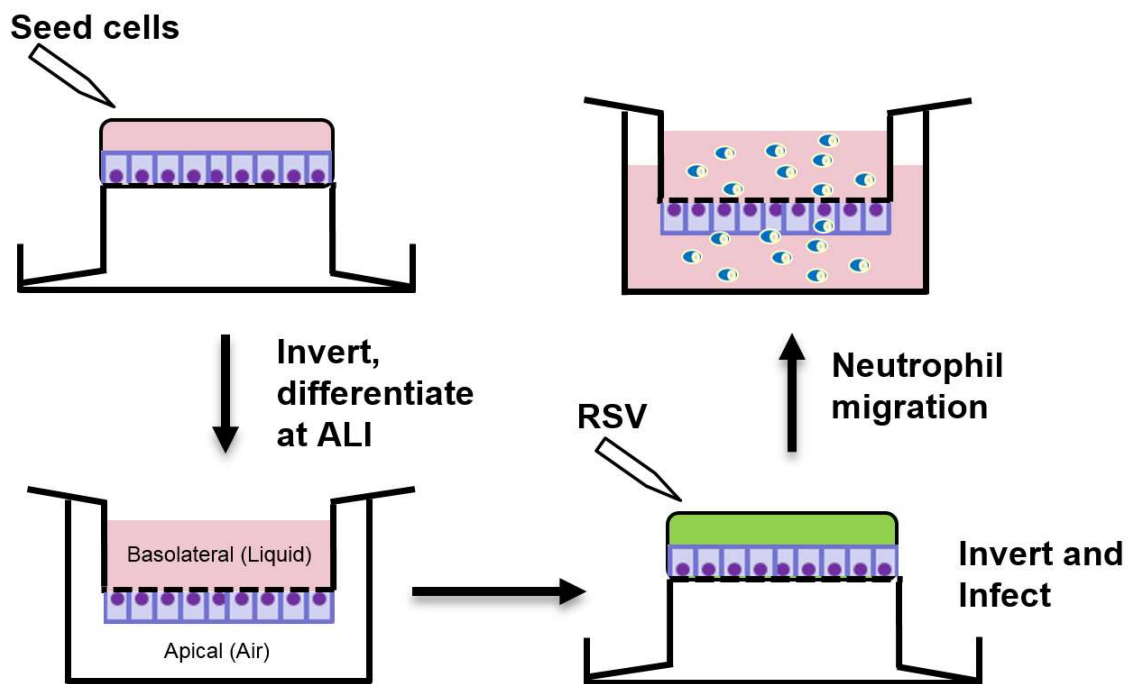


Figure 2-3 Schematic illustrating method of inverted air-liquid interface neutrophil migration model.

Adapted from Deng et al(201).

2.6.1 Inverted Air-Liquid Interface culture seeding

Transparent 24 well plate inserts with a 3µm pore size (Greiner cat #662630) were coated on both sides with 1% (v/v) collagen (Nutacon) in sterile H₂O overnight then left to dry completely in a class 2 laminar flow hood prior to seeding. Coated inserts were inverted in a sterile 6-well plate for seeding on the underside of the pore surface. 1 x 10⁶ epithelial cells were seeded onto each insert in 70µl of F-media. The plate containing the inverted inserts was then placed in the incubator for 4 hours at 37°C and 5% CO₂. After incubation, the inserts were reinverted into a new 24 well plate, with 500µl F-media below each insert on the apical side of cells and 100µl of 'top media' above (basolateral side) and returned to the incubator. Top media is formulated of F-media supplemented with (v/v) 5% Matrigel (Corning) and 1% Collagen (nutacon). After 2 days, media from the apical side of the inserts was removed and the basolateral fluid replaced with a 100µl of air liquid interface media (ALI media). The basolateral media was changed every second day. After 7 days at air liquid interface, AECs had not yet ciliated and displayed a basal cell phenotype. Basal AEC cultures, used for microscopy experiments described in **Chapter 3**, were used within 7 days of air-lift. Ciliation began to occur around day 16 and AEC cultures were used at a median age of 28 days (±3 days). Ciliated AECs were used in transepithelial migration experiments in **Chapters 4, 5 and 6**.

2.6.1.1 Validation of AEC culture integrity and ciliation

Trans-epithelial electrical resistance (TEER) of cells cultured at ALI was measured with an EVOM² Epithelial Voltohmmeter (World Precision Instruments, Sarasota, Florida, US) prior to infection. Electrodes were sterilised by dipping into 70% ethanol, left to air-dry and then dipped into sterile culture medium until calibrated. TEER values were calculated by multiplying the ohmic resistance value given by the voltmeter by the surface area of the PET culture insert (33.6mm²). AEC cultures with a TEER reading below 300 were not used in experiments.

Ciliation was visually assessed using high speed video microscopy after at least 28-days at ALI and prior to migration experiments. To assess, AEC cultures were flooded apically with 400 µl ALI media and the holding plate subsequently placed on an inverted Nikon Eclipse TiE microscope stage (Nikon, Kingston-upon-Thames, UK) with the temperature set to 37° C and

CO₂ to 5%. Each culture was inspected using a x20 long working distance objective (numerical aperture=0.45) and a Hamamatsu ORCA camera (Hamamatsu, Japan) attached to a Nikon Eclipse TiE microscope. The level of epithelial culture ciliation was noted and over 50% of the well surface displaying motile cilia was considered adequate ciliation. For a video of a ciliated AEC culture see **Appendix 8** and Video 1 on the USB stick attached.

2.6.2 Inverted Air Liquid Interface Transepithelial Migration protocol

AEC cultures were infected with green fluorescent protein (GFP) tagged RSV for 24 or 72 hours prior to migration experiments. When ready, AEC cultures were inverted in a sterile 12-well plate then infected apically with an inoculum of RSV MOI 5 in 20µl ALI media. An inoculum of MOI 5 was investigated due to previous investigation in a similar model by Herbert et al, and was found to produce reliable infection in ciliated AEC cultures(185).The inoculum was applied to the apical surface of cells and the plate of inverted AEC cultures placed in the incubator at 37°C 5% CO₂ for 1 hour. The same volume of ALI media without virus was applied in the same manner to control 'mock' infected AEC cultures. After the hour incubation, the apical liquid was aspirated, and AEC cultures reinverted back into the 24-well plate and 100µl ALI media replenished on the basolateral side. Viral infection of AEC cultures was confirmed visually by the presence of GFP expressing AECs using an inverted Nikon Eclipse TiE microscope (Nikon, Kingston-upon-Thames, UK) with the temperature set to 37° C and CO₂ to 5%. Each culture was inspected in the FITC channel using a x20 long working distance objective (numerical aperture=0.45) and a Hamamatsu ORCA camera (Hamamatsu, Japan). RSV infection was confirmed by presence of at least 5 GFP plaques. Images of RSV infected GFP expressing AEC cultures are available in **Appendix 9**.

On the day of neutrophil migration, 600µl of fresh HBBS (+) was placed below the AEC cultures and left to incubate for 1 hour prior to migration to collect any secretory factors produced by the epithelium. AEC Cultures were then placed in an ultra-low binding 24 well plate (Greiner cat#662970) and the apical supernatants removed, spun down at 1200rpm for 5 mins to remove any epithelial cell debris, and 400µl replaced under their respective culture in the new plate. To initiate migration, media on the basolateral side of the AEC cultures were aspirated and replaced with 5 x 10⁵ isolated neutrophils in 100µl HBSS(+) supplemented with

1% autologous serum (201). Autologous serum was isolated by centrifuging 1ml whole blood for 10 minutes at 3000 x g and removing the straw-coloured plasma layer.

The culture plate was then returned to the incubator for either 1 hour or 4 hours. After incubation, apical and basolateral media were collected and centrifuged at 1200 rpm for 5 minutes to concentrate cell populations.

2.6.2.1 Post migration processing

AEC cultures were then fixed and stained for microscopy analysis (see **Section 2.6.4.2**) or treated with 100µl 5% Accutase (Gibco) solution for 10 minutes at room temperature to dissociate epithelium and adhered neutrophils into a single cell solution. Neutrophils were then analysed for markers of their function (**Section 2.7**).

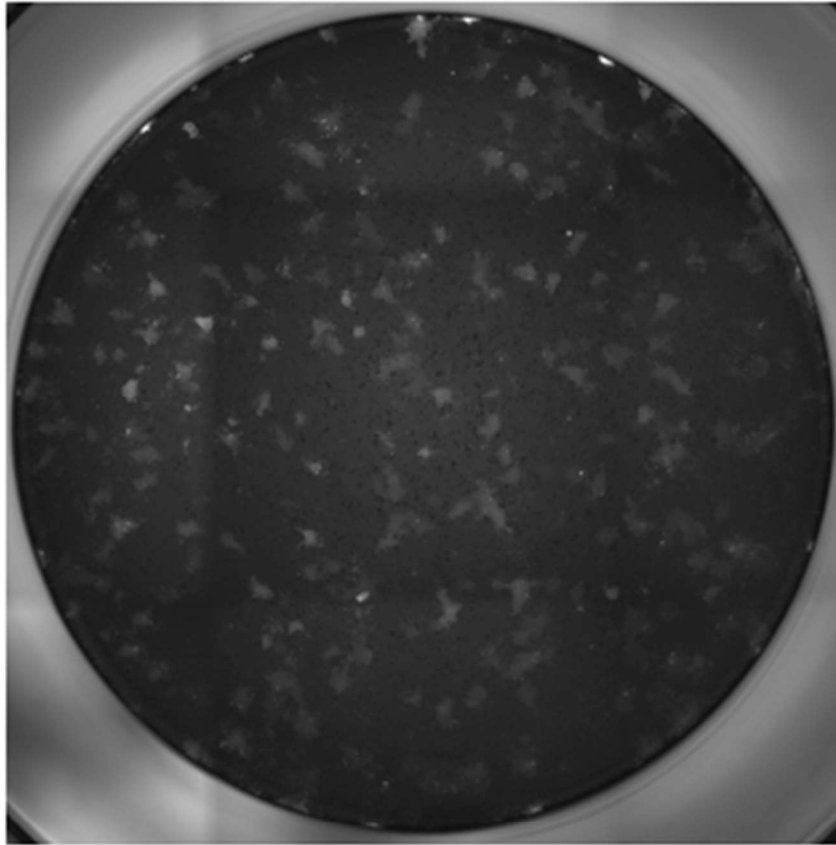
2.6.2.2 Controls used in transepithelial migration experiments

‘Mock infected’ AECs were used to control for the presence of AECs without RSV infection. Mock infected AECs were inoculated with an equivalent volume of media without virions at the same time as RSV infection of AECs took place as described in **Section 2.6.2**. Apart from the lack of virus, mock and RSV infected AECs were given the same interventions and treated the same. ‘RSV Sup’ AECs were used to distinguish between the influence of RSV infected AECs and the inflammatory mediators present in the RSV infected AEC supernatant. RSV Sup AEC wells consisted of mock infected AECs with supernatant collected from RSV infected AECs placed apically. The reverse, placing mock infected supernatant apically to RSV infected AECs, would be counterintuitive – as the RSV infected AECs would simply generate inflammatory markers during the experiment which would contaminate the apical ‘mock’ supernatant.

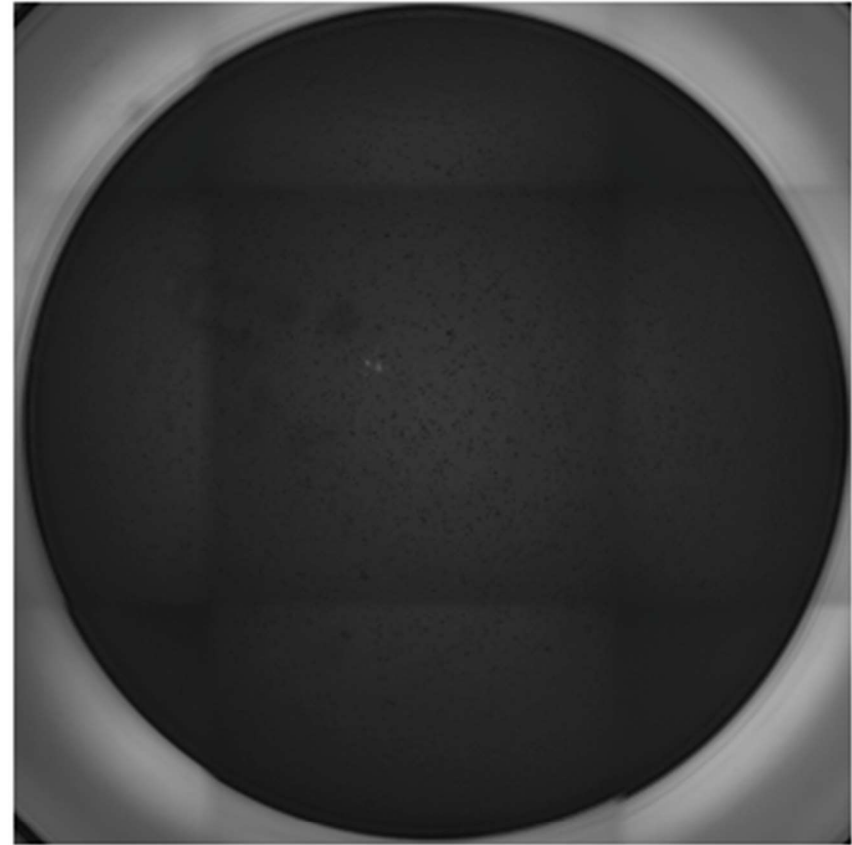
Mock infected AECs with a 1ng/ml N-Formylmethionine-leucyl-phenylalanine (fMLP) in HBSS (+) solution placed apically was used as a positive control for neutrophil migration. fMLP was chosen as a known potent chemoattractant to neutrophils which is exogenous in origin and for its documented use in model systems such as this(185,201,207). It was not possible to incorporate a negative control for migration within the culture model. However, neutrophils unexposed to AECs and suspended in media alone throughout the experiment ‘No AEC’ control, was used as a control for interaction with AECs.

2.6.3 Preparation of AEC supernatants for neutrophil function experiments

To understand the relative contribution of chemokines and cytokines to neutrophil function in the transepithelial migration assay, supernatants from mock and RSV infected AEC cultures were collected and stored for future experiments. 600µl of fresh HBBS (+) was placed on the apical side of the AEC cultures grown at air liquid interface. After 1 hour of incubation, the apical supernatants were removed, centrifuged at 1200rpm for 5 mins to remove any epithelial cell debris, and supernatant recovered from duplicate cultures pooled. The pooled supernatants were stored on ice for 1 hour then transferred to a -20 freezer for 2 weeks. After this time, no replicative virus was detected in the supernatant as verified by plaque assay described in **Section 2.2.1**.



RSV



Prepared 72hr RSV infected
AEC supernatant

Figure 2-4 Representative plaque assay images of GFP RSV stocks and neat supernatant collected from 72 hour infected ciliated AECs
Supernatants were stored and processed as described in **Section 2.6.3**. Plaque Assay was performed according to **Section 2.2.1**

2.6.4 Quantification of neutrophils migrating through RSV infected AECs

2.6.4.1 Neutrophils apically dissociated from AECs

Prior to migration, isolated neutrophils were stained with a fluorescent red viability dye, calcein red orange (Life Technologies) as described in **Section 2.7.2.1**. The inverted air liquid interface transepithelial migration protocol (**Section 2.6.2**) was followed with the substitution of red stained neutrophils. After the experiment, neutrophils from the apical side of the AEC cultures were transferred to a black plastic 96 well plate (Greiner #655892), and a standard curve of known concentrations of red fluorescent neutrophils constructed. The plate was read on an OPTIMA co-star fluorescence enabled plate reader at 570nm and standard curve constructed and interpolated using Microsoft Excel to calculate numbers of viable neutrophils recovered on the apical side.

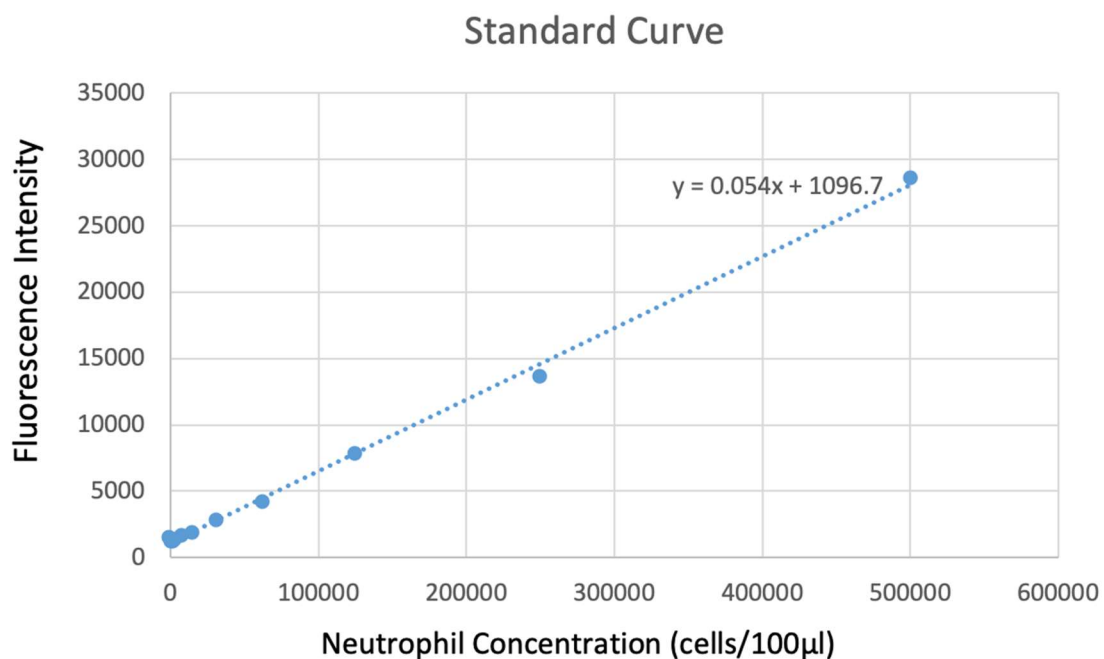


Figure 2-5 Representative Standard Curve of known concentrations of Calcein RedOrange stained fluorescent neutrophils.

These standard curves were used to interpolate numbers of viable neutrophils recovered from the apical side of AEC cultures after transepithelial migration assay

2.6.4.2 Neutrophils adherent to AECs

In order to measure the numbers of adherent neutrophils, AEC cultures were fixed, stained and mounted for microscopy. Firstly, the PET membranes on which the AEC are cultured, were carefully cut off the well insert base using a scalpel blade then mounted epithelium side up onto a normal microscope slide and PBS washed. For the purposes of this staining protocol, a 'PBS wash' consists of washing the AEC culture surface with 100µl sterile PBS in triplicate. AEC cultures were then fixed for 30 minutes in a 4% PFA (v/v) in PBS solution and then PBS washed again. 20µl Hoechst solution (Sigma) was applied to the AEC cultures and incubate for 10 minutes in the dark. After a final PBS wash, AEC cultures were then immersed in propyl gallate solution (Sigma) and sealed under a coverslip. Prepared slides were kept in the fridge until imaging using a 20x Plan Achromat Objective and a Zeiss LSM710 confocal microscope. The pinhole was set at 1 airy unit (AU) to provide the optimal fluorescence signal to background ratio. A Z-stack image with slice thickness of 0.5µm was captured in the GFP, Calcein RedOrange and DAPI channel to image the RSV infected cells, neutrophils and cell nuclei respectively. The numbers of neutrophils, viable at the time of fixation, adherent to the AECs were counted from the captured images using an ImageJ script available in **Appendix 5**.

2.6.5 Matching AEC donor to neutrophil donor

A potential advantage of this novel *in vitro* transepithelial migration model is the possibility that AECs grown to ciliation and neutrophils isolated for migration experiments may be sourced from the same individual donor. In practice however, it is incredibly difficult to coordinate sampling and growing airway cells and ensuring the same individual is willing, available and eligible in line with our ethical considerations to donate blood at a later but not yet determined date when AEC cultures are ready for experiments. To examine the impact of donor matching between AEC and neutrophil donor on interaction with AECs, neutrophil adherence to Mock and RSV infected AECs was measured for autologous and allogenic neutrophils. These data are shown in **Figure 2-6**). There was no significant difference in numbers of adherent autologous and allogenic neutrophils to Mock and RSV infected ciliated AECs after 1 hour transepithelial migration conducted as described in **Sections 2.6.2** and **2.6.4.2**. In addition, allogenic and autologous neutrophil CD11b expression was measured after 1 hours transepithelial migration through mock infected AECs to examine the impact of

donor matching on the neutrophil. Neutrophils were taken from the basolateral, apical and AEC adherent compartments of the transepithelial assay, CD11b measured by Flow cytometry as described in **Section 2.7.3** and then compared between autologous and allogenic neutrophils recovered from the same locations in replicate AEC cultures. Neutrophils not exposed for airway and left untouched in media for the assay duration were used as a non-migration control (No AEC). These data are also shown in **Figure 2-6**. There was no significant difference in neutrophil CD11b expression between allogenic and autologous neutrophils in any location (basolateral, apical or AEC adherent) or the non-AEC controls. These comparisons form part of further analyses presented in **Chapter 5** and are reanalysed here for the purpose of evaluating donor matching.

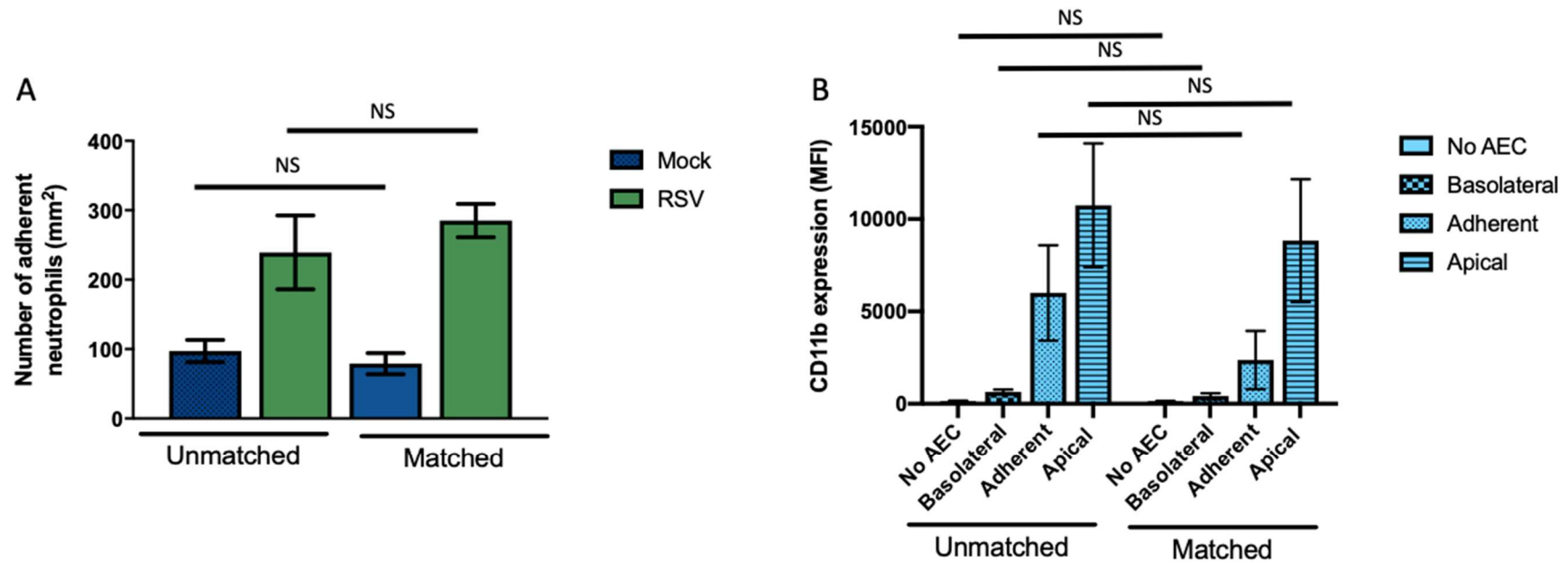


Figure 2-6 Comparative numbers of neutrophils adherent to AECs and comparative neutrophil CD11b expression after 1 hours transepithelial migration between neutrophils autologous to the AEC donor (matched) and allogenic to the AEC donor (unmatched).

(A) Numbers of neutrophils counted adherent to 72 hour Mock infected (blue) and 72 hour RSV infected (Green) AECs after 1 hour transepithelial migration assay where neutrophils were allogenic to the AEC donor (Unmatched) or autologous to the AEC donor (Matched). N=15 (B) CD11b expression on autologous and allogenic neutrophils after 1 hours transepithelial migration assay. Neutrophils were recovered from basolateral (checked), AEC adherent (dotted) and apical (striped) compartments of the assay alongside non AEC controls (solid bars) where neutrophils were allogenic to the AEC donor (Unmatched) or autologous to the AEC donor (Matched). Comparison between neutrophils from matched and unmatched experiments was performed using Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons. N=6.

2.6.6 Quantifying neutrophil distribution

To investigate whether neutrophil adherence to AECs was uniform (null hypothesis =0) or whether they clustered in particular areas, a method of measuring neutrophil distribution was required. To this end, fixed and stained AECs were imaged across a 50µm Z range (0.3µm steps) using a 20x Plan Achromat Objective and a Zeiss LSM710 confocal microscope. For these experiments, AEC cultures were fixed and stained as per **Section 2.6.4.2**. Images were analysed using ImageJ to first identify neutrophils within the image, determine their 3D coordinates, this was achieved by thresholding the red channel of the z stack and analysing particles using a 3D objects plugin(208). These coordinates were then exported into RStudio and analysed using a spatial statistics package to then calculate the distance between the centre-point of each neutrophil and the centre-point of its five nearest neighbours(209). However, it is expected that if more neutrophils are adherent to RSV infected AECs in comparison to mock, that they would be closer together by crowding alone. To account for this, the distance apart each neutrophil would be if neutrophils were evenly spread over the AEC area was calculated from the numbers of neutrophils counted as adherent to the same AECs. ImageJ macro code for determining 3D coordinates and subsequent R script for distance analysis is available in **Appendix 6**.

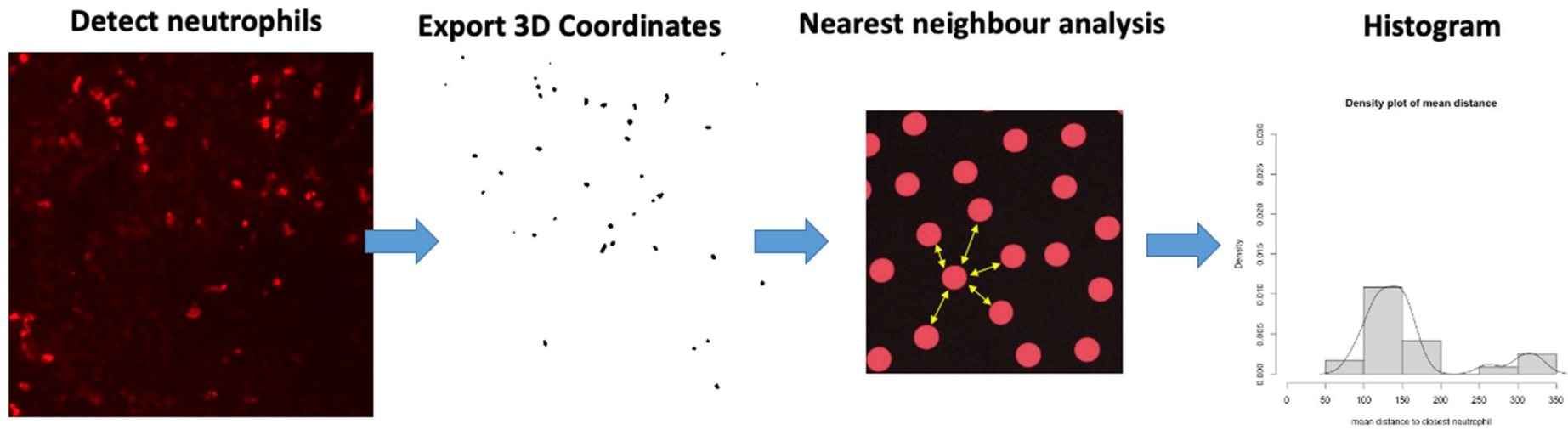


Figure 2-7 Schematic of the steps of nearest neighbour analysis to measure distribution of neutrophil adherence to AECs.

The first step is to detect neutrophils in the red channel of the z stack, then determine the 3D coordinates of their centre of mass. With these coordinates the distance from each neutrophil to its nearest 5 neighbours was calculated and plotted in a histogram of mean distance to closest neighbours.

2.7 Neutrophil function

Assays of neutrophil chemotaxis, activation and apoptosis were used to identify changes in neutrophil function in response to transepithelial migration, exposure to RSV or its surface glycoproteins and secreted chemokines and cytokines produced by primary human AECs in response to RSV infection.

2.7.1 2D Chemotaxis Assays

The day before a chemotaxis assay, all plasticware was placed in a standard incubator to allow for gas equilibration.

Neutrophils, direct from isolation or from a migration assay, were suspended at a concentration of 1.5×10^6 in Matrigel™ (Corning Cat#354234) at a concentration of 4.5mg/ml on ice. 6µl of this solution was added to the top of each filling port of an ibitreat chemotaxis µ-slide (ibidi gmbh), and then aspirated through from the opposite chamber to fill the channel. These slides were placed in a humidified incubator at 37°C for 15 minutes to allow the Matrigel to solidify. 65µl of ALI media was added to neutral control wells. Chemoattractant or undiluted supernatants collected from AEC cultures were added to the right-hand chambers at a volume of 65µl. The slides were then plugged to avoid change in pressure or pH during imaging and mounted for the stage of a Zeiss Axiovert 135 microscope. The cell chamber was visualised using a 10x phase objective and an image captured every 30 seconds for an hour. Time lapse videos were used to track at least 200 neutrophils per condition.

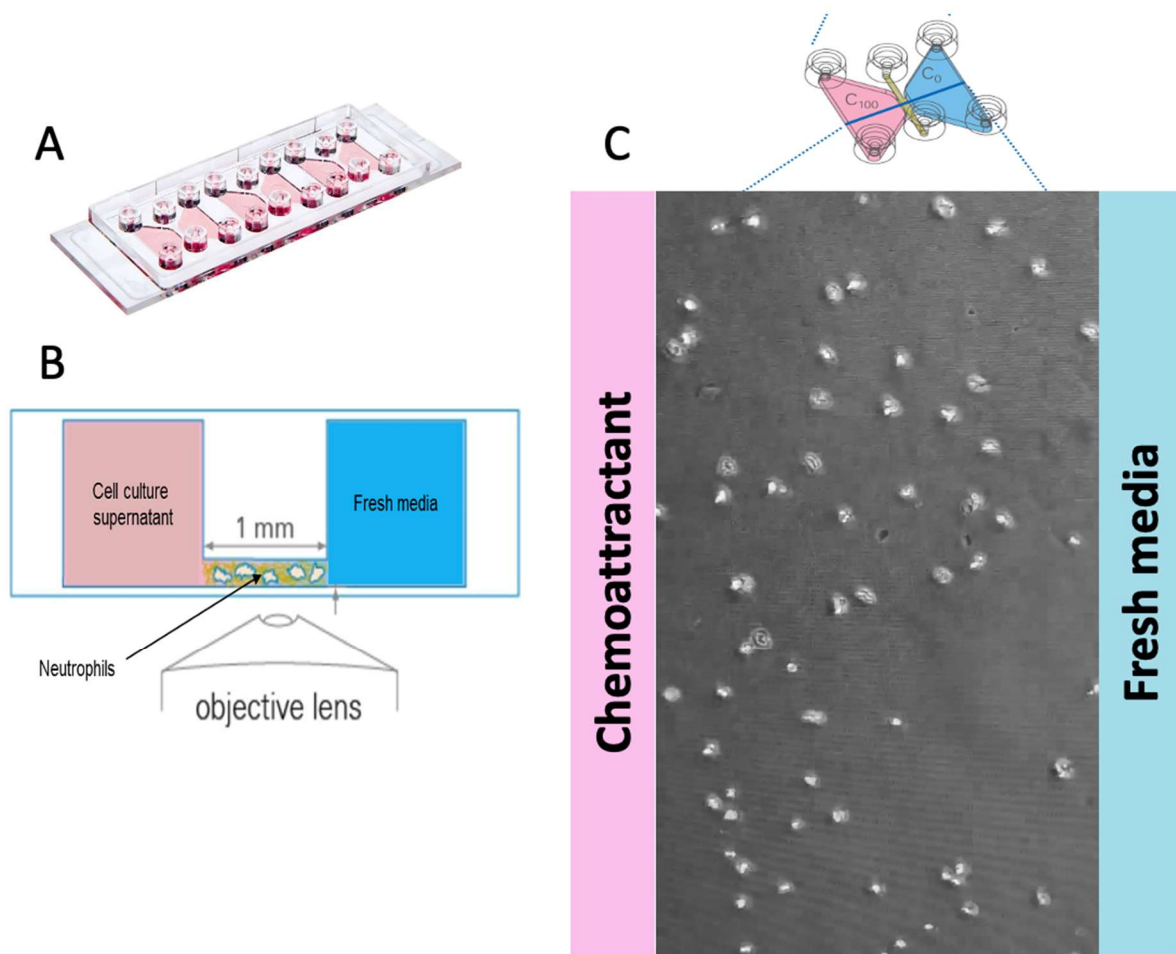


Figure 2-8 Schematic illustrating 2D chemotaxis experimental procedure

(A) Chemotaxis slide layout (B) structure of the chamber containing neutrophils illustrating how a concentration gradient of chemoattractant is established (C) representative image of cells during chemotaxis assay.

2.7.2 Time-lapse imaging of neutrophil trans-epithelial migration in 3D

To gain a fuller understanding of neutrophil function during interaction with the RSV infected airway, live imaging techniques were optimised to observe the trans-epithelial model of neutrophil migration on a microscope. I also used fluorescence and brightfield microscopy techniques to investigate neutrophil functions of chemotaxis, phagocytosis and apoptosis as described above. Staining of both neutrophils and epithelial cells was also performed to investigate neutrophil tropism and clustering on the epithelial surface. To investigate the initial influx of neutrophils across the respiratory epithelium, I modified the transepithelial migration model to be able to visualise this event in real time.

2.7.2.1 Neutrophil staining

Isolated neutrophils were centrifuged at 1250 rpm for 5 minutes and suspended in 500µl HBSS(-) in a sterile microtube. 1µl of CellTrace™ calcein red-orange stain (Life Technologies) was added and the cell solution mixed thoroughly before leaving the cells to incubate for 30 minutes in the dark. Cells were centrifuged at 1250rpm in a micro-centrifuge for 5 minutes, washed twice in HBSS(-) and once in (+) before being suspended in HBSS(+) for use.

2.7.2.2 Assay modifications

A neutrophil transepithelial migration experiment was modified, with basal AEC cultures grown at ALI for 7 days, placed in a black plastic 24 well plate with a glass coverslip bottom (Greiner). Imaging took place using a Zeiss CSU22 Spinning Disk microscope system and a LD Plan Achromat 20x objective within the Institute of Child Health Flow Cytometry and Imaging Facility.

2.7.2.3 Analysis

Stacks were split into constituent channels and saved for further analysis using an ImageJ macro which is available in **Appendix 4**. Neutrophils were identified in each image' red stack using Icy, programmed to run a spot detection protocol for each frame of the stack, detecting light objects close to 10 µm diameter on a dark background. Then each spot was tracked using the spot tracking plugin using a 3D tracking algorithm(210). Representative screenshots of this analysis technique is shown for 2D analysis in **Figure 2-9**. Tracks were then visually checked

for tracking accuracy and where erroneous tracks were found, were either corrected manually or removed from analysis. Raw track coordinates were exported for further analysis using RStudio package Motility Lab(211). Data detailing displacement, speed and linearity were exported combined with replicate data sets for analysis using RStudio and GraphPad Prism v8.0.

2.7.3 Staining for markers of neutrophil activation

Neutrophils collected from transepithelial migration assays were washed once in 500µl of FACS buffer, centrifuged at 3000rpm for 5 minutes then the cell pellet was suspended in 50µl (1/50 dilution) of TruStain FcX blocker for 10 minutes at 4°C. After this the neutrophils were washed in 500ul FACS buffer and centrifuged at 1400rpm for 5 minutes. The neutrophil pellet was then resuspended in 50µl of FACS buffer containing 1/250 dilution CD11b-FITC, 1/250 dilution CD64-PE-Cy7, 1/250 dilution CD62L-APC-Cy7, 1/50 dilution of MPO-APC and 1/50 dilution of NE-PE conjugate antibody, then incubated for 20 minutes at 4°C in the dark. Cells were then fixed in 1% (v/v) paraformaldehyde (PFA) solution for 20 mins at 4°C then suspended in permeabilisation buffer (Thermofisher Cat #00-8333-56). Afterwards the cells were centrifuged at 1400rpm for 5 minutes. To perform intracellular staining, the cell pellet was then suspended in 50µl of permeabilisation buffer containing 1/250 dilution CD11b-FITC, 1/250 dilution CD64-PE-Cy7, 1/250 dilution CD62L-APC-Cy7, 1/50 dilution of MPO-APC and 1/50 dilution of NE-PE conjugate antibody, then incubated for 20 minutes at 4°C in the dark. Cells were then washed once more in 500µl FACS buffer and processed using a CytoFlex flow cytometer at the UCL GOS ICH Flow cytometry and imaging facility. Details of antibodies and suppliers used are contained in **Table 2 – 1)**

Table 2-1 Antibodies used in flow cytometry analysis

Target	Target Function	Fluorophore	Dilution	Supplier
TruStain FcX blocker	Non specific Fc binding	n/a	1/50	Biolegend
CD11b (ICRF44)	Integrin	FITC	1/50	Insight Biotechnology
CD11b (ICRF44)	Integrin	APC	1/50	Cambridge Bioscience
Neutrophil Elastase	Secreted effector enzyme	PE	1/5	Abcam
Myeloperoxidase	Secreted effector enzyme	APC	1/5	Biolegend
CD64	Fc receptor	PE-CY7	1/7	Beckton Dickinson
CD62L	L-selectin	APC-CY7	1/5	Biolegend
CD66c	CEACAM6	PE	1/5	Biolegend

2.7.3.1 Flow cytometric neutrophil activation analysis

Cells were gated for inclusion of whole cells and exclusion of debris, then were arranged on several dot plots of CD11b – FITC, NE – PE, MPO – APC, CD62L-APC-Cy7, CD64-PE-Cy7, each on a y axis vs forward scatter (FSC). Single stained control cell populations were used for compensation. Cell counts, marker mean fluorescence intensity (MFI) and proportions positive for each marker were exported to Microsoft Excel and combined with replicate datasets. Statistical analysis was performed using GraphPad Prism v8.0.

2.7.3.2 Quantification of secretory markers of neutrophil activation

Specific markers of neutrophil activation, Neutrophil Elastase (NE) and Myeloperoxidase (MPO) are secretory agents expelled by neutrophils during degranulation to act on exogenous pathogens. After neutrophil transepithelial migration assays, supernatant from the apical and basolateral compartments of AEC cultures was separated from cells by centrifugation at 3000rpm for 5 minutes and stored at -80. Stored supernatants were thawed and concentration of NE and MPO determined by enzyme linked immunosorbent assay (ELISA). ELISA kits for NE (#ab270204) and MPO (#ab119605) were purchased from AbCam and assays were performed according to the manufacturer's instructions.

2.7.4 Apoptosis Assays

Programmed cell death by apoptosis is a key function of neutrophils to regulate the immune response and ensure the timely resolution of infection and inflammation. It has been reported that RSV infection prolongs apoptosis in neutrophils(212).

Traditional apoptosis assays either use visual examination of cell morphology using cytology staining or flow cytometry using Annexin V and a viability stain, such as propidium iodide (PI). Annexin V binds to phosphatidyl serine, a component of the cell membrane which is not present on the outer envelope when the cell is healthy, but does appear on the outer membrane of the cell once the cell is undergoing apoptosis (**Figure 2-10**)(213). Using annexin V conjugated to a fluorophore such as FITC alongside a fluorescent viability dye such as PI it is possible to separate populations of live cells, apoptotic cells, late apoptotic and dead cells using a flow cytometer.

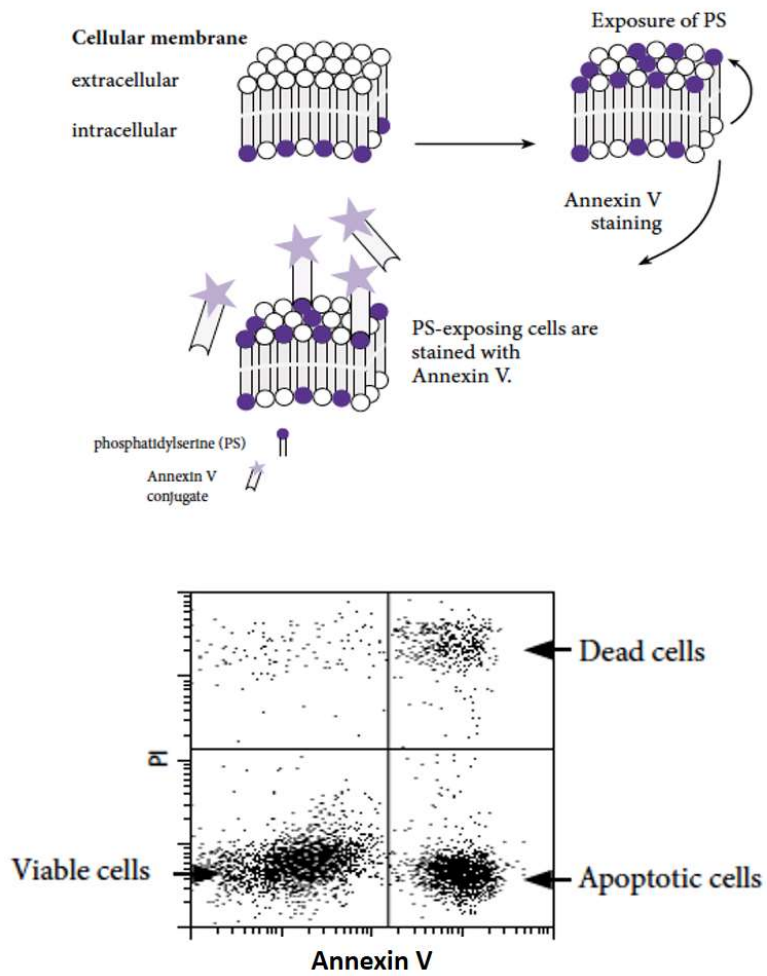


Figure 2-10 Schematic to show mechanism of Annexin V and PI costaining apoptosis assay

(Top) Illustration demonstrating the mechanism of Annexin V binding to phosphatidyl serine on the outer membrane of apoptosis cells. (Bottom) Representative dot flow of separation of viable, apoptotic and dead cells using this method. Images produced by MACS.

2.7.4.1 Staining impure cell populations for CD11b

Cell samples retrieved from migration models may also contain airway epithelial cells. To separate these populations and ensure only the examination of neutrophils, collected cells were stained first for CD11b, an integrin receptor present on neutrophils and other granulocytes but not airway epithelium. Cells retrieved were washed once in 500µl of FACS buffer, then the pellet suspended in 50µl (1/50 dilution) of TruStain FcX blocker for 10 minutes at 4°C. After this the cells were again washed in 500µl FACS buffer, then centrifuged at 1400rpm for 5 minutes. The cell pellet was then suspended in 50µl of 1/250 dilution CD11b-APC conjugate antibody in FACS buffer, then incubated for 20 minutes at 4°C in the dark. Cells were then washed once more in 500µl FACS buffer and used in assay.

2.7.4.2 Annexin V – FITC apoptosis assay

Neutrophils were centrifuged at 1250 rpm for 5 minutes and washed once in 500µl FACS buffer. Neutrophils recovered from the transepithelial migration model, where the retrieved population of neutrophils are contaminated with AECs, were stained for CD11b expression as described above. Neutrophils were then suspended in 50µl Annexin binding buffer with 0.3µl of Annexin V-FITC (MACS) and left to incubate for 15 minutes in the dark at room temperature. Cells were then flash stained with 300µl Annexin Binding buffer containing 1µg/ml Propidium Iodide. All samples, including unstained and single Annexin V or PI controls, were processed using a Beckton Dickinson FACSCalibur Flow Cytometer. At least 30,000 events were collected for each sample. In the case of an impure neutrophil population, neutrophils were first identified as being CD11b positive before subsequent analysis. Early apoptosis was characterised by Annexin V positivity without PI positivity. Cell death was characterised by positivity of both Annexin V and PI. Live cells were defined as negative for both Annexin and PI.

2.7.4.3 Apoptosis assay analysis

Analysis was performed with FlowJo V8.0 (Treestar). First, samples were displayed as a dot plot showing forward scatter (FSC) vs side scatter (SSC), and debris removed by gating. If samples were from a migration assay, a histogram of fluorescence intensity in the CD11b-APC

channel was constructed, and cells positive for APC fluorescence gated for further analysis. Then, PI was plotted vs FITC both logarithmic scales, and compensation performed on single stain controls to separate overlapping fluorescence profiles. Cells were quadrant gated as PI negative FITC negative (live cells), PI negative FITC positive (Early apoptotic cells) and PI positive (Late apoptotic, necrotic and dead cells). Counts and percentages of these 3 parameters were exported to Microsoft Excel and combined with other datasets. Statistical analysis was performed using GraphPad Prism v8.0.

2.8 Supernatant analysis

To gain an insight into the effect of soluble factors secreted by AECs into the apical and basolateral surface media, enzyme linked immunosorbent assays (ELISAs) were performed to ascertain the concentration of secreted chemokines IL-8 and IP-10. Supernatants were collected from AECs as described in **Section 2.6.3**. ELISA kits were purchased from Sigma Aldrich for IL-8 (CXCL-8 #RAB0119) and IP-10 (CXCL-10 #RAB0319), and assays performed to the manufacturer's instructions.

2.9 Flow Cytometry

Flow cytometry was conducted on one of two machines. Apoptosis assays were analysed on a BD Biosciences FACSCalibur equipped with a 488nm and a 638nm argon-ion lasers. Activation and expression marker experiments were analysed using a Beckman Coulter CytoFLEX equipped with 405 nm, 488 nm, 561 nm and 638 nm LED lasers.

2.9.1 Flow cytometry reagents

FACS buffer for reagents was made up from sterile PBS supplemented with 2% Foetal Bovine Serum (Gibco) and 200mM Ultrapure EDTA solution (Gibco). FACS buffer supplemented with 1% PFA was used as FACS fixing reagent. Propidium Iodide (Sigma), used for cell death assays, was stored at 50mg/ml and used at a concentration of 1mg/ml.

2.10 Imaging and Histology

2.10.1 Histology staining.

Neutrophils samples were cytospun at 600rpm for 5 minutes onto microscope slides for staining. A commercial DiffQuik™ stain (Sigma), a variation of romanowsky staining favoured for visualising neutrophils, was purchased from Thermofisher and staining performed according to manufacturer's instructions. Histology pictures were captured using a Zeiss Axioplan microscope equipped with a colour Hamamatsu 4.0 ORCA camera in the Institute of Child Health Flow Cytometry and Imaging Facility.

2.10.2 Image Analysis

Image analysis was performed using Fiji, Icy and RStudio. Fiji and Icy are both open source softwares for visualisation and analysis of biological data. Where automated scripts and macros were used, these have been included in an appendix indicated in the necessary chapter. In all other instances, softwares used and steps taken on each process have been outlined in the relevant analysis section.

2.11 Statistical Analysis

Statistical analysis and graphical representation of data was performed using GraphPad Prism v8.0 or RStudio. All datasets were tested first for normal distribution by Shapiro-Wilk Test. Data following the normal distribution were presented in this thesis as mean \pm standard error of the mean (SEM), with the exception of neutrophil distribution analysis (**Chapter 3**) which is presented as median + IQR. These normally distributed data were compared using a simple students t-test if only 2 groups present or One Way ANOVA with Tukeys post hoc test to correct for multiple testing if more than 2. Where two independent variables influence were to be assessed, Two Way ANOVA with Greenhouse-Geisser correction were performed. After consulting with a statistician, neutrophil activation data in **Chapter 5** was analysed using a linear model of mixed effects to examine the significance of infection state and location within the AEC culture on observed differences in activation. An R script was written for this purpose by Professor Cortina Borja and this is available in **Appendix 7**.

Non-normally distributed data were compared using the Mann-Whitney U test when there were 2 experimental groups. When there were more than 2 groups Kruskal- Wallis test was used for unpaired data or Friedman test when data were paired, with Conover Inman post hoc test.

Throughout this thesis a statistically significant difference was accepted as a probability (p) value of <0.05 . Statistical advice was kindly provided by Professor Mario Cortina-Borja (UCL Great Ormond Street Institute of Child Health, London, UK).

Chapter 3 Profiling the kinetics and directionality of neutrophil migration across RSV infected airway epithelial cells

3.1 Introduction

In **Chapter 1**, I outlined the characteristic clinical findings during bronchiolitis, including the work of McNamara et al who demonstrated neutrophilic infiltration of the airways of infants hospitalised with severe RSV bronchiolitis, demonstrated by increased neutrophil cellularity on bronchoalveolar lavage (BAL)(214). The first logical question to further understand this observation, is ‘what causes neutrophils to infiltrate the airway?’ Neutrophils, classically, are the first recruited immune cells to arrive at a site of infection(120). Much research has gone into detailing the highly specialised methods of migration, chemotaxis and tissue infiltration employed by neutrophils traversing the epithelium. But what mediates this movement of neutrophils in RSV bronchiolitis? Neutrophil responses have been well documented in bacterial infection of the airway(215), but their response to viral infections are less well explored.

Recruitment of neutrophils to the virally infected lung is regulated by specific chemoattractant gradients produced by either the airway in response to detection of RSV, or by viral processes within infected epithelial cells, although relative contribution of either is difficult to determine. In the lungs of infants with RSV bronchiolitis it was shown that CXC chemokines, ones which preferentially attract neutrophils, were the most abundant, in particular CXCL10/interferon inducible protein 10 (IP-10) and CXCL8/interleukin 8 (IL-8)(79). CC chemokines, such as CCL2/monocyte chemotactic protein 1 and CCL3/macrophage inflammatory protein-1 alpha) which provoke a monocyte/macrophage infiltration, were also present in lesser quantities(79). After initial infection, there is increasing evidence that the inflammatory state of the airway is maintained by the sustained release of chemokines from immune cells. A study of ventilated infants with RSV bronchiolitis showed interleukin 9 (IL-9), a type 2 cytokine, was produced in large quantities in the BAL of children with RSV bronchiolitis and immunofluorescence staining showed the greatest expression of IL-9 was present in the infiltrating neutrophils (78). This is

particularly relevant for the long term respiratory health of infants with RSV bronchiolitis, as IL-9 has been implicated as a key cytokine in asthma susceptibility later on in life(216).

In **Chapter 2**, I described a novel model of transepithelial migration across RSV infected primary airway epithelial cells, and we previously published a paper using this model in 2020. In it, we showed an increase in CD11b expression on neutrophils which have travelled through RSV infected ciliated AECs (185).

An area which has not yet been studied is the movement of neutrophils themselves as they interact with the RSV infected airway. This is an impossible task in human trials and most animal models, and an exciting challenge for translational *in vitro* models of the airway epithelium, like the one I described in **Chapter 2**. Lammerman et al, used sophisticated two photon imaging techniques to track neutrophil 'swarming' towards a dying neutrophil during sterile injury of mouse ear, and showed this movement was mediated by leukotriene B₄(LTB₄) secretion by neutrophils(134). Similar sophisticated imaging techniques of neutrophils have been demonstrated in other animal models and suggested that neutrophils may respond to tissue damage in the absence of infection(217). These studies clearly demonstrate organisation in neutrophil movement, dictated by their environment, within damaged tissue. One difficulty encountered in translating these techniques to profile the kinetics of neutrophil response to RSV is the lack of relevant animal models. However, using a sophisticated *in vitro* model to explore the dynamics of neutrophil infection would be extremely valuable. In this chapter I will demonstrate the modification and use of the transepithelial migration model, described in **Chapter 2**, to investigate neutrophil interaction with RSV infected AECs.

3.2 Hypothesis

Neutrophil kinetics during transepithelial migration across primary airway epithelial cells is altered by RSV infection.

3.3 Aims

In this chapter I aim to

1. Establish a modified transepithelial migration model to allow imaging of neutrophil movement through RSV infected basal AECs
2. Quantify neutrophil migration across and adherence to RSV infected basal AECs in comparison to mock infected basal AECs.
3. Compare neutrophil migration across and adherence to RSV and mock infected basal AECs in comparison to ciliated AECs.
4. Assess neutrophil behaviour, spatially and temporally, in migration across and adherence to RSV infected basal AECs
5. Assess antiviral effects of neutrophils on RSV infected AECs

3.4 Chapter specific methods

The model used in this chapter consists of human airway epithelial cells (AECs) grown at an air liquid interface for 7 days, as described in methods **Section 2.7.2**, herein referred to as basal AECs. This creates a flatter culture system that is easier to image using 3D microscopy. All AECs used in this chapter are basal AECs with the exception of the comparative work in **Section 3.5.6-7** comparing neutrophil migration and adherence with ciliated AECs, matured at ALI for 28 days. Basal AEC cultures were infected with RSV 24 or 72 hours prior to the addition of neutrophils. Neutrophils, isolated from healthy adults' venous blood, were first stained with calcein red-orange for identification purposes as described in **Section 2.7.2.1**. Neutrophil transepithelial migration was performed as described in **Section 2.6.1**. Mock infected AECs with RSV infected AEC supernatant were used as a control (RSV Sup), as were mock infected AECs with fMLP 100nM placed apically as a positive control for neutrophil chemotaxis. Quantification of numbers of migrating neutrophils and adherent neutrophils were performed as described in **Section 2.6.4**. All microscopy experiments in this chapter save one (**Section 3.5.9.1**), were performed using an inverted Zeiss LSM 710 confocal microscope as described in **Section 2.7.2**. Fast time-lapse analysis of neutrophil migration through RSV infected AECs presented in **Section 3.5.9.1** was performed using a Zeiss Spinning disk microscope as described in **Section 2.7.2.2**. Adherent neutrophil distribution analysis was performed as described in **Section 2.6.6**. Analysis code from ImageJ and RStudio for this chapter are available in **Appendix 4-6**.

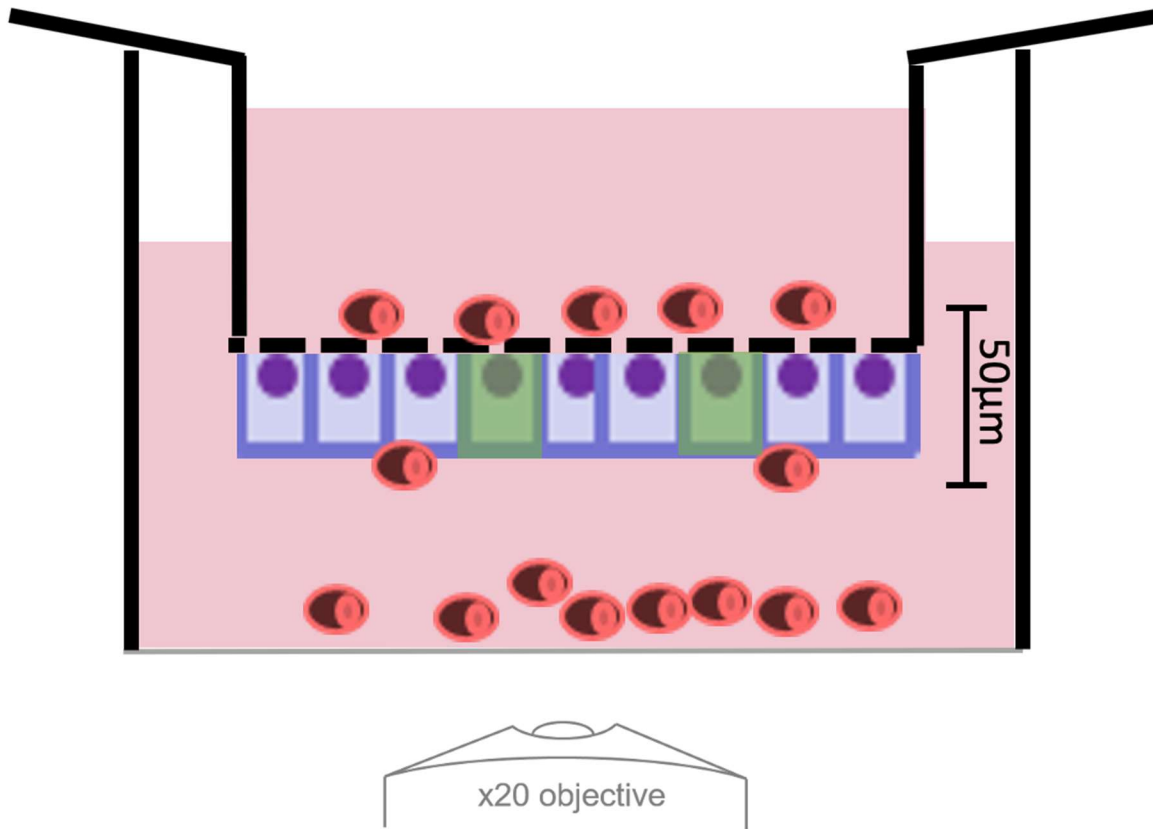


Figure 3-1 Schematic of transepithelial migration model as optimised for efficient microscopy.

Picture indicates a Thincert™ (Greiner) with 3µm pore transparent PET culture membrane (dashed line), sitting in the well of a black plastic 24 well plate with a coverslip bottom (Greiner). Human primary airway epithelial cells (AECs) (purple cells) are grown on the underside of the culture membrane for 7 days post ALI and infection is performed with RSV (green cells) 72 hours before addition of neutrophils. Scale bar indicates the range of image stacks taken and 50µm distance. Neutrophils (red) indicate the position of neutrophils after 1 hour of migration – basolateral (top), adherent to the epithelium (middle) and apical dissociated (bottom).

3.5 Results

3.5.1 Epithelial integrity is maintained in AEC cultures infected with RSV

To confirm basal AEC cultures did not lose their integrity once infected with RSV, trans-epithelial electrical resistance (TEER) measurements were performed prior to addition of neutrophils to the migration assay. These data are presented in **Figure 3-2**.

Healthy AEC cultures infected with RSV for 24 hours had an average (mean) TEER of $416 \Omega\text{cm}^2$ ($\pm 101 \Omega\text{cm}^2$) and those mock infected for 24 hours had average (mean) TEER of $398 \Omega\text{cm}^2$ ($\pm 115 \Omega\text{cm}^2$). Healthy AEC cultures infected with RSV for 72 hours had an average (mean) TEER of $442.7 \Omega\text{cm}^2$ ($\pm 149 \Omega\text{cm}^2$) and those mock infected for 72 hours had average (mean) TEER of $430 \Omega\text{cm}^2$ ($\pm 85.6 \Omega\text{cm}^2$). There was no significant difference in mean TEER between mock and RSV infected basal AEC cultures.

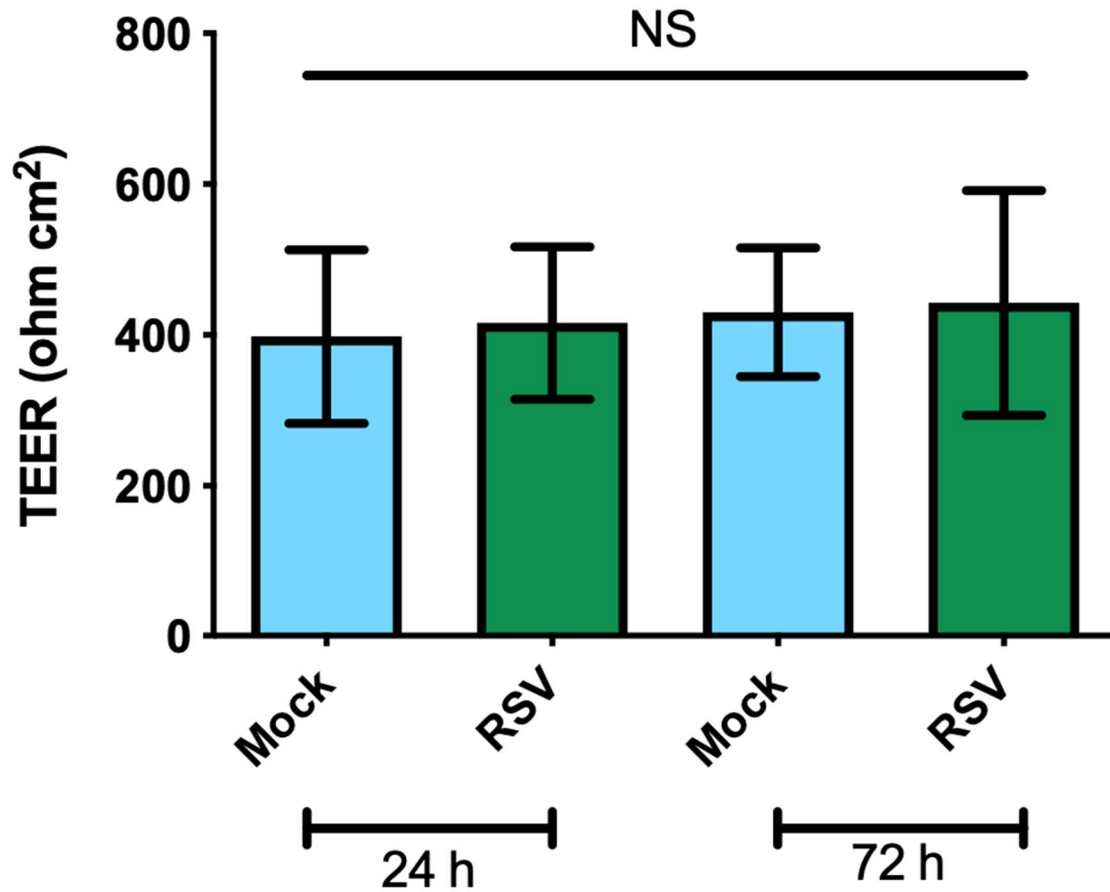


Figure 3-2 TEER of AEC cultures infected with RSV for 24 or 72 hours.

Trans-epithelial electrical resistance (TEER) of AECs cultured at ALI was measured with an EVOM² Epithelial Voltohmmeter voltmeter (World Precision Instruments, Sarasota, Florida, US) using two unequal length electrodes. Final TEER values were calculated by multiplying the ohmic resistance value given by the voltmeter by the surface area of the culture membrane. Comparisons between groups was performed using One-Way ANOVA with Tukeys adjustment for multiple comparisons (N=6).

3.5.2 Supernatant collected from RSV infected AEC cultures increases neutrophil migration through uninfected basal respiratory epithelium

After 1 hour incubation, I found no significant difference between the number of viable migrated neutrophils recovered from the apical side of AEC cultures infected with RSV for 24 hours an average of (mean \pm SEM) (22,876 cells/well \pm 12713), which was 4.4% of the total added, compared to the relative mock infected AEC cultures (19,184 cells/well \pm 10806) or 3.8% of total. There was also no significant difference after 4 hours incubation, with viable neutrophil counts measured at 174,382 cells/well \pm 39445(34.9%) in RSV infected AEC cultures and 162,105 cells/well \pm 37137 (32.4%) in the mock infected.

However, I did recover significantly greater numbers of viable neutrophils from mock infected AEC cultures exposed to supernatant collected from AEC cultures infected with RSV for 24 hours (RSV Sup only), with an average 70,016 cells/well \pm 21115 compared to the mock with 19,184 cells/well \pm 10806 (p value of 0.006) and in comparison to RSV with 22,876 cells/well \pm 12713 (p value of 0.0058) after 1 hour incubation.

Allowing the RSV infection to progress to 72 hours, there was no significant difference in number of viable neutrophils recovered from the RSV infected AEC cultures (40,415 cells/well \pm 15143) compared to the mock (59,900 cells/well \pm 18885) after 1 hour incubation or after 4 hours incubation, with neutrophil counts at 4 hours measured at 177,507 cells/well \pm 49480 on the apical side, compared to the mock at 222,558 cells/well \pm 41274.

Comparing between 24 hour and 72 hour infected AEC cultures, increased numbers of viable neutrophils recovered from the RSV Sup only AEC cultures after 4 hours incubation from 211,133 cells/well \pm 46,690 (4.2% of total) at 24 hours post RSV infection to 307,208 cells/well \pm 32,818 (61.4%) at 72 hours post RSV infection. This difference was significant with a p value of 0.0034.

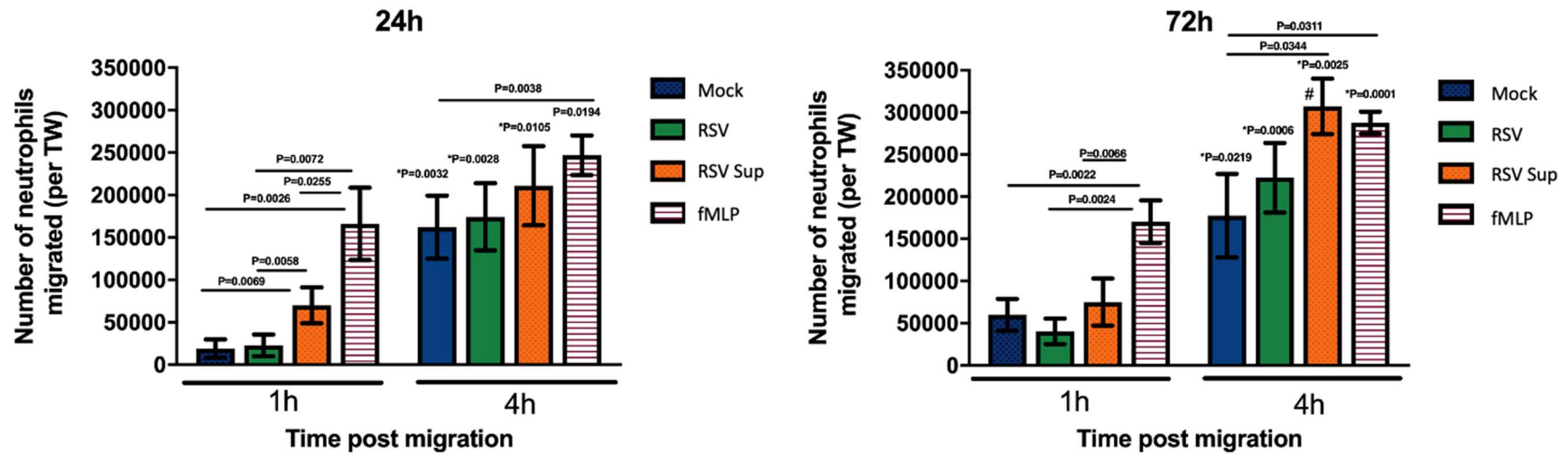


Figure 3-3 Numbers of viable neutrophils recovered from the apical side of basal AEC cultures infected with RSV.

Viable neutrophil counts were calculated by fluorescence intensity from a standard curve of known values. Data shown from AEC cultures infected with RSV, or mock media only (control) at 24 hours (left) or 72 hours (right). RSV SUP denotes mock infected epithelium with supernatant recovered from an RSV infected culture placed on the apical side. fMLP 100nM was used as a positive control for chemotaxis. Statistical analysis was performed with Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons

, where statistical significance was found p values are shown on the figure. * indicated significance in comparison to the 1 hour equivalent measure. # indicated significant in comparison to the equivalent measure from 24 hour infected epithelium studies. N=15.

3.5.3 RSV infection increases neutrophil adherence to basal AECs

After counting numbers of neutrophils migrating across RSV infected AEC cultures, I then wanted to investigate numbers adherent to AECs after migration. I chose to do this after 1 hour of transepithelial migration as previous studies and my own work have shown the loss of epithelial integrity at the 4 hour time point(185). The numbers of neutrophils adherent to mock and RSV infected AEC cultures are shown in **Figure 3-4**. Representative images of fixed and stained AEC cultures are shown in **Figure 3-5**.

I found that, after 1 hour incubation, there were significantly more neutrophils adherent to the AEC cultures infected with RSV (791.1 ± 106.8 cells/cm²) for 24 hours in comparison to the respective mock infected AEC cultures (449.8 ± 81.82 cells/cm²) after 24 hours with a p value of 0.0135. Similarly, AEC cultures infected with RSV for 72 hours showed significantly greater numbers adherent (711.1 ± 74.3 cells/cm²) compared to the respective mock control (486.6 ± 61.5 cells/cm²), with a p value of 0.0152. In addition to this, significantly fewer neutrophils remained adherent to the RSV Sup (462.7 ± 43.3 cells/cm²) (mock infected AECs with 72 hour RSV infected AEC supernatant underneath) cultures in comparison to the 72 hour RSV infected (711.1 ± 74.3 cells/cm²) with a p value of 0.0056. Allowing the RSV infection to progress to 72 hours did not lead to significant change in the numbers of adherent neutrophils (p=0.257).

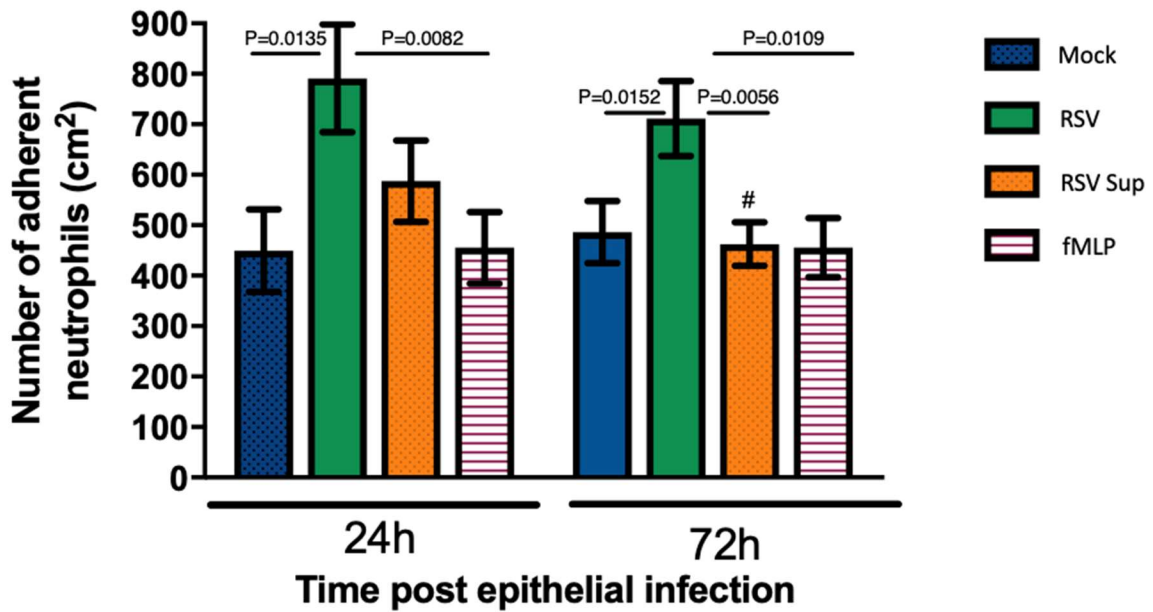


Figure 3-4 Neutrophils adherent to apical surface of basal AEC cultures infected with RSV.

Data shown from AECs infected with RSV, or mock media only control for 24hours (left) or 72 hours (right). RSV Sup denotes mock infected epithelium with supernatant recovered from an RSV infected AEC culture placed on the apical side. fMLP 100nM was used as a positive control for chemotaxis. Statistical analysis was performed with Individual comparisons performed with Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons; where statistical significance was found P values are shown on the figure. # indicates significant in comparison to the equivalent measure from 24hour infected epithelium studies. N=15

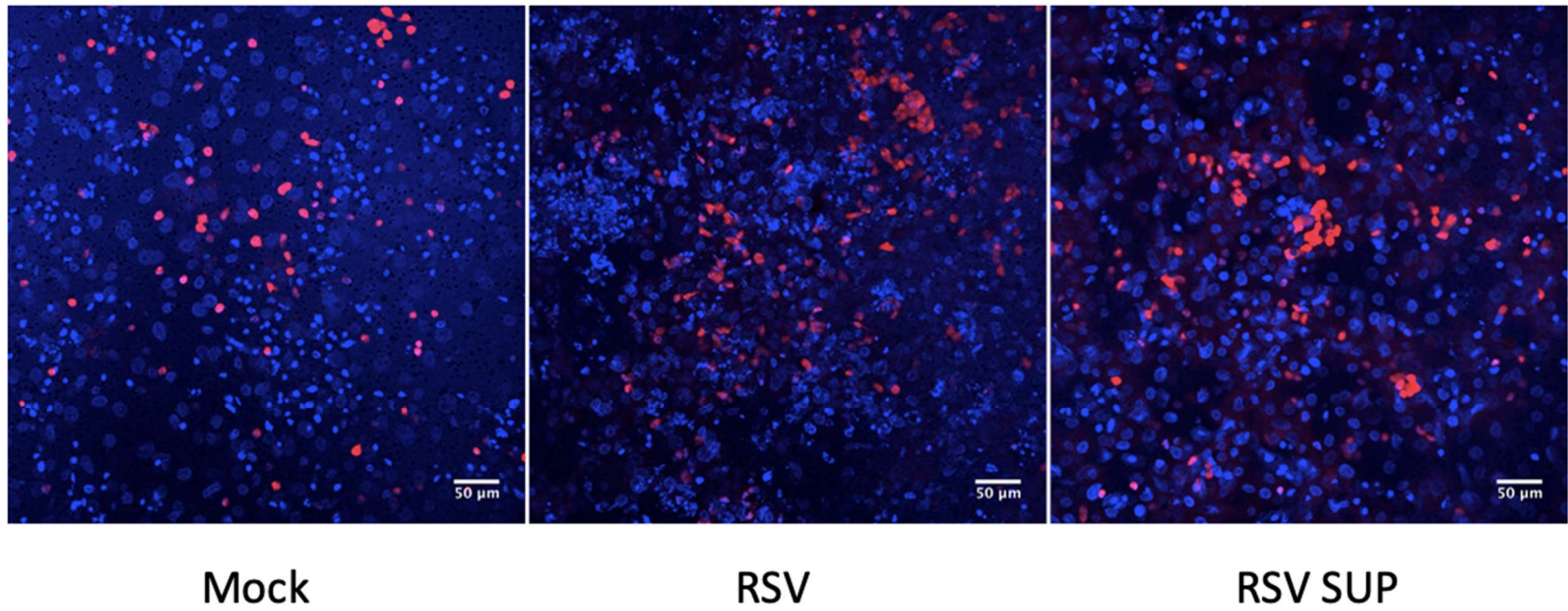


Figure 3-5 Representative images of basal AEC cultures after 1 hour of neutrophil migration

AECs were stained with Hoechst stain for nucleus (blue) and neutrophils stained with calcein red orange (pink). Images shown from AECs infected with RSV, or mock media only control for 72 hours (right). RSV Sup denotes mock infected AECs with supernatant recovered from an RSV infected culture placed on the apical side.

3.5.4 Greater numbers of neutrophils migrate through RSV infected ciliated AECs in comparison to mock infected AECs

To compare the numbers of neutrophils migrating through RSV infected ciliated AECs, neutrophils found on the apical side of transepithelial migration assay AEC cultures after 1 hours migration were quantified using fluorescence and a standard curve. I did not perform migration counts after 4 hours migration as in my basal AEC experiments and in published literature I saw formation of holes in the epithelium which may influence whether what I observe is true migration or simply the effect of gravity. RSV SUP denotes mock infected ciliated AECs with supernatant from RSV infected AECs placed apically. fMLP denotes mock infected AECs with 1ng/ml fMLP in HBBS placed apically. Data is presented as mean (\pm SEM)

After 1 hour migration through 24 hour infected AECs, 18930 (\pm 3107) neutrophils were recovered apical to mock infected AECs, 41099 (\pm 10248) recovered from RSV infected AECs, 39031 (\pm 4557) from fMLP AECs and 25255 (\pm 7311) from RSV SUP AECs. There was a significant difference between numbers of neutrophils recovered from the RSV infected AECs (41099 \pm 10248) compared to the mock infected AECs (18930 \pm 3107) with a p value of 0.0356. There was a significant difference between numbers of neutrophils on the apical side of mock infected AECs (18930 \pm 3107) in comparison to the fMLP AECs (39031 \pm 4557) with a p value of 0.0043.

In 72 hour infected AECs, 23881 (\pm 5282) neutrophils were recovered apical to mock infected AECs, 57026 (\pm 12317) recovered from RSV infected AECs, 40365 (\pm 9034) from fMLP AECs and 33150 (\pm 8135) from RSV SUP AECs. There was a significant difference between number of neutrophils recovered from the apical surface of the RSV infected AEC cultures (57026 \pm 12317) compared to the mock (23881 \pm 5282) with a p value of 0.0334.

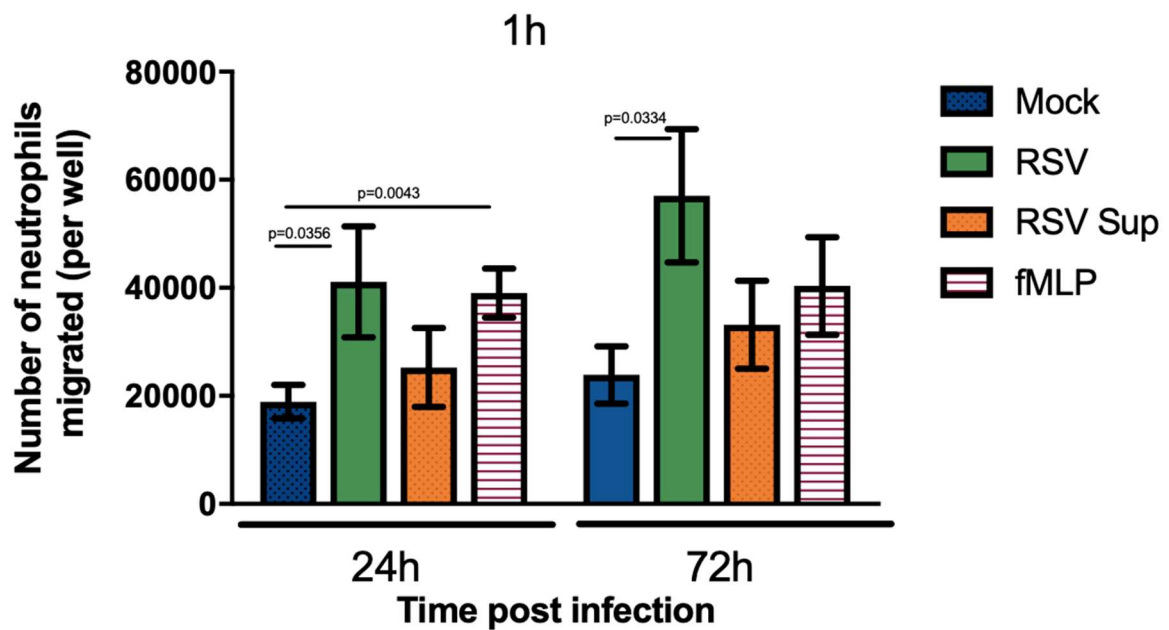


Figure 3-6 Numbers of neutrophils recovered from the apical side of ciliated AEC cultures infected with RSV.

Count numbers were calculated by fluorescence intensity measured off a standard curve. Data shown from AEC cultures infected with RSV, or mock media only control for 24hours (left) or 72 hours (right). RSV SUP denotes mock infected AECs with supernatant recovered from an RSV infected culture placed on the apical side. fMLP 100nM was used as a positive control for chemotaxis. Individual comparisons performed with Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons, where statistical significance was found p values are shown on the figure. N=12.

3.5.5 RSV infection increases neutrophil adherence to ciliated AECs

After examining the numbers of neutrophils found apically, I then wanted to quantify neutrophils remaining adherent to ciliated AECs after transepithelial migration. Neutrophils adherent to mock, RSV and RSV SUP cultures are shown in **Figure 3-7**. Representative confocal images of fixed and stained AEC cultures are presented in **Figure 3-8**.

After 1 hour migration through 24 hour infected AECs, there were 671 cells/cm² (± 115) adherent to mock infected AECs, 1710 cells/cm² (± 157) adherent to RSV infected AECs, 310 cells/cm² (± 130) adherent to fMLP cultures and 1170 cells/cm² (± 155) adherent to RSV Sup AECs. There were significantly more neutrophils remaining adherent to the 24 hour RSV infected AECs (1710 cells/cm² ± 157) in comparison to the 24 hour mock infected AECs (671 cells/cm² ± 15) with a p value of <0.0001 .

After migration through 72 hour infected AECs, there were 875 cells/cm² (± 109) adherent to mock infected AECs, 2638 cells/cm² (± 275) adherent to RSV infected AECs, 555 cells/cm² (± 230) adherent to fMLP cultures and 1059 cells/cm² (± 116) adherent to RSV Sup AECs. There were significantly greater numbers adhered to the 72 hour infected RSV AECs (2638 cells/cm² ± 275) than the mock (875 cells/cm² ± 109), with a p value of <0.0001 .

Significantly fewer neutrophils remained adhered to the 24 hour RSV Sup AECs (1059 cells/cm² ± 116), in comparison to the 24 hour RSV infected AECs (1710 cells/cm² ± 157) with a p value of 0.0097. In addition to this, significantly fewer neutrophils remained adhered to the 72 hour infected RSV Sup AECs (1059 cells/cm² ± 116), in comparison to the 72 hour RSV infected (2638 cells/cm² ± 275) with a p value of <0.0001 .

When comparing the numbers of neutrophils adherent to AECs infected with RSV for 24 and 72 hours, there were significantly more neutrophils adherent to the 72 hour infected AECs (2638 cells/cm² ± 275) than the 24 hour infected AECs (1710 cells/cm² ± 157) with a p value of 0.00184.

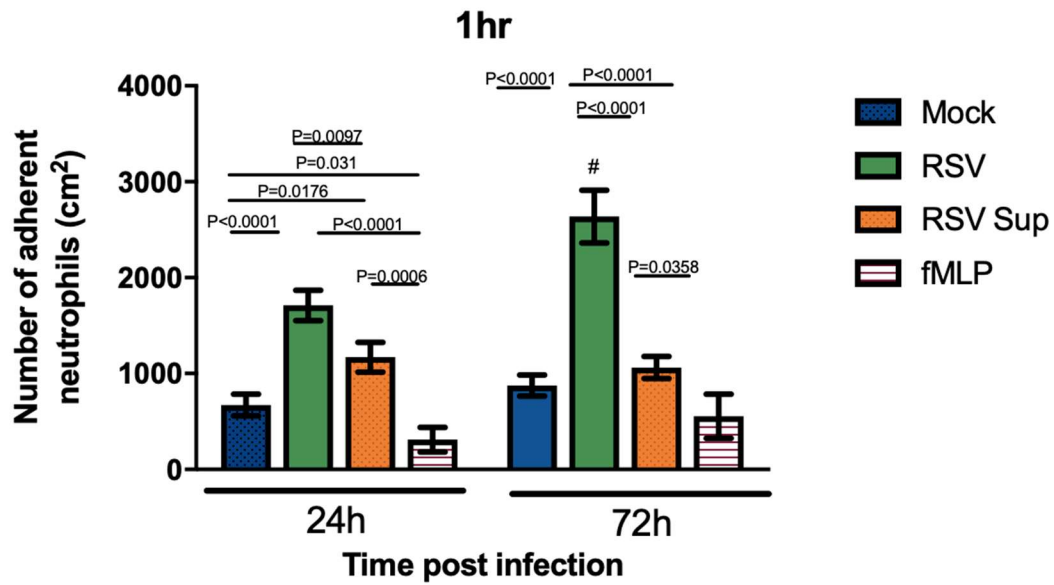


Figure 3-7 Neutrophils adherent to apical surface of ciliated epithelial AECs infected with RSV.

Data shown from AECs infected with RSV, or mock media only control for 24 hours (left) or 72 hours (right). RSV Sup demotes mock infected AECs with supernatant recovered from an RSV infected AEC culture placed on the apical side. fMLP 100nM was used as a positive control for chemotaxis. Individual comparisons performed with Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons, where statistical significance was found p values are shown on the figure. # indicated significant in comparison to the equivalent measure from 24 hour infected AEC studies. N=12.

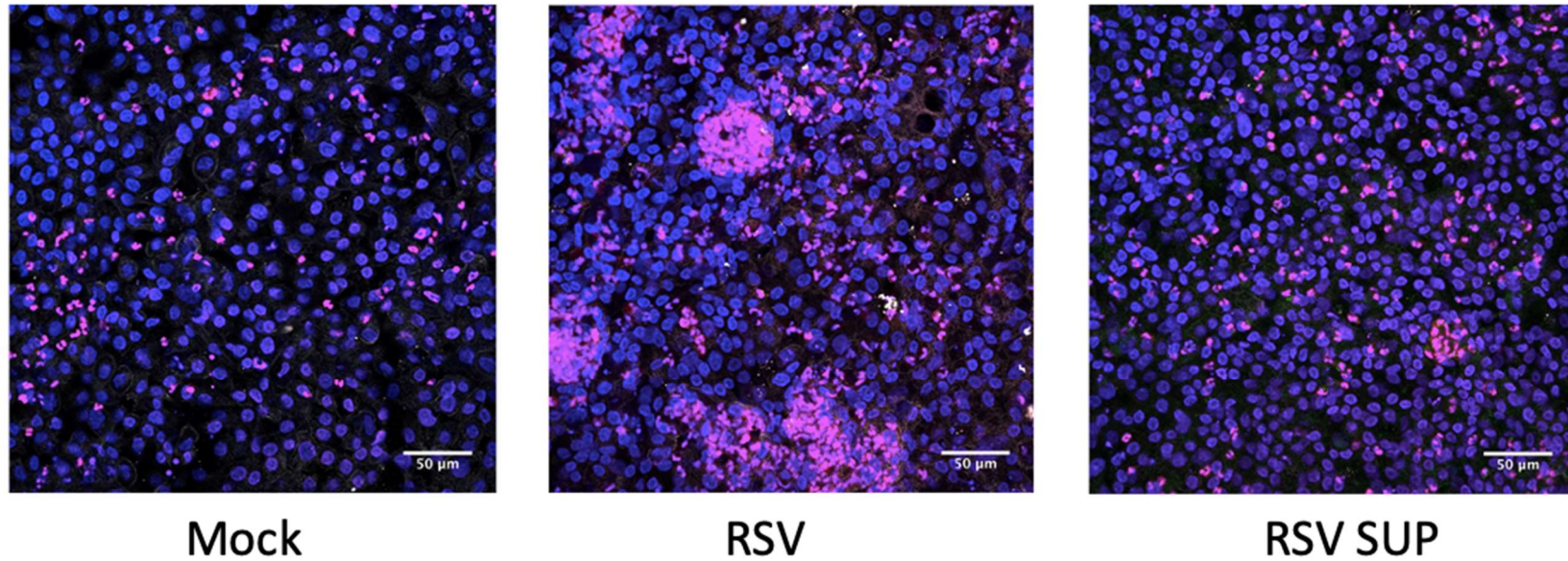


Figure 3-8 Representative images of ciliated epithelial AECs after 1 hour of neutrophil migration

AEC cultures were stained with Hoechst stain for nuclei (blue) and neutrophils stained with calcein red orange (pink). Data shown from AEC cultures infected with RSV, or mock media only control for 72 hours. RSV SUP denotes mock infected epithelium with supernatant recovered from RSV infected AEC culture placed on the apical side.

3.5.6 Similar numbers of neutrophils migrate through basal AECs in comparison to ciliated AECs

To determine any differences in apical migration numbers of neutrophils during transepithelial migration across basal and ciliated AECs, I performed comparative analysis between the two datasets using Two Way ANOVA with Greenhouse Geisser post hoc test for multiple comparisons. These data are presented in **Figure 3-9**. Descriptive statistics and comparative analyses of neutrophil numbers within different infection conditions of basal and AEC cultures have been described previously in the preceding sections.

Examining neutrophils found apically to RSV infected AECs, there was no significant difference in number of neutrophils found on the apical side of 24 hour mock, RSV or RSV Sup infected basal or ciliated AECs. There were significantly greater numbers of neutrophils recovered on the apical side of 24 hour mock infected basal AECs with 1ng/ml fMLP placed apically (39031 ± 4557) (fMLP) in comparison to 24 hour mock infected ciliated AECs with 1ng/ml fMLP placed apically (166116 ± 42635) (fMLP) with a p value of $p=0.0004$.

I also found no significant difference in number of neutrophils found on the apical side of 72 hour mock, RSV or RSV Sup infected basal or ciliated AECs. Like at 24 hours, there were significantly greater numbers of neutrophils recovered on the apical side of 72 hour mock infected basal AECs with 1ng/ml fMLP placed apically (40365 ± 9034) (fMLP) in comparison to 72 hour mock infected ciliated AECs with 1ng/ml fMLP placed apically (170454 ± 25212) (fMLP) with a p value of $p=0.0003$.

3.5.7 Greater numbers of neutrophils adhere to RSV infected ciliated AECs in comparison to RSV infected basal AECs

Comparing neutrophils remaining adhered to basal and ciliated AECs after 1 hours migration, there was no significant difference in number of neutrophils found adhered to 24 hour mock, RSV Sup or fMLP infected basal or ciliated AECs. There were significantly fewer numbers of neutrophils adhered to 24 hour RSV infected basal AECs ($791 \text{ cells/cm}^2 \pm 107$) in comparison to 24 hour RSV infected ciliated AECs ($1710 \text{ cells/cm}^2 \pm 157$) with a p value of $p=0.0002$. I also found no significant difference between number of neutrophils found adhered to 72 hour mock, RSV Sup or fMLP infected basal or ciliated AECs. However, there were significantly fewer numbers of neutrophils adhered to 72 hour RSV infected basal AECs ($711 \text{ cells/cm}^2 \pm 74.3$) in comparison to 72 hour RSV infected ciliated AECs ($2638 \text{ cells/cm}^2 \pm 275$) with a p value of $p<0.0001$.

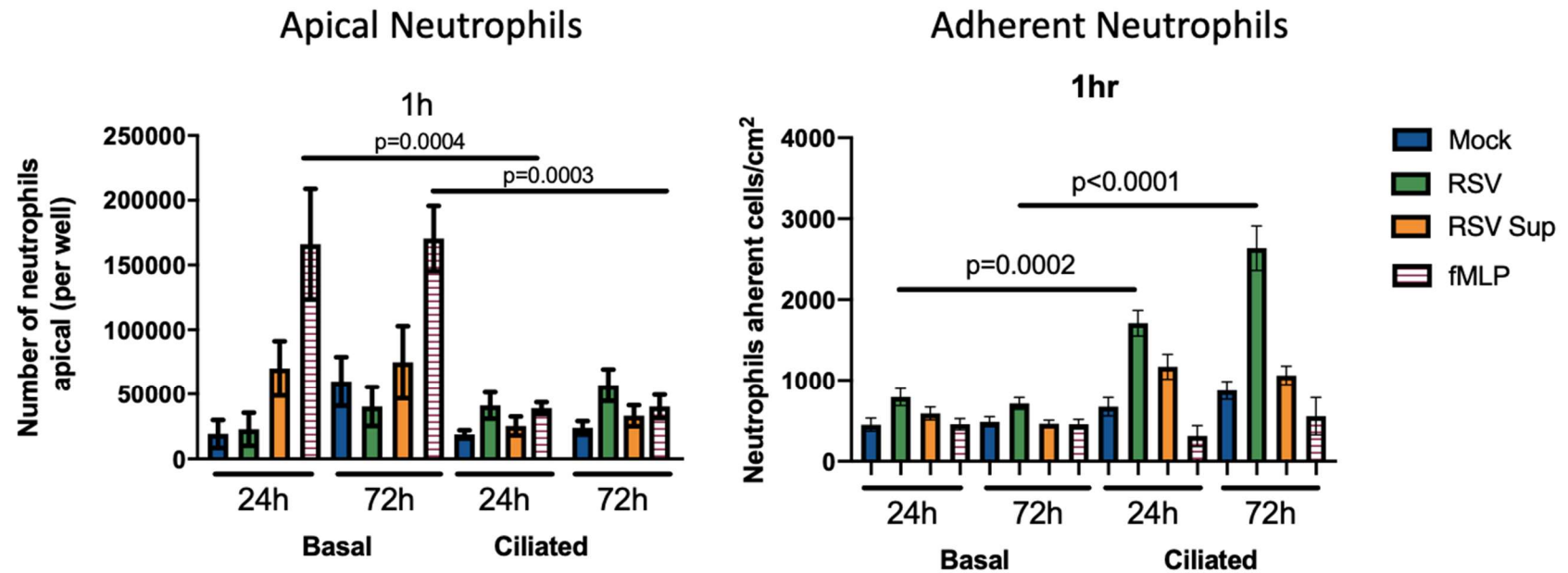


Figure 3-9 Neutrophil numbers present on the apical side of and adherent to basal AECs and ciliated AECs

Data shown from AEC cultures infected with RSV, or mock media only control for 24hours (left) or 72 hours (right). RSV SUP denotes mock infected epithelium with supernatant recovered from an RSV infected culture placed on the apical side. fMLP 100nM was used as a positive control for chemotaxis. Statistical analysis performed with Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons, where statistical significance was found p values are shown on the figure.

(Left) Count numbers were calculated by fluorescence intensity measured off a standard curve. (Right) Adhered neutrophils were counted from confocal images of fixed and stained AECs using an Image J macro. N=12-15.

3.5.8 Neutrophils adherent to the RSV infected AECs are more likely to be 'clustered' compared to those adherent to mock infected AECs

After comparing numbers of neutrophil migrating across and adhering to ciliated and basal AECs, the remainder of this chapter will be utilising basal AECs. As I have shown that more neutrophils adhere to RSV infected basal epithelium compared to mock infected cells, I then wanted to investigate whether the distribution of these neutrophils was uniform (null hypothesis =0) or whether they clustered in particular areas.

I found that after 1 hour the median distance from an adherent neutrophil to the nearest neighbouring neutrophils was significantly shorter in RSV infected AEC cultures (median \pm IQR) ($61.32\mu\text{m} \pm 30.9$) in comparison to neutrophils adherent to the mock infected AECs ($119.4\mu\text{m} \pm 51.6$). This difference was significant when compared using a Mann Whitney U test with Wilcoxon rank with a p value of <0.0001 .

As anticipated, I found expected median distance from an adherent neutrophil to its nearest neighbours assuming even distribution, was shorter in RSV infected AEC cultures ($140\mu\text{m} \pm 24.3$) in comparison to neutrophils adherent to the mock infected epithelium ($169.4\mu\text{m} \pm 57.8$). Comparing the observed distances between neutrophils adherent to RSV infected AECs ($61.32\mu\text{m} \pm 30.9$) and the expected distance if they were evenly distributed ($140\mu\text{m} \pm 24.3$), I found a significant difference using a Mann Whitney U test with Wilcoxon rank with a p value of <0.0001 . Similarly, comparing the observed distances between neutrophils adherent to mock infected AECs ($119.4\mu\text{m} \pm 51.6$) and the expected distance if they were evenly distributed ($169.4\mu\text{m} \pm 57.8$), I found a significant difference using a Mann Whitney U test with Wilcoxon rank with a p value of 0.004.

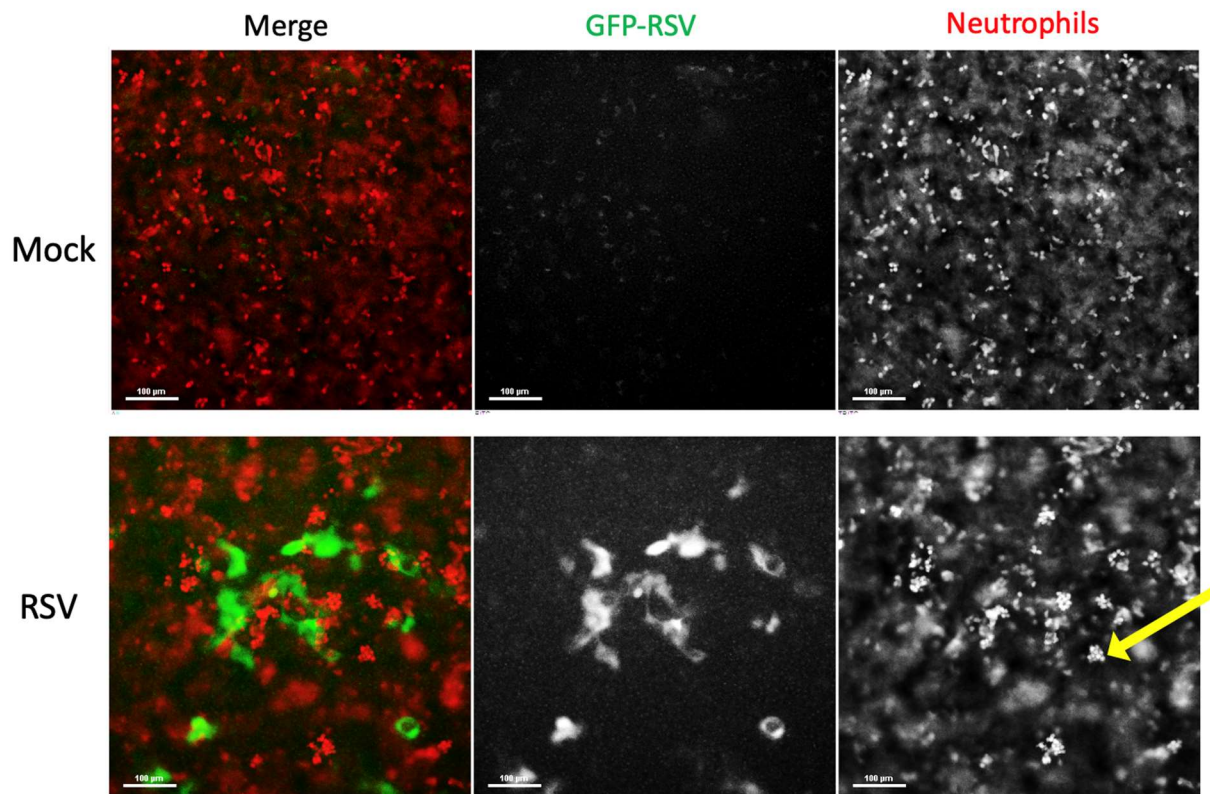


Figure 3-10 Neutrophils adhere to RSV infected AECs in clusters.

Representative 2 channel image of 72 hour mock (top) and RSV (bottom) infected AECs after incubation with neutrophils for 1 hour. Arrow indicated observed 'cluster' formation of neutrophils.

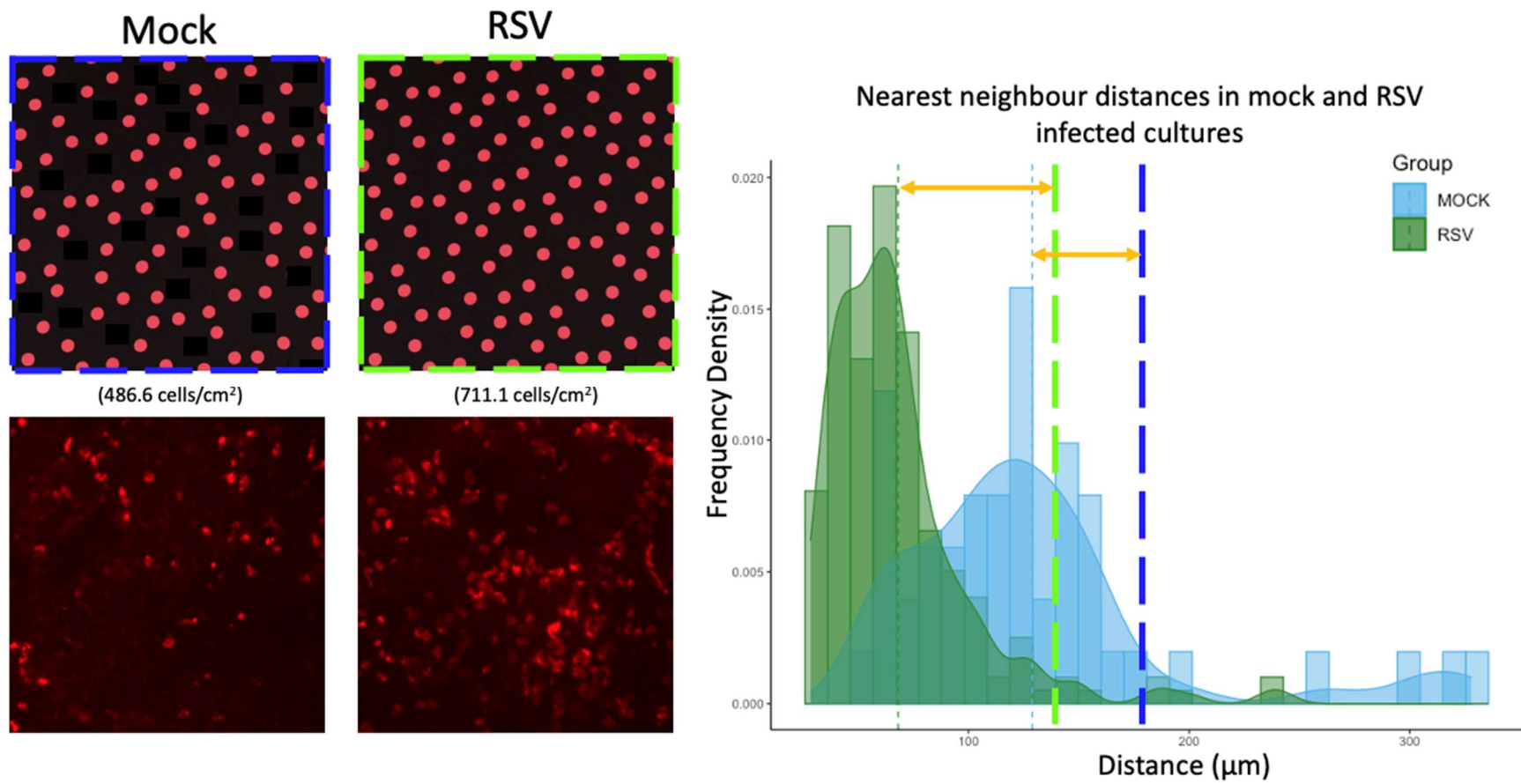


Figure 3-11 Nearest Neighbour analysis of the distribution of adherent neutrophils to the RSV and Mock Infected basal AECs after 1 hours migration.

Top left – diagram to illustrate calculation of even distribution of neutrophils adherent to mock and RSV infected AECs based on adherence counts data presented in **Figure 3-4**.

Bottom left - Cultured AECs were fixed and stained after 1 hour neutrophil migration, red channel extracted for clarity and to determine neutrophil position. Red = Neutrophils stained with calcein red orange. Images were processed using Image J and neutrophil position coordinates analysed using RStudio

(Right) Histogram to show distance to nearest neighbouring neutrophil calculated from neutrophils adherent to the RSV or mock infected AECs after 1 hours migration. Thin dashed lines show median distance to nearest neighbour in neutrophils adherent to mock (blue) and RSV (green) infected AECs. Fat dashed lines show median distance to nearest neighbour if distribution was assumed to be even based on numbers of neutrophils counted adherent to mock (blue) and RSV (green) infected AECs. Difference between the observed median distance (thin line) and the expected distance if evenly distributed (fat lines) for mock (blue) and RSV (green) is indicated with double headed yellow arrows. Images were processed using Image J and neutrophil position coordinates analysed using RStudio. Median distances were compared using a Mann Whitney U test with Wilcoxon rank. N=5

3.5.9 Spatial and temporal analysis of neutrophil trans-epithelial migration

3.5.9.1 Neutrophil clustering in fast time-lapse appears as swarming pattern.

To investigate the kinetics of clustering observed in **Figure 3-7**, a modified transepithelial migration assay was performed as described in **Section 2.7.2.1** using a Zeiss spinning disk CSU22 system. In order to maintain 50 μ m Z-range and accommodate fast time-lapse, it was not possible to also image a mock control for this experiment and so this data is purely observational. Time lapse images of neutrophil and accompanying analysis of nearest neighbour measurement is available in **Appendix 8**, Video 2 on the attached USB stick. At each time point, a Mann Whitney U test with Wilcoxon test was performed to compare the distances to nearest neighbour observed, to the expected values calculated assuming even distribution. After 10 minutes of migration the observed median difference between neutrophils and their nearest neighbours was 102.9 μ m \pm 55.8 (Median \pm IQR), and the expected value was (140 μ m \pm 24.3). This difference was significant with a p value of 0.0008.

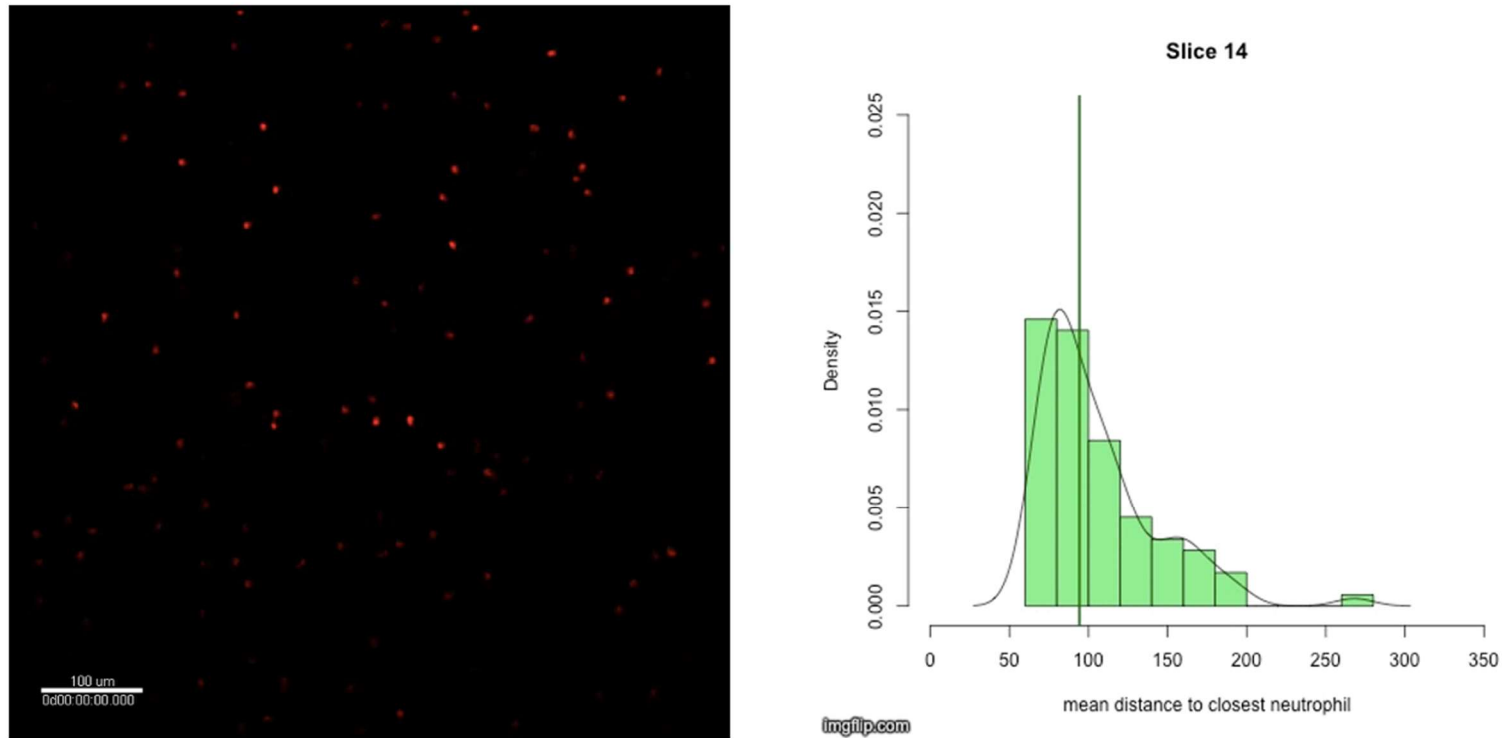


Figure 3-12 Fast time lapse of neutrophil cluster formation and nearest neighbour analysis over time

(Left) video of neutrophil cluster formation over time for 1 hour, red = neutrophils stained with calcein red orange.

(Right) Sequence of histograms produced from measured distances from neutrophils to their nearest neighbours, vertical line indicates the median value at each time point.

A z-stack image was captured every 45seconds. Videos of this figure are provided in **Appendix 8**, Video 2 the accompanying USB stick.

3.5.10 Neutrophils move faster and further when migrating through the mock infected epithelium compared to the RSV.

To examine neutrophil movement during transepithelial migration across RSV infected AECs in comparison to Mock infected, a protocol enabling imaging both Mock and RSV AEC cultures simultaneously was developed using a Zeiss LSM710 microscope. This procedure is described in **Section 2.7.2** alongside details of analysis technique. Analysis code is available in **Appendix 6**. Videos 3 (Mock) and 4 (RSV) are available on the accompanying USB stick for visualisation purposes (See **Appendix 8**).

Over the 1 hour, neutrophils' mean speed when moving through RSV infected AECs was less ($2.28\mu\text{m}/\text{sec} \pm 0.008$) than the mean speed of neutrophils moving through the mock infected AECs ($4.18\mu\text{m}/\text{sec} \pm 0.14$) with a p value of $p < 0.0001$.

There was no significant difference in linearity, or 'track straightness' between neutrophils observed migrating through RSV infected epithelium or the mock.

Neutrophils moving through mock infected AECs showed greater total displacement ($137.19\mu\text{m} \pm 4.51$) in contrast to those moving through RSV infected AECs ($107.053\mu\text{m} \pm 4.86$) which was significant with a $p < 0.0001$.

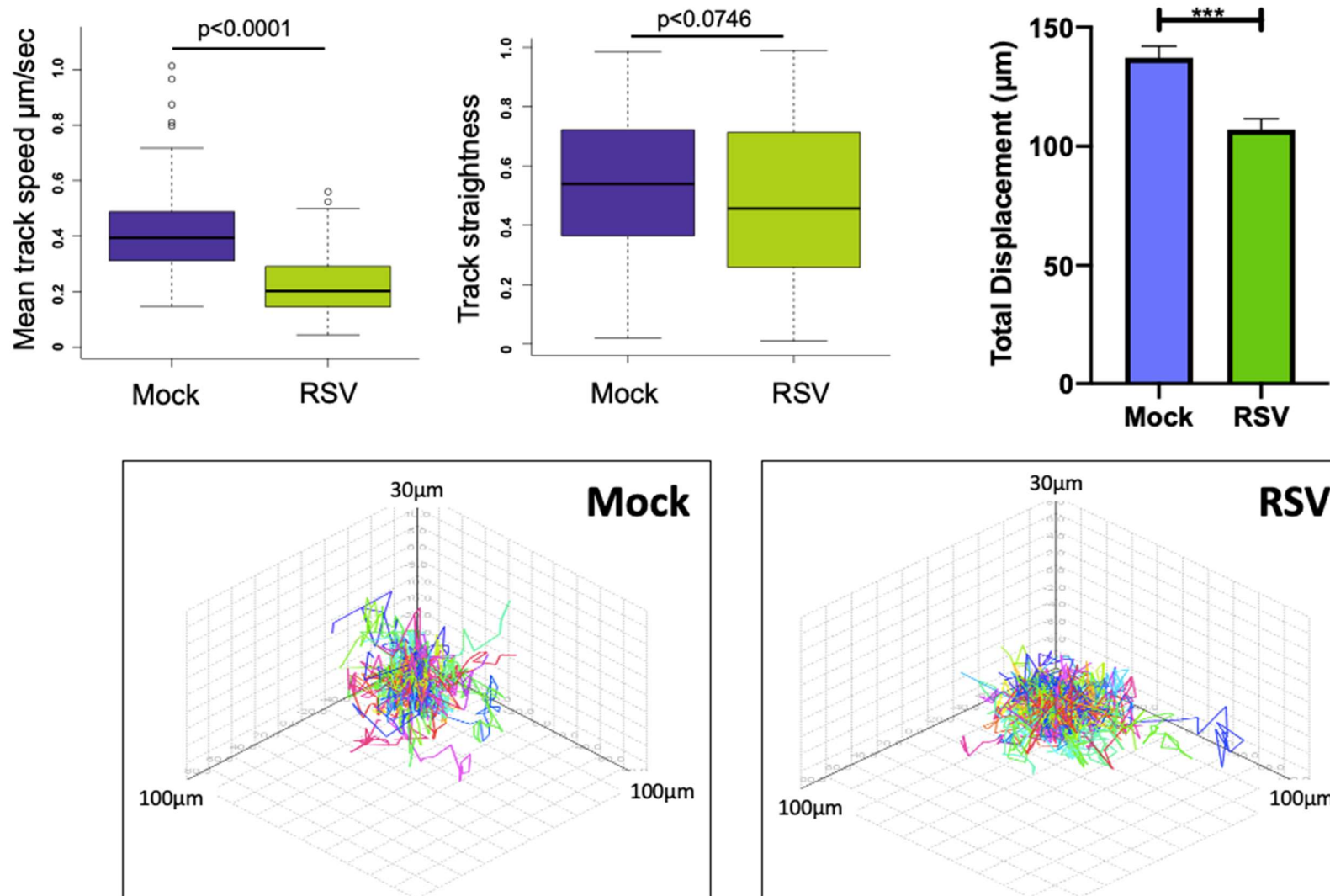


Figure 3-13 Analysis of neutrophil movement during migration through RSV infected basal AECs

Top left - Box and whisker plot to compare mean speed of neutrophils during transepithelial migration assay through the mock (blue) and RSV (green) infected basal AECs. Top middle - Box and whisker plot to compare track straightness (linearity) of neutrophils while migrating through the mock (blue) and RSV (green) infected basal AECs. Top right - Bar chart to compare total displacement of neutrophils after 1 hours migration through the mock (blue) and RSV (green) infected basal AECs. Bottom – origin plots for all tracks measured in Mock (left) and RSV infected (right) basal AEC cultures, describing relative movement in X, Y and Z planes. Statistical analysis between groups were performed using a Mann Whitney U test with Wilcoxon rank, where significance was found this is indicated on the chart. *** $p < 0.0001$. N=6.

3.5.10.1 Bidirectional neutrophil transepithelial migration

Examining the movement of neutrophils in the Z axis, the possibility that neutrophils may traverse the membrane in two directions was investigated. Individual tracks from earlier analysis were individually examined and some are shown in **Figure 3-14**.

Figure 3-14 shows the z coordinate for three example neutrophil tracks selected from neutrophils moving through RSV infected epithelium. Almost all tracks visualised during this analysis resembled track 1 (purple) in their Z coordinates, an initial descent and then small ups and downs in the region of the epithelium. A small number behaved more like track 2 (green), largely similar to track 1, but with spikes of detection above the culture membrane, which could suggest presence in the basolateral chamber. Likewise, a small number resemble track 3 (orange), whereby the z coordinates indicate prolonged positioning above the culture membrane.

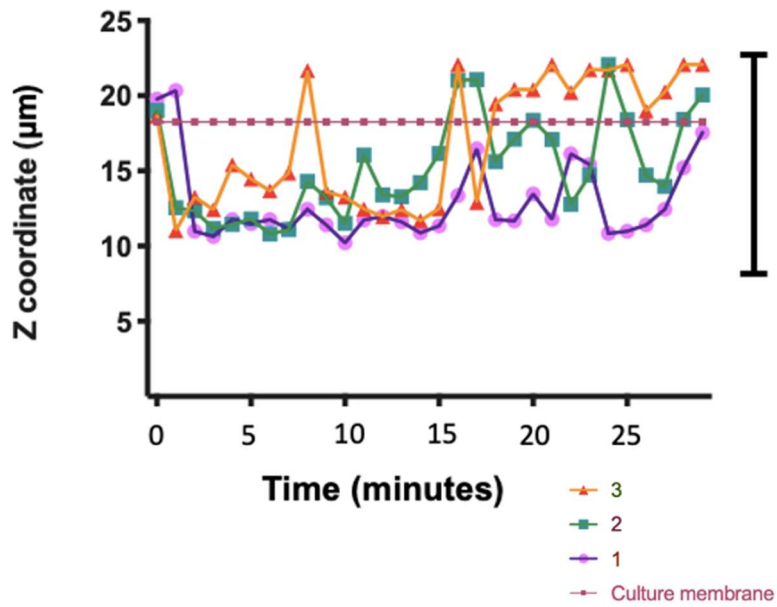


Figure 3-14 Individual track trajectories in the z-axis of neutrophils migrating through RSV infected basal AECs.

Line graph detailing the z position (x axis) over time (y axis) of three selected tracks discovered in dataset described in **Figure 3-8**.

3.5.11 Neutrophil transepithelial migration causes destruction of RSV infected AECs

Having examined the effect on neutrophils of transepithelial migration across RSV infected basal AECs, I then wanted to observe the effect of neutrophil migration on RSV infected AECs using time lapse microscopy.

As shown in **Figure 3-11**, as time progresses and neutrophil transepithelial migration continues, an AEC expressing GFP RSV disappears from the image, indicated by a yellow circle. (This is observable in Video 4 available in **Appendix 8** and the attached USB stick). This phenomenon was observed in multiple migration experiments.

3.5.11.1 Neutrophil trans-epithelial migration across RSV infected ciliated AECs reduces the infectious viral load

As part of a previous study to assess the influence of migration on viral load, RSV infected AECs, infected for 72 hours, and apical media were collected 4 hours after neutrophil transepithelial migration then infectious viral titre determined by plaque assay as described in **Section 2.2.1**. RSV infected AECs through which neutrophils had been added exhibited significantly lower viral titre of 1.6×10^4 pfu/ml, compared to 4.4×10^5 pfu/ml in RSV infected AECs without neutrophil migration with a p value of 0.026. This was a mean \pm SEM difference in viral titre of $-2.7 \times 10^4 \pm 1.2 \times 10^4$ pfu/ml.

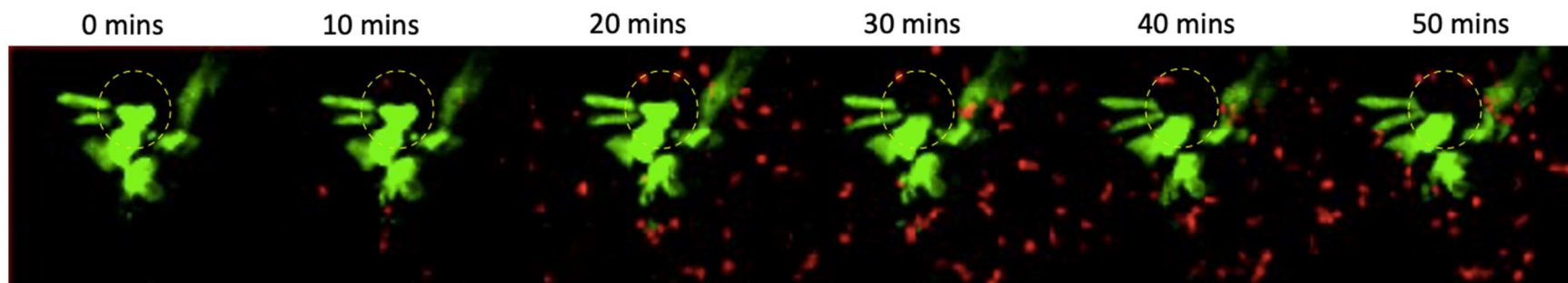


Figure 3-15 Disappearance of RSV infected AEC during neutrophil migration through RSV infected basal AECs.

Representative stills of a time lapse stack detailing neutrophil migration through RSV infected basal AECs. Green indicates RSV infected cells expressing GFP and red indicates neutrophils stained with calcein red orange. Yellow dashed circle shows position of disappearing epithelial cell.

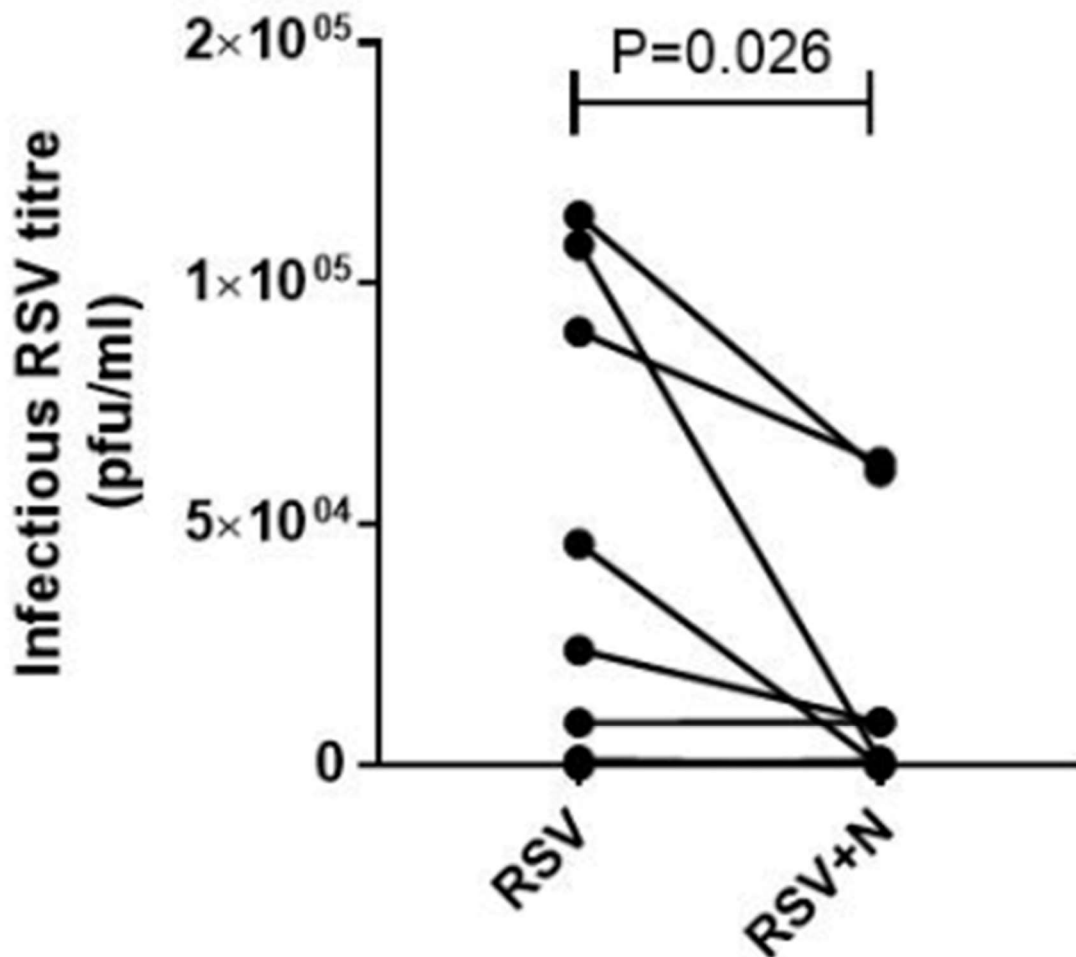


Figure 3-16 Infectious RSV titre determined by plaque assay after 4 hours neutrophil transepithelial migration

AECs infected with RSV for 72 hours and apical supernatants were lysed after 4 hours transepithelial migration assay (RSV +N) and infectious viral titre assessed by plaque assay. RSV infected AECs with no neutrophils added were used as a control (RSV). Viral titre was compared using a paired students two tailed t-test and probability value is shown on the chart. This figure has been previously published in the European Respiratory Journal (185). Statistical comparison was performed using a paired two tailed students t-test. N=7.

3.5.11.2 RSV infection of basal epithelial cells increases concentration of IL-8 and IP-10 secreted apically

IL-8 and IP-10 was measured as described in **Section 2.8**. IL-8 concentration measured in supernatant from mock infected AEC cultures infected for 24 hours was 2528 pg/ml \pm 543 and 4010 pg/ml \pm 1217 in supernatant from RSV infected AEC cultures infected for 24 hours. There was no significant difference in IL-8 concentration between supernatants recovered from Mock or RSV AEC cultures infected for 24 hours. IP-10 concentration measured in supernatant from mock infected AEC cultures infected for 24 hours was 64.8 pg/ml \pm 5.05 and 71.6 pg/ml \pm 6.11 in supernatant from RSV infected AEC cultures infected for 24 hours. Likewise, there was no significant difference in IP-10 concentration between supernatants recovered from Mock or RSV AEC cultures infected for 24 hours.

At 72 hours, concentration of IL-8 in supernatants recovered from RSV infected AEC cultures (13164 pg/ml \pm 3054) was significantly higher than recovered from mock infected AEC cultures (6385 pg/ml \pm 1956) with a p value of 0.0241. Similarly, concentration of IP-10 in supernatants recovered from RSV infected AEC cultures (137 pg/ml \pm 17.6) was significantly higher than recovered from mock infected AEC cultures (55.6 pg/ml \pm 1.40) with a p value of 0.0103.

Examining the supernatants recovered from mock infected AEC for 72 hours in comparison to those mock infected for 24 hours, there was no significant difference in IL-8 concentration. The same was true for IP-10 concentration.

However, the supernatants recovered from AEC cultures infected with RSV for 72 hours (13164 pg/ml \pm 3054) in comparison to those infected for 24 hours (4010 pg/ml \pm 1217) showed significantly greater concentrations of IL-8 with a p value of 0.0122. The same difference was observed in IL-10 concentration as supernatants recovered from RSV AEC cultures infected for 72 hours (137 pg/ml \pm 17.6) showed a significantly higher concentration of IP-10 in comparison to those infected for 24 hours (71.6 pg/ml \pm 6.11) with a p value of 0.0089.

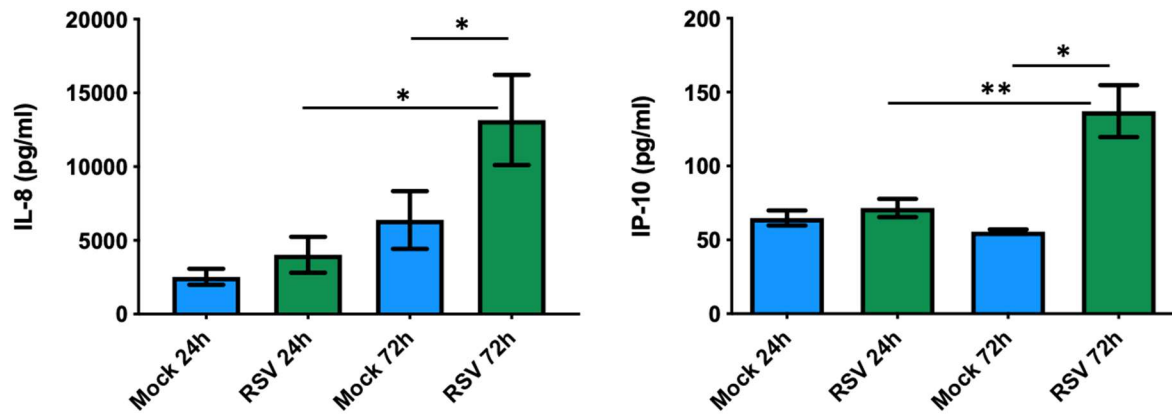


Figure 3-17 Concentration of IL-8 and IP-10 in the apical supernatants collected from flooded basal RSV infected AECs

Concentration of IL-8 (left) and IP-10 (right) in apical supernatants collected one hours incubation of fresh media with basal epithelium infected with RSV for 24 (RSV 24) or 72 hours (RSV 72) or Mock infected control epithelium infected for 24 (Mock 24) or 72 hours (Mock 72).

Groups were compared with One-Way ANOVA with Tukey adjustment for multiple testing. *= $p < 0.05$. **= $p < 0.002$. N=6.

3.5.11.3 Supernatants collected from basal AEC cultures infected with RSV for 72 hours are chemoattractant to neutrophils

Neutrophil chemotaxis experiments were performed as described in **Section 2.7.1** with supernatants collected as described in **Section 2.6.3**.

Examining the average speed of neutrophils during chemotaxis, there was no significant difference between the neutrophils exposed to media only, mock infected epithelial supernatants or supernatants collected from AEC cultures infected with RSV for 24 hours. On average, neutrophils exposed to supernatants collected from AEC cultures infected with RSV for 72 hours ($0.2051087 \mu\text{m}/\text{sec} \pm 0.001$) were faster than those migrating toward supernatant collected from 72 hour infected mock AECs ($0.1101605 \mu\text{m}/\text{sec} \pm 0.0006$) with a p value of 0.05.

Likewise, comparing the maximum measured speed of neutrophils during chemotaxis, there was no significant difference between the neutrophils exposed to media only, mock infected AEC supernatants or supernatants collected from AEC cultures infected with RSV for 24 hours. At their fastest recorded speed, neutrophils exposed to supernatants collected from AECs infected with RSV for 72 hours ($0.568 \mu\text{m}/\text{sec} \pm 0.049$) were faster than those migrating toward supernatant collected from 72 hour infected mock AECs ($0.3768 \mu\text{m}/\text{sec} \pm 0.014$) with a p value of 0.018.

Neutrophils moving towards supernatant collected from RSV infected AECs showed a slightly straighter linearity of movement compared to mock AEC supernatants, however this was not statistically significant. There was no significant difference in linearity between the neutrophils exposed to media only, mock infected AEC supernatants or supernatants collected from AECs infected with RSV.

Measuring net displacement, (distance travelled relative to the first detected position), there was no significant difference between the neutrophils exposed to media only, mock infected AEC supernatants or supernatants collected from AECs infected with RSV.

However measuring cumulative, or total, displacement neutrophils exposed to supernatants collected from AECs infected with RSV for 72 hours ($368 \mu\text{m} \pm 49.9$) moved further than those

exposed to supernatant collected from 72 hour infected mock AECs ($228 \mu\text{m} \pm 14.85$) with a p value of 0.0468. There was no significant difference between neutrophils exposed to supernatants collected from AEC cultures infected with RSV for 24 hours or those exposed to supernatant collected from 24 hour infected mock AECs. However, neutrophils exposed to supernatants collected from AEC cultures infected with RSV for 24 hours ($349 \mu\text{m} \pm 49.9$) moved further than those exposed to media alone ($182.7 \mu\text{m} \pm 8.96$) with a p value of 0.0157.

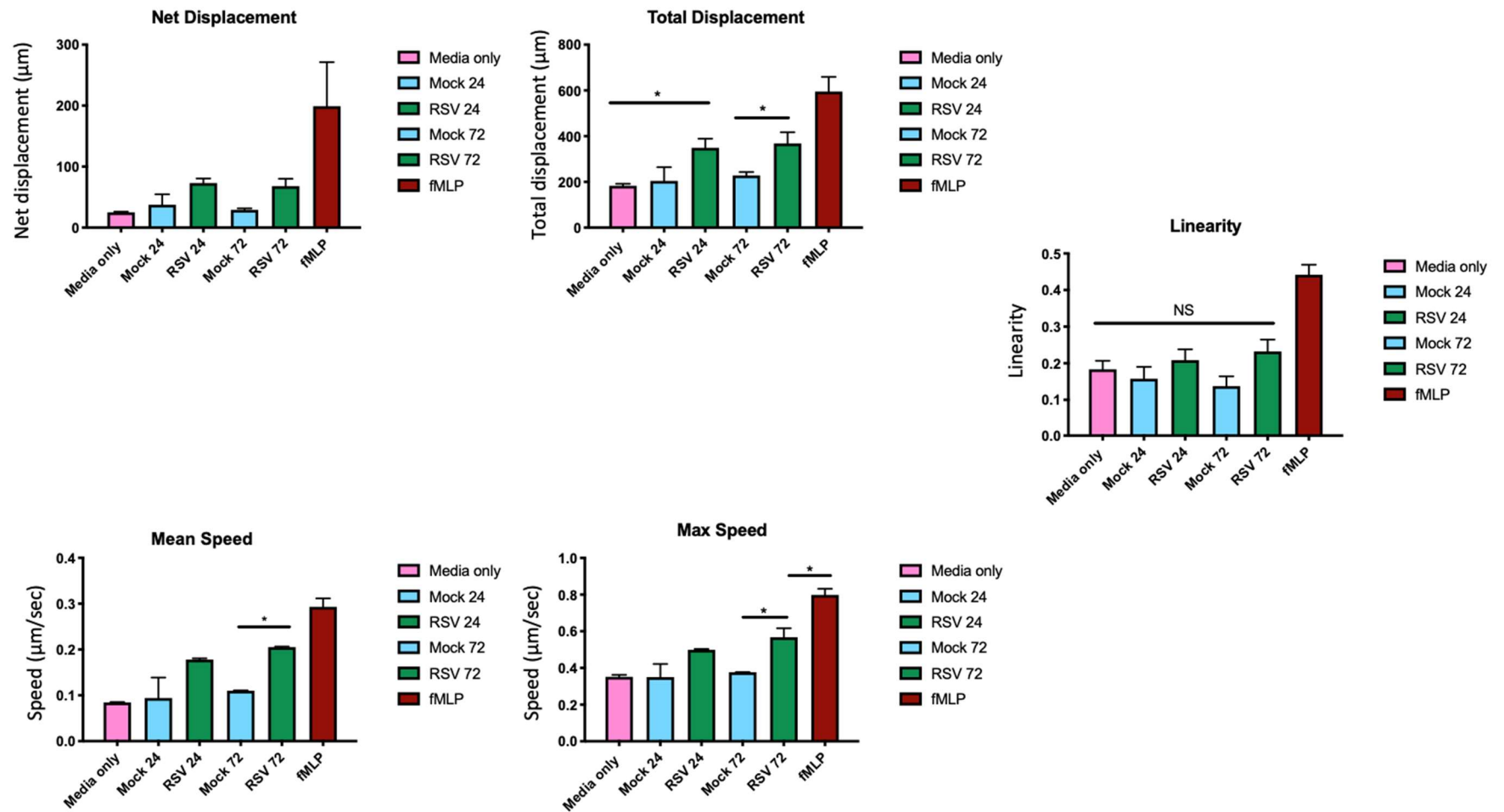


Figure 3-18 Analysis of neutrophil chemotaxis towards supernatants collected from RSV infected basal AEC supernatants.

Neutrophils moving in response to a concentration gradient of supernatant recovered from) apical supernatants collected one hours incubation of fresh media with basal AECs infected with RSV for 24 (RSV 24) or 72 hours (RSV 72) or Mock infected control AECs infected for 24 (Mock 24) or 72 hours (Mock 72). Media Alone and fMLP were used as negative and positive controls for chemotaxis respectively.

Groups were compared with One-Way ANOVA with Tukey adjustment for multiple testing *= $p < 0.05$. **= $p < 0.002$. N=4.

3.6 Discussion

Having described in **Chapter 2** the *in vitro* model of transepithelial migration, I wanted to investigate the nature of the interaction between neutrophils and RSV infected AECs in more detail. Also, in **Chapter 2**, I demonstrated that neutrophils had been isolated to a high degree of purity and primary nasal AEC cultures had been grown to a good degree of consistency. As this model is new, and relatively uncharacterised, with respect to the role and function of neutrophils, I first sought to profile neutrophil transepithelial migration across the RSV infected basal AECs. In this chapter I investigated neutrophil localisation in relation to RSV infected AECs after 1 hour migration and then profile neutrophil movement during transepithelial migration across RSV infected AECs.

In order to allow microscopy, modifications to the transepithelial migration assay included using basal AECs, primary human AECs matured at air liquid interface for 7 days, instead of AECs allowed to differentiate for 28 days. These flatter basal cultures allowed a z-stack, traversing the entire AEC layer, to be taken repeatedly, and better rendering of the apical surface, in comparison to thicker ciliated cultures. Previous studies have examined chemotaxis of neutrophils using a quantitative transwell based chemotaxis assay, which assesses difference in 'chemotaxis' by comparing numbers of cells moving towards a stimulus(9). This study examined the movement and speed of individual cells in relation to AECs, which has not been done before in a 3D system.

In this chapter, I demonstrated no difference in the number of viable neutrophils found apically to RSV infected basal AECs in comparison to the mock infected basal AECs at any time or infection point. This was unexpected, as previous clinical studies of children with bronchiolitis have shown massive increase in BAL neutrophil concentration in infants with RSV bronchiolitis in comparison to controls(214). I also counted more neutrophils migrating through mock infected AECs with RSV AEC supernatant on their apical side (RSV Sup) in comparison to RSV infected AECs. As the supernatant composition between these groups was the same, this observation may be due to a soluble factor secreted by RSV infected AECs. It could be hypothesised that in both culture systems the apical supernatant is equally chemo

attractive, but RSV infected epithelium poses greater resistance to transepithelial migration. This increase in resistance could be due to processes of viral infection. A known histopathological characteristic of RSV is cell syncytia formation, which reduces cell-cell junctions through which migration may occur(54). RSV infection of AECs is also known to upregulate ICAM-1, an adhesion molecule which interacts with neutrophils through CD11b and LFA-1(114,185), leading to greater neutrophil adherence to RSV infected AECs in comparison, which may sequester some of the additional neutrophils found in the RSV Sup AECs.

Comparing numbers of neutrophils transepithelially migrating across basal AECs and ciliated AECs, it was interesting that in basal AECs there was no significant difference in numbers of neutrophils which migrated through to the apical side of mock infected AECs and RSV infected AECs; but in ciliated AECs, a significantly greater number of neutrophils were found on the apical side of RSV infected AECs, in comparison to mock infected AECs. This difference could be attributed to differentiation related factors of the AECs, with a thicker ciliated epithelium neutrophils may have greater opportunity to 'wander' around the RSV infected AECs surface in comparison to basal cells instead of moving directly through, or it could also be that ciliated cells produce greater quantity of chemoattractant cytokines upon infection with RSV which in turn increases numbers of neutrophils migrating.

Comparing neutrophil migration between basal and ciliated AECs, there was no significant difference in number of neutrophils found on the apical side of mock, RSV or RSV Sup basal AECs in comparison to the same infection condition in ciliated AECs. However, there were significantly greater numbers of neutrophils found apically to basal AECs with fMLP placed apically (a potent neutrophil chemoattractant) in comparison to ciliated AECs, indicating basal AECs without RSV infection are more permissive to neutrophil migration. This difference could be attributed to a difference in the AECs due to differentiation, a thicker ciliated epithelium may pose greater resistance to neutrophilic migration in comparison to thinner basal cells. This observation may have clinical relevance, as if it is reflected *in vivo*, if there is damage to ciliated AECs in the airway and these are replaced with basal cells, greater neutrophilic infiltration may occur, although in my model I did not see this in response to RSV infection of

AECs. It is also true that these counts were comparing are viable counts, and the discrepancy in neutrophil migration numbers between basal and ciliated AECs may actually be accounted for by a greater proportion of neutrophils traversing basal AECs being apoptotic or dead, when they reach the apical side. Unfortunately, I was not able to conduct these comparisons myself during the course of this PhD.

In this chapter I observed greater numbers of neutrophils adherent to RSV infected AECs in comparison to mock infected, or RSV SUP controls in both basal AECs and ciliated AECs. Comparing neutrophil adherence between basal and ciliated AECs, I showed vastly greater numbers of neutrophils remaining adherent to RSV infected ciliated AECs in comparison to basal. Potentially, this was to be expected as it has been shown previously that RSV targets ciliated cells for infection and so there may be greater quantities of live virus and viral antigen presented on AEC MHC molecules for neutrophils to detect as greater levels of infection were found(59,66). Ciliated AEC cultures were also thicker so had greater numbers of cells generating cytokines and have a bumpy apical surface that has a greater surface area to potentiate neutrophil adhesion.

In my analysis of RSV infected basal AECs, it was observed that neutrophils adhere in a 'clustering' pattern, leaving some areas of the AECs with a high density of neutrophil adherence and other areas with fewer neutrophils. Previous *in vitro* studies have shown damage to the RSV infected airway observed after extended period (4 hours) of neutrophil migration(185), and in these studies 'gaps' in the AECs were observed. High concentration of neutrophils adherent to the AECs in a particular location, as shown in this chapter, with an associated high concentration of neutrophil generated toxic effector molecules in close proximity to the airway could go some way to account for this damage pattern.

Examining the formation of these neutrophil clusters or collections, I observed the convergence of neutrophils during their transepithelial migration of the airway. This is one of the only instances where neutrophil behaviour, as seen in sophisticated imaging of *in vivo* models notably in mice and zebrafish, have been observed in an *in-vitro* cell culture system of the airway(134,217). Similar 'swarming' behaviour of neutrophils have been shown in studies of sterile inflammation(134). Lammerman et al demonstrated using an *in vivo* model of mouse

ear damages with a laser beam as a simulation of sterile inflammation, that self-organisation of neutrophils was triggered by a dying neutrophil and the signal was propagated to neighbouring neutrophils by Leukotriene B4 (LTB4)(134). The movement pattern observed in this study was very similar to seen in our video in **Figure 3-12**. It is highly probable that some common mechanism is involved in both these observations, but it is interesting that Lammerman's experiments were not correlated to infection but damage to the host tissue. This raises the idea that it may not be a factor produced by AECs and could be neutrophils themselves both triggering and maintaining the clustering behaviour. This is an exciting future direction arising from my research.

In my 2D chemotaxis experiments stimulating neutrophil chemotaxis with AEC supernatants alone in the absence of AECs, mean speed of neutrophils was greater towards RSV infected supernatants in comparison to the mock. However, measuring neutrophil speed in our 3D transepithelial migration model, neutrophils moving across RSV infected airway moved more slowly than those moving across mock infected epithelium. Analysing the supernatant from mock and RSV infected AECs prior to migration, elevated levels of IL-8 and IP-10, potent neutrophil chemoattractants, were observed in the supernatants of RSV infected AEC cultures in comparison to those isolated from the mock. Therefore, it is logical to hypothesise a factor linked to the epithelium is responsible for slowing down the neutrophils, such as interaction of integrins and ICAM1 in RSV infected AECs. This may also account for the greater horizontal movement of neutrophils across RSV infected AECs in comparison to those moving through mock infected AECs. This difference could also be partially explained by neutrophils being in close proximity to the airway where local concentration gradients of soluble mediators at the AEC apical surface may facilitate a "patrolling" function of neutrophils on the AEC apical surface.

In **Figure 3-11** I showed the disappearance of an RSV infected AEC during neutrophil transepithelial migration. It is important to interpret this in the context that neutrophil migration reduces infective viral titre in a ciliated model of transepithelial migration(185). This observation of neutrophil migration of during RSV infection of AECs has demonstrated a potential mechanism for this reduction in viral titre.

In this chapter I showed preliminary evidence that neutrophils may initially migrate across RSV infected AECs, then return to the basolateral side sometime later. Unfortunately, due to hardware limitations in our imaging facility we were unable at this time to further investigate this. In a clinical study of infants with RSV bronchiolitis, Halfhide et al demonstrated the presence of viral RNA in neutrophils isolated from the blood of patients with RSV bronchiolitis, which was an unexpected finding considering we have found no evidence that RSV infection induces a viremia in these patients(180). This initial observation may provide clues as to how neutrophils in peripheral circulation may obtain viral RNA.

3.7 Summary

In this chapter I established a modified transepithelial migration model to allow imaging of neutrophil movement through RSV infected basal AECs. Using this model, I was able to quantify neutrophil migration across and adherence to RSV infected basal and ciliated AECs in comparison to mock infected basal AECs and in comparison to each other. I showed no difference in numbers of neutrophils found apically to RSV infected basal AECs in comparison to mock infected basal AECs, but I did show greater numbers found apically to RSV infected ciliated AECs in comparison to mock infected ciliated AECs. In both basal and ciliated AEC I showed greater numbers of neutrophils remain adherent to RSV infected AECs in comparison to mock infected AECs, but that overall greater numbers remain adherent to RSV infected ciliated AECs in comparison to RSV infected basal AECs. I then assessed neutrophil movement, spatially and temporally, during transepithelial migration across RSV infected basal AECs, and I showed neutrophils move faster and further during transepithelial migration across mock infected AECs than they do across RSV infected AECs. Finally, I observed the disappearance of RSV infected AECs on time lapse microscopy in correlation with reduction of viral titre in neutrophil transepithelial migration experiments. Indeed, neutrophil kinetics during transepithelial across primary airway epithelial cells are altered by RSV infection.

Chapter 4 Altered neutrophil apoptosis during RSV infection

4.1 Background

The appropriate resolution of inflammation can be considered just as important to clinical recovery from infection, as the initial pathogen response. The removal of infiltrating immune cells, which are obsolete, once infection is resolved is important to ensure inappropriate damage is not inflicted on host cells by continuing inflammation. A failure to achieve this homeostatic balance has long been suggested as contributing to severe diseases in those infected with RSV, as it has been hypothesised that an over-exuberant anti-viral immune response may contribute to epithelial cell damage, mucus production and necrotic immune cells causing small airway closure(218,219). A failure of appropriate apoptosis may contribute to this.

Neutrophil lifespan is regulated by programmed cell death, usually apoptosis (220). Apoptosing neutrophils can be taken up by tissue resident phagocytes, for example resident macrophages, therefore limiting the release of destructive neutrophil products into the airway, which would occur if the neutrophils were allowed to necrose (221). A number of neutrophilic and chemotactic factors present in airway secretion of infants with RSV bronchiolitis, inhibit neutrophil apoptosis (220,222), thereby prolonging cell survival. Wang et al also studied neutrophils recovered from the airways of infants with RSV bronchiolitis and suggested that neutrophil apoptosis may, conversely, be accelerated in this condition (223). This study isolated neutrophils from the upper airways and peripheral blood of RSV infected infants and showed higher levels of apoptosis and cell death, as measured by propidium iodide (PI) and annexin V, in comparison to neutrophils isolated from blood of healthy age matched control children(224). However this group isolated neutrophils from the airways of infants with RSV by instilling saline to the nostrils and performing a nasopharyngeal aspirate, hereby sampling only neutrophils in the upper airways which are easily dislodged by saline(224). This study was limited by not knowing exactly how long these neutrophils had been resident in the airways, so it is difficult to determine whether their apoptosis or death had been shortened or prolonged relative to when they entered the airway from the blood. Jones et al showed that

nasal lavage fluid collected from infants with RSV bronchiolitis when added to purified healthy adult neutrophils neat in an *in vitro* system, significantly delays neutrophil apoptosis(225). Because of this, Jones et al postulated that the prolonged survival of neutrophils in the infant airway contributes to the observed accumulation of neutrophils in the airways of infants with severe RSV(212).

Conventionally, propidium iodide (PI) and Annexin-V co-staining have been used to assess apoptosis (226). This methodology exploits the apoptosis mediated expression of phosphatidyl serine on the outer envelope of the neutrophil cell membrane to which annexin V is a natural ligand. In these experiments, by coupling Annexin V to a fluorophore, fluorescein (FITC), one can identify cells that have begun the process of apoptosis even if no morphological changes have yet occurred. Propidium Iodide is a fluorescent orange dye, which binds to DNA and is impermeant to live cells, meaning it can be used to identify dead cells. By staining with both dyes one can separate populations of dead cells (PI positive), apoptotic cells (Annexin positive, PI negative) and live cells (Annexin negative, PI negative). This is the approach which was used in this study.

The interaction between neutrophils and RSV has been subject to research for many years, and it has long been suggested the clinical syndrome of RSV bronchiolitis may be a consequence of immunopathology (227). Despite continued research to elucidate this, no specific immunopathological process has been found. McNamara et al have demonstrated that neutrophils are abundant in the lower airways of children with severe RSV bronchiolitis, accounting for ~80% of cells recovered from the tracts (82). These finding have been replicated by other groups (228).

4.2 Hypothesis

Neutrophil apoptosis is reduced by RSV viral products during RSV infection of the airway

4.3 Aims

The specific aims of this chapter are to determine whether:

1. RSV reduces neutrophil apoptosis
2. RSV G glycoprotein reduces neutrophil apoptosis
3. Neutrophil apoptosis is reduced in presence of soluble factors secreted from infected airway epithelial cells
4. Neutrophil apoptosis is reduced in presence of RSV infected ciliated respiratory epithelium
5. Trans-epithelial migration through RSV infected ciliated respiratory epithelium reduces neutrophil apoptosis

4.4 Chapter specific methods

Neutrophils were isolated from peripheral venous blood from healthy volunteers and prepared as per **Section 2.4**. Apoptosis was determined by Annexin V-FITC and PI co-staining as described in **Section 2.7.4**. Experiments including upright or inverted air liquid interface respiratory epithelial cultures were performed for a minimum of three airway donors and neutrophil donors were matched if possible. RSV infected AEC supernatants were prepared as per **Section 2.6.3**, and corresponding chemoattractant and viral analysis with the same supernatants in **Section 2.8**. Throughout this chapter, mock infection is a term used to describe the same process of infecting the AECs used but a media only solution and not live virus used instead. These cultures are used as controls for viral infection. A negative control of HBSS only and a positive control of HBSS containing 5ng/ml GM-CSF, which prolongs neutrophil survival, were used in experiments. In **Section 4.5.6**, upright ciliated AEC cultures were used, and these were grown as previously described by Lee et al(229). Where neutrophils in suspension with AECs were analysed for apoptosis, neutrophils were first distinguished from epithelial cells by antibody staining with CD11b-APC conjugate as described in **Section 2.7.4.1**. Apoptosis of naïve neutrophils incubated with media alone (HBSS) or GMCSF over time is presented in **Appendix 10**.

4.5 Results

4.5.1 RSV maintains neutrophil viability and reduces neutrophil apoptosis

Neutrophils were incubated in HBSS containing increasing concentrations (multiplicity of infection (MOIs)) of RSV for up to 24hrs. After 1 hour, 4 hours and 24 hours proportion of neutrophils live, apoptotic and dead were measured, a representative dot plot is shown in **Figure 4-1** and proportion breakdown demonstrated in **Figure 4-2**.

I found that after 1 hour incubation, neutrophils exposed to RSV at the lower MOI 0.1 ($1.8875\% \pm 0.67$) and 1 ($1.978\% \pm 0.47$) showed a significant smaller proportion of apoptotic neutrophils in comparison to HBSS alone ($3.3075\% \pm 0.96$), with p values of 0.0428 and 0.0075 respectively. Neutrophils exposed to RSV MOI 5 showed $2.674\% (\pm 1.04)$ apoptotic.

After 4 hours incubation, neutrophils incubated with MOI 0.1 showed $7.74\% (\pm 2.33)$ apoptotic, with RSV MOI 1 showed $11.17\% (\pm 2.84)$ apoptotic and neutrophils with RSV MOI 5 showed $8.99\% (\pm 1.66)$ apoptotic. Neutrophils incubated with GMCSF showed $5.58\% (\pm 1.76)$ apoptotic and neutrophils exposed to HBSS alone showed $18.01\% (\pm 6.78)$. There was no significant difference between the proportions of, live, apoptotic or dead neutrophils either RSV MOI 0.1, 1 or 5 groups and HBSS alone or GMCSF.

After 24 hours, neutrophils incubated with the higher concentrations of RSV MOI 1 and 5 showed a significantly smaller proportion of cells were apoptotic, RSV 1 ($39.6\% \pm 4.85$) and RSV 5 ($30.2\% \pm 10.11$), in a dose dependent manner in comparison to HBSS only controls ($57.7\% \pm 4.27$), with p values of 0.0062 and <0.0001 respectively. Neutrophils incubated with RSV MOI 1 and 5 also showed a greater proportion of neutrophils were still viable, RSV 1 ($39.36\% \pm 8.75$) and RSV 5 ($46.88\% \pm 6.00$), compared to controls with p values of 0.0118 and 0.0350 respectively. There was no significant difference in proportion of apoptosis or viability between neutrophils incubated with RSV MOI 0.1 ($58.83\% \pm 1.06$) or HBSS only ($57.7\% \pm 4.27$).

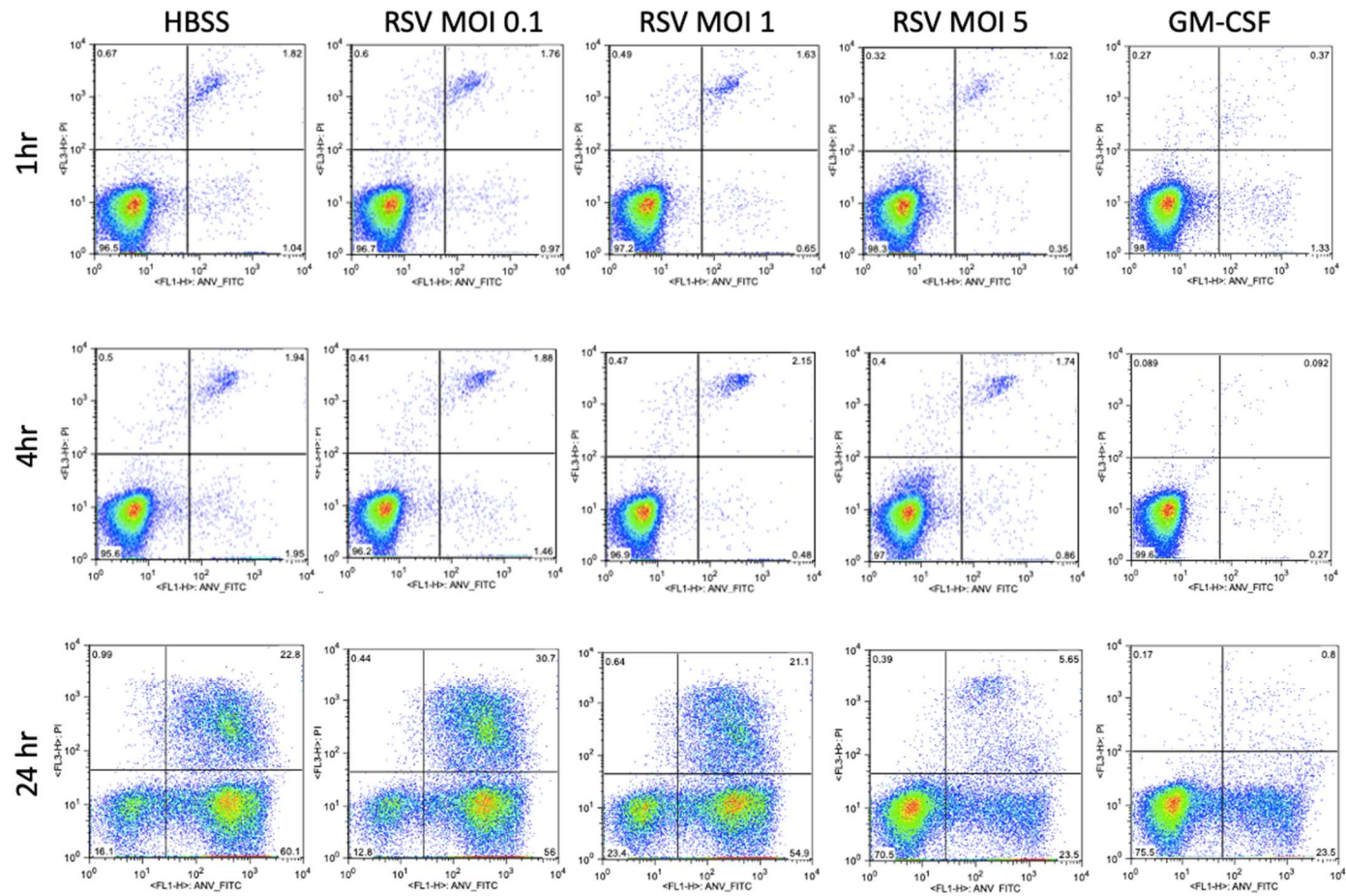


Figure 4-1 Apoptosis of adult neutrophils incubated with RSV at MOI 0.1, 1 and 5.

Representative dot plots at 1, 4 and 24hours of neutrophils incubated with HBSS only, RSV MOI 0.1, RSV MOI 1, RSV MOI 5 and 5ng/ml GM-CSF. Apoptosis measured by Annexin V-FITC and Propidium Iodide (PI) costaining.

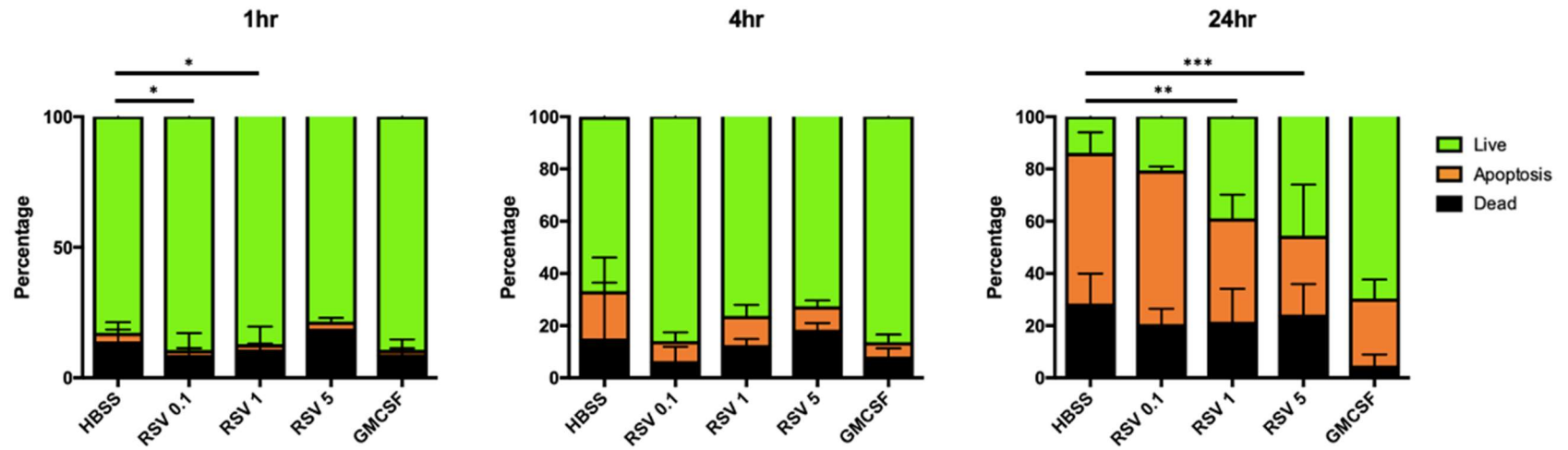


Figure 4-2 Apoptosis of adult neutrophils incubated with RSV at MOI 0.1, 1 and 5.

Naïve neutrophils from healthy adult donors incubated with RSV (MOI 0.1, 1, 5) or controls. Percentage of live, apoptotic and dead cells measured at 1 hour, 4 hour and 24 hour time points. Relative proportions of neutrophils which were live (green), apoptotic (orange) and dead (black) after 1, 4 and 24 hours. (* $p < 0.05$ ** $p < 0.002$ *** $p < 0.0001$, Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons. N=5.

4.5.2 RSV G maintains neutrophil viability and reduces neutrophil apoptosis

To investigate the contribution of RSV G glycoprotein to the delay in apoptosis seen following incubation with RSV, we incubated neutrophils in HBSS supplemented with 1mg/ml, 10mg/ml or 20mg/ml purified RSV G protein. HBSS and GM-CSF were used as negative and positive controls respectively.

I found no significant difference between the proportions of live, apoptotic or dead neutrophils G protein concentrations or GM-CSF at 1 hour and 4 hours, compared to HBSS alone.

However, after 24 hours, we found a significantly smaller proportion of apoptotic neutrophils when incubated with 10mg/ml ($29.73\% \pm 5.17$) and 20mg/ml ($32.2\% \pm 5.64$) RSV G protein in comparison to HBSS alone ($62.1\% \pm 12.42$), with p values of 0.00621 and 0.0329 respectively. There was no significant difference between proportions of neutrophils apoptotic after incubation with G protein 1mg/ml ($46.4\% \pm 9.3$) in comparison to HBSS alone. There was also a smaller proportion of apoptotic neutrophils found in the groups incubated with GM-CSF ($28.27\% \pm 4.2$) in comparison to HBSS alone ($62.1\% \pm 12.42$) with a p value of 0.0061.

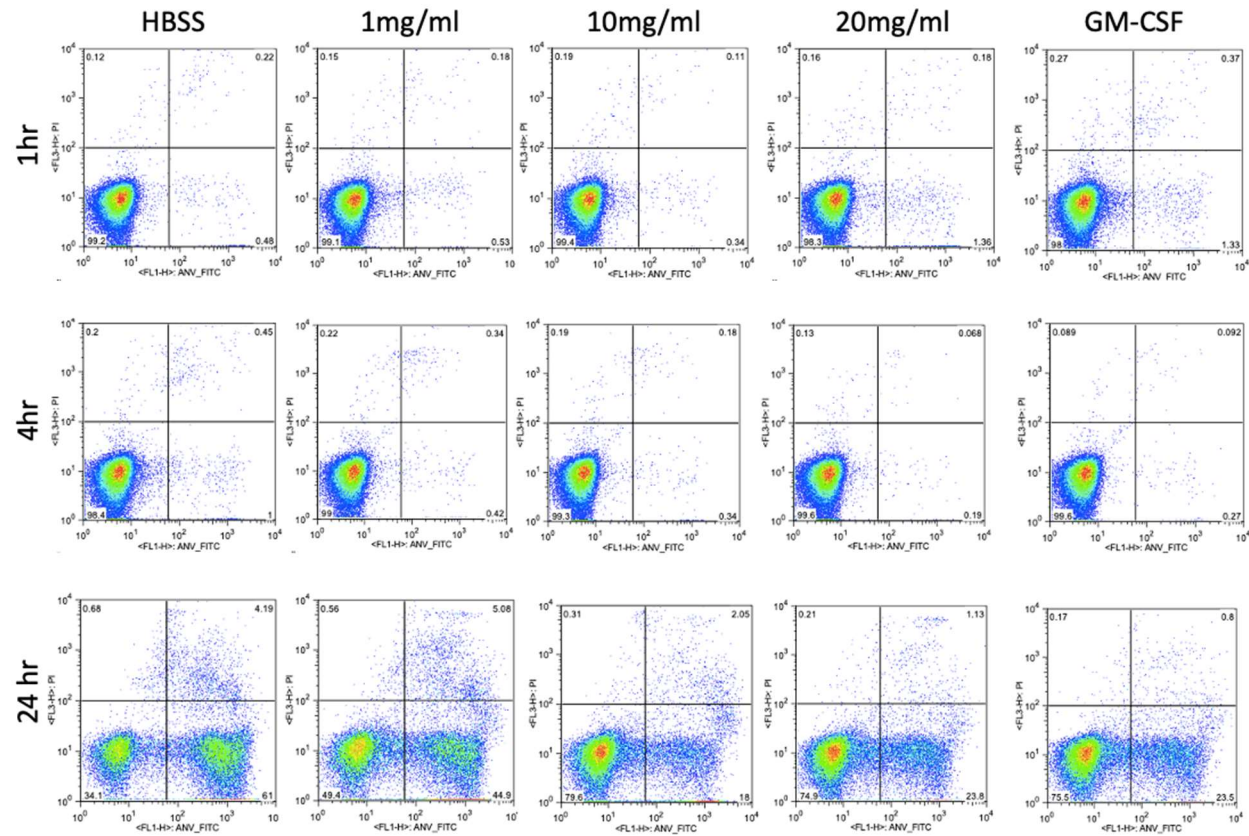


Figure 4-3 Apoptosis of adult neutrophils incubated with RSV G protein concentration of 1mg/ml, 10mg/ml and 20mg/ml.

Representative dot plots at 1, 4 and 24hours of neutrophils incubated with HBSS alone or G protein at a concentration of 1mg/ml, 10mg/ml and 20mg/ml. Apoptosis measured by Annexin V-FITC and Propidium Iodide (PI) costaining.

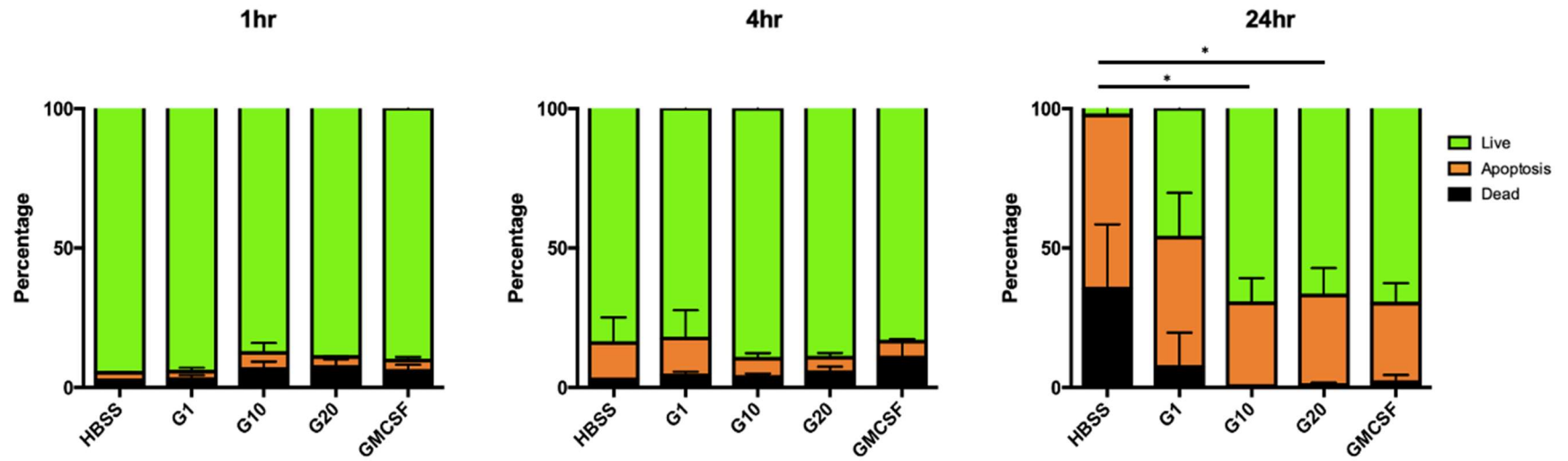


Figure 4-4 Apoptosis of adult neutrophils incubated with RSV G protein concentration of 1mg/ml, 10mg/ml and 20mg/ml.

Neutrophils from healthy adult donors were incubated with purified G protein at a concentration of 1mg/ml, 10mg/ml and 20mg/ml or HBSS only as a control. Relative proportions of neutrophils which were live (green), apoptotic (orange) and dead (black) after 1, 4 and 24 hours. (* $p < 0.05$ using Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons). N=5.

4.5.3 Increasing the Ig content reverses the reduction in neutrophil apoptosis

I then investigated whether we could reverse the delay in neutrophil apoptosis by incubating the neutrophils with a monoclonal antibody that blocked the binding site of RSV G-protein (131-2G) (230).

In preliminary experiments after 24 hours incubation, we found an observable difference between the proportion of apoptotic neutrophils treated with RSV MOI 5 alone (7.85%) and those also treated with RSV MOI 5 plus human anti-G antibody (60.1%) (**Figure 4-5**). Similarly, there was a significant difference between the proportion of apoptotic neutrophils treated with RSV MOI 5 alone (7.85%) and those also treated with RSV MOI 5 plus human immunoglobulin isotype control (60.2%) (**Figure 4-5**), suggesting that the effect of human anti-G antibody is not specific to its interaction with RSV G protein.

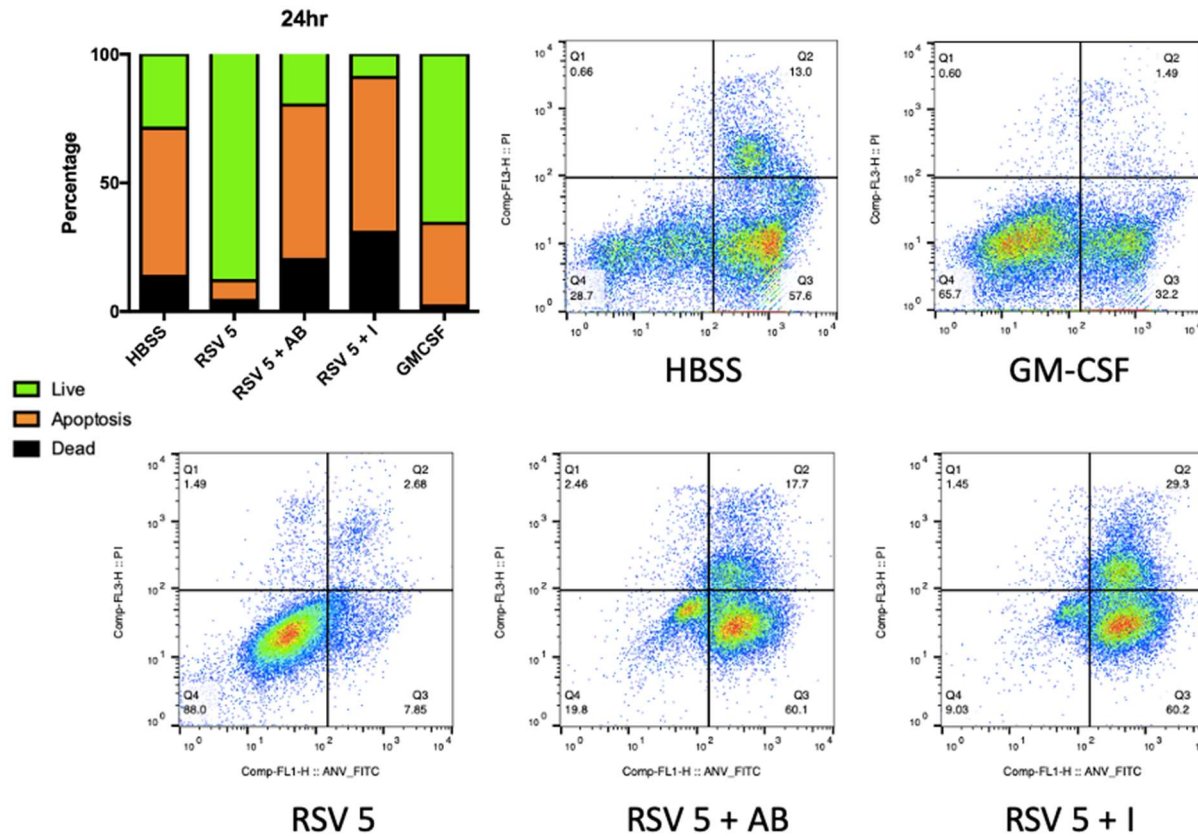


Figure 4-5 Apoptosis of adult neutrophils incubated with RSV and 1mg/ml Anti-RSV G antibody (131-2G).

Top left. Relative proportions of neutrophils which are live (green), apoptotic (orange) or dead (black) after 24hours incubation with HBSS alone, 5ng/ml GMCSF, RSV, RSV + AntiG antibody, RSV + isotype control.

Representative dot plots after 24hrs of incubation of neutrophils incubated with (top row) HBSS alone and 5ng/ml GMCSF and (bottom row) RSV + AntiG antibody (AB), RSV + isotype control (I). Apoptosis measured by Annexin V-FITC and Propidium Iodide (PI) costaining. N=2.

4.5.4 Supernatant from cultured primary AECs lead to greater neutrophil viability and reduced neutrophil apoptosis, regardless of whether they had been infected with RSV or not.

Neutrophils were incubated with undiluted supernatants harvested from RSV or Mock infected ciliated AEC cultures, which had been infected for 24 or 72 hours. ALI media only and GMCSF were used as negative and positive controls respectively.

After 1 hour and 4 hours, I found that there was no significant difference between the proportions of, live, apoptotic or dead neutrophils between Mock and RSV supernatant incubated groups or ALI media alone.

However, after 24 hours, I found a significantly smaller proportion of neutrophils were apoptotic when incubated with supernatants from Mock 72 hour ($17.7\% \pm 1.81$) and RSV 72 hour ($35.07\% \pm 5.69$) infected ciliated AEC cultures or GMCSF ($28.27\% \pm 4.2$), compared to ALI media only controls ($63.13\% \pm 6.25$), with p values of <0.05 . I found no significant difference in the proportion of apoptotic neutrophils when incubated with supernatants from mock 24 hour or RSV 24 hour infected ciliated AEC cultures, compared to media only controls.

Interestingly after 24 hours, the proportion of viable neutrophils was significantly increased when incubated with supernatants from all infected ciliated AEC cultures (Mock 24, Mock 72, RSV 24 and RSV 72) compared to those incubated with media alone, $p < 0.002$.

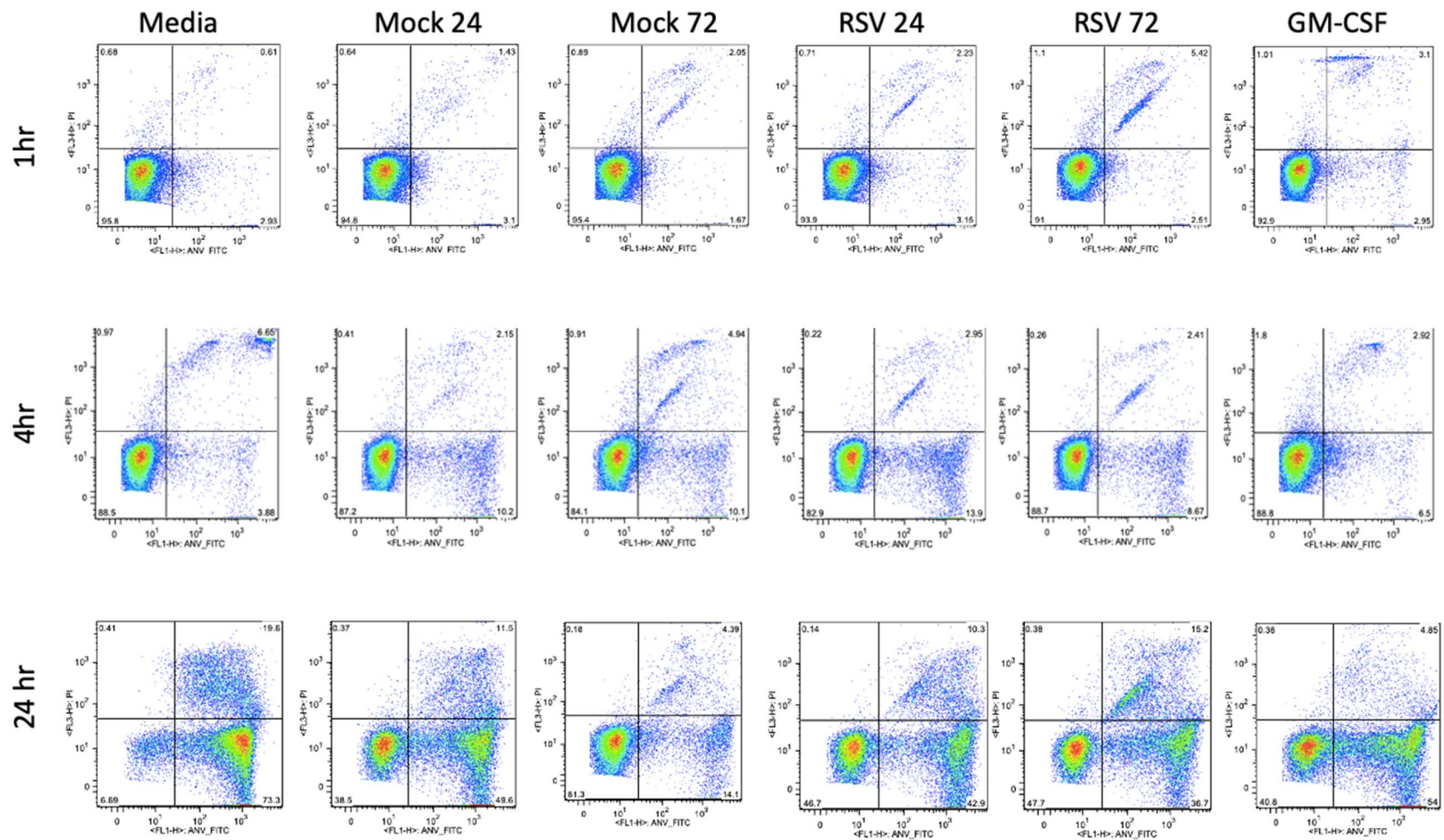


Figure 4-6 Apoptosis of adult neutrophils incubated with RSV and mock infected AEC supernatants.

Naïve neutrophils from healthy adult donors incubated with supernatant collected from mock or RSV infected AEC cultures or media only control. Apoptosis measured as a percentage of all whole cells retrieved by positive Annexin V staining and negative PI staining. Representative dotplots at 1, 4 and 24 hours of neutrophils incubated with supernatant collected from mock or RSV infected AEC cultures or media only control.

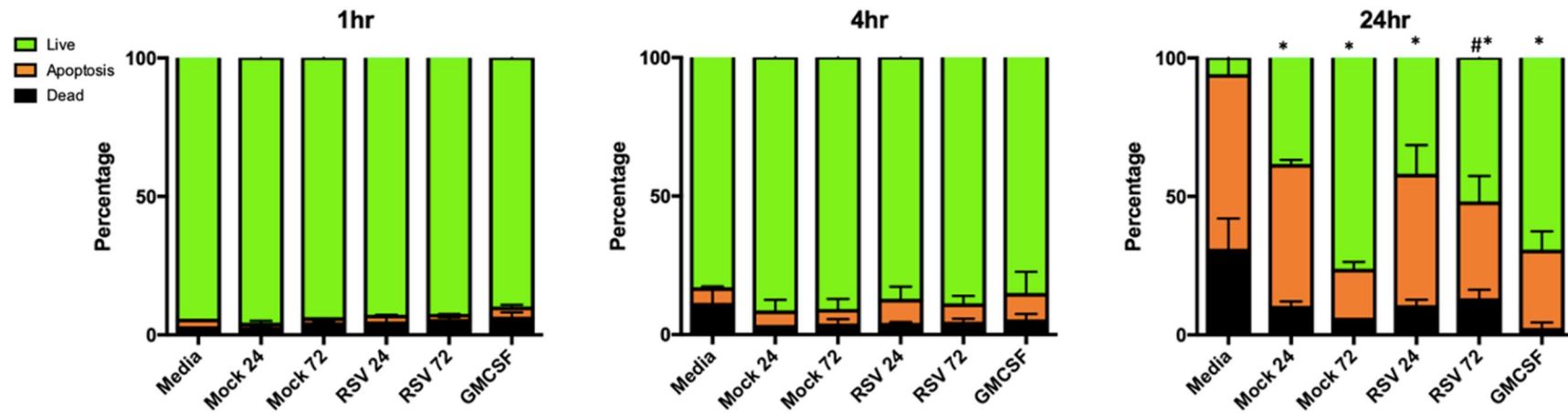


Figure 4-7 Apoptosis of adult neutrophils incubated with RSV and mock infected AEC supernatants.

Naïve neutrophils from healthy adult donors were incubated with supernatant collected from mock or RSV infected AECs or media only control. Apoptosis measured as a percentage of all whole cells retrieved by positive Annexin V staining and negative PI staining. Relative proportions of neutrophils which were live (green), apoptotic (orange) and dead (black) after 1, 4 and 24 hours. (* $p < 0.05$ difference compared to media alone. # $p < 0.05$ compared to mock equivalent, Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons). N=5.

4.5.5 Comparing the effect of RSV and supernatants from RSV infected AECs on apoptosis of neutrophils

24 hour datasets of neutrophil incubation with RSV alone and RSV infected AEC supernatants are combined in **Figure 4-7** for comparison. Descriptive statistics and original analyses are found in sections previously and **Figure 4-2** and **Figure 4-6**.

Comparing the proportion of neutrophils apoptotic after incubated from 24 hours with RSV MOI 1 and supernatants collected from RSV and mock infected AECs, I found no significant difference in comparison to mock and RSV infected AEC supernatants infected for 24 hours or 72 hours. Similarly, I found no significant difference between proportions of neutrophils apoptotic in neutrophils incubated with RSV MOI 5 and mock and RSV infected AEC supernatants infected for 24 hours or 72 hours.

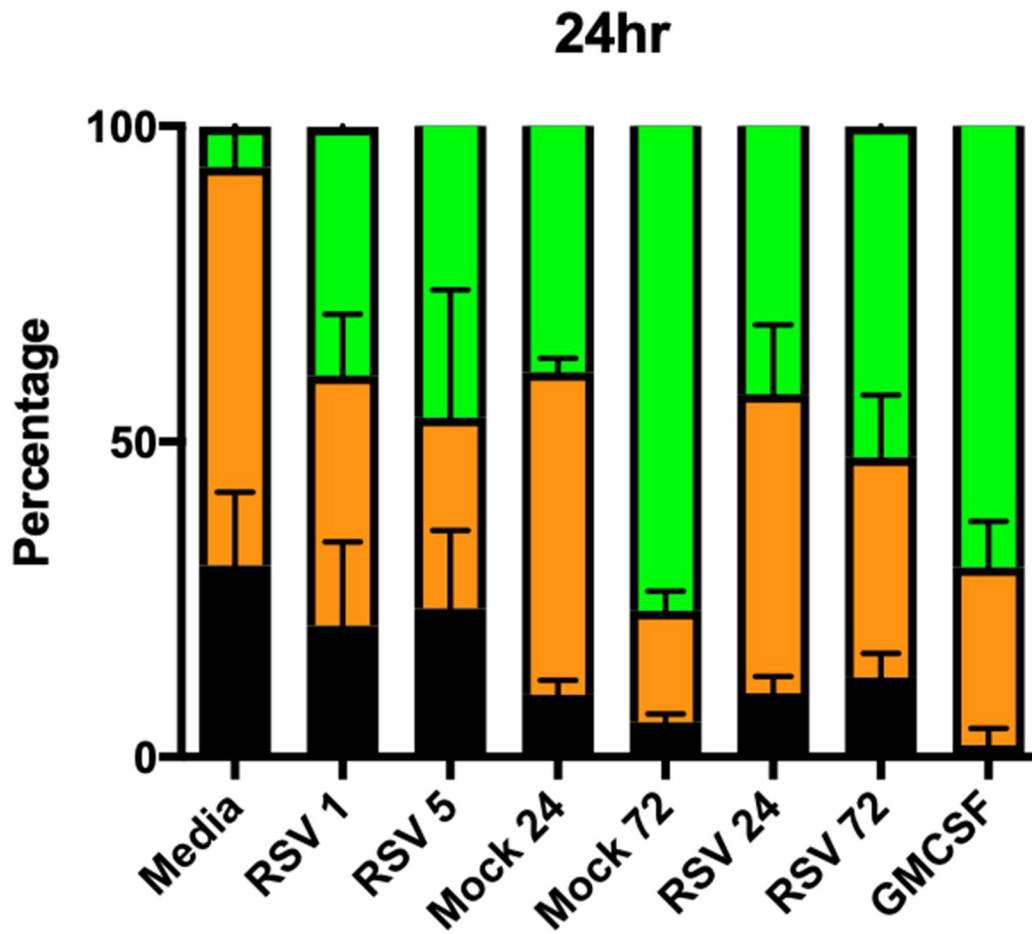


Figure 4-8 Apoptosis adult neutrophils incubated with RSV MOI 1 and 5 and supernatants collected from mock and RSV infected AECs.

Naïve neutrophils from healthy adult donors incubated with RSV (MOI 1, 5) or supernatant collected from mock or RSV infected AECs or controls. Percentage of live, apoptotic and dead cells measured at 24 hour time points. Relative proportions of neutrophils which were live (green), apoptotic (orange) and dead (black) after 1, 4 and 24 hours. (* $p < 0.05$ ** $p < 0.002$ *** $p < 0.0001$, Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons.). N=5.

4.5.6 Exposure to the apical surface of RSV infected ciliated epithelium increases neutrophil apoptosis

To investigate interactions of neutrophils with RSV infected AECs without migration, neutrophils were directly added to the apical side of a ciliated AEC cultures infected for 24 hours or 72 hours with RSV MOI 5 or mock control. The proportion of apoptotic neutrophils was determined at 0, 1 hour and 4 hours. A 24 hour time point was not possible in this culture system due to damage caused to the epithelium post addition of neutrophils.

We found that after 4h, there was no difference in the percentage of apoptotic neutrophils recovered from AEC cultures infected with RSV for 24 hours ($11.1\% \pm 5.1\%$) compared with $4.8\% \pm 2.7\%$ in the mock-infected cocultures. However, there were significantly more apoptotic neutrophils after 4 hours of exposure to AEC cultures infected with RSV for 72 hours with $46.7\% \pm 11.4\%$, compared with $6.2\% \pm 0.9\%$ in the mock-infected cocultures ($p < 0.0001$). Apoptosis appeared to be the dominant form of cell death, as we did not detect an increase in dead neutrophils at any time point or test condition.

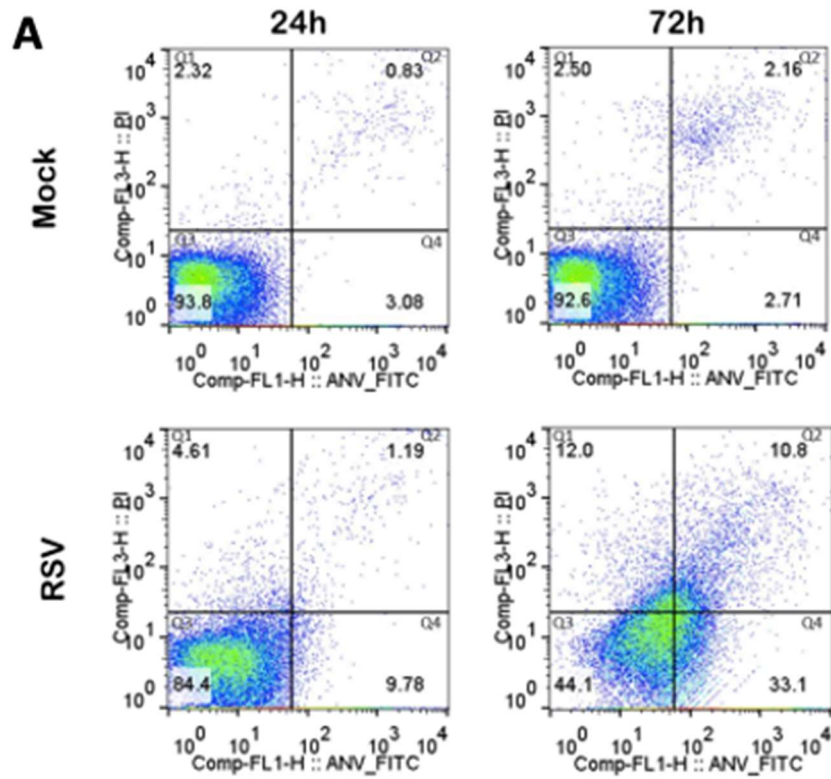


Figure 4-9 Neutrophil viability and apoptosis following exposure to RSV infected ciliated AEC cultures.

Representative dotplots of neutrophils incubated for 1 hour with RSV and mock infected ciliated AECs infected for 24 or 72 hours. Apoptosis measured by Annexin V-FITC and Propidium Iodide (PI) costaining.

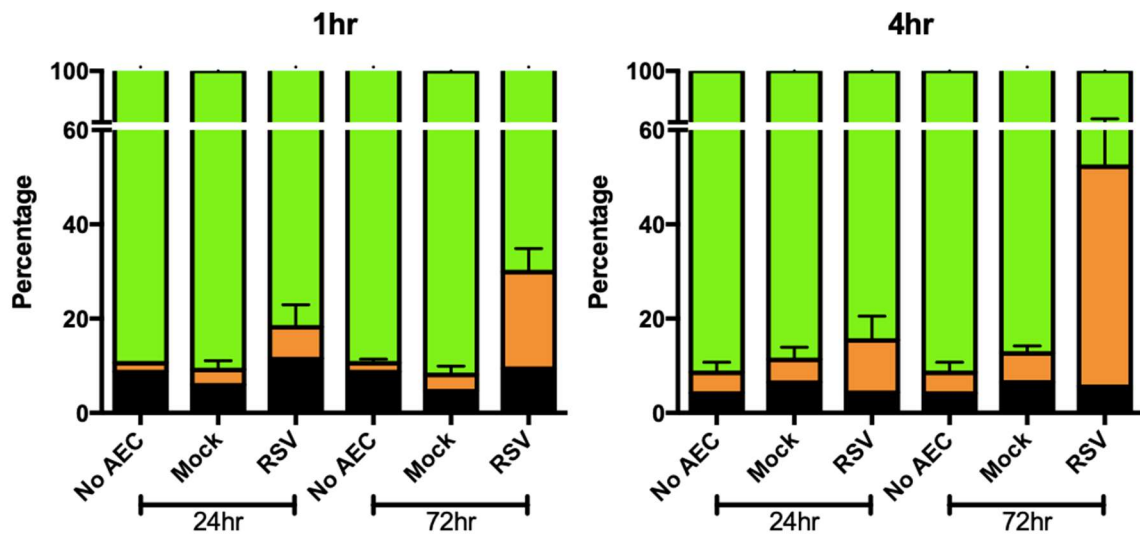


Figure 4-10 Neutrophil viability and apoptosis following exposure to RSV infected ciliated AEC cultures.

Proportions of neutrophils live (green), apoptotic (orange) and dead (black), after 1 or 4 hours incubation on the apical surface of ciliated AEC cultures infected with RSV or mock for 24 or 72 hours. Bars indicate standard error of the mean. Apoptosis measured by Annexin V-FITC and Propidium Iodide (PI) costaining. Comparisons between groups performed using Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons. N=6.

4.5.7 Neutrophil trans-epithelial migration experiments shows that different populations, defined by migration patterns, have different viability and apoptosis.

To investigate the changing viability, cell death and apoptosis of neutrophils during transepithelial migration across RSV infected AECs, neutrophils were added to the basolateral side of a ciliated AECs infected for 24 hours or 72 hours with RSV. Transepithelial migration assays were undertaken as described in **Section 2.6.2**. Unlike previous experiments, this experiment was not conducted for 4 hours due to previous work demonstrating gaps appearing in AEC cultures after 4 hours transepithelial migration(185). The presence of these gaps may allow neutrophils to move from basolateral to apical compartments without interacting with AECs and make findings difficult to interpret.

After 1 hour, three populations of neutrophils were recovered as illustrated in **Figure 4-10**.

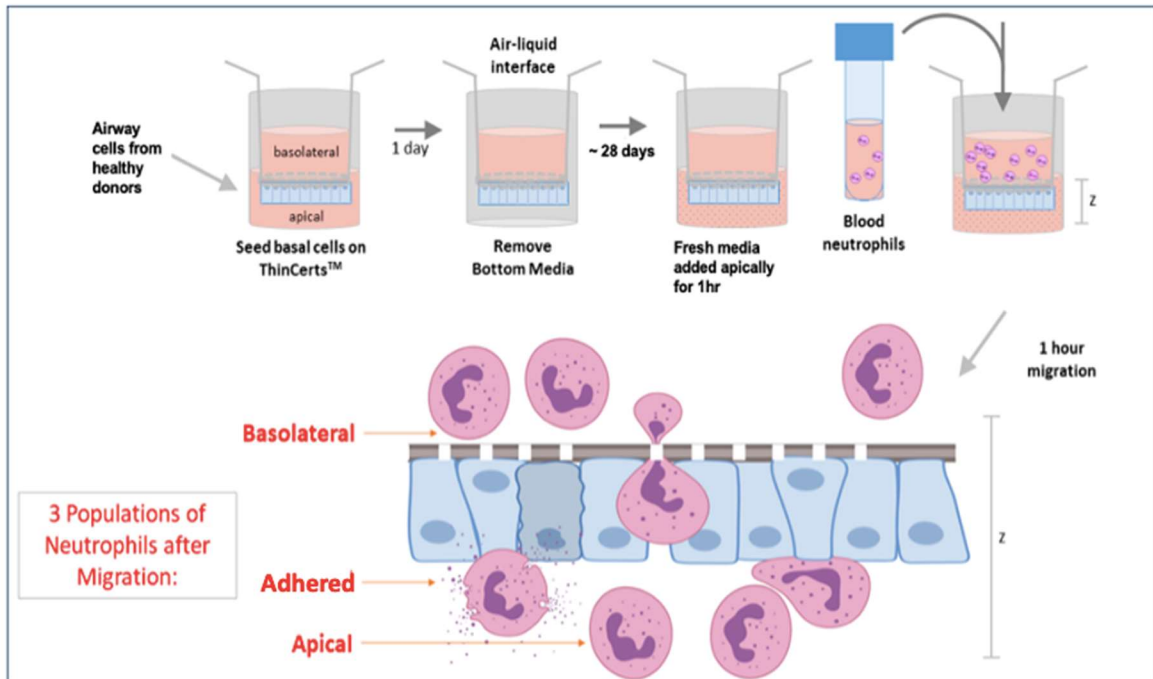


Figure 4-11 Schematic of inverted ALI model of transepithelial migration

Culture process of preparing ciliated AEC cultures and step by step methodology explanation, detailing the 3 populations of neutrophils isolated from this model.

4.5.7.1 More Neutrophils on the basolateral side of RSV infected ciliated AECs are apoptotic and fewer are viable compared with Mock infected AECs and media control

I found a significantly greater proportion of neutrophils recovered from the basolateral compartment of the RSV infected AEC cultures, for 24 hours and 72 hours, were apoptotic ($6.1\% \pm 3.11$) and ($14.63\% \pm 6.71$) compared to neutrophils incubated with no airway epithelial cells ($0.21\% \pm 0.13$) (no AEC) ($p < 0.05$) and when compared to their respective mock controls, 24 hour infected ($1.63\% \pm 1.31$) and 72 hour ($1.95\% \pm 1.6$) ($p < 0.05$ for 24 hour and $p < 0.002$ for 72 hour infected AEC cultures). There was no significant difference between the proportion of apoptotic neutrophils recovered from the mock AEC cultures infected for 24 hours ($1.63\% \pm 1.31$) and 72 hour ($1.95\% \pm 1.6$), and the neutrophils incubated with no airway epithelial cells (no AEC) ($0.21\% \pm 0.13$).

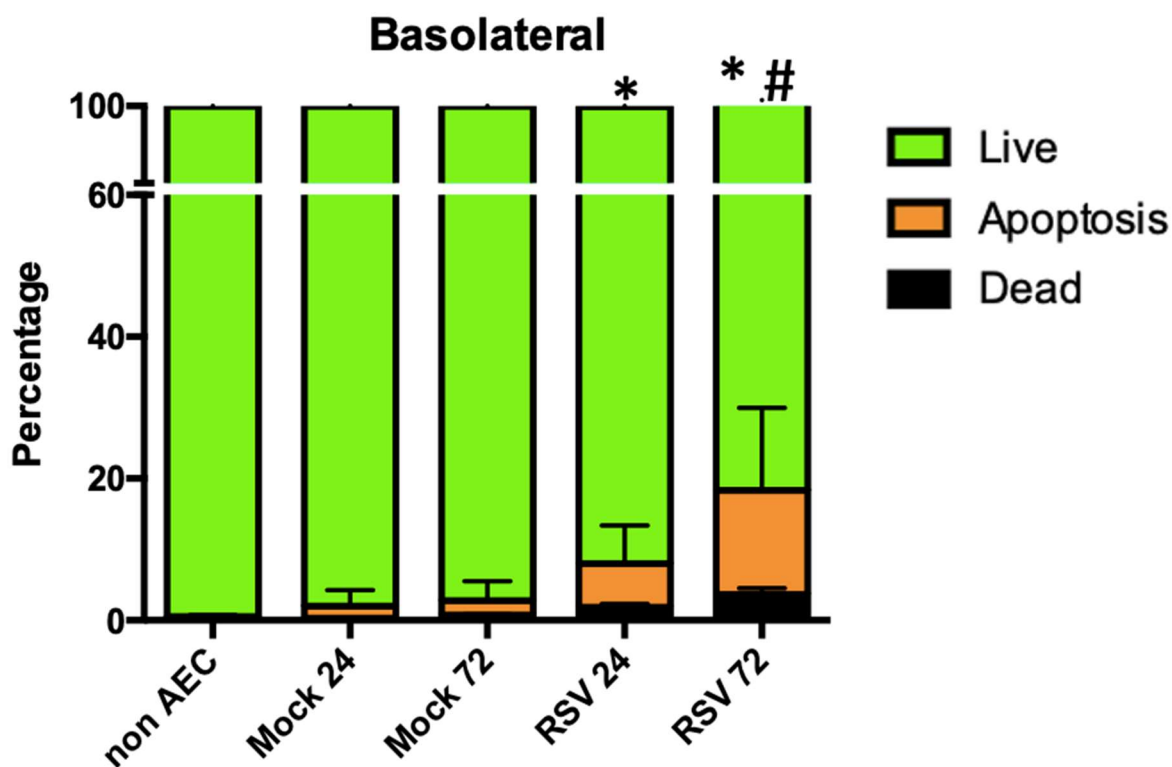


Figure 4-12 Neutrophils recovered from basolateral side of ciliated AEC cultures.

Neutrophils were applied to the basolateral side of ciliated AEC cultures infected with RSV or mock for 24 or 72 hours prior to migration. Neutrophils incubated in media alone was used as a control (non AEC). Relative proportions of neutrophils which were live (green), apoptotic (orange) and dead (black) after 1 hour incubation. (* $p < 0.05$ difference compared to media alone. # $P < 0.05$ compared to mock equivalent, Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons). N=5.

4.5.7.2 Neutrophils which migrate through and dissociate from the RSV infected ciliated epithelium show greater proportions apoptotic

I found a significantly greater proportion of neutrophils recovered from the apical compartment of the RSV infected AEC cultures, infected for 24 hours and 72 hours, were apoptotic (11.4% \pm 1.94) and (9.49% \pm 2.65) compared to neutrophils incubated with no airway epithelial cells (0.21% \pm 0.13) (no AEC) with p values of $p < 0.05$. There was no significant difference between the proportion of neutrophils apoptotic recovered from the mock AEC cultures infected for 24 hours and 72 hours, and the neutrophils incubated with no airway epithelial cells (non AEC).

Furthermore, the proportion of apoptotic neutrophils recovered from the apical side of RSV infected AEC cultures was significantly greater than those recovered from their respective mock controls with a p value of 0.0216 for 24 hour infected cultures and 0.00665 for 72 hour infected AEC cultures.

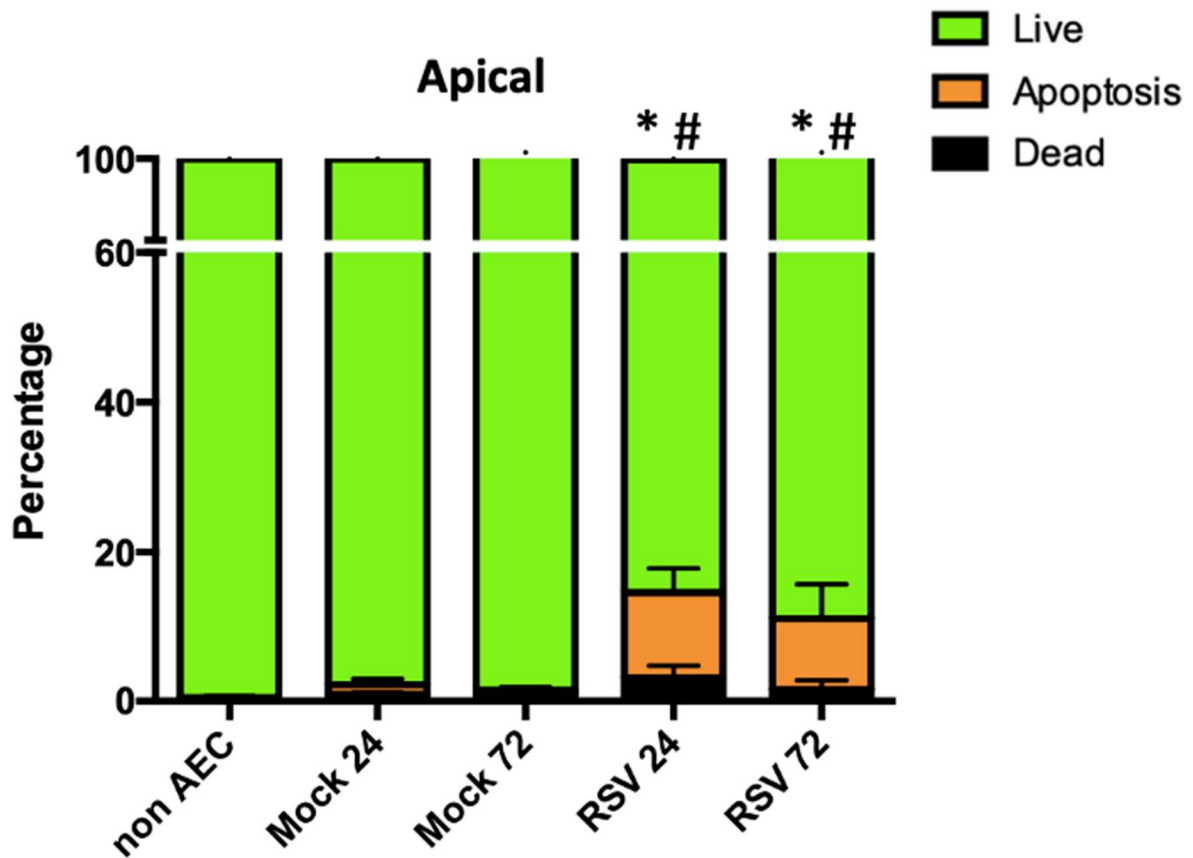


Figure 4-13 Neutrophils recovered from apical side of ciliated AEC cultures.

Neutrophils were applied to the basolateral side of ciliated AEC cultures infected with RSV or mock for 24 or 72 hours prior to migration, ones which migrated through the epithelial layer and dissociated into the apical media were collected and analysed for markers of apoptosis. Neutrophils incubated in media alone was used as a control (non AEC). Relative proportions of neutrophils which were live (green), apoptotic (orange) and dead (black) after 1 hour incubation. (* $p < 0.05$ difference compared to media alone. # $P < 0.05$ compared to mock equivalent, Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons) N=5.

4.5.7.3 Neutrophils remaining adhered to the RSV infected ciliated epithelium show reduced proportions of apoptosis

I found that a significantly smaller proportion of neutrophils incubated with both RSV infected AEC cultures, infected for 24 hours ($5.28\% \pm 2.03$) and 72 hours ($0.81\% \pm 0.3$), and no airway epithelial cells ($0.21\% \pm 0.13$) (non AEC) were apoptotic compared to neutrophils incubated with their respective mock infected AEC cultures ($15.4\% \pm 13.3$) and ($10.52\% \pm 4.74$) with p values of $p < 0.05$. There was no significant difference between the proportion of neutrophils apoptotic recovered from the RSV AEC cultures infected for 72 hours, and the neutrophils incubated with no airway epithelial cells (non AEC).

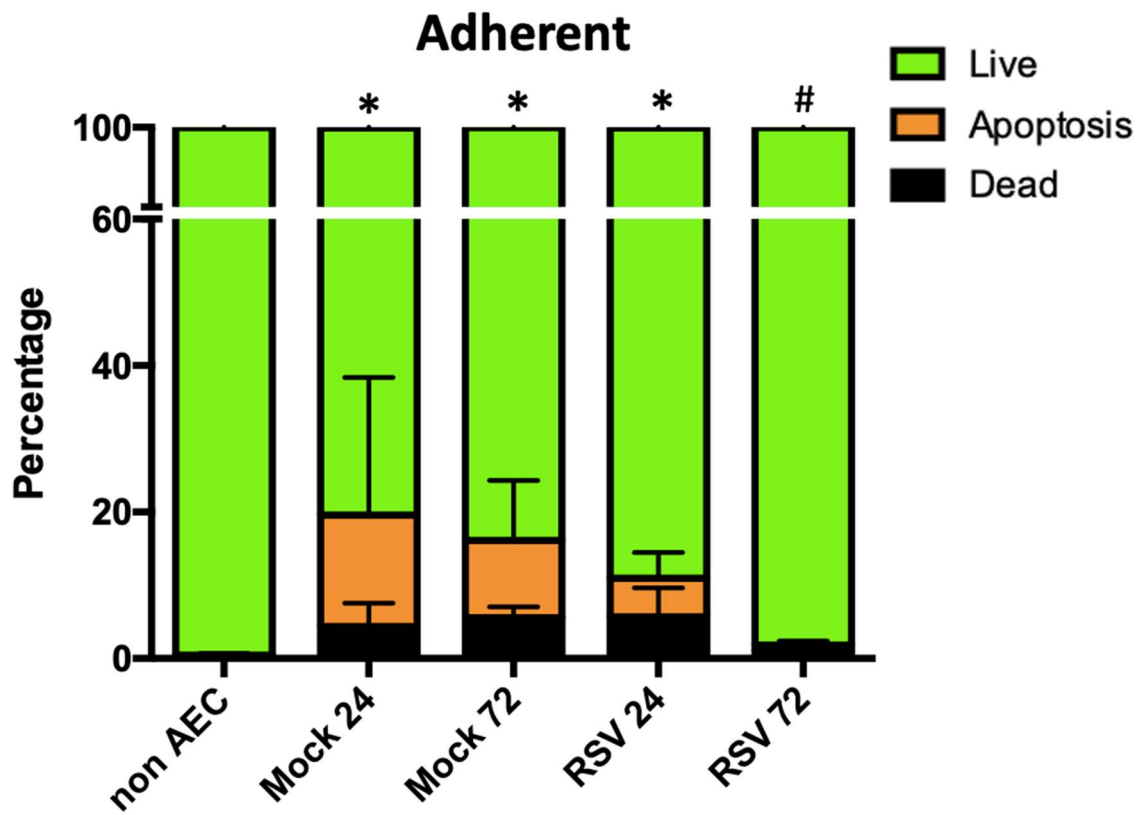


Figure 4-14 Neutrophils adherent to ciliated AEC cultures.

Neutrophils were applied to the basolateral side of ciliated AEC cultures infected with RSV or mock for 24 or 72 hours prior to migration. After 1 hour incubation, neutrophils remaining adherent to the ciliated epithelium were isolated and stained for markers of apoptosis Annexin V-FITC and Propidium Iodide. Neutrophils incubated in media alone was used as a control (non AEC). Relative proportions of neutrophils which were live (green), apoptotic (orange) and dead (black).(* $p < 0.05$ difference compared to media alone. # $P < 0.05$ compared to mock equivalent, Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons) N=5.

4.5.7.4 Apoptosis of neutrophils basolateral, apical and adhered to AECs during transepithelial migration

Comparing between neutrophils recovered from 24 hour mock infected AECs, 1.62% (± 1.3) of those on the basolateral side were apoptotic, 15.24 (± 10.3) of those adhered were apoptotic and 13.3 (± 0.47) on the apical side were apoptotic. There was no significant difference between these measures. Of neutrophils recovered from 72 hour mock infected AECs, 1.95% (± 1.6) of those on the basolateral side were apoptotic, 10.5 (± 4.74) of those adhered were apoptotic and 0.5 (± 0.21) on the apical side were apoptotic. There was no significant difference between these measures. Examining neutrophils recovered from 24 hour RSV infected AECs, 6.1% (± 3.11) of those on the basolateral side were apoptotic, 5.28% (± 2.03) of those adhered were apoptotic and 11.4 (± 1.94) on the apical side were apoptotic. There was no significant difference between these measures. Comparing neutrophils recovered from 72 hour RSV infected AECs, 14.63% (± 6.7) of those on the basolateral side were apoptotic, 0.8 (± 0.31) of those adhered were apoptotic and 9.49 (± 2.66) on the apical side were apoptotic. There was no significant difference in proportion apoptotic between basolateral and adhered neutrophils, however neutrophils on the apical side of 72 hour infected AECs showed significantly greater proportions of apoptosis in comparison to those adhered with a p value of 0.0313.

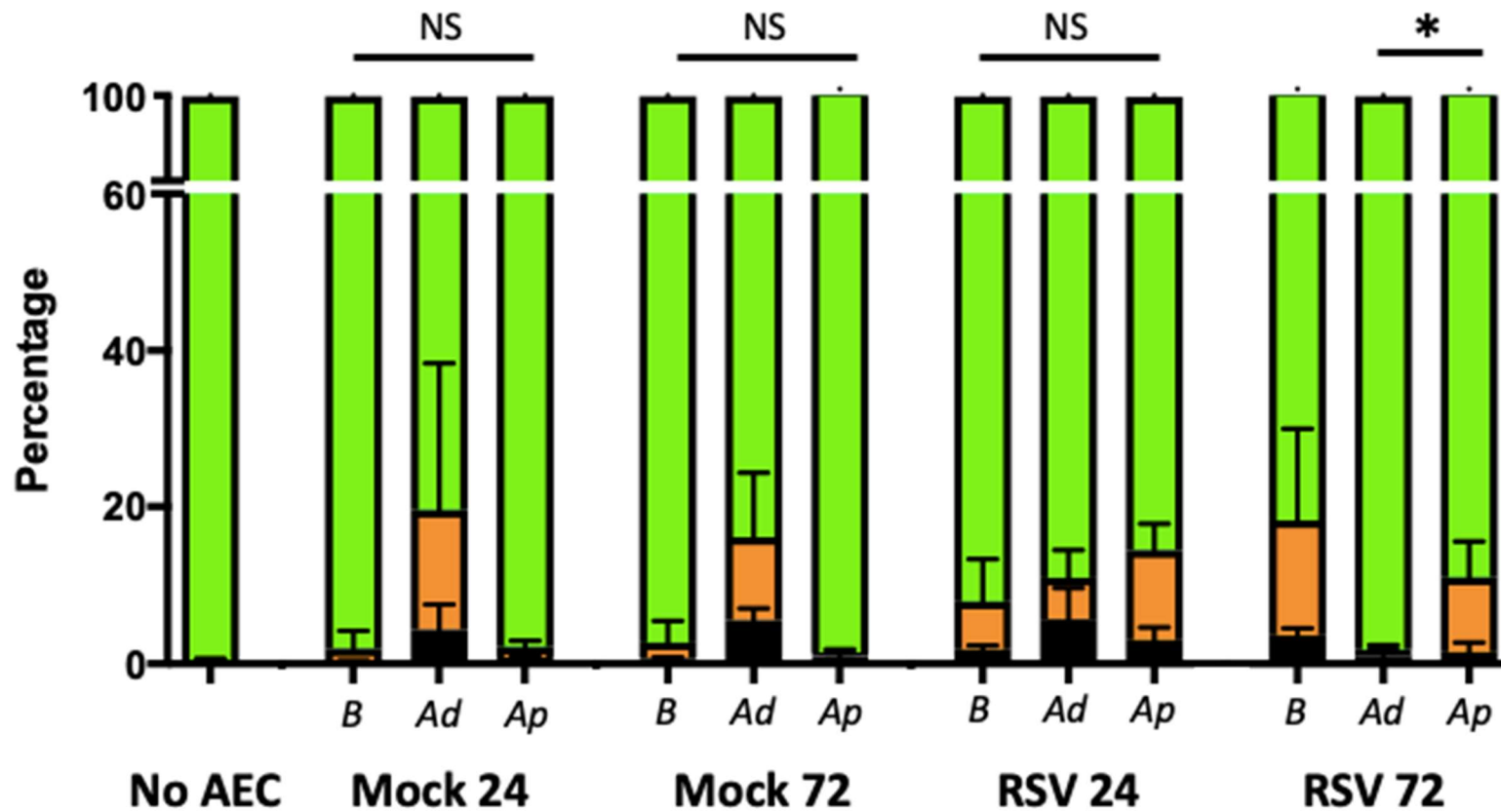


Figure 4-15 Proportion of neutrophils live, apoptotic and dead after 1 hour transepithelial migration across RSV and mock infected AECs.

Neutrophils were applied to the basolateral side of ciliated epithelial cultures infected with RSV or mock for 24 or 72 hours prior to migration. After 1 hour incubation, neutrophils remaining basolateral (B), apical (Ap) and adherent (Ad) to the ciliated epithelium were isolated and stained for

apoptosis using Annexin V-FITC and Propidium Iodide. Neutrophils incubated in media alone was used as a control (non AEC). Relative proportions of neutrophils which were live (green), apoptotic (orange) and dead (black). (* $p < 0.05$ difference with Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons.) N=5.

4.6 Discussion

In **Chapter 2**, I described and optimised an *in vitro* model of ciliated airway enabling the study of neutrophil transepithelial migration during RSV infection. Previous studies into the apoptosis of neutrophils during RSV infection have been based on clinical sampling, which poses significant limitations as discussed in the introduction of this chapter. This is the first study of its kind where we can continuously measure neutrophil viability and apoptosis during an infection comprising RSV infected ciliated AECs and autologous neutrophils.

There are many factors at play in the infected airway of infants with RSV infection which could influence neutrophil survival, we can broadly separate them into the virus itself, the airway epithelium and the host inflammatory response to infection.

Examining the effect of the virus itself, I showed prolonged incubation of neutrophils with RSV reduced neutrophil apoptosis and increased neutrophil viability. Likewise, I saw the same effect was precipitated by incubating neutrophils with purified RSV G protein. However, on attempting to block this interaction, it was demonstrated a specific RSV G-protein blocking antibody had the same apparent reversal effect on neutrophil apoptosis as the isotype control.

When incubating neutrophils with supernatants collected from ciliated AECs infected with RSV, it was shown apoptosis was reduced and viability increased, compared to neutrophils incubated with media alone. It is important to note in these supernatants collected from RSV infected AECs, viral particles were presumably present but infectious replicating RSV was not as demonstrated in **Figure 2-4**. Apoptosis was also reduced, and viability increased in neutrophils incubated with mock infected ciliated AEC supernatants. This could indicate that the respiratory epithelium produces soluble factors which prolong the life of immune cells as demonstrated here and by Jones et al (225), independently of, but enhanced by RSV infection.

To investigate the interaction of neutrophils with RSV infected AECs, neutrophils were combined with the apical surface of RSV infected ciliated AECs grown upright on a small pore size so as not to allow migration. Conversely to what was seen in my experiments with RSV, G protein and cell culture supernatants, it was shown after 1 and 4 hours incubation a greater proportion of neutrophils incubated of RSV infected AECs were apoptotic compared to those

incubated with mock controls. This was unexpected but is consistent with Wang et al, where a greater proportion of recovered neutrophils from the airways were apoptotic (223).

After this, an inverted transepithelial migration model was used to investigate neutrophil migration during RSV infection through differentiated ciliated AECs in a physiological basolateral to apical direction. From this, I recovered three populations of neutrophils during migration. Those which are found on the basolateral side of the AECs, those which migrate across the AECs and remain in adherence with to it, and those which migrate across the AECs and dissociate from it on the apical side (See **Figure 4-11**). In **Chapter 3** I investigated the difference in numbers of neutrophils found apically and adhered to mock and RSV infected AECs and the context of this on my apoptosis findings is discussed further in **Chapter 7** General Discussion.

Of the neutrophils recovered from the basolateral side of AECs, the proportion of neutrophils which were apoptotic and the proportion which were viable were significantly higher in RSV infected AECs compared to mock infected AECs. This was in keeping with what I observed in the non-migration AEC model. Interestingly this also suggests AECs may be secreting factors into the basolateral compartment as well as apically, which are likely to be different in composition and affected by RSV infection. This hypothesis is explored more in **Chapter 5**. As discussed in **Chapter 3**, I suggested the potential for reverse migration of neutrophils in the trans-epithelial migration model, and it may be that some neutrophils recovered on the basolateral side of AECs have in fact already traversed through the AECs to the apical side and returned.

Of the neutrophils which migrated through and dissociated from the epithelium the proportion of neutrophils which were apoptotic was significantly higher and the proportion which were viable significantly lower in RSV infected AECs compared to mock infected AECs. This again was in keeping with what I saw in my non-migration model. As I demonstrated in **Chapter 3**, I know fewer neutrophils migrate to the apical side of mock infected ciliated AECs than those infected with RSV. This finding could be a consequence of neutrophil exhaustion. After recruitment to the site of infection, neutrophils migrate into the airway, fulfill their purpose, become obsolete and apoptose (231,232).

Examining the neutrophils which remained adherent to ciliated AECs, I demonstrated that in RSV infected AECs a smaller proportion of those adherent were apoptotic and a greater proportion were viable in comparison to mock infected controls. As demonstrated in **Chapter 3**, I know greater numbers of neutrophils adhere to RSV infected AECs than adhere to mock-infected AECs, during migration and this finding, which is the converse of what happens in the migrated neutrophils may simply be a consequence of the neutrophils which are starting to apoptose, dissociating from the AECs as they do so. If these neutrophils remain bound to the AECs after migrating through the RSV infected AECs from the basolateral side and do not apoptose, what mechanism is keeping them viable?

It is possible that the mechanical interaction of neutrophil and AECs is driving prolonged neutrophil survival, and this attachment has been demonstrated to be mediated in part by ICAM-1 on epithelial cells and LFA-1 on neutrophils previously(185). However, if this were the sole determinant to neutrophil survival, I would expect to see a higher proportion of apoptosis in neutrophils which migrate then dissociate as the binding driving survival is broken. However, it may be a secondary effect of this adherence instead. By adhering neutrophils in close proximity to the epithelium, neutrophils are exposed to much higher concentrations of cytokines secreted by the airway epithelial cells and/or much higher concentrations of new RSV virions. RSV infected AECs are also known to expel a secretory form of RSV G protein, which is a truncated version of entire (membrane bound) RSV G protein, similar to that I showed in this chapter is likely to contribute to prolonged neutrophil survival(233).

The data presented here has clearly demonstrated the benefit of in-vitro models for the investigation of clinical phenomena and has allowed us to advance from a perspective of knowing there are many live neutrophils in the airways of children with RSV Bronchiolitis, to profiling neutrophil viability and apoptosis as a dynamic consequence of the neutrophilic immune response as it progresses over time. However, the extrapolation of these findings to the clinical situation in RSV bronchiolitis is limited because I used adult neutrophils and AECs to investigate a disease which almost solely affects infants. Nonetheless all age-groups experience RSV infection, although the severe consequences are predominantly found in the

very young and very old. These hypotheses are further explored using a cord blood neutrophils in **Chapter 6**.

Chapter 5 Altered neutrophil activation during RSV infection

5.1 Introduction

In **Chapter 2**, I described an optimised transepithelial migration assay for the *in vitro* assessment of the interaction between the RSV infected airway and the neutrophil. After examining neutrophil chemotaxis and migration in **Chapter 3**, I will now progress to exploring neutrophil activation in the *in vitro* model of RSV Bronchiolitis. The term neutrophil 'activation' is widely accepted to be dependent on a prior 'priming' step which was discussed earlier in **Chapter 1**, and constitutes initiation of degranulation and reactive oxygen species generation (122,234). However, how this is measured in scientific studies varies across the board, with several cell surface markers and granule products measured as a correlate of neutrophil activation(71,235). In this study I examine both membrane-bound receptors correlating with change in neutrophil function as well as granule-based factors which can be secreted exogenously or remain in internal lysozymes for the process of internalised pathogen destruction. Mechanisms and pathways of neutrophil activation in bacterial and fungal infections have been well described (236,237), but less is known about the mechanisms or consequences of neutrophil activation in viral infections such as RSV.

In clinical studies, Halfhide et al demonstrated that neutrophils retrieved from the blood and airways of infants with RSV bronchiolitis showed greater expression of CD11b than neutrophils from the blood or airways of healthy age matched control children(75). This study used membrane-based expression markers as a correlate of neutrophil activation, as well as a granular product MPO, which was shown to be raised in the BAL of children with RSV bronchiolitis in comparison to controls. This study also found a difference in TLR4 expression on blood neutrophils, with term infants with RSV bronchiolitis showing significantly lower TLR4 expression than control children.

Cortjens et al conducted a study in The Netherlands of neutrophil activation in infants with severe viral respiratory infection(238). Although this study was investigating other viruses as well as RSV, it is of interest to this study, as Cortjens proposes the existence of subsets of neutrophils with different activation profiles which enhance or suppress inflammation. The

infants in this study, with severe viral infection appeared to lack the 'suppressive' subset of neutrophil in their blood which suggests that although some inflammation may be necessary for viral clearance, allowing it to continue unchecked may lead to worsening of disease(238). Neutrophil activation in this study was determined by membrane-based markers CD16 and CD62L.

In a laboratory model of RSV bronchiolitis, Herbert et al have shown that neutrophils which migrate across an RSV infected differentiated human nasal epithelial cell culture show greater expression of CD11b than those traversing 'mock' infected differentiated human AEC cultures(185).

These studies suggest that RSV infection modulates the "activation" of neutrophils and therefore their subsequent ability for pathogen destruction, but there are a number of ways this could be mediated. Although suggested by Kirsebom et al in their mouse model work, there is no definitive evidence save one report, that RSV itself replicates inside neutrophils during natural infection(239). However, neutrophils retrieved from the blood and airways of infants with RSV bronchiolitis have been previously shown to be positive for RSV RNA by PCR, indicating either RSV virions or fragments of RNA have been taken up inside neutrophils(240). Furthermore, it has been shown that in the same study that RSV mRNA transcripts are detectable in peripheral blood neutrophils of children with bronchiolitis but it is not clear by what mechanism this uptake occurs(240). It is possible that encountering live virus itself initiates neutrophil priming or influences subsequent neutrophil activation, more specifically by means of RSV F glycoprotein which has been shown to interact with the neutrophil surface marker TLR4(71). Mechanical binding to the infected epithelium during migration by means of surface integrins may also play a part, as may the inflammatory environment of the airway – either generated by the neutrophils themselves or the infected airway epithelium.

On degranulation, neutrophils secrete the granular protease neutrophil elastase (NE), which has previously been found to be present at high concentration in the airway secretions of infants with RSV bronchiolitis(241). NE is a multipotent enzyme which is able to degrade a wide variety of structural and immune regulated protein not limited to elastin after which it is named, but also fibronectin, α_1 -antitrypsin and tissue resident inhibitors of

metalloproteinase enzymes(122,126). The effect of excessive or over exuberant release of NE, and other granular protease release, may result in collateral damage to immature airway and has been hypothesised to have a role in long term respiratory sequelae of bronchiolitis, inducing airways hyperreactivity and hyperplasia of goblet cells of the bronchioles(242,243).

In this study I will measure membrane bound correlates of neutrophil activation, CD11b and CD64 as well as secretory factors neutrophil elastase (NE) and myeloperoxidase (MPO). CD11b is an integrin expressed on neutrophils, important for transendothelial and transepithelial migration. It is known to interact with ICAM-1, expressed on the apical side of the airway epithelium, mediating attachment of neutrophils(137). Herbert et al also described that RSV infection of ciliated airway epithelial cells (AECs) increases adherence of neutrophils and blocking LFA-1, present on neutrophils, reduces the adherence of neutrophils to RSV infected AECs (185). It is hypothesised LFA-1 also binds to ICAM-1 on the apical surface of AECs, expression of which has been shown previously to be upregulated by RSV infection of the airway(185,244). CD64 is a high affinity Fc receptor, specific to the Fc γ section of the IgG heavy chain, and binds IgG1, IgG3 and aggregated IgG(245). This receptor mediates phagocytosis of bacteria and other microorganisms(246). It is present on neutrophils at low levels constitutively, but is increased during 'activation' of neutrophils when exposed to pro inflammatory markers such as IFN- γ (247). Because of this function, CD64 expression measured on neutrophils isolated from cord blood has also been evaluated as a useful clinical correlate of neonatal sepsis risk, as other well known markers of sepsis are raised by parturition(248).

Understanding which components in the RSV infected airway contribute to changes in neutrophil activation, in particular which is the overriding factor, will contribute to our understanding of the role neutrophils play during RSV infection of the airway.

5.2 Hypotheses

RSV, the supernatants collected from RSV infected AEC cultures and purified RSV proteins increase the activation state of adult neutrophils when cultured together. Furthermore, migration of neutrophils through a RSV infected AECs increases their activation state.

5.3 Aims

1. Determine the effect of live RSV alone on neutrophil activation markers
2. Determine the effect of supernatants collected from RSV infected AECs alone on neutrophil activation markers
3. Determine the effect of RSV F or G protein alone on neutrophil activation markers
4. Determine the activation status of neutrophils during transepithelial migration through RSV infected AECs

5.4 Specific methods

Adult neutrophils from healthy donors were used throughout this Chapter. GFP RSV A2 was propagated and purified as described in **Section 2.3**, Supernatants collected from RSV infected AECs were prepared as stored as described in **Section 2.6.3**. Purified RSV F or G protein were obtained as described in **Section 2.2.2**. Neutrophil transepithelial migration experiments in this chapter were performed as described in **Section 2.6.2** using ciliated AECs matured at ALI for 28 days. All AECs used in this chapter are ciliated AECs. Mock infected AECs with RSV infected AEC supernatant was used as a control (RSV Sup). In all experiments, 5×10^5 purified neutrophils prepared with 1% autologous serum were used per infection condition. Neutrophil activation was examined by flow cytometry staining as described in **Section 2.7.3**. Where neutrophils in suspension with AECs were analysed, neutrophils were first distinguished from epithelial cells by antibody staining with CD11b-APC conjugate as described in **Section 2.7.4.1**. Statistical analysis was performed using a linear model of mixed effects using RStudio (code available in **Appendix 7**), where intradonor variation was to be controlled for, or Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons.

5.5 Results

5.5.1 Incubation with live RSV increases neutrophil activation markers

To start to understand which components of the RSV infected airway are influencing neutrophil activation and to what contributing extent, firstly I examined how incubation for 1 hour with purified RSV affected expression of CD11b, NE and MPO. Results are presented as mean (\pm SEM).

After one hour of incubation, the mean CD11b expression density on neutrophils exposed to RSV MOI 0.1, RSV MOI 1 and RSV MOI 5 was 127.3 (\pm 2.02), 133.3 (\pm 3.84) and 151 (\pm 4.51) respectively. Mean CD11b expression on neutrophils exposed to media alone was 238.3 (\pm 27.9). There was a significant difference between neutrophils incubated with RSV MOI 0.1, 1 and 5 in comparison to the media only control with p values of 0.0015, 0.002 and 0.0005 respectively.

The mean NE expression density on neutrophils exposed to RSV MOI 0.1, RSV MOI 1 and RSV MOI 5 was 19.03 (\pm 2.1), 23.9 (\pm 2.98) and 20.2 (\pm 1.6) respectively. Mean NE expression on neutrophils exposed to media alone was 15.7 (\pm 0.95). Mean NE expression density was significantly increased on neutrophils incubated with RSV MOI 1 (23.9 \pm 2.98) in comparison with those exposed to media alone (15.7 \pm 0.95) with a p value of 0.0301. There was no significant difference in NE expression density between neutrophils incubated with RSV MOI 0.1 or RSV MOI 5 and media alone.

Conversely, the mean MPO expression density on neutrophils exposed to RSV MOI 0.1, RSV MOI 1 and RSV MOI 5 was 22.46 (\pm 1.97), 18.7 (\pm 1.1) and 23.03 (\pm 1.77) respectively. Mean MPO expression on neutrophils exposed to media alone was 13.27 (\pm 0.84). There was a significant difference between mean MPO expression density on neutrophils incubated with RSV MOI 0.1 (22.46 \pm 1.97), RSV MOI 1 (18.7 \pm 1.1) and RSV MOI 5 (23.03 \pm 1.77) in comparison with those exposed to media alone (13.27 \pm 0.84) with p values of 0.0041, 0.0373 and 0.003 respectively.

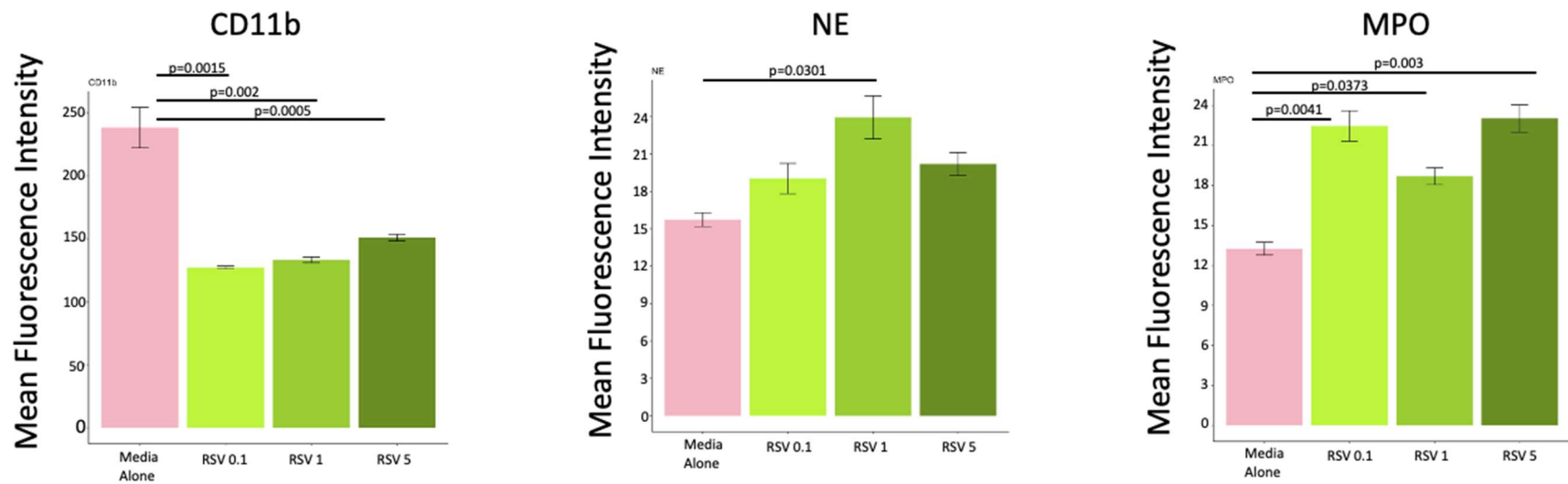


Figure 5-1 Neutrophil activation markers after incubation with live RSV concentrations.

(Top) Mean fluorescence intensity of CD11b, Neutrophil Elastase (NE) and Myeloperoxidase (MPO) in neutrophils incubated with media alone, RSV MOI 0.1, 1 and 5 for 1 hour. (Bottom) Fold change in expression density of CD11b, NE and MPO as calculated from the media alone control. Bars indicate standard error of the mean. A linear model of mixed effects was used to assess significance, where significance was found – the p value is indicated on the bar chart. N=6.

5.5.2 RSV F and G glycoproteins have no effect on neutrophil activation markers

Next, to investigate whether these changes were mediated by viral proteins alone I examined how incubation for 1 hour with purified RSV surface glycoproteins, F and G affected expression of CD11b, NE and MPO.

After one hour of incubation, the mean CD11b expression density on neutrophils exposed to G protein 1 μ g/ml, F protein 1 μ g/ml, G protein 10 μ g/ml and F protein 10 μ g/ml was 224.3 (\pm 11.1), 205.3 (\pm 20.48), 239.67 (\pm 25.82) and 158 (\pm 61.3) respectively. Mean CD11b expression on neutrophils exposed to media alone was 231.67 (\pm 28.26). There was a no significant difference between these measures.

The mean NE expression density on neutrophils exposed to G protein 1 μ g/ml, F protein 1 μ g/ml, G protein 10 μ g/ml and F protein 10 μ g/ml was 18.9 (\pm 2.23), 21.57 (\pm 2.29), 18.97 (\pm 3.39) and 18.83 (\pm 3.29) respectively. Mean NE expression on neutrophils exposed to media alone was 14.63 (\pm 0.67). There was a no significant difference between these measures. NE expression on neutrophils showed a trend of increase after incubation with F protein 1 μ g/ml and 10 μ g/ml compared to media alone, but this increase was not significant. The same trend was observed after incubation with G protein 1 μ g/ml and 10 μ g/ml compared to media alone but, again, this difference was not significant.

The mean MPO expression density on neutrophils exposed to G protein 1 μ g/ml, F protein 1 μ g/ml, G protein 10 μ g/ml and F protein 10 μ g/ml was 10.59 (\pm 0.66), 11 (\pm 0.44), 10.67 (\pm 1.08) and 10.85 (\pm 0.73) respectively. Mean MPO expression on neutrophils exposed to media alone was 10.0 (\pm 0.66). There was a no significant difference between these measures.

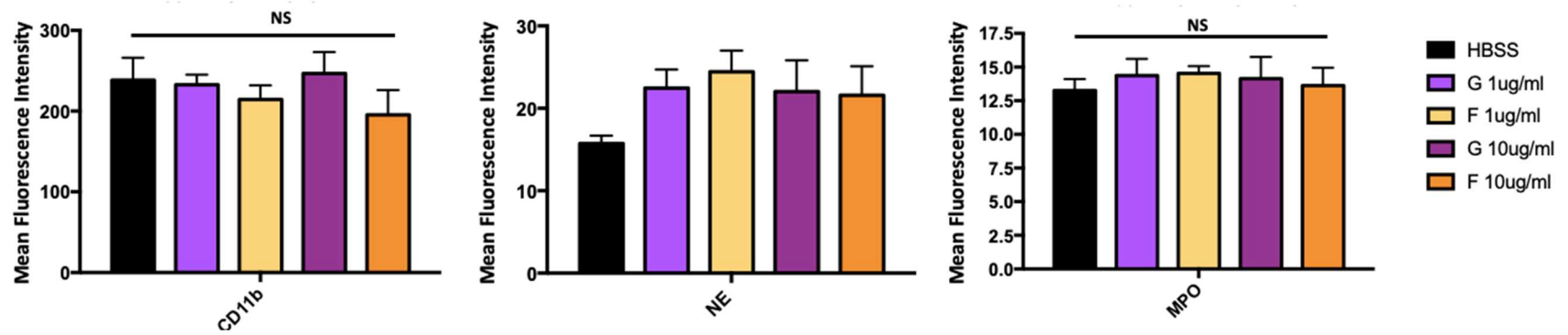


Figure 5-2 Neutrophil activation markers after incubation with RSV F and G protein.

(Top) Mean fluorescence intensity of CD11b, Neutrophil Elastase (NE) and Myeloperoxidase (MPO) in neutrophils incubated with media alone (HBSS), purified RSV G protein at a concentration of 1 μ g/ml or 10 μ g/ml or purified RSV F protein at a concentration of 1 μ g/ml or 10 μ g/ml.

(Bottom) Fold change in expression density of CD11b, NE and MPO as calculated from the media alone control.

Bars indicate standard error of the mean. A linear model of mixed effects was used to assess significance, where significance was found – the p value is indicated on the bar chart. NS = not significant. N=6.

5.5.3 Supernatants from RSV infected ciliated primary AEC cultures increase neutrophil activation

To investigate whether the soluble inflammatory products of the airway influenced neutrophil expression of CD11b, NE and MPO, neutrophils were incubated with apical supernatants collected from primary ciliated AECs previously infected with RSV for 24 or 72 hours or mock infection.

After one hour of incubation, the mean CD11b expression density on neutrophils exposed to supernatant from AECs infected with mock for 24 or 72 hours was 254.3 (\pm 37.5) and 237.7 (\pm 22.8) respectively. Mean CD11b expression density on neutrophils exposed to supernatant from AECs infected with RSV for 24 or 72 hours was 300.67 (\pm 16.38) and 352.3 (\pm 32.45) respectively. Mean CD11b expression on neutrophils exposed to media alone was 238.3 (\pm 27.9). There was a no significant difference between these measures. There was a significant difference in mean CD11b expression between neutrophils incubated with media alone 238.3 (\pm 27.9) and neutrophils incubated with supernatant from AEC cultures infected with RSV for 24 hours 300.67 (\pm 16.38) with a p value of 0.0327. Similarly, there was a significant difference in mean CD11b expression between neutrophils incubated with media alone 238.3 (\pm 27.9) and neutrophils incubated with supernatant from AECs infected with RSV for 72 hours 352.3 (\pm 32.45) with a p value of 0.0015.

Mean NE expression density on neutrophils exposed to supernatant from AECs infected with mock for 24 or 72 hours was 25.06 (\pm 4.78) and 24.96 (\pm 3.47) respectively. Mean NE expression density on neutrophils exposed to supernatant from AECs infected with RSV for 24 or 72 hours was 30 (\pm 4.46) and 38.83 (\pm 2.46) respectively. Mean NE expression on neutrophils exposed to media alone was 15.73 (\pm 0.95). There was a significant difference in mean NE expression between neutrophils incubated with media alone 15.73 (\pm 0.95) and neutrophils incubated with supernatant from AECs infected with RSV for 24 hours 25.06 (\pm 4.78) with a p value of 0.0208. Likewise, there was a significant difference in mean NE expression between neutrophils incubated with media alone 15.73 (\pm 0.95) and neutrophils incubated with supernatant from AECs infected with RSV for 72 hours 24.96 (\pm 3.47) with a p

value of 0.0017. In addition to this, there was a significant difference in NE expression of neutrophils incubated with supernatants from 72 hour RSV infected AEC cultures compared to those incubated with mock 72 hour infected AEC cultures, $p=0.0316$.

Mean MPO expression density on neutrophils exposed to supernatant from AECs infected with mock for 24 or 72 hours was $21.97 (\pm 0.17)$ and $21.23 (\pm 1.77)$ respectively. Mean MPO expression density on neutrophils exposed to supernatant from AECs infected with RSV for 24 or 72 hours was $23.7 (\pm 0.78)$ and $22.46 (\pm 1.17)$ respectively. Mean MPO expression on neutrophils exposed to media alone was $13.26 (\pm 0.84)$. There was a significant difference in mean MPO expression between neutrophils incubated with supernatant from AECs infected with RSV for 24 hours ($23.7 (\pm 0.78)$) and those incubated with media alone ($13.26 (\pm 0.84)$) with a p value of <0.0001 . Likewise, there was a significant difference in mean MPO expression between neutrophils incubated with supernatant from AECs infected with RSV for 72 hours ($22.46 (\pm 1.17)$) and those incubated with media alone ($13.26 (\pm 0.84)$) with a p value of <0.0001 . The same was true after incubation with supernatants collected from mock infected AEC cultures, infected for 24 hours ($21.97 (\pm 0.17)$) and 72 hours ($21.23 (\pm 1.77)$), compared to the media alone ($13.26 (\pm 0.84)$) with p values of 0.0005 and 0.0008 respectively.

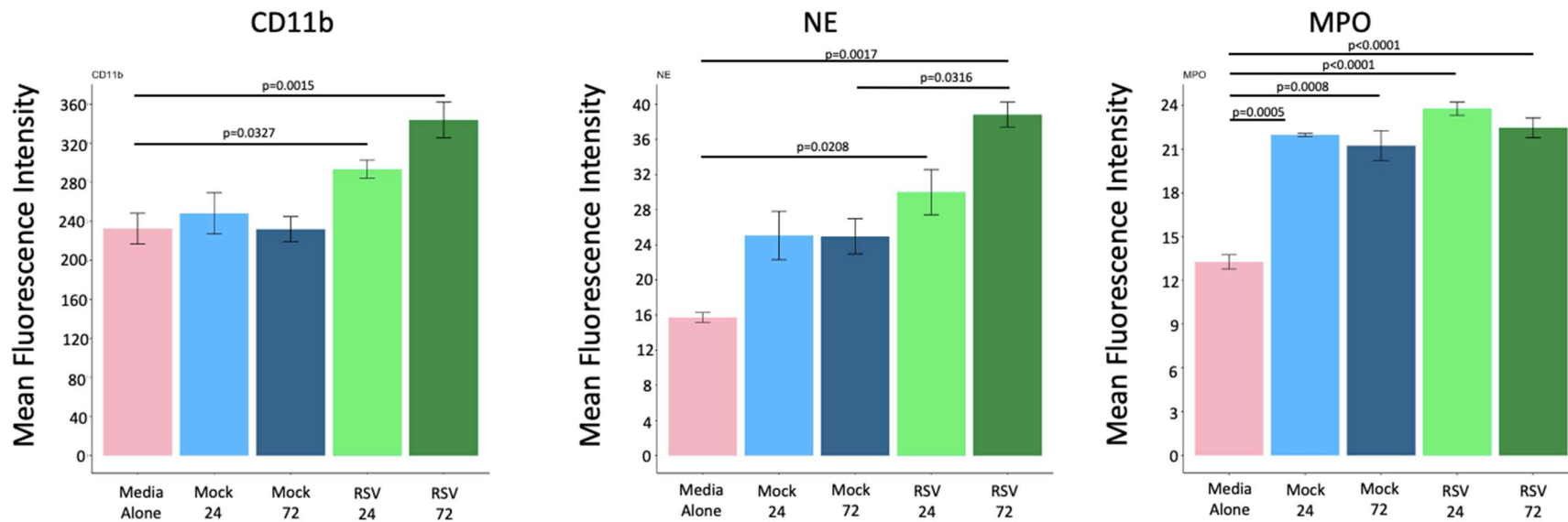


Figure 5-3 Neutrophil activation markers after incubation with supernatants from cultured primary ciliated AECs.

(Top) Mean fluorescence intensity of CD11b, Neutrophil Elastase (NE) and Myeloperoxidase (MPO) in neutrophils incubated with media alone, supernatants from Mock infected AEC cultures "infected" for 24 hours (M24) or 72 hours (M72) or supernatants from RSV infected AEC cultures infected for 24 hours (R24) OR 72 hours (R72). (Bottom) Fold change in expression density of CD11b, NE and MPO as calculated from the media alone control.

Bars indicate standard error of the mean. Mixed and random effects model were used to assess significance, where significance was found – the p value is indicated on the bar chart. NS = not significant. # = in comparison to the media only control. N=6.

5.5.4 Neutrophil activation during transepithelial migration through the RSV infected ciliated AECs

5.5.4.1 Migration through RSV infected ciliated AECs increases neutrophil CD11b and CD64 expression

Having quantified the numbers of neutrophils migrating to the apical side of ciliated AECs and adhering to ciliated AECs in **Chapter 3**, I then wanted to examine the activation of neutrophils basolateral, adherent and apical to RSV infected AECs following transepithelial migration.

The 72 hour timepoint of AEC infection was chosen as that is the timepoint I saw the greatest adherence of neutrophils to the epithelium. All infection durations of AECs herein will be 72 hour unless expressly indicated otherwise. CD64 was also chosen to be examined in neutrophil migration experiments as it has been described in previous *in vivo* models as the most specific marker of neutrophil activation in the lungs(239).

After a 1 hour transepithelial migration assay, adult neutrophils isolated from the basolateral side of mock infected AECs had mean CD11b expression of 524 (± 103.5); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean CD11b expression of 682 (± 29.3) and for neutrophils recovered from the basolateral surface RSV infected AECs, mean CD11b expression was 1097 (± 94). There was no significant difference in CD11b expression on neutrophils on the basolateral side of Mock and RSV Sup AECs in comparison to those incubated with media alone which was 138 ± 12.4 . Neutrophils recovered from the basolateral side of RSV infected AEC cultures showed significantly greater expression of CD11b (1097 ± 94) in comparison to those recovered from basolateral side of mock infected AEC cultures (524 ± 103.5) with a p value of 0.0012.

Adult neutrophils recovered adherent to mock infected AECs had mean CD11b expression of 4187 (± 1577); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean CD11b expression of 6380 (± 2296) and for neutrophils recovered from the adherent to RSV infected AECs, mean CD11b expression was 11976 (± 601). There was no significant difference in CD11b expression between those adhered to the Mock (4187 ± 1577) and RSV Sup (6380 ± 2296) AECs. Neutrophils adherent to RSV infected AECs showed

significantly greater expression of CD11b (11976 ± 601) in comparison to those adherent to mock infected AEC cultures (4187 ± 1577) with a p value of 0.01213. Neutrophils recovered adherent to RSV infected AECs (11976 ± 601) showed significantly greater expression of CD11b in comparison to neutrophils isolated from basolateral side of RSV infected AECs (1097 ± 94), with a p value of $p < 0.0001$.

Adult neutrophils isolated from the apical compartment had mean CD11b expression of 9791 (± 2154); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean CD11b expression of 19515 (± 728) and for neutrophils recovered from the apical surface of RSV infected AECs, mean CD11b expression was 28588 (± 3853). Mean CD11b expression was significantly greater in neutrophils which had migrated through RSV infected AECs (28588 ± 3853) in comparison to those which had migrated through mock infected AECs (9791 ± 2154) with a p value of 0.01462. Neutrophils recovered apically showed significantly greater mean expression of CD11b in comparison to neutrophils recovered from their respective membrane adherent compartments, with p values of 0.0313, 0.00607 and 0.01797 (Mock, RSV, RSV Sup).

Overall analysis using linear model of mixed effects to control for neutrophil donor variation found there is an interaction between infection and neutrophil location within the cultured AECs, such that infection mediates CD11b expression. Models used are available in **Appendix 7**. Examining all groups of neutrophils recovered from RSV infected AECs in comparison to those recovered from Mock infected AECs using a linear mixed-effects model, neutrophils recovered from RSV infected AECs showed significantly greater mean CD11b expression with a p-value of 0.0072. In the same analysis, comparison of neutrophils recovered from mock infected AECs and RSV Sup groups returned no significant difference in mean CD11b expression.

After 1 hours transepithelial migration assay, adult neutrophils isolated from the basolateral side of mock AECs showed mean CD64 expression of 2014 (± 247); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean CD64 expression of 2279 (± 100.4) and for neutrophils recovered from the basolateral surface RSV infected AECs, mean

CD64 expression was 4430 (\pm 502). There was no significant difference in mean CD64 expression on neutrophils on the basolateral side of Mock, RSV Sup or RSV infected AECs.

Adult neutrophils recovered adherent to mock infected AECs had mean CD64 expression of 45000 (\pm 1720); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean CD64 expression of 4532 (\pm 1065) and for neutrophils adherent to RSV infected AECs, mean CD64 expression was 43103 (\pm 5703). There was no significant difference in CD64 expression between neutrophils adhered to RSV or Mock infected AECs. Neutrophils adherent to RSV Sup AEC cultures showed significantly lesser expression of CD64 (4532 \pm 1065) in comparison to those adhered to Mock (45000 \pm 1720) or RSV (43103 \pm 5703) infected epithelium with p values of <0.0001 and 0.009 respectively. Neutrophils recovered adherent to the mock infected AECs showed significantly greater expression of CD64 in comparison to neutrophils recovered basolateral to mock infected AECs (2014 \pm 247), with a p value of 0.00240. Likewise, neutrophils recovered adherent to RSV infected AECs (43103 \pm 5703) showed significantly greater expression of CD64 in comparison to neutrophils recovered basolateral to RSV infected AECs compartment (4430 \pm 502), with a p value of 0.0352. There was no significant difference in CD64 expression between neutrophils adhered to the RSV Sup AECs and those recovered basolateral to RSV Sup AECs.

Adult neutrophils isolated from the apical compartment had mean CD64 expression of 49969 (\pm 12507); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean CD64 expression of 58513 (\pm 13302) and for neutrophils recovered from the apical surface of RSV infected AECs, mean CD64 expression was 58599 (\pm 11316). There was no significant difference in mean CD64 expression between neutrophils recovered from Mock, RSV Sup or RSV Infected AECs. There was no significant difference in CD64 expression of neutrophils recovered from the apical side of Mock, and RSV infected AECs in comparison to neutrophils from their respective membrane adherent compartments. However, neutrophils from the apical side of RSV Sup AECs (58513 \pm 13302) showed significantly greater expression of CD64 than adherent neutrophils under the same conditions (4532 \pm 1065) with a p value of 0.01554.

Overall analysis using linear model of mixed effects to control for neutrophil donor variation found there is no significant interaction between infection and neutrophil location within the culture, suggesting that neither location within the culture (ie basolateral, adhered, apical) nor infection mediates CD64 expression.

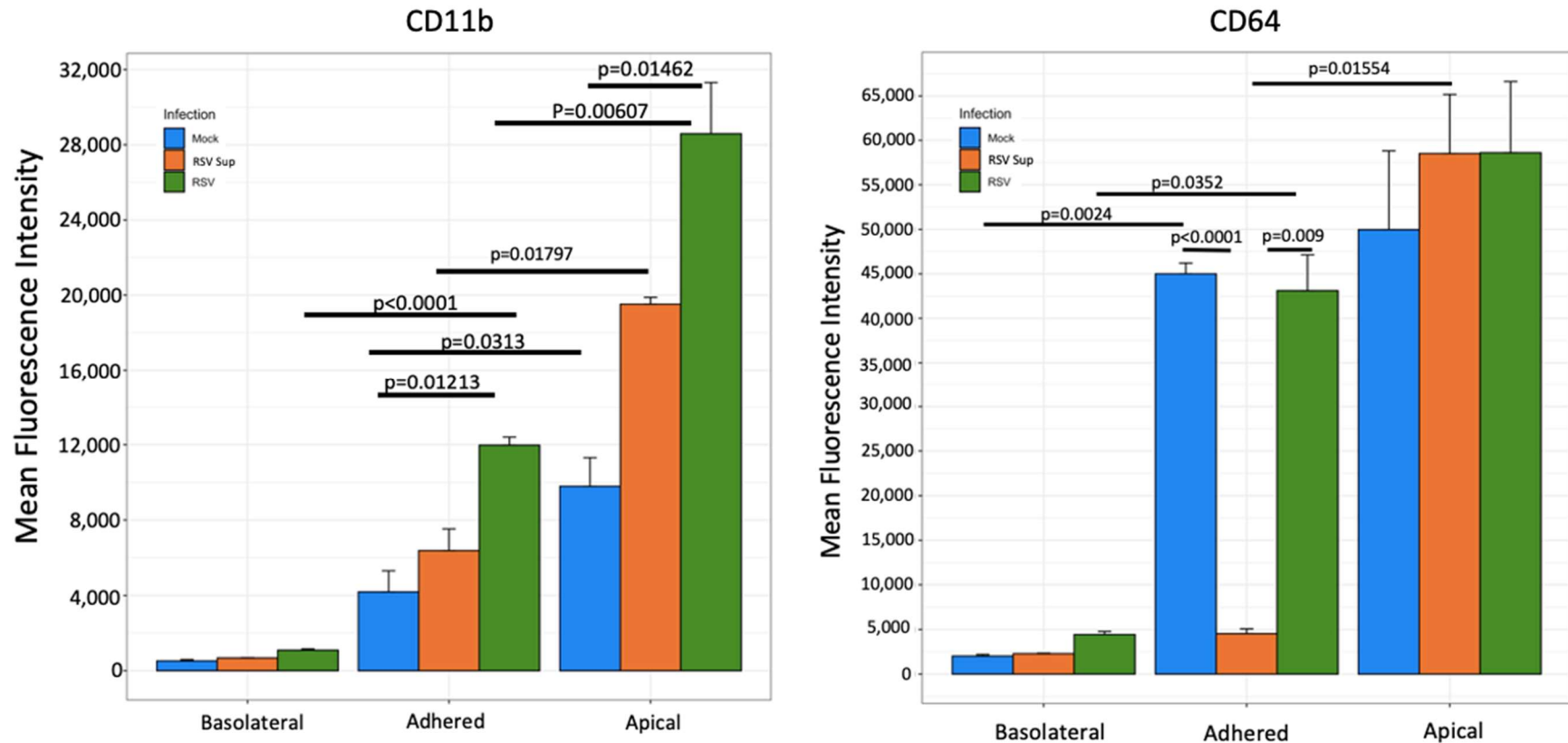


Figure 5-4 Neutrophil activation markers CD11b and CD64 after transepithelial migration in different sites, during transepithelial migration of neutrophils through RSV infected ciliated AECs.

Mean fluorescence intensity of CD11b (left) and CD64 (right) in neutrophils incubated with media alone (No AEC), and those recovered from transepithelial migration assays through RSV infected ciliated AECs infected for 72 hours (RSV), Mock infected control epithelium (Mock) or Mock infected epithelium with the supernatant recovered from RSV infected ciliated culture on the apical side (RSV Sup) as a control. Graphs are separated to distinguish neutrophils recovered from the basolateral, adhered and apical (migrated) locations within the transepithelial migration assay.

Bars indicate standard error of the mean. A linear model of mixed effects was used to compare interactions between infection and location groups and control for intradonor variability. Individual comparisons performed with Two-Way ANOVA with pairing and Geiser greenhouse correction. * = in comparison to respective basolateral group # = in comparison to respective adhered group. N=6.

5.5.4.2 Migration through RSV infected ciliated epithelium increases neutrophil elastase and myeloperoxidase expression of neutrophils

After a 1 hour transepithelial migration assay, adult neutrophils isolated from the basolateral side of mock infected AECs had mean NE expression of 5289 (± 1333); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean NE expression of 2558 (± 31.8) and for neutrophils recovered from the basolateral surface RSV infected AECs, mean NE expression was 8153 (± 1673). There was no significant difference between NE expression on neutrophils on the basolateral side of Mock or RSV Sup AECs. Neutrophils recovered from the basolateral side of RSV infected AECs showed significantly greater expression of NE (8153 \pm 1673) in comparison to those recovered from basolateral side of mock infected AECs (5289 \pm 1333) with a p value of 0.0145496.

Adult neutrophils recovered adherent to mock infected AECs had mean NE expression of 34324 (± 3747); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean NE expression of 13006 (± 12432) and for neutrophils recovered from the adherent to RSV infected AECs, mean NE expression was 105435 (± 30991). There was no significant difference in mean NE expression between neutrophils adherent to RSV or Mock infected AECs. Neutrophils adherent to RSV Sup AECs showed significantly lower mean expression of NE (13006 \pm 12432) in comparison to those adherent to Mock (34324 \pm 3747) or RSV infected AECs (13006 (± 12432) with p values of 0.0001 and 0.00903 respectively. Neutrophils adherent to AECs showed significantly greater mean expression of NE in comparison to neutrophils recovered from each respective basolateral compartment, with p values of 0.00151, 0.0214 and 0.0493 (Mock, RSV, RSV Sup).

Adult neutrophils isolated from the apical compartment of mock infected AECs had mean NE expression of 40764 (± 8022); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean NE expression of 44021 (± 4164) and for neutrophils recovered from the apical surface RSV infected AECs, mean NE expression was 295001 (± 113326). NE expression was significantly greater in neutrophils which had migrated through RSV infected AECs (295001 \pm 113326), in comparison to those which had migrated through mock infected epithelium (44021 \pm 4164) with a p value of 0.00666. Neutrophils on the apical side of RSV

infected AECs showed greater mean expression of NE than neutrophils adherent to RSV infected AECs with a p value of 0.072. Similarly, neutrophils on the apical side of RSV Sup AECs showed greater mean expression of NE than neutrophils adherent to RSV Sup AECs with a p value of 0.04947.

Overall analysis using linear model of mixed effects to control for neutrophil donor variation found there is an interaction between infection and neutrophil location within the culture, such that infection mediates NE expression. Models used are available in **Appendix 7**. Examining all groups of neutrophils recovered from RSV infected AEC cultures in comparison to those recovered from Mock infected AECs, neutrophils recovered from RSV infected AEC cultures showed significantly greater NE expression with a p-value of 0.0125.

After a 1 hour transepithelial migration assay, adult neutrophils isolated from the basolateral side of mock infected AECs had mean MPO expression of 5769 (± 889); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean MPO expression of 4451 (± 96.8) and for neutrophils recovered from the basolateral surface RSV infected AECs, mean MPO expression was 9320 (± 1701). There was no significant difference in mean MPO expression between neutrophils on the basolateral side of Mock, RSV Sup or RSV infected AECs.

Adult neutrophils recovered adherent to mock infected AECs had mean MPO expression of 45119 (± 3310); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean MPO expression of 4183 (± 1558) and for neutrophils recovered from the adherent to RSV infected AECs, mean MPO expression was 31333 (± 10036). There was no significant difference in mean MPO expression between neutrophils adherent to RSV or Mock infected AECs. Neutrophils adherent to RSV Sup AECs showed significantly lower expression of MPO (4183 ± 1558) in comparison to those adherent to Mock infected (45119 ± 3310) AECs with a p value of <0.0001 . Neutrophils recovered adherent to RSV (31333 ± 10036) and mock infected AECs (45119 ± 3310) showed significantly greater expression of MPO in comparison to neutrophils recovered from the basolateral side of their respective AEC cultures with p values of 0.00021 and 0.0208. There was no significant difference in MPO expression between

neutrophils adherent to RSV Sup AECs and neutrophils recovered from the basolateral side of RSV Sup AECs.

Adult neutrophils isolated from the apical compartment of mock infected AECs had mean MPO expression of 43472 (\pm 10900); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean MPO expression of 62852 (\pm 15289) and for neutrophils recovered from the apical surface RSV infected AECs, mean MPO expression was 53559 (\pm 11269). There was no significant difference in mean MPO expression between neutrophils isolated apically from Mock, RSV Sup or RSV infected AECs. There was no significant difference in mean MPO expression on neutrophils from the apical side of mock infected AECs in comparison to neutrophils isolated adherent to mock infected AECs. However, neutrophils from the apical side of RSV infected AECs and RSV Sup AECs showed significantly greater expression of MPO than neutrophils adherent to RSV infected AECs and RSV Sup AECs, with p values of 0.02089 and 0.01803 respectively.

Overall analysis using linear model of mixed effects to control for neutrophil donor variation found there is an interaction between infection and neutrophil location within the culture, such that location mediates MPO expression in apical and adherent groups. In comparison to neutrophils recovered on the basolateral side of the culture, neutrophils recovered from the apical side of AEC cultures and those adherent showed significantly greater MPO expression with p values of 0.0001 and 0.0011 respectively.

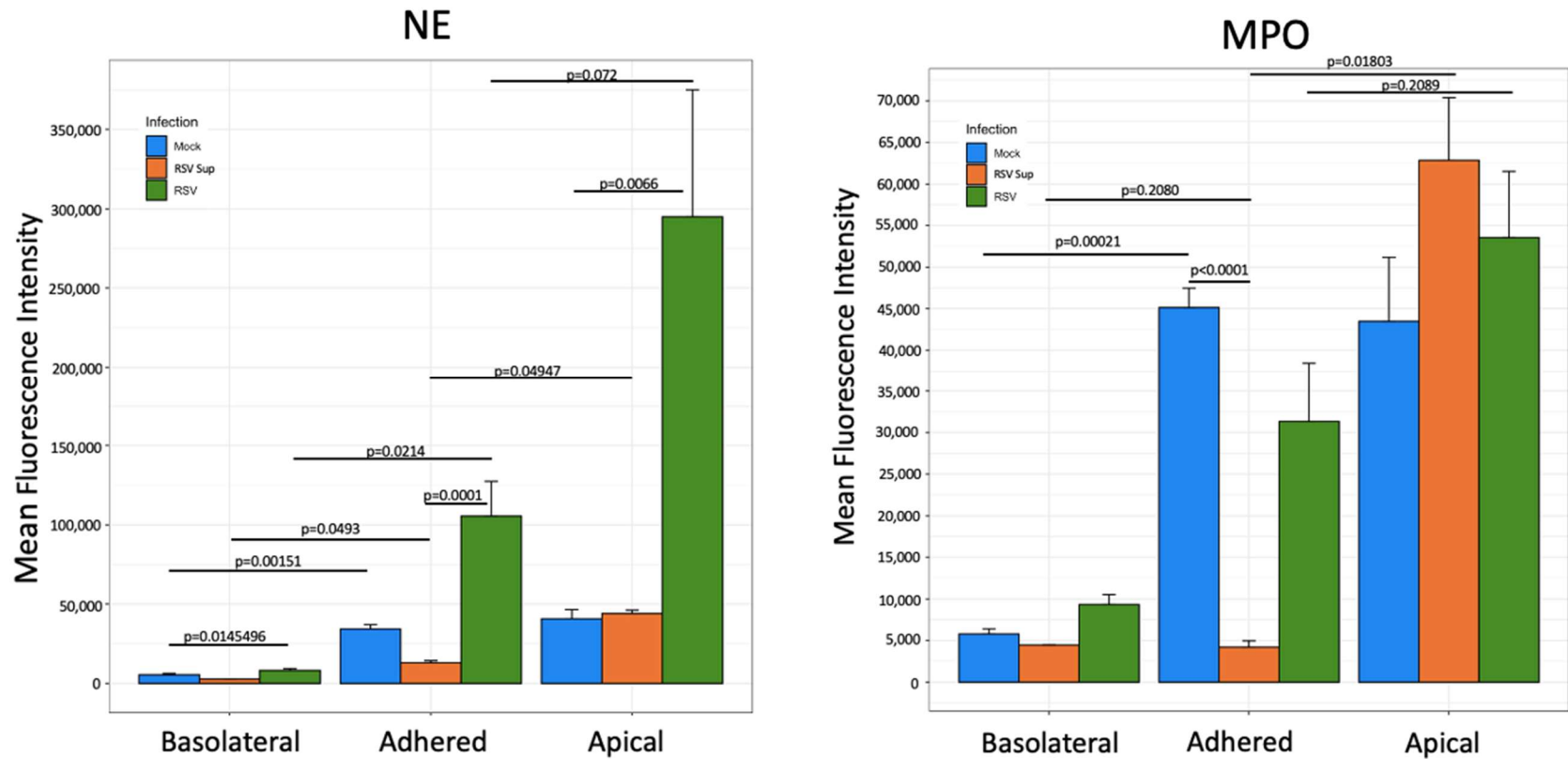


Figure 5-5 Neutrophil expression of neutrophil elastase and myeloperoxidase after transepithelial migration through RSV infected ciliated AECs.

Mean fluorescence intensity of CD11b (left) and CD64 (right) in neutrophils incubated with media alone (No AEC), and those recovered from transepithelial migration assays through RSV infected ciliated AECs infected for 72 hours (RSV), Mock infected control AECs (Mock) or Mock infected AECs with the supernatant recovered from RSV infected ciliated AEC on the apical side (RSV Sup) as a control. Graphs are separated to distinguish neutrophils recovered from the basolateral, adhered and apical (migrated) locations within the transepithelial migration assay.

Bars indicate standard error of the mean. A linear mixed effects model was used to compare interactions between groups and control for donor variability. Individual comparisons performed with Two-Way ANOVA with pairing and Geiser greenhouse correction. * = in comparison to respective basolateral group # = in comparison to respective adhered group. N=6.

5.5.5 Neutrophil degranulation during transepithelial migration through the RSV infected AECs

As NE and MPO are also secreted by neutrophils in response to infection, I then wanted to measure their concentration in AEC supernatants to determine to what extent this was the case.

Examining NE concentration in apical supernatants recovered from AECs after 1 hour transepithelial migration, NE concentration apical to 24 hour mock infected AECs was 0.64mU/ml (± 0.17), apical to 24 hour RSV Sup AECs NE concentration was 0.44mU/ml (± 0.099) and apical to 24 hour RSV infected AECs was 0.608mU/ml (± 0.1). Likewise, NE concentration apical to 72 hour mock infected AECs was 0.52mU/ml (± 0.14), apical to 72 hour RSV Sup AECs NE concentration was 0.54 mU/ml (± 0.13) and apical to 72 hour RSV infected AECs was 0.66mU/ml (± 0.15). There was no significant difference in NE concentration in apical supernatants recovered from mock, RSV Sup or RSV AECs at either 24 or 72 hours.

MPO concentration apical to 24 hour mock infected AECs was 1.26mU/ml (± 0.297), apical to 24 hour RSV Sup AECs MPO concentration was 1.46mU/ml (± 0.25) and apical to 24 hour RSV infected AECs was 1.3mU/ml (± 0.2). Likewise, MPO concentration apical to 72 hour mock infected AECs was 1.29mU/ml (± 0.22), apical to 72 hour RSV Sup AECs NE concentration was 1.51 mU/ml (± 0.19) and apical to 72 hour RSV infected AECs was 1.65mU/ml (± 0.37). Likewise, there was no significant difference in MPO concentration in apical supernatants recovered from mock, RSV Sup or RSV AECs at either 24 or 72 hours.

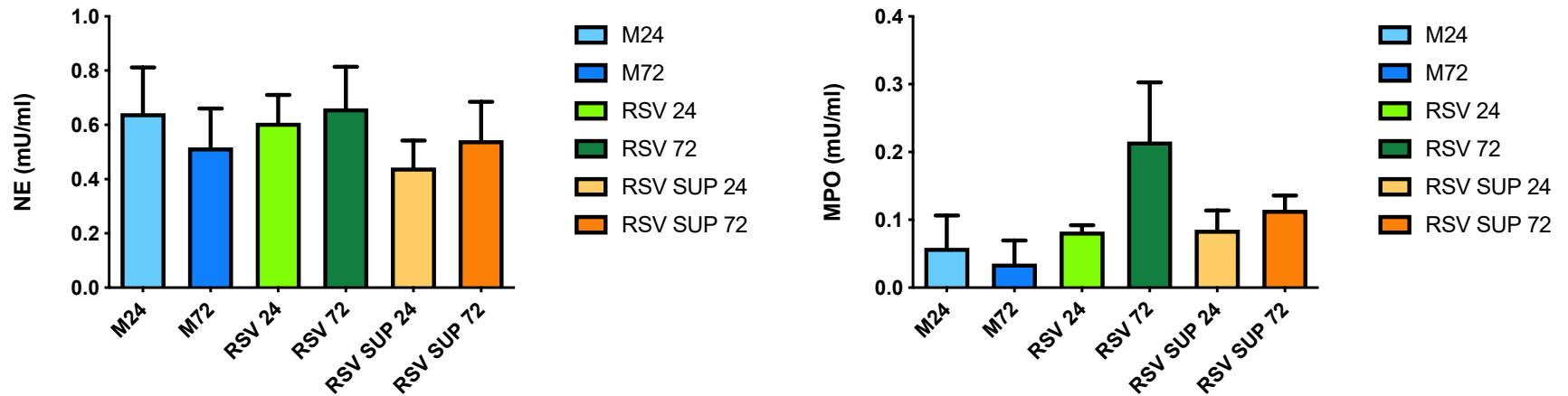


Figure 5-6 Concentration of Neutrophil Elastase and Myeloperoxidase in the apical supernatants collected after neutrophil migration through RSV infected AECs

Concentrations of NE (left) and MPO (right) in the apical supernatants collected after neutrophil migration through ciliated AECs infected with RSV for 24 (RSV 24) or 72 hours (RSV 72), Mock infected control AECs infected for 24 (Mock 24) or 72 hours (Mock 72) or Mock infected AECs with the supernatant recovered from RSV infected ciliated AEC cultures on the apical side (RSV SUP 24) (RSV SUP 72) as a control. Comparisons between groups was performed using a One-Way ANOVA with Tukeys adjustment for multiple comparisons. N=6.

5.6 Discussion

In this chapter I aimed to first determine the effect of live RSV alone on neutrophil activation markers. I showed that incubation of neutrophils with RSV alone reduces CD11b expression but increases expression of NE and MPO. Secondly, I aimed to determine the effect of supernatants collected from RSV infected AECs alone on neutrophil activation markers, and I showed that supernatants from RSV infected AECs increased neutrophil expression of CD11b, NE and MPO. Indeed, I also showed supernatant collected from mock AECs increased MPO expression on neutrophils. Thirdly, I aimed to determine the effect of RSV F or G protein alone on neutrophil activation markers. Investigating this led me to conclude there was no effect on CD11B, NE or MPO expression of incubation with RSV F or G protein. Finally, I aimed to determine the activation status of neutrophils during transepithelial migration through RSV infected AECs. I used a novel *in vitro* model of neutrophil transepithelial migration across ciliated primary AECs infected with RSV to investigate this aim, and I will discuss the results in this section.

Using the transepithelial migration model, I found neutrophils, which had migrated through RSV infected AECs and dissociated (apical) had significantly higher mean expression of CD11b in comparison to those which had migrated through the mock infected AECs or mock infected AECs with RSV infected AEC supernatant placed apically (RSV Sup). I saw the same relationship of greater expression in the adherent and basolateral neutrophils from RSV infected AECs in comparison to mock and RSV Sup controls, and examining the relationship of CD11b expression as determined by neutrophil location or infection using a linear model of mixed effects, I found there is a significant interaction between infection and neutrophil location within the culture, such that infection mediates CD11b expression dependent on whether the neutrophils were basolateral, adhered or apical to the AECs. I also saw an increase in CD11b mediated by incubation of neutrophils with supernatants collected from RSV infected primary ciliated AECs. It is important to note the supernatants used in these neutrophil alone experiments do not contain detectable replicative RSV, so it is likely this upregulation is mediated by inflammatory markers produced by AECs. CD11b is a surface integrin and upregulation would allow greater interaction with other neutrophils and, indeed, the infected

epithelium. However, the magnitude of upregulation of CD11b on neutrophils when incubated with RSV supernatant alone is less than seen in neutrophils exposed to the apical side of infected airway, and indeed I saw similar upregulation of CD11b in mock AEC cultures. It is not clear however whether CD11b is upregulated in response to migration or whether greater expression of CD11b on neutrophils allows them to migrate through RSV infected AECs. The clinical relevance of these findings are discussed further in **Chapter 7**.

Conversely to these findings, I showed that incubating naïve neutrophils with RSV alone induced downregulation of CD11b which was not seen in the transepithelial migration model. I also examined the effect of RSV specific proteins F and G on neutrophil CD11b expression and found no effect. This leads to the supposition this observed downregulation could potentially be due to another RSV protein, which is unlikely as F and G form the majority of the outer envelope of RSV(249). It is more likely this finding may be down to purity of my RSV preparation. RSV in these experiments was propagated in Hep2 cells, and although cellular debris was removed by centrifugation and ultrafiltration – cytokines produced by infected Hep2 cells may remain in the viral preparation. The presence of these mean that any difference seen in neutrophil function as a result of RSV preparation alone, is in fact a consequence due to RSV plus potential Hep2 derived cytokines. However, these experiments form part of a larger body of work and can be interpreted in the context of neutrophil response to primary human AEC supernatants also.

Examining neutrophil elastase expression, I found neutrophils which had migrated through RSV infected AECs and dissociated (apical) had significantly higher levels of mean NE expression in comparison to those which had migrated through mock infected AECs or RSV Sup AECs. I saw the same relationship in the adherent and basolateral neutrophils from RSV infected AECs in comparison to mock and RSV Sup controls and there was no significant difference between the Mock and RSV Sup neutrophils in the basolateral, adhered or apical compartments. Examining the statistical relationship of NE expression as determined by neutrophil location or infection using our linear model of mixed effects, I found there is a significant interaction between infection and neutrophil location within the AEC culture, such that infection mediates NE expression dependent on whether the neutrophil is basolateral,

apical or adhered. In these experiments I showed both RSV infection and transepithelial migration across AECs were necessary to produce high NE expression. This is not unexpected as NE is a secretory factor produced in large quantities as a response to infection, however interestingly this upregulation of NE was not seen in the RSV Sup groups which were exposed to supernatant from RSV infected cultures but not to RSV infected AECs. An upregulation of NE was also seen in neutrophils incubated alone with supernatant recovered from RSV infected AECs and indeed in neutrophils incubated with live virus, but, as observed with CD11b, the magnitude of upregulation observed with exposure to infected airway epithelium was much greater. Measuring NE concentration in the apical supernatant post migration also showed a greater degranulation of neutrophils on the apical side of RSV infected AECs in comparison to the mock, and to a lesser degree in the RSV Sup cultures. This may indicate that RSV infected AEC supernatant increases production of secretory NE, but interaction with RSV infected AECs allows greater quantities to be produced and stored internally. The physical interaction with AECs infected with RSV may form part of a 'priming' step necessary for complete neutrophil activation, in keeping with previously discussed literature(127).

An interesting observation is the low level of expression of specific activation markers, in particular CD64 and MPO, on neutrophils adherent to RSV Sup AECs. This difference was not seen in CD11b expression, or in NE expression, and was also not observed in the apical neutrophils. CD64, as discussed at the start of this chapter, is an Fc receptor implicated in opsonophagocytosis and is regarded as a sensitive measure of neutrophil activation in neonates and murine models of RSV Bronchiolitis, which makes sense as upregulation of Fc receptors will facilitate greater ability to phagocytose pathogens(239,248). MPO is a granular enzyme which is both secreted by neutrophils in response to infection and present in the phagosome to enable degradation of engulfed pathogens(250). It is not clear from this study, why these markers are markedly not upregulating on neutrophils once adhered to mock infected AECs in the presence of RSV infected AEC supernatant. I have shown that neutrophils do migrate through RSV Sup AEC cultures and do adhere to the epithelium in **Figure 3-7** and **Figure 3-8**. However, the similarities in the expression of CD64 and MPO of adhered neutrophils are very striking and suggest these changes are more than artefact and potentially

due to the same mechanism at play. Conversely, it could be that exposure of mock infected AECs to the supernatant from RSV infected AECs causes an unanticipated change in the AECs, potentially mediating the release of a different pattern of inflammatory markers.

I also found upregulation of neutrophil expression of CD11b, and neutrophil elastase on neutrophils on the basolateral side RSV infected cultures in comparison to those found on the mock or RSV Sup groups. This could be in part defined by the inflammatory environment in the basolateral compartment and suggests the AEC cultures may produce a polarised inflammatory response with distinct differences in the apical and basolateral compartments. It could also be a suggestion of reverse migration of neutrophils which move across RSV infected AECs before returning to the basolateral compartment where they are detected with increased CD11b expression, and this possibility is explored more in a basal cell model in **Chapter 3**.

In retrospect, some of the experiments I conducted investigating neutrophil activation could have benefitted from an additional positive control, particularly in the absence of AECs. Traditionally, exogenous bacterial factors, such as fMLP, have been used as a positive control for neutrophil activation (234,251,252). Other bacterial peptides, such as LPS (endotoxin), TNF and PMA, have been used to the same end, but each factor induces 'neutrophil activation' measurable by different outcomes, in the case of LPS increase in surface integrins and down regulation of selectins (251,253–255). For example fMLP, exploited in **Chapter 3** to control for neutrophil movement, is a potent chemotactic agent but also causes upregulation of CD11b/CD18 integrins on neutrophils allowing for increased neutrophil interaction with the endothelium(234). However conflicting reports have commented on low concentration efficacy of inducing neutrophil activation in absence of a priming step(256). Viral factors, and viral infection induced host factors modulating neutrophil activation remain an area for further research. Although these experiments are still interpretable in the context of the rest of the experiments in this chapter, it is important to note that inclusion of a positive control such as LPS or fMLP in the non-AEC would strengthen future naive neutrophil activation experiments.

In each activation marker studied here (CD11b, CD64, NE and MPO), I have seen an increase in expression as a neutrophil moves through the culture from no AEC to basolateral, to adherent in all groups, but the pattern of this is not always the same. Certain markers increase stepwise, in the case of CD11b I see in all adherent groups significantly greater expression than seen on the basolateral side, and further increase in the apical groups. However, other markers seem to peak at the expression level they achieve at adherence, for example CD64 and MPO where there is no difference between the expression density found apically and that of adherent neutrophils, in both mock and RSV infected AECs. I hypothesise here, it is likely that these specific markers are key to the actions of the neutrophil at this specific location. For example, CD11b mediates passage through the epithelium interacting with ICAM-1 on the airway epithelial surface and so it makes sense those which have migrated all the way through to the apical side may have migrated faster, facilitated by greater expression or upregulation of CD11b, than those which are adherent (257). It is important to note that this is an endpoint measure and I cannot tell whether neutrophils adherent at 1 hour would remain adherent later, or whether it is likely that neutrophils now present in the apical compartment were once adherent and this is a necessary step in migrating through the epithelium from basolateral to apical. Conversely, MPO expression is elevated both in neutrophils in adherence to mock and RSV infected AECs and those recovered from the apical compartment, this could be due to the role of neutrophils in removing virally infected cells occurring within close proximity to the epithelium. It is also possible that MPO is key for dissociation of neutrophils from the epithelial surface after migration, which has been previously suggested and indeed demonstrated in murine models of migration (258).

Chapter 6 Cord blood neutrophils and RSV infected AECs

6.1 Introduction

In **Chapters 3, 4** and **5** I have used adult donor neutrophils to explore the interactions of neutrophils with RSV infected AECs. I then wanted to study the response of neutrophils which are more representative of the age group affected by RSV bronchiolitis, and compare any differences seen to what I have illustrated using adult neutrophils. Although it would have been ideal to use neutrophils from infants under one year old, the age we see most cases of RSV bronchiolitis, it is simply not ethically or logistically possible to obtain the volume of blood required from neonates in order to conduct these experiments. Many previous studies have shown that neonatal neutrophil responses to pathogens are not the same as seen in adults, as we discussed in **Section 1.4.4**. Instead of neonatal, this chapter used cord blood neutrophils as a correlate of neonatal neutrophils as they can be easily obtained in sufficient quantities by venepuncture of the placental vein, post-delivery of the baby.

The use of cord blood derived neutrophils as a substitute for neonatal venous neutrophils has been widely used in previous studies investigating neonatal neutrophil responses to inflammation and infection(164,169,259,260).

In this chapter I repeated with cord blood neutrophils, a number of experiments described in previous chapters. Unlike with adult neutrophils, it is not possible to match AEC and neutrophil donors within an experiment. However, as my optimisation experiments in **Section 2.6.5** showed very little difference in neutrophil response where matching was possible and where it was not. Furthermore, these experiments were powered to compare the responses of adult and cord blood neutrophils, not AECs and therefore it seemed appropriate to control for AECs in order to compare like for like.

6.2 Aim

To study the interaction between the RSV infected AECs and neutrophils from patient groups which reflect the age range of children affected by RSV bronchiolitis, in order to explore differences in neutrophil response between adults and neonates.

6.3 Specific aims

In this chapter I aim to specifically address the following aims

1. To isolate a pure population of neutrophils from cord blood of healthy term infants

To study cord blood neutrophils in order to characterise and compare with adult neutrophils:

2. migration numbers of neutrophils from cord blood of healthy term infants in a transepithelial migration model of RSV infected ciliated AECs
3. proportion of neutrophils in the different compartments of the model, which had undergone apoptosis
4. activation state of neutrophils

6.4 Chapter specific methods

All primary human AEC cultures used in this chapter were grown at air liquid interface (ALI) for 28 days until ciliated and then were infected with RSV MOI 5 or 'mock' media only control 72 hours prior to the experiment as described in **Section 2.6.1**. Mock infected AECs with RSV infected AEC supernatant placed on the apical side was used as a control (RSV SUP). Neutrophils isolated from cord blood or healthy adult volunteers were suspended at a concentration of 5×10^6 /ml in HBSS+ supplemented with 1% autologous serum. Transepithelial migration was conducted as described in **Section 2.6.2** for 1 hours incubation. Neutrophils were distinguished from AECs by staining with CD11B-APC as described in **Section 2.7.4.1**. Absolute counts of neutrophils within a 100 μ l volume were measured using a CYTOFLEX cytometer and these values extrapolated for volume of solution recovered using GraphPad Prism v8.0. Neutrophil activation markers were determined by antibody staining and flow cytometry as described in **Section 2.7.3**.

6.4.1 Cord blood collection and isolation

I recruited expectant mothers on the day of an elective caesarean section to the stem cell study (see **Section 2.3.3**) on labour ward at University College Hospital from May 2018 – March 2020. Up to 20ml of cord blood was collected immediately once the midwife had completed her post birth checks, by venepuncture of the umbilical vein of the placenta. Blood

was only collected when there were intact membranes, no signs of infection or neonatal distress, no steroids had been prescribed and if gestational age was 37 weeks or more.

Cord blood samples were transported on ice from labour ward at UCH to the laboratories at Great Ormond Street Institute of Child Health. Neutrophils were isolated from cord blood in the same manner as was as described for adult venous blood in **Section 2.4**. Where required, autologous serum was isolated alongside cord blood for use.

6.5 Results

6.5.1 Cord blood donor characteristics

In total 20 women consented to participate in the study, of which, 17 cord blood samples were collected. The reasons the other samples were not collected related to maternal complications making them ineligible for inclusion in the study, or the delay of their caesarean section start time, such that there wasn't sufficient labtime left to conduct subsequent experiments. The mean maternal age was 35.8 (range 26 – 42) and the mean gestational age was 38.93 weeks (range 37.9 – 40.9). The mean volume of blood collected was 12mls (range 5mls – 20mls).

6.5.2 Cord blood neutrophil isolation and purity

Neutrophils were isolated from cord blood at 97.8% purity by the negative immunoselection method determined in **Section 2.4**. Neutrophils were identified by CD66c and CD11b positivity using flow cytometry. Additionally, neutrophils were confirmed morphologically using DiffQuik™ staining and microscopy (**Figure 6-1**).

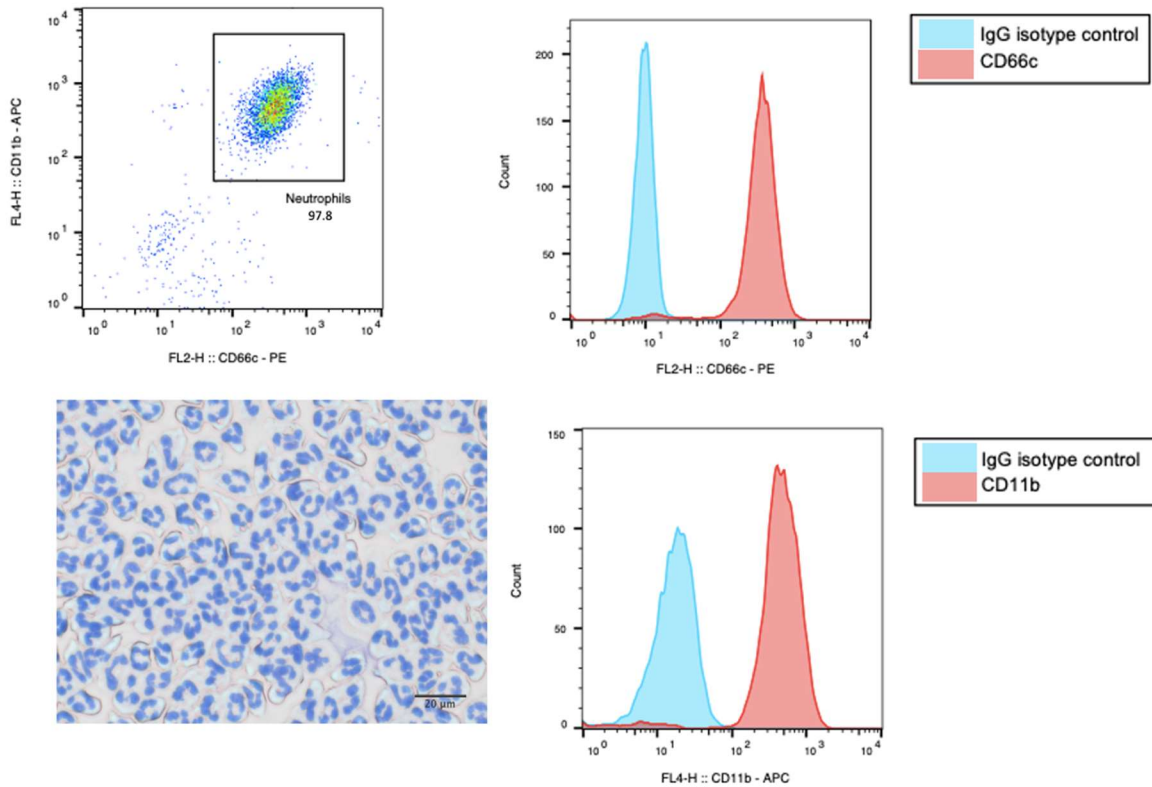


Figure 6-1 Purification of neutrophils isolated from cord blood of healthy term infants

Neutrophils isolated using the direct whole blood isolation kit (Stem Cell technologies). (Top left) Dotplot showing 97.8 percentage purity by proportion of cells which are both CD66c and CD11b positive;

(Bottom left) Representative image of a cytopun neutrophil sample with DiffQuik™ staining showing characteristic polymorphous nucleus of neutrophils. (Right) Histogram to show CD66c (top) and CD11b (bottom) expression of cord blood neutrophils.

6.5.3 Greater numbers of cord blood neutrophils migrate through the RSV infected epithelium in comparison to adult neutrophils

Firstly, to determine whether neutrophilic migration through the infected AECs was comparable to what I have seen in adult neutrophils, a migration experiment was performed. In contrast to previous data shown, this time counts were performed using a flow cytometer so as to encompass all neutrophils not just those which are viable. These data are presented in **Figure 6-2**, counts between groups were compared using a Two-Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons.

After 1 hour migration through mock infected AECs, there were a mean neutrophil count (\pm SD) of 301,215 (\pm 93,993.99) adult, and 189,838 (\pm 64017.5) cord blood neutrophils recovered from the basolateral side of AEC cultures. There was no significant difference between these measures. A mean neutrophil count of 4,673 (\pm 2319.8) adult, and 53,275 (\pm 7331.1) cord blood neutrophils were adherent to mock infected AEC cultures. There was no significant difference between these measures. A mean neutrophil count of 54,458 (\pm 6760.136) adult, and 96,268 (\pm 28592.29) cord blood neutrophils were recovered from the apical side of mock infected AEC cultures. There was no significant difference between these measures.

There was a mean neutrophil count of 311,568 (\pm 34,647.35) adult, and 16,428 (\pm 2,342.45) cord blood neutrophils recovered from the basolateral side of mock infected AECs with RSV infected AEC supernatant placed beneath them (RSV SUP). This difference was significant with $p < 0.0001$. A mean neutrophil count of 4,742 (\pm 2,930.281) adult, and 33,075 (\pm 6,240.249) cord blood neutrophils were adherent to mock infected AEC cultures with RSV infected AEC supernatant placed beneath them (RSV SUP). There was no significant difference between these measures. A mean of 49,387 (\pm 18,799.64) adult, and 343,244 (\pm 67,293.91) cord blood neutrophils were recovered from the apical side of mock infected AEC cultures. This difference was significant with $p < 0.0001$.

There was a mean neutrophil count of 171,641 (\pm 54789.61) adult, and 23,870 (\pm 6,718.591) cord blood neutrophils on the basolateral side of RSV infected AEC cultures. This difference

was significant with $p < 0.05$. A mean neutrophil count of $15,065.33 (\pm 2,388.171)$ adult, and $41,972 (\pm 7,665.655)$ cord blood neutrophils were adherent to RSV infected AEC cultures. There was no significant difference between these measures. A mean neutrophil count of $56,586 (\pm 27,953)$ adult, and $336,684 (\pm 48,129.32)$ cord blood neutrophils were recovered from the apical side of mock infected AEC cultures. This difference was significant with $p < 0.0001$.

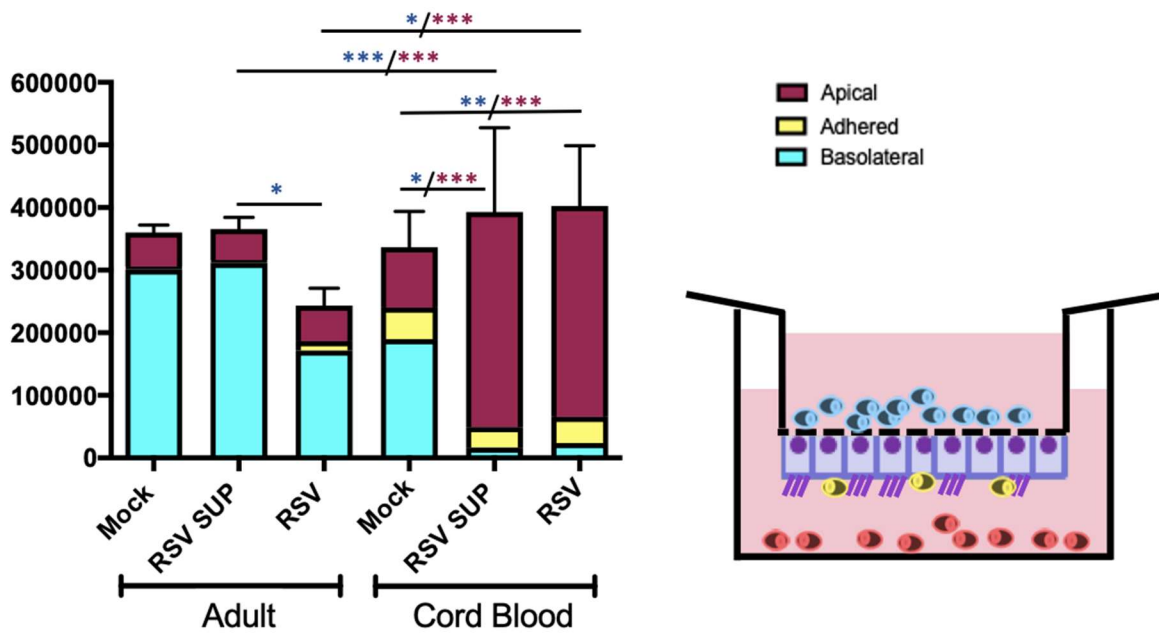


Figure 6-2 Numbers of neutrophils recovered after transepithelial migration assay through RSV infected ciliated AECs.

Neutrophils isolated from healthy adult venous blood or cord blood from healthy infants born at term were added to the basolateral side of ciliated adult AEC cultures infected with RSV for 72 hours. After 1 hours migration, neutrophils on the basolateral and apical sides of AECs, and those adhered to the AECs were counted by flow cytometry. Stacked bars show the numbers of neutrophils isolated from the basolateral (blue), adhered (yellow) and apical (purple) compartments of the culture, as illustrated on the schematic (left). Differential counts were analysed using Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons. * = $p < 0.05$, ** = $p < 0.002$, *** = $p < 0.0001$. Blue stars indicate differences found between basolateral counts, purple stars indicate differences found between apical counts. N=4.

6.5.4 Neutrophil trans-epithelial migration experiments show that neutrophils from adults and cord blood donors have different viability and apoptosis, dependent on location within the culture.

Next, adult and cord blood neutrophils, recovered from a migration assay, were assessed for viability and apoptosis to determine if similar proportions of cord blood neutrophils were live, dead or apoptotic in comparison to adult neutrophils. The focus of these experiments has been on apoptosis as a form of programmed cell death, however the assay methodology also enabled comparisons between viable and dead neutrophil populations. These data are shown in **Figure 6-3**, **Figure 6-4** and **Figure 6-5**.

6.5.4.1 A greater proportion of adult neutrophils on the basolateral side of RSV infected ciliated cultures are apoptotic and fewer are viable compared with cord blood neutrophils

First, I examined the apoptosis of neutrophils remaining on the basolateral side of RSV and mock AEC cultures infected for 72 hours. Of the adult neutrophils recovered from AEC cultures after 1 hours migration, those recovered from RSV infected AEC cultures were 14.63% (± 6.7) (Mean \pm SEM) apoptotic, those recovered from mock infected AEC cultures were 1.95% (± 1.59) apoptotic and those recovered from mock infected AEC cultures with RSV infected AEC sup on the apical side (RSV Sup) 1.797% (± 0.5831) were apoptotic. In the non AEC control, 0.17% (± 0.08) of neutrophils were apoptotic. More adult neutrophils recovered from RSV infected AEC cultures were apoptotic were than those recovered from non AEC, mock and RSV Sup cultures with p values of 0.0199, 0.047 and 0.046 respectively.

Of the cord blood neutrophils recovered from AEC cultures after 1 hour of migration, those recovered from RSV infected AEC cultures were 1.78% (± 0.49) apoptotic, from mock infected AEC cultures were 1.29% (± 0.21) apoptotic, from mock infected AEC cultures with RSV infected AEC sup on the apical side (RSV Sup) 1.08% (± 0.55) were apoptotic. In the non AEC control, 0.067% (± 0.12) of neutrophils were apoptotic. There was no significant difference in proportion of cord blood neutrophils apoptotic between those recovered from Non AEC neutrophils, RSV infected AEC cultures, Mock infected AEC cultures, or RSV Sup AEC cultures.

Comparing between adult and cord blood neutrophils recovered from the basolateral side of RSV infected AEC cultures, adult neutrophils showed a significantly higher proportion were apoptotic in comparison to cord blood neutrophils with a p value of 0.0461. There was no significant difference in proportion of neutrophils apoptotic between adult neutrophils recovered from the basolateral side of non AEC, Mock infected AEC cultures and RSV Sup AEC cultures in comparison to their respective cord blood neutrophils.

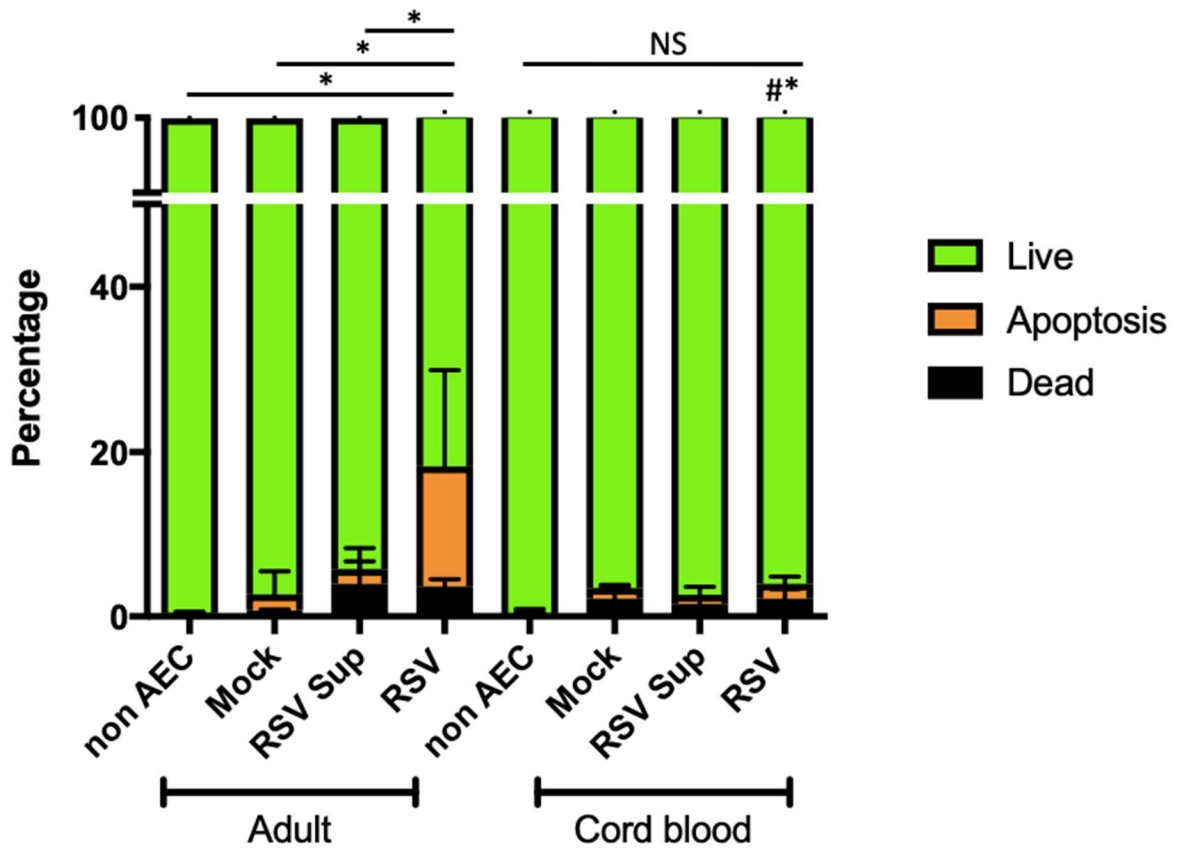


Figure 6-3 Adult and cord blood neutrophils recovered from basolateral side of ciliated AEC cultures infected with RSV for 72 hours.

Adult and cord blood neutrophils were applied to the basolateral side of ciliated AEC cultures infected with RSV or 'mock' media only control 72 hours prior to addition of neutrophils. Neutrophils incubated in media alone were used as a control (non AEC). Relative proportions of neutrophils which were live (green), apoptotic (orange) and dead (black) after 1 hour incubation. were analysed using Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons. N=4. * = $p < 0.05$, ** = $p < 0.002$, *** = $p < 0.0001$. # indicates in comparison to its respective measurement in adult neutrophils.

6.5.4.2 A greater proportion of cord blood neutrophils adherent to RSV infected ciliated cultures are apoptotic and fewer are viable compared with adult neutrophils

Of the adult neutrophils adherent to AEC cultures after 1 hour of migration, those from RSV infected AEC cultures were 0.803% (± 0.31) (Mean \pm SEM) apoptotic, from mock infected AEC cultures were 10.5% (± 4.7) apoptotic and from mock infected AEC cultures with RSV infected AEC sup on the apical side (RSV Sup) 33.6% (± 7.4) were apoptotic. In the non AEC control, 0.17% (± 0.08) of neutrophils were apoptotic. More adult neutrophils recovered from RSV Sup AEC cultures were apoptotic than those recovered from non AEC, Mock and RSV infected AEC cultures with p values of <0.0001 , 0.0036 and <0.0001 respectively.

Of the cord blood neutrophils adherent to AEC cultures after 1 hour of migration, those recovered from RSV infected AEC cultures were 14.6% (± 1.8) apoptotic, those recovered from mock infected AEC cultures were 7.99% (± 1.08) apoptotic and those recovered from mock infected AEC cultures with RSV infected AEC sup on the apical side (RSV Sup) 14.4% (± 2.26) were apoptotic. In the non AEC control, 0.067% (± 0.12) of neutrophils were apoptotic. More cord blood neutrophils recovered from RSV Sup AEC cultures, mock and RSV infected AEC cultures were apoptotic than those recovered from non AEC, with p values of 0.008, 0.284 and 0.009 respectively.

Comparing adult and cord blood neutrophils adherent to RSV infected AEC cultures, adult neutrophils showed a significantly lower proportion apoptotic in comparison to cord blood neutrophils with a p value of 0.0192. Likewise, adult neutrophils adherent to RSV Sup AEC cultures showed a significantly greater proportion apoptotic in comparison to cord blood neutrophils with a p value of 0.0198. There was no significant difference in proportion of neutrophils apoptotic between adult neutrophils in non AEC or mock infected AEC cultures in comparison to their respective cord blood neutrophils.

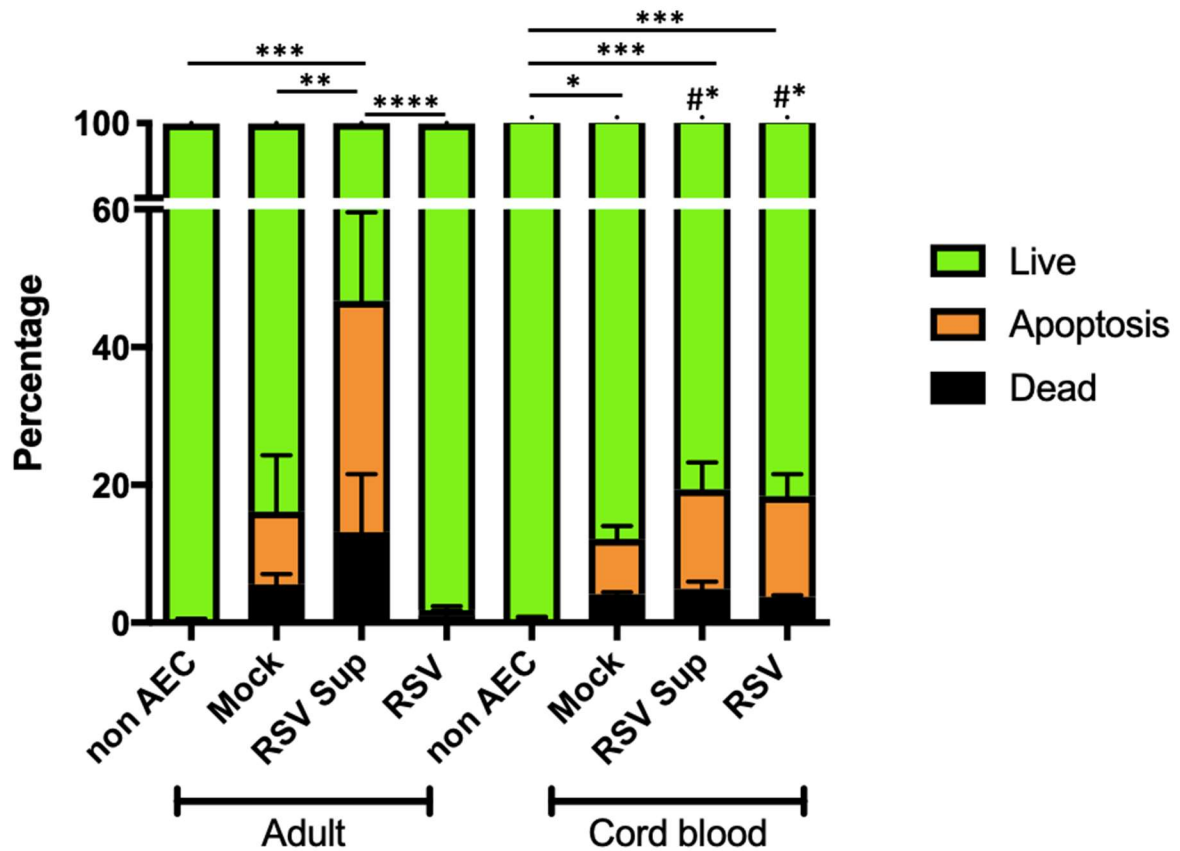


Figure 6-4 Adult and cord blood neutrophils adherent to ciliated AEC cultures infected with RSV for 72 hours.

Adult and cord blood neutrophils were applied to the basolateral side of ciliated AEC cultures infected with RSV or 'mock' media only control 72 hours prior to addition of neutrophils. Neutrophils incubated in media alone was used as a control (non AEC). Relative proportions of neutrophils recovered adherent to the AEC culture which were live (green), apoptotic (orange) and dead (black) after 1 hour incubation. Comparisons between groups were performed using Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons. N=4. * = $p < 0.05$, ** = $p < 0.002$, *** = $p < 0.0001$. # indicates in comparison to its respective measurement in adult neutrophils.

6.5.4.3 A greater proportion of cord blood neutrophils found apically in RSV infected ciliated AECs are apoptotic and fewer are viable compared with adult neutrophils

Of the adult neutrophils recovered from AEC cultures after 1 hour migration, those recovered from RSV infected AECs were 9.49% (± 2.7) (Mean \pm SEM) apoptotic, those recovered from mock infected AEC cultures were 0.51% (± 0.21) apoptotic and those recovered from mock infected AEC cultures with RSV infected AEC sup on the apical side (RSV Sup) 2.48% (± 2.11) were apoptotic. In the non AEC control, 0.17% (± 0.08) of neutrophils were apoptotic. Adult neutrophils recovered from RSV infected AEC cultures were more apoptotic than those recovered from non AEC and mock infected AEC cultures with p values of 0.0196 and 0.0237 respectively.

Of the cord blood neutrophils recovered adherent to AEC cultures after 1 hour of migration, those recovered from RSV infected cultures were 43.3% (± 10.68) apoptotic, from mock infected AEC cultures were 19.4% (± 3.08) apoptotic and from mock infected AEC cultures with RSV infected AEC sup on the apical side (RSV Sup) 30% (± 6.26) were apoptotic. In the non AEC control, 0.067% (± 0.12) of neutrophils were apoptotic. More cord blood neutrophils recovered from RSV Sup AEC cultures and RSV infected AEC cultures were apoptotic than were those recovered from non AEC, with p values of 0.0078 and 0.0002 respectively. Likewise, more cord blood neutrophils recovered from RSV infected AEC cultures were apoptotic than those recovered from mock infected AEC cultures, with a p value of 0.042.

Comparing adult and cord blood neutrophils recovered apically to RSV infected AEC cultures, adult neutrophils showed a significantly lower proportion were apoptotic in comparison to cord blood neutrophils with a p value of 0.0024. Likewise, adult neutrophils recovered apically to RSV Sup AEC cultures showed a significantly lower proportion apoptotic in comparison to cord blood neutrophils with a p value of 0.014. There was no significant difference in proportion of neutrophils apoptotic between adult neutrophils in non AEC or mock infected AEC cultures in comparison to their respective cord blood neutrophils.

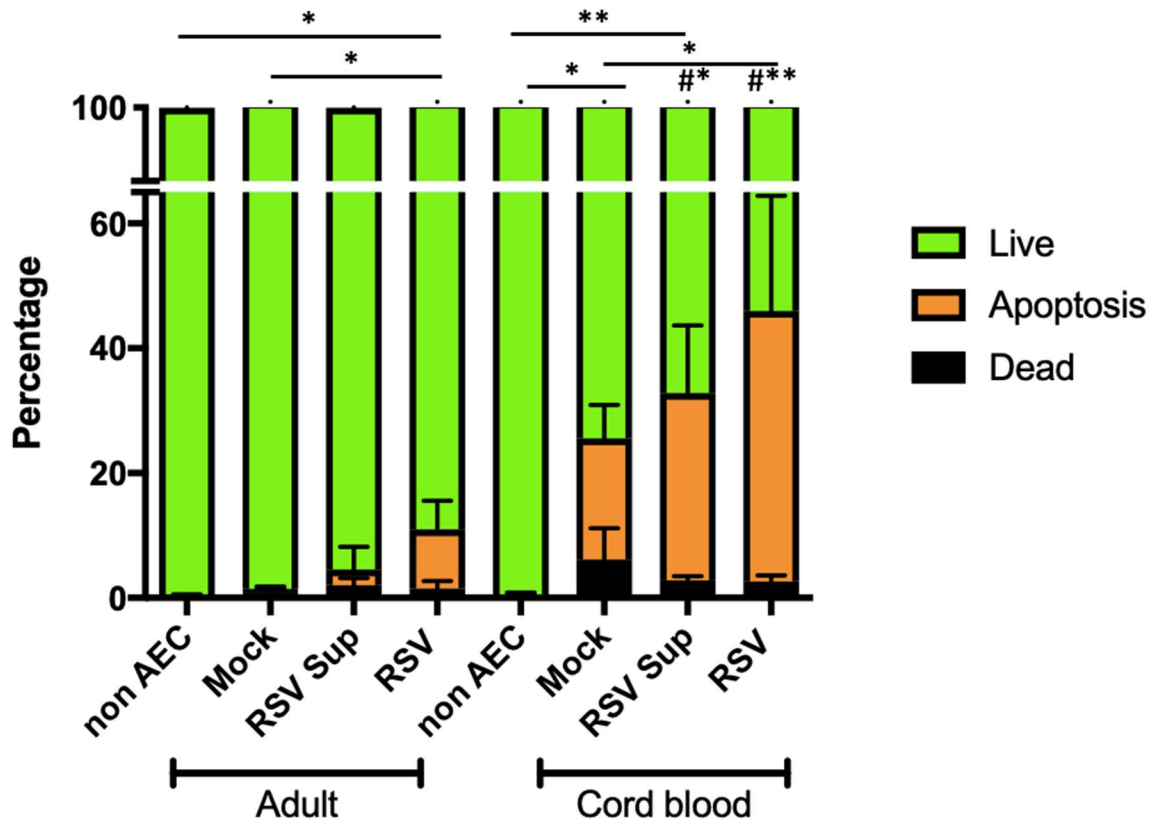


Figure 6-5 Adult and cord blood neutrophils recovered apically to ciliated AEC cultures infected with RSV for 72 hours.

Adult and cord blood neutrophils were applied to the basolateral side of ciliated AEC cultures infected with RSV or 'mock' media only control 72 hours prior to addition of neutrophils. Neutrophils incubated in media alone was used as a control (non AEC). Relative proportions of neutrophils recovered apically to the AEC culture which were live (green), apoptotic (orange) and dead (black) after 1 hour incubation. Comparisons between groups were performed using Two Way ANOVA with Greenhouse-Geisser post hoc test for multiple comparisons. N=4. * = $p < 0.05$, ** = $p < 0.002$, *** = $p < 0.0001$. # indicates in comparison to its respective measurement in adult neutrophils.

6.5.5 Cord blood and adult neutrophils display different activation profiles when exposed to the RSV infected epithelium in a transepithelial migration model

Next, cord blood neutrophils recovered from a migration assay were assessed for activation markers to compare to the observed changes to adult neutrophil activation after transepithelial migration presented in **Chapter 5**. A transepithelial migration assay was performed for 1 hours migration through AECs infected with mock or RSV for 72 hours. Activation marker staining was performed with CD11b-APC, NE-PE, MPO-FITC and CD64-PE-CY7.

6.5.5.1 Interaction with RSV infected ciliated AECs increases CD11b expression on adult but not cord blood neutrophils

Figure 6-6 has been previously shown in **Chapter 5** of this thesis and the analysis is given in **Section 5.5.4.1**. Data are presented as mean fluorescence intensity (\pm SEM). When interpreting the graphs in **Figure 6-6**, it is important to note the difference in axes range between the two plots. This difference will be discussed further later in this chapter.

One hour after they were added to the basolateral surface of the mock infected AECs, cord blood neutrophils isolated from the basolateral compartment, had mean CD11b expression of 55,383 (\pm 7881.7); mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed mean CD11b expression of 91,927.8 (\pm 10822) and for neutrophils recovered from the basolateral surface RSV infected AECs, mean CD11b expression was 73,4441 (\pm 701.6). There was no significant difference in CD11b expression between cord blood neutrophils recovered from the basolateral side of Mock and RSV infected AECs. There was a significantly greater expression of CD11b found on neutrophils recovered from RSV Sup AEC cultures in comparison to those recovered from mock infected AEC cultures with a p value of 0.0152.

For cord blood neutrophils isolated adherent to AECs, mean CD11b expression on neutrophils from of mock infected AECs was 39,358 (\pm 6629.7), from mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) mean CD11b expression was 75,880.7 (\pm 15999) and for neutrophils adherent to RSV infected AECs, mean CD11b expression was 56,704 (\pm 6586). There was no significant difference in CD11b expression between cord blood neutrophils adherent to mock and RSV infected AECs. There was a significantly greater expression of CD11b found on neutrophils adherent to RSV Sup cultures in comparison to those adherent to mock with a p value of 0.0153.

For cord blood neutrophils isolated apically to AECs, mean CD11b expression on neutrophils from of mock infected AECs was 42,258.5 (\pm 6870), from mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) mean CD11b expression was 50,306 (\pm 6603) and for neutrophils recovered from RSV infected AECs, mean CD11b expression was 79,122.8 (\pm 16761). There was no significant difference in mean CD11b expression between cord blood neutrophils isolated apically to mock and RSV Sup AEC cultures. There was a significantly

greater mean expression of CD11b found on neutrophils recovered apically to RSV infected AECs in comparison to those recovered from mock with a p value of 0.0144.

Comparing my no AEC controls between adult and cord blood neutrophils, mean CD11b expression was 15,209 (± 373.7) on cord blood neutrophils and 147 (± 25.7) on adult neutrophils. Mean CD11b expression was significantly higher on cord blood neutrophils with a p value of <0.0001 . To control for this observed difference in baseline expression of CD11b, expression of CD11b was calculated as mean fold change from the no AEC control and these data are shown in **Figure 6-7**.

Comparing fold change in CD11b expression between adult and cord blood neutrophils on the basolateral side of AECs, there was no significant difference in CD11b mean fold change expression on neutrophils recovered from mock infected AECs. Likewise, there was no significant difference in CD11b mean fold change expression between adult and cord blood neutrophils isolated from the basolateral side of mock infected AECs with RSV infected AEC supernatant on the apical side (RSV Sup). Adult neutrophils after 1 hour incubation on the basolateral side of RSV infected AECs showed significantly greater mean fold change of CD11b expression (8.209 ± 1.2) in comparison to cord blood neutrophils (4.829 ± 0.046) with a p value of 0.014.

Adult neutrophils adherent to mock infected AECs showed a mean fold change of CD11b expression $51.44 (\pm 6.96)$ and cord blood neutrophils adherent showed mean fold change of $2.59 (\pm 0.044)$. This difference was significant with a p value of 0.0005. Adult neutrophils adherent to mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed a mean fold change of CD11b expression $43.4 (\pm 15.62)$ and adherent cord blood neutrophils showed mean fold change of $4.99 (\pm 1.052)$. This difference was significant with a p value of 0.0045. Adult neutrophils adherent to RSV infected AECs showed a mean fold change of CD11b expression (74.8 ± 3.8) and cord blood neutrophils adherent showed (3.44 ± 0.39). This difference was significant with a p value of <0.0001 .

Adult neutrophils recovered apically to mock infected AECs showed a mean fold change of CD11b expression (97.7 ± 3.97) and cord blood neutrophils apically showed (2.778 ± 0.45). This difference was significant with a p value of 0.0253. Adult neutrophils recovered apically

to mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed a mean fold change of CD11b expression (132.8 ± 4.95) and cord blood neutrophils apically showed (3.3 ± 0.43). This difference was significant with a p value of 0.0021. Adult neutrophils recovered apically to RSV infected AECs showed a mean fold change of CD11b expression (179.3 ± 54.01) and cord blood neutrophils apically showed (5.2 ± 1.1). This difference was significant with a p value of <0.0001

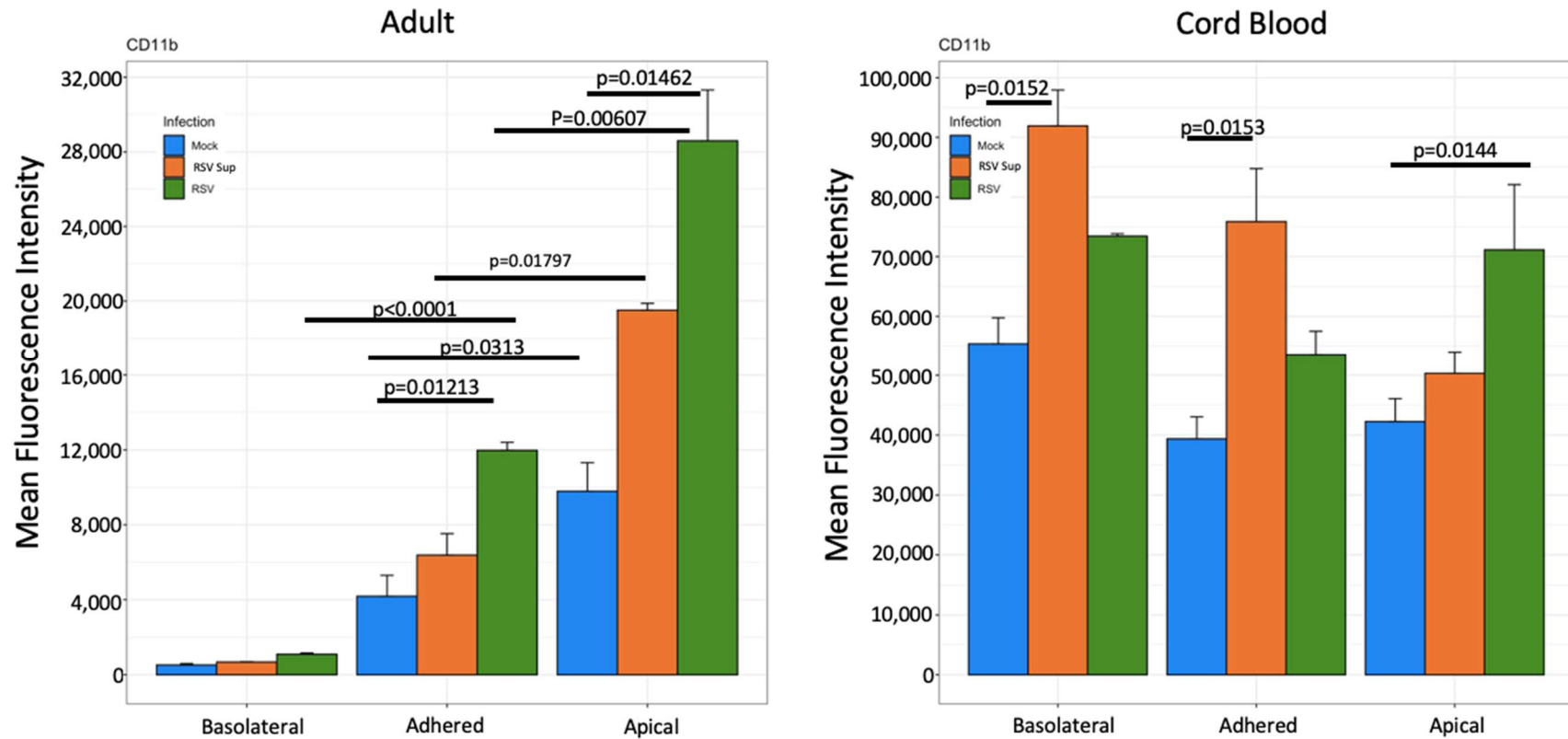


Figure 6-6 Neutrophil activation marker CD11b expression on adult and cord blood neutrophils after transepithelial migration assay

Mean fluorescence intensity of CD11b on adult (left) and cord blood (right) recovered from transepithelial migration assays through RSV infected ciliated AECs infected for 72 hours (RSV), Mock infected control AECs (Mock) or Mock infected AECs with the supernatant recovered from RSV infected ciliated culture on the apical side (RSV Sup) as a control. Graphs are separated to distinguish neutrophils recovered from the basolateral, membrane bound (adhered) and apical (migrated) locations within the transepithelial migration assay.

Bar colours (Blue = Mock, Orange = RSV Sup, Green = RSV, Pink = Non AEC) A linear mixed effects model was used to compare interactions between groups and control for donor variability. Individual comparisons performed with Two-Way ANOVA with pairing and Geiser greenhouse correction. # = in comparison to respective adult neutrophil group. N=5.

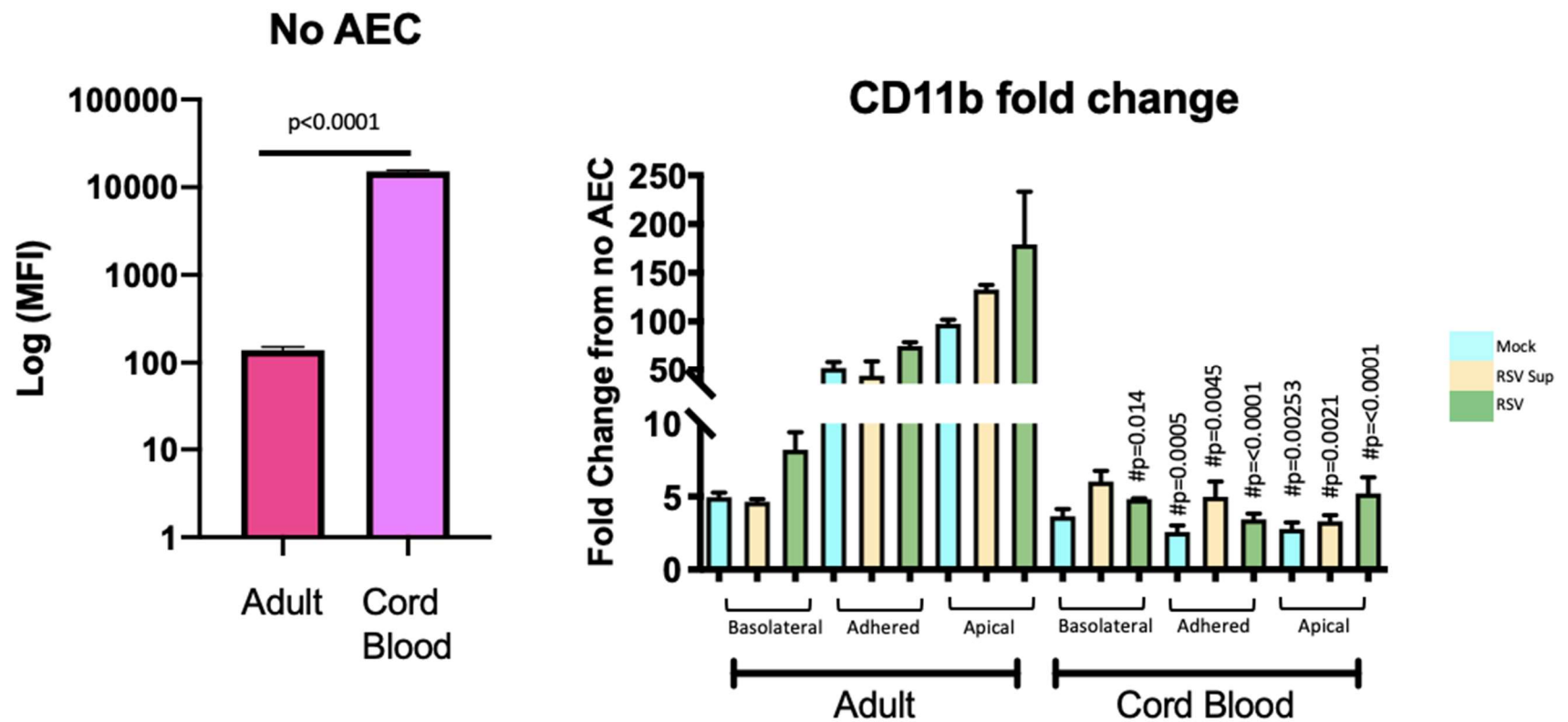


Figure 6-7 Comparing neutrophil activation marker CD11b expression on adult and cord blood neutrophils after transepithelial migration assay

(Left) Graph to show difference in non AEC control groups between adult and cord blood neutrophils presented on a log₁₀ scale of mean fluorescence intensity of CD11b expression.

(Right) Fold change of CD11b expression mean fluorescence intensity calculated using the respective adult or cord blood non AEC control as baseline.

Bar colours (Blue = Mock, Orange = RSV Sup, Green = RSV, Pink = Non AEC). Comparisons between groups were performed with Two-Way ANOVA with pairing and Geiser greenhouse correction. # = in comparison to respective adult neutrophil group. N=5.

6.5.5.2 Migration through ciliated epithelium increases CD64 expression on adult but not cord blood neutrophils

Figure 6-8 has been previously shown in **Chapter 5** of this thesis and the analysis is given in **Section 5.5.4.1**. Data presented as mean fluorescence intensity (\pm SEM).

One hour after they were added to the basolateral surface of the mock infected AECs, cord blood neutrophils isolated from the basolateral compartment, had mean CD64 expression on of 249,958 (\pm 98,449), from mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) mean CD64 expression was 140,636 (\pm 85380) and for neutrophils recovered from RSV infected AECs, mean CD64 expression was 130,175.25 (\pm 53880). There was no significant difference in mean CD64 expression between cord blood neutrophils recovered from the basolateral side of Mock, RSV Sup and RSV infected AECs.

For cord blood neutrophils adherent to AECs, mean CD64 expression on neutrophils from of mock infected AECs was 189,770 (\pm 12510), from mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) mean CD64 expression was 434,765 (\pm 19555) and for neutrophils recovered from RSV infected AECs, mean CD64 expression was 30,7381 (\pm 143203). There was no significant difference in mean CD64 expression between cord blood neutrophils adherent to Mock and RSV infected AECs. There was a significantly greater expression of mean CD64 found on neutrophils adherent to RSV Sup AECs in comparison to those adherent to mock with a p value of 0.0474.

For cord blood neutrophils isolated apically to AECs, mean CD64 expression on neutrophils from of mock infected AECs was 298,878 (\pm 14367), from mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) mean CD64 expression was 55826 (\pm 2455) and for neutrophils recovered apically from RSV infected AECs, mean CD64 expression was 82434 (\pm 26861). There was no significant difference in mean CD64 expression between cord blood neutrophils isolated apically to Mock and RSV Sup cultures infected cultures. There was a significantly greater expression of mean CD64 found on neutrophils recovered apically to RSV Sup infected AECs in comparison to those recovered from mock with a p value of 0.0491.

Comparing my no AEC controls between adult and cord blood neutrophils, mean CD64 expression was 349,029 (± 11785) on cord blood neutrophils and 3383 (± 649) on adult neutrophils. Mean CD64 expression was significantly higher on cord blood neutrophils with a p value of <0.0001 . To control for this observed difference in baseline expression of CD64, expression of CD64 was calculated as mean fold change from the no AEC control and this data is shown in **Figure 6-9**.

Comparing fold change in mean CD64 expression between adult and cord blood neutrophils on the basolateral side of AECs, there was no significant difference in mean CD64 expression mean fold change on neutrophils recovered from RSV infected AECs. Likewise, there was no significant difference in CD64 expression mean fold change between adult and cord blood neutrophils isolated from the basolateral side of Mock infected AECs with RSV infected AEC supernatant on the apical side (RSV Sup). Adult neutrophils after 1 hour incubation on the basolateral side of mock infected AECs showed significantly greater mean fold change of mean CD64 expression (0.59 ± 0.1) in comparison to cord blood neutrophils (7.7 ± 2.17) with a p value of 0.0359.

Adult neutrophils recovered adherent to mock infected AECs showed a mean fold change of mean CD64 expression (14.8 ± 5.2) and cord blood neutrophils adherent showed (0.52 ± 0.35). This difference was significant with a p value of 0.0276. Adult neutrophils recovered adherent to mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed a mean fold change of mean CD64 expression (17.3 ± 3.9) and cord blood neutrophils adherent showed (1.339 ± 0.3). This difference was significant with a p value of 0.0058. Adult neutrophils recovered adherent to RSV infected AECs showed a mean fold change of mean CD64 expression (17.3 ± 4.7) and cord blood neutrophils adherent showed (1.004 ± 0.3). This difference was significant with a p value of 0.0059.

Adult neutrophils recovered apically to mock infected AECs showed a mean fold change of mean CD64 expression (13.3 ± 0.7) and cord blood neutrophils apically showed (0.82 ± 0.39). This difference was significant with a p value of <0.0001 . Adult neutrophils recovered apically to mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed a mean fold change of CD64 expression (1.339 ± 0.3) and cord blood neutrophils apically showed (0.16

± 0.06). This difference was significant with a p value of <0.0001 . Adult neutrophils recovered apically to RSV infected AECs showed a mean fold change of mean CD64 expression (12.74 ± 2.384) and cord blood neutrophils apically showed (0.23 ± 0.07). This difference was significant with a p value of <0.0001 .

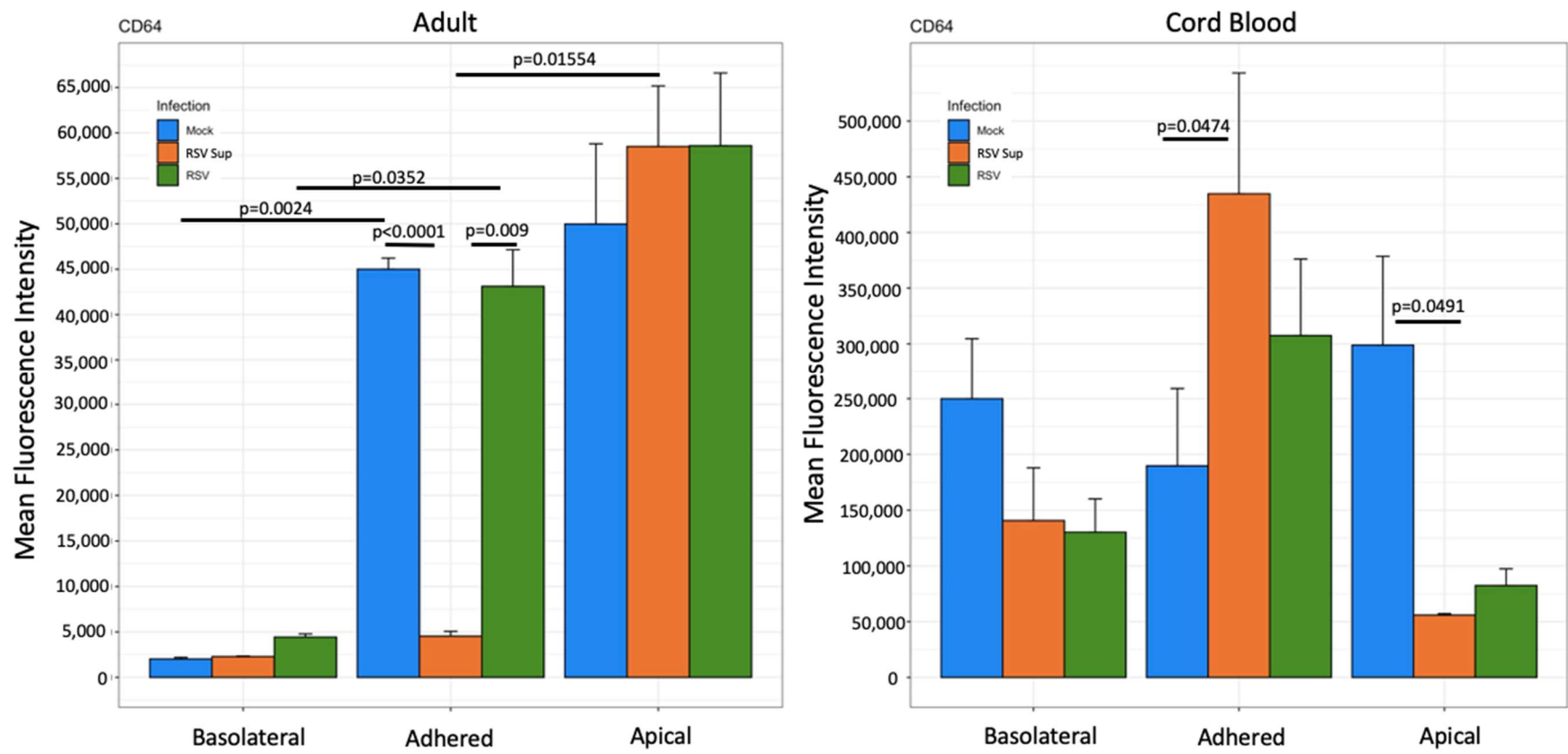


Figure 6-8 Neutrophil activation marker CD64 expression on adult and cord blood neutrophils after transepithelial migration assay

Mean fluorescence intensity of CD64 on adult (left) and cord blood (right) recovered from transepithelial migration assays through RSV infected ciliated AECs infected for 72 hours (RSV), Mock infected control AECs (Mock) or Mock infected AECs with the supernatant recovered from RSV infected ciliated culture on the apical side (RSV Sup) as a control. Graphs are separated to distinguish neutrophils recovered from the basolateral, membrane bound (adhered) and apical (migrated) locations within the transepithelial migration assay.

Bar colours (Blue = Mock, Orange = RSV Sup, Green = RSV, Pink = Non AEC) Comparisons between groups were performed with Two-Way ANOVA with pairing and Geiser greenhouse correction. # = in comparison to respective adult neutrophils. N=5.

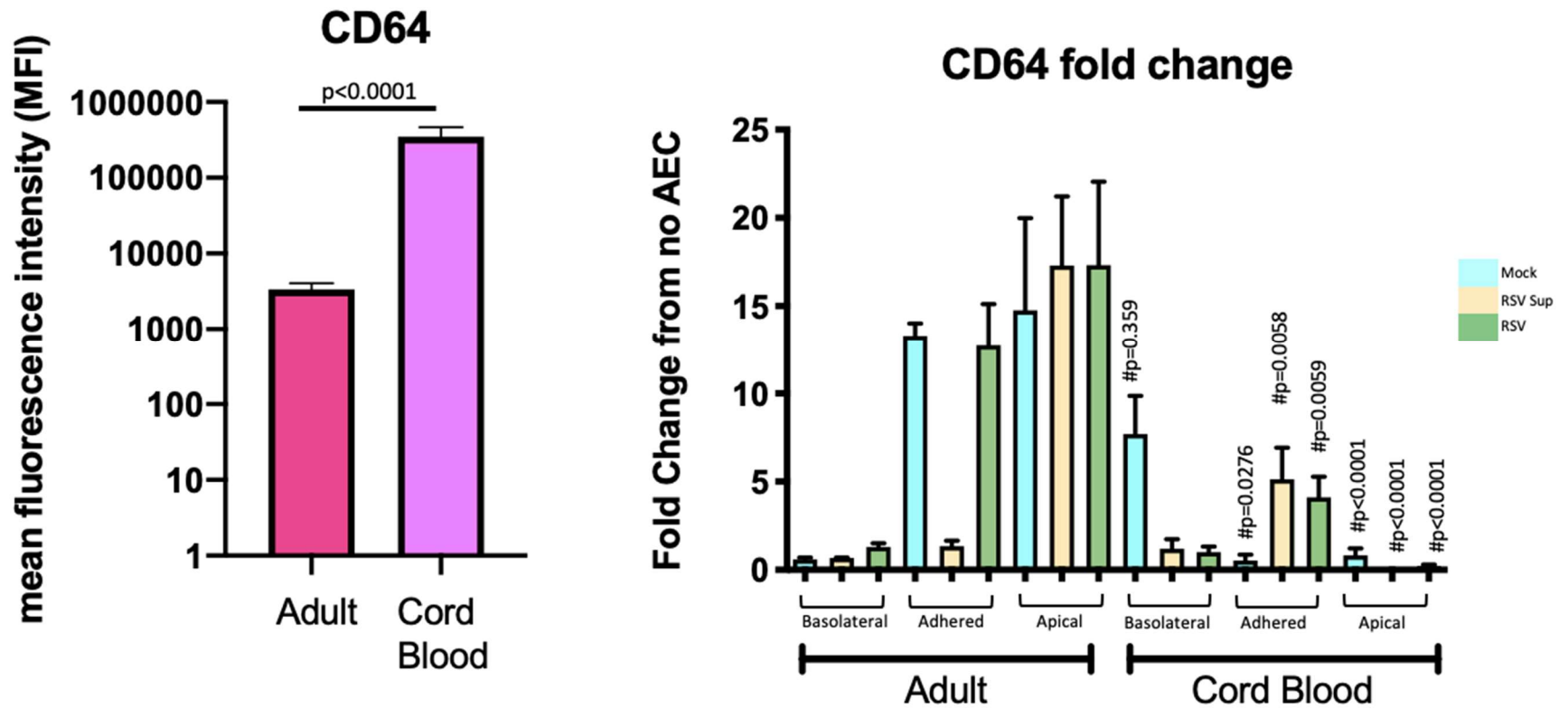


Figure 6-9 Comparing neutrophil activation marker CD64 expression on adult and cord blood neutrophils after transepithelial migration assay

(left) Graph to show difference in non AEC control groups between adult and cord blood neutrophils presented on a log₁₀ scale of mean fluorescence intensity of CD64 expression.

(right) Fold change of CD64 expression mean fluorescence intensity calculated using the respective adult or cord blood non AEC control as baseline.

Bar colours (Blue = Mock, Orange = RSV Sup, Green = RSV, Pink = Non AEC). Comparisons between groups were performed with Two-Way ANOVA with pairing and Geiser greenhouse correction. # = in comparison to respective adult neutrophils. N=5.

6.5.5.3 Migration through RSV infected ciliated epithelium increases NE expression on adult but not cord blood neutrophils

Figure 6-10 has been previously shown in **Chapter 5** of this thesis and the analysis is given in **Section 5.5.4.2**. Data presented as mean fluorescence intensity (\pm SEM).

One hour after they were added to the basolateral surface of the mock infected AECs, cord blood neutrophils isolated from the basolateral compartment, had mean NE expression 96633 (\pm 24754), from mock infected AECs; with RSV infected AEC supernatant apically (RSV Sup) mean NE expression was 104839 (\pm 44341) and for neutrophils recovered from RSV infected AECs, mean NE expression was 101364 (\pm 28951). There was no significant difference in NE expression between cord blood neutrophils recovered from the basolateral side of Mock, RSV Sup and RSV infected AECs.

For cord blood neutrophils adherent to AECs, mean NE expression on neutrophils from of mock infected AECs was 77938 (\pm 12510), from mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) mean NE expression was 246427 (\pm 102269) and for neutrophils recovered from RSV infected AECs, mean NE expression was 134837 (\pm 41312). There was no significant difference in mean NE expression between cord blood neutrophils recovered from adherent to Mock and RSV infected AECs. There was a significantly greater mean expression of NE found on neutrophils recovered adherent to RSV Sup AECs in comparison to those recovered from mock with a p value of 0.0073.

For cord blood neutrophils isolated apically to AECs, mean NE expression on neutrophils from of mock infected AECs was 175701 (\pm 80995), from mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) mean NE expression was 37890 (\pm 3220) and for neutrophils recovered from RSV infected AECs, NE expression was 57985 (\pm 33247). There was a significantly greater mean expression of NE found on neutrophils recovered apically to mock infected AECs in comparison to those recovered from RSV Sup with a p value of 0.0216. There was a significantly greater mean expression of NE found on neutrophils recovered apically to mock infected AECs in comparison to those recovered from RSV with a p value of 0.0437.

Comparing my no AEC controls between adult and cord blood neutrophils, NE expression was 67212 (\pm 9111) on cord blood neutrophils and 1525 (\pm 95) on adult neutrophils. NE expression was measured significantly higher on cord blood neutrophils with a p value of <0.0001 . To control for this observed difference in baseline expression of NE, expression of NE was calculated as fold change from the no AEC control and these data are shown in **Figure 6-11**.

Comparing mean fold change in NE expression between adult and cord blood neutrophils on the basolateral side of AECs, there was no significant difference in NE expression fold change on neutrophils recovered from RSV infected AECs. Likewise, there was no significant difference in NE expression fold change between adult and cord blood neutrophils isolated from the basolateral side of Mock infected AECs with RSV infected AEC supernatant on the apical side (RSV Sup) or those found on the basolateral side of RSV infected AECs.

Adult neutrophils recovered adherent to mock infected AECs showed a fold change of NE expression (14.64 ± 1.7) and cord blood neutrophils adherent showed (1.16 ± 0.7). This difference was significant with a p value of 0.002. Adult neutrophils recovered adherent to mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed a fold change of NE expression (8.4 ± 1.5) and cord blood neutrophils adherent showed (3.67 ± 1.5). This difference was not significant. Adult neutrophils recovered adherent to RSV infected AECs showed a fold change of NE expression (23.9 ± 2.9) and cord blood neutrophils adherent showed (1.9 ± 0.58). This difference was significant with a p value of <0.0001 .

Adult neutrophils recovered apically to mock infected AECs showed a fold change of NE expression (19.01 ± 0.7) and cord blood neutrophils apically showed (2.6 ± 1.2). This difference was significant with a p value of <0.0001 . Adult neutrophils recovered apically to mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed a fold change of NE expression (17.9 ± 0.6) and cord blood neutrophils apically showed (0.56 ± 0.04). This difference was significant with a p value of <0.0001 . Adult neutrophils recovered apically to RSV infected AECs showed a fold change of NE expression (28.5 ± 2.7) and cord blood neutrophils apically showed (0.827 ± 0.49). This difference was significant with a p value of <0.0001 .

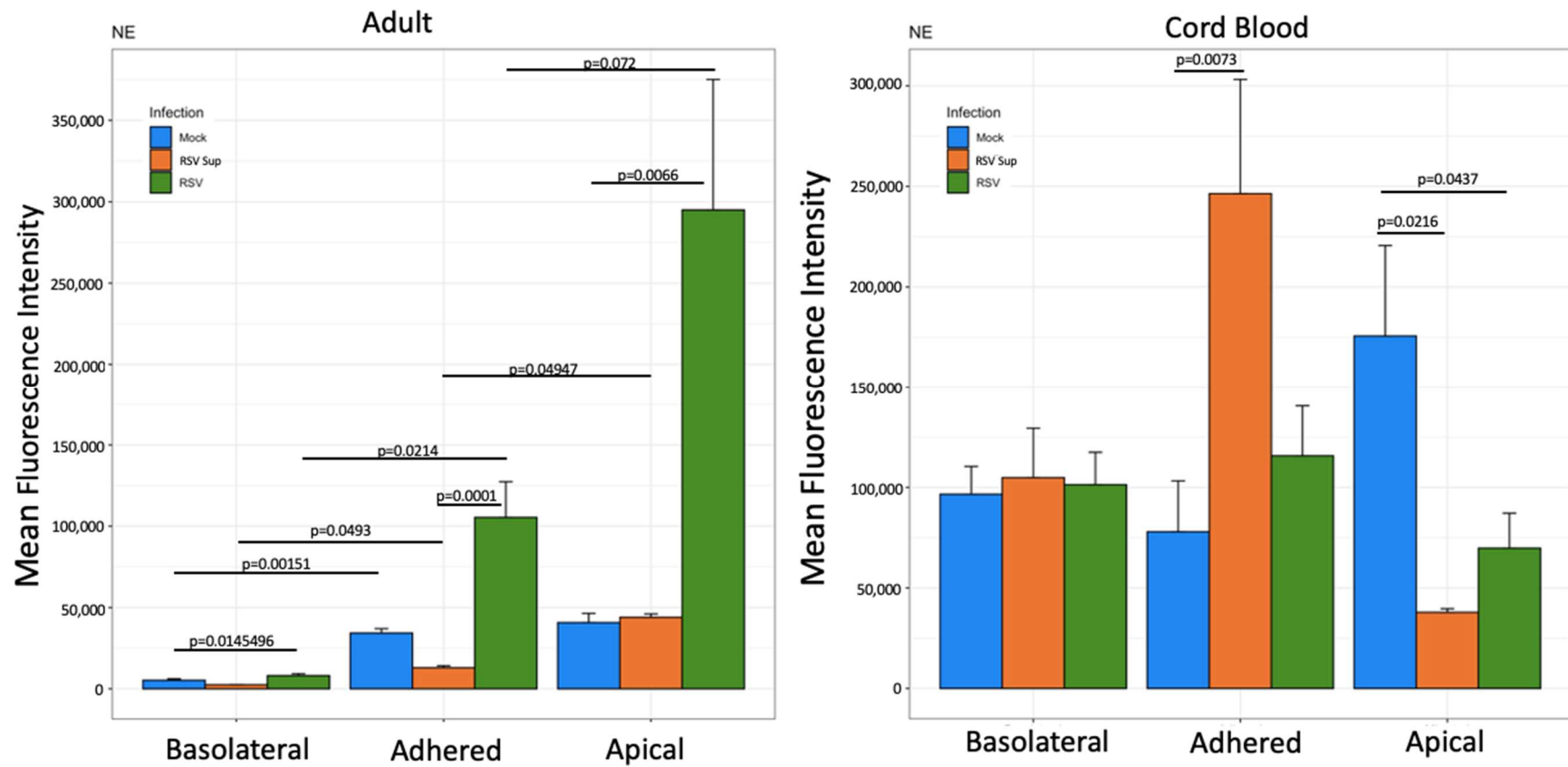


Figure 6-10 Neutrophil activation marker NE expression on adult and cord blood neutrophils after transepithelial migration assay

Mean fluorescence intensity of NE on adult (left) and cord blood (right) recovered from transepithelial migration assays through RSV infected ciliated AECs infected for 72 hours (RSV), Mock infected control AECs (Mock) or Mock infected AECs with the supernatant recovered from RSV infected ciliated AECs on the apical side (RSV Sup) as a control. Graphs are separated to distinguish neutrophils recovered from the basolateral, membrane bound (adhered) and apical (migrated) locations within the transepithelial migration assay.

Bar colours (Blue = Mock, Orange = RSV Sup, Green = RSV, Pink = Non AEC). Comparisons between groups were performed with Two-Way ANOVA with pairing and Geiser greenhouse correction. # = in comparison to respective adult neutrophil. N=5.

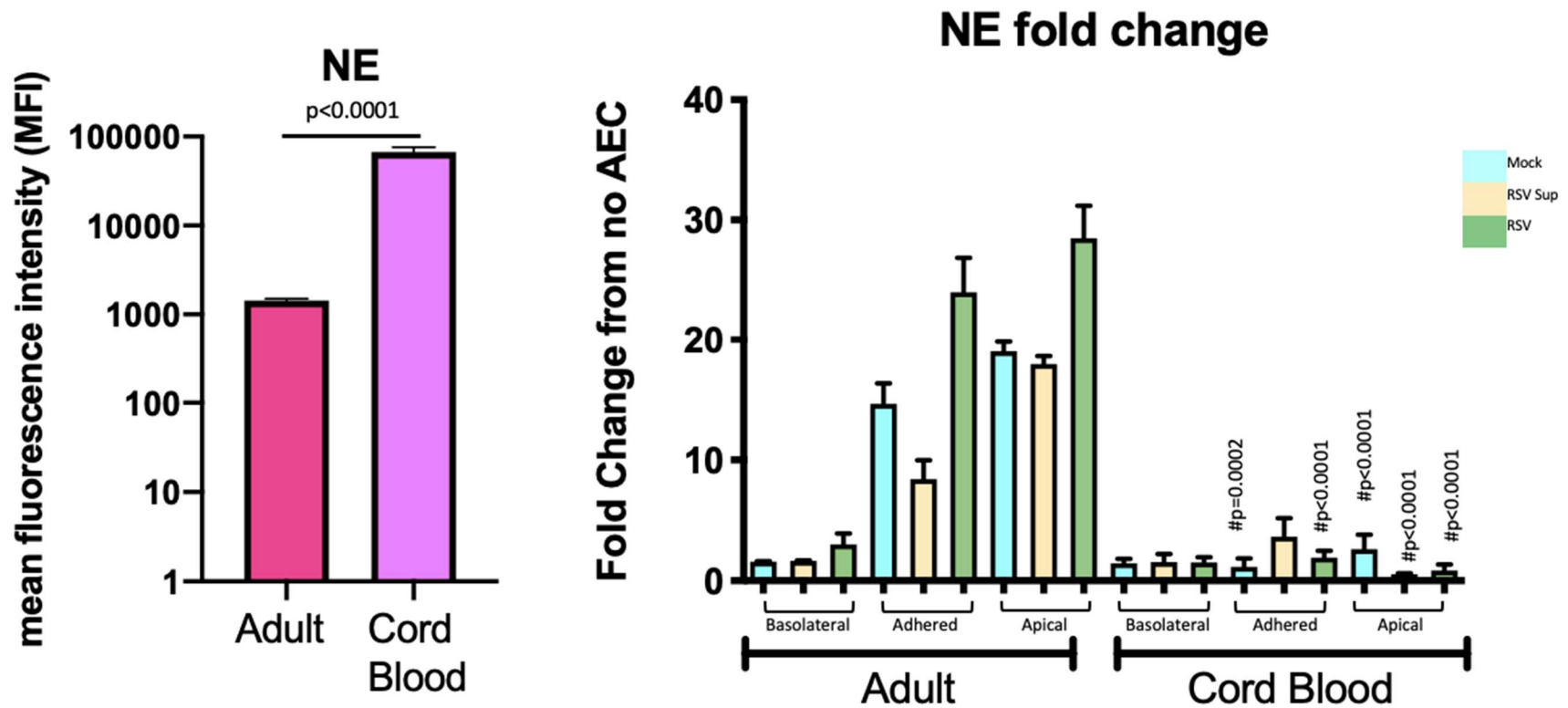


Figure 6-11 Comparing neutrophil activation marker NE expression on adult and cord blood neutrophils after transepithelial migration assay

(Left) Graph to show difference in non AEC control groups between adult and cord blood neutrophils presented on a log₁₀ scale of mean fluorescence intensity of NE expression.

(Right) Fold change of NE expression mean fluorescence intensity calculated using the respective adult or cord blood non AEC control as baseline. Mean fold change calculated from mean fluorescence intensity of NE on adult and cord blood neutrophils recovered from transepithelial migration assays through RSV infected ciliated epithelium infected for 72 hours (RSV), Mock infected control epithelium (Mock) or Mock infected epithelium with the supernatant recovered from RSV infected ciliated culture on the apical side (RSV Sup) as a control.

Bar colours (Blue = Mock, Orange = RSV Sup, Green = RSV, Pink = Non AEC) Comparisons between groups were performed with Two-Way ANOVA with pairing and Geiser greenhouse correction. # = in comparison to respective adult neutrophil. N=5.

6.5.5.4 Interaction with RSV infected ciliated epithelium increases MPO expression on adult but not cord blood neutrophils

Figure 6-12 has been previously shown in **Chapter 5** of this thesis and the analysis is given in **Section 5.5.4.2**. Data presented as mean fluorescence intensity (\pm SEM).

One hour after they were added to the basolateral surface of the mock infected AECs, cord blood neutrophils isolated from the basolateral compartment, had mean MPO expression of 126,765 (\pm 36553), from mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) MPO expression was 142273 (\pm 60022) and for neutrophils recovered from RSV infected AECs, MPO expression was 126719 (\pm 35914). There was no significant difference in MPO expression between cord blood neutrophils recovered from the basolateral side of Mock, RSV Sup and RSV infected AECs.

For cord blood neutrophils adherent to AECs, MPO expression on neutrophils from of mock infected AECs was 111,856 (\pm 75041), from mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) mean MPO expression was 482,016 (\pm 225849) and for neutrophils recovered from RSV infected AECs, MPO expression was 164304 (\pm 58633). There was no significant difference in MPO expression between cord blood neutrophils recovered from adherent to Mock and RSV infected AECs. There was a significantly greater expression of MPO found on neutrophils recovered adherent to Mock infected AECs in comparison to those recovered from RSV Sup cultures with a p value of 0.0008. There was a significantly greater expression of MPO found on neutrophils recovered adherent to RSV infected AECs in comparison to those recovered from RSV Sup cultures with a p value of 0.0024.

For cord blood neutrophils isolated apically to AECs, MPO expression on neutrophils from of mock infected AECs was 243316 (\pm 129636), from mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) MPO expression was 17942 (\pm 1244) and for neutrophils recovered from RSV infected AECs, MPO expression was 86898 (\pm 70509). There was no significant difference in MPO expression between cord blood neutrophils isolated apically to Mock and RSV Sup cultures infected cultures. There was a significantly greater expression of MPO found on neutrophils recovered apically to mock infected AECs in comparison to those recovered from RSV Sup AECs with a p value of 0.0186.

Comparing my no AEC controls between adult and cord blood neutrophils, MPO expression was 60,438 (\pm 19216) on cord blood neutrophils and 2619 (\pm 532) on adult neutrophils. MPO expression was measured significantly higher on cord blood neutrophils with a p value of <0.0001 . To control for this observed difference in baseline expression of MPO, expression of MPO was calculated as fold change from the no AEC control and this data is shown in **Figure 6-13**.

Comparing fold change in MPO expression between adult and cord blood neutrophils on the basolateral side of AECs, there was no significant difference in MPO expression fold change on neutrophils recovered from mock infected AECs. Likewise, there was no significant difference in MPO expression fold change between adult and cord blood neutrophils isolated from the basolateral side of Mock infected AECs with RSV infected AEC supernatant on the apical side (RSV Sup).

Adult neutrophils adherent to mock infected AECs showed a mean fold change of MPO expression (14.64 ± 1.6) and cord blood neutrophils adherent showed (1.16 ± 0.67). This difference was significant with a p value of 0.0002. Adult neutrophils recovered adherent to mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed a fold change of MPO expression (8.4 ± 1.6) and cord blood neutrophils adherent showed (3.66 ± 1.52). This difference was not significant. Adult neutrophils recovered adherent to RSV infected AECs showed a fold change of MPO expression (23.98 ± 2.85) and cord blood neutrophils adherent showed (1.9 ± 0.5). This difference was significant with a p value of <0.0001 .

Adult neutrophils recovered apically to mock infected AECs showed a fold change of MPO expression (97.7 ± 3.97) and cord blood neutrophils apically showed (2.6 ± 1.2). This difference was significant with a p value of <0.0001 . Adult neutrophils recovered apically to mock infected AECs with RSV infected AEC supernatant apically (RSV Sup) showed a fold change of MPO expression (132.8 ± 4.95) and cord blood neutrophils apically showed (0.5 ± 0.04). This difference was significant with a p value of <0.0001 . Adult neutrophils recovered apically to RSV infected AECs showed a fold change of MPO expression (179.3 ± 54.01) and cord blood

neutrophils apically showed (0.86 ± 0.49). This difference was significant with a p value of <0.0001 .

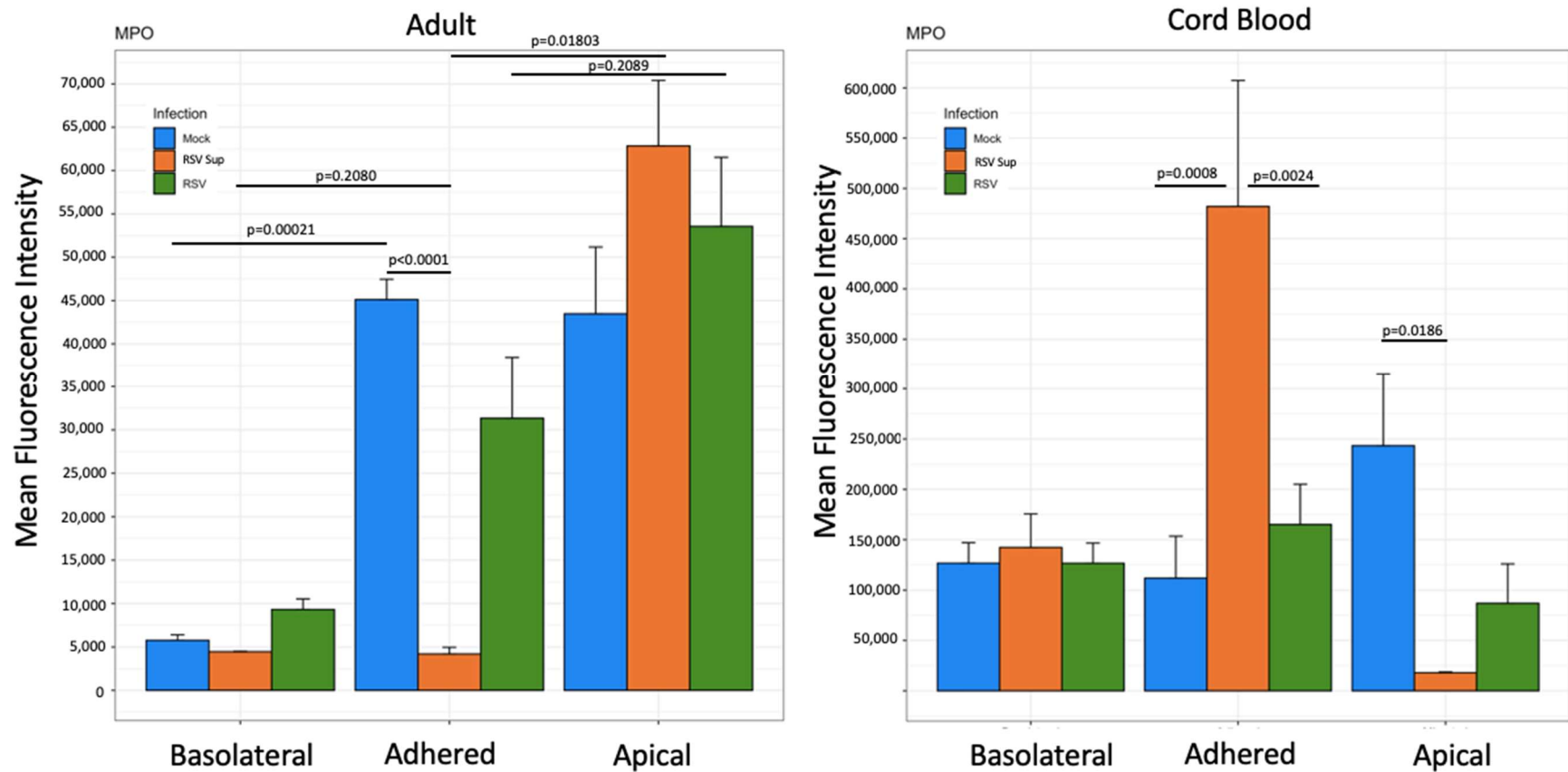


Figure 6-12 Neutrophil activation marker MPO expression on adult and cord blood neutrophils after transepithelial migration assay

Mean fluorescence intensity of MPO on adult (left) and cord blood (right) recovered from transepithelial migration assays through RSV infected ciliated AECs infected for 72 hours (RSV), Mock infected control AECs (Mock) or Mock infected AECs with the supernatant recovered from RSV infected ciliated AECs on the apical side (RSV Sup) as a control. Graphs are separated to distinguish neutrophils recovered from the basolateral, membrane bound (adhered) and apical (migrated) locations within the transepithelial migration assay.

Bar colours (Blue = Mock, Orange = RSV Sup, Green = RSV, Pink = Non AEC) Comparisons between groups were performed with Two-Way ANOVA with pairing and Geiser greenhouse correction. # = in comparison to respective adult neutrophils. N=5.

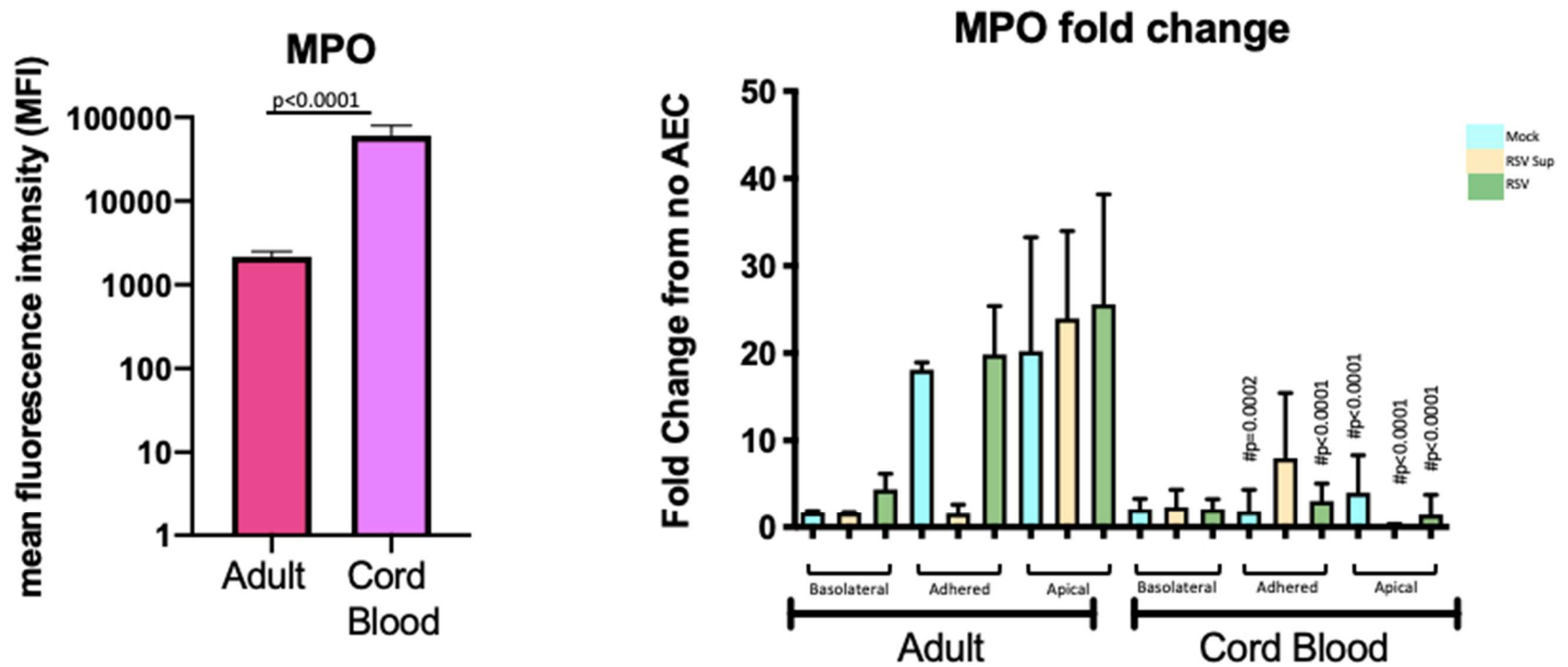


Figure 6-13 Comparing neutrophil activation marker MPO mean expression on adult and cord blood neutrophils after transepithelial migration assay

(Left) Graph to show difference in non AEC control groups between adult and cord blood neutrophils presented on a log₁₀ scale of mean fluorescence intensity of MPO expression.

(Right) Fold change of MPO expression mean fluorescence intensity calculated using the respective adult or cord blood non AEC control as baseline.

Bar colours (Blue = Mock, Orange = RSV Sup, Green = RSV, Pink = Non AEC). Comparisons between groups were performed with Two-Way ANOVA with pairing and Geiser greenhouse correction. # = in comparison to respective adult neutrophils. N=5.

6.5.6 Neutrophil degranulation during transepithelial migration through the RSV infected epithelium

As NE and MPO are also secreted by neutrophils in response to infection, I then wanted to measure their concentration in AEC supernatants after adult and cord blood neutrophil transepithelial migration to determine the extent of difference between the two. Data from adult neutrophil transepithelial migration presented in **Figure 6-14** has been previously shown in **Chapter 5** of this thesis and the analysis is given in **Section 5.5.5**.

Examining NE concentration in apical supernatants recovered from AECs after 1 hour of cord blood neutrophil transepithelial migration, NE concentration apical to 24 hour mock infected AECs was 1.26mU/ml (± 0.3), apical to 24 hour RSV Sup AECs NE concentration was 1.46mU/ml (± 0.25) and apical to 24 hour RSV infected AECs was 1.3mU/ml (± 0.2). Likewise, NE concentration apical to 72 hour mock infected AECs was 1.29mU/ml (± 0.216), apical to 72 hour RSV Sup AECs NE concentration was 1.51 mU/ml (± 0.19) and apical to 72 hour RSV infected AECs was 1.65mU/ml (± 0.37). There was no significant difference in NE concentration after cord blood transepithelial migration in apical supernatants recovered from mock, RSV Sup or RSV AECs at either 24 or 72 hours.

Comparing NE concentration apical to 24 hour RSV infected AECs after 1 hours transepithelial migration of adult and cord blood neutrophils, NE concentration after adult neutrophil migration was 0.608mU/ml ± 0.1 and after cord blood neutrophil migration was 1.3mU/ml ± 0.2 . This difference was significant with a p value of 0.0466. Similarly, examining NE concentration apical to 72 hour RSV infected AECs after 1 hours transepithelial migration of adult and cord blood neutrophils, NE concentration after adult neutrophil migration was 0.66mU/ml ± 0.15 and after cord blood neutrophil migration was 1.65mU/ml ± 0.37 . This difference was significant with a p value of 0.0213.

After cord blood transepithelial migration, MPO concentration apical to 24 hour mock infected AECs was 0.066mU/ml (± 0.046), apical to 24 hour RSV Sup AECs MPO concentration was 0.21mU/ml (± 0.044) and apical to 24 hour RSV infected AECs was 0.29mU/ml (± 0.071). Likewise, MPO concentration apical to 72 hour mock infected AECs was 0.04mU/ml (± 0.03), apical to 72 hour RSV Sup AECs MPO concentration was 0.82 mU/ml (± 0.7) and apical to 72

hour RSV infected AECs was 1.23mU/ml (± 0.89). There was no significant difference in MPO concentration in apical supernatants recovered from mock, RSV Sup or RSV AECs at either 24 or 72 hours. Comparing MPO concentration in the apical supernatant after 1 hours transepithelial migration of mock, RSV and RSV Sup AECs, there was no significant difference between adult and cord blood neutrophil transepithelial migration.

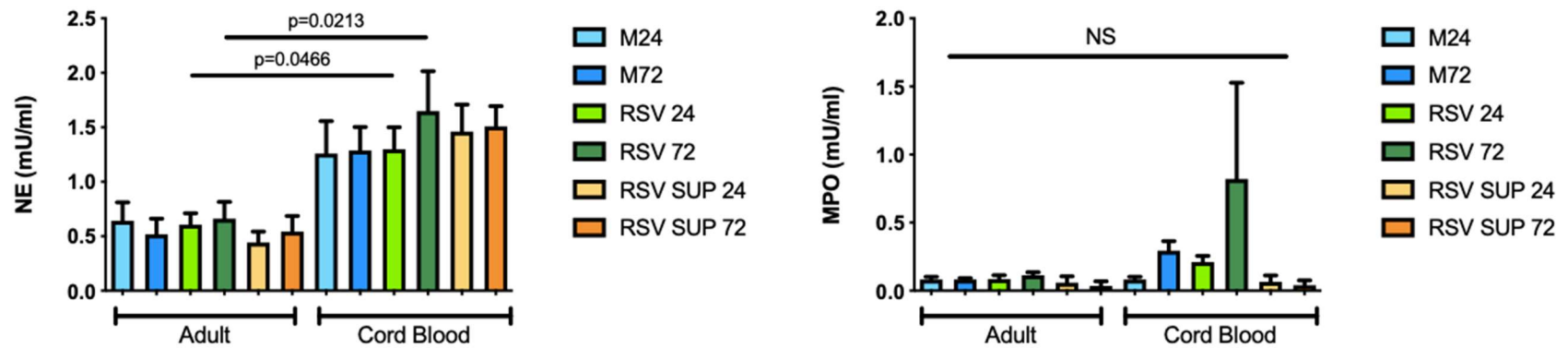


Figure 6-14 Concentration of Neutrophil Elastase and Myeloperoxidase in the apical supernatants collected after neutrophil migration of adult and cord blood neutrophils through RSV infected AECs

Concentrations of NE (left) and MPO (right) in the apical supernatants collected after neutrophil migration through ciliated AECs infected with RSV for 24 (RSV 24) or 72 hours (RSV 72), Mock infected control AECs infected for 24 (Mock 24) or 72 hours (Mock 72) or Mock infected AECs with the supernatant recovered from RSV infected ciliated AECs on the apical side (RSV SUP 24) (RSV SUP 72) as controls. Individual comparisons performed with Comparisons between groups was performed using One-Way ANOVA with Tukeys adjustment for multiple comparisons. N=5-6.

6.6 Discussion

Data presented in this chapter demonstrate that cord blood neutrophils show distinctly different migratory and apoptotic profiles to adult neutrophils in a transepithelial migration model of the RSV infected lung. Firstly, my experiments revealed that greater numbers of cord blood neutrophils migrate through RSV infected AECs in comparison to adult neutrophils. This difference was not anticipated, given previous reports that neonatal neutrophils have depressed chemotactic and migratory functions *in vitro*(161,261). Secondly, cell viability analysis showed that a greater proportion of cord blood neutrophils adherent to and dissociated apically from RSV infected AECs were apoptotic in comparison to adult neutrophils. Finally, analysing neutrophil activation showed adult neutrophils were able to upregulate activation markers in response to migration whereas cord blood neutrophils rarely increased more than two-fold in comparison to no AEC controls.

Adults have been documented previously to show similar neutrophil counts in the blood to infants born at full term, however, as discussed in **Chapter 1**, there have been many reports claiming impaired neonatal neutrophil functions of chemotaxis, formation of the lysosome and phagosome and migration during infection(139,164,165,262). Using cord blood neutrophils as a surrogate for infant immune cells is a common approach in studies investigating infant responses to infection, however their suitability for this purpose has not been formally investigated nor their comparative function to infant neutrophils characterised(263,264). However general consensus is that neonatal neutrophil function is comparable to that of an adult after a few weeks of age, with one study investigating neonatal neutrophil chemotaxis showing similar speeds to adult neutrophils developing by 2 weeks post birth(265,266).

If these differences in chemotaxis and migratory function seen in neonates are also true in cord blood neutrophils, why in this study do cord blood neutrophils show heightened migration numbers through RSV infected AECs in comparison to mock infected AECs, and in comparison, to adult neutrophils migrating through RSV infected AECs? It is important to note the same large numbers of cord blood neutrophils were seen migrating across mock infected AECs with RSV infected AEC supernatant placed apically (RSV Sup). In clinical studies,

McNamara et al showed a greater neutrophilic cellularity in the BAL of children with RSV bronchiolitis in comparison to age matched uninfected children(81). My results here follow the same pattern, with large numbers of migrating neutrophils correlated with the presence of RSV infected AEC supernatants on the apical side of AEC cultures. This is not consistent with previously discussed literature documenting a chemotactic deficit in neonatal neutrophils(266). However, it does illustrate a potential difference between adult and neonatal neutrophils in their responses to RSV infection, which none of these previous studies examined. It is clear neutrophil function which differs from the norm is implicated in pathogenesis of severe disease, and sheer numbers of migrating neutrophils may go some way to explain clinical findings of airway neutrophilia, airway damage and mechanical blockage of the small airways due to debris which includes dead and apoptotic neutrophils.

I also observed greater numbers of cord blood neutrophils remaining adherent to RSV, mock and RSV Sup AEC cultures in comparison to adult neutrophils. Neonatal neutrophil adherence to AECs has not been extensively studied in sophisticated *in vitro* systems, however differences in neutrophil adherence has been postulated to be influenced by method of delivery with infants born by elective caesarean section exhibiting greater neutrophil adherence to nylon fibre in comparison to those born vaginally or by emergency caesarean section(267). All cord blood donors in this study were consented prior to elective caesarean section and so this was not evaluable in my study, but any potential difference was controlled for. It has also been demonstrated that neutrophil adherence to an epithelial cell line (A549) acts as a driver to the epithelial cells to produce more IL-8, a potent chemoattractant(268). This phenomenon may be at play in our system, with increased neutrophil adherence driving continued neutrophil migration across AEC cultures facilitated by increased IL-8 production. A future study using this model measuring these key mediators in the supernatant would be important in investigating this hypothesis.

But which characteristics of cord blood neutrophils in comparison to adult neutrophils facilitates increased adherence to AECs? Herbert et al described adherence in the same model to be mediated by LFA-1, a β_2 integrin which, at least in part, mediated neutrophil adherence to the RSV infected AECs(185). However, several studies have illustrated similar levels of

expression of LFA-1 between adult and neonatal neutrophils(269,270). It has been shown previously that neonatal neutrophils do not upregulate their CD11b expression in response to the exogenous bacterial peptide fMLP to the same magnitude as adults (271). Despite finding reliably that cord blood neutrophils displayed greater expression of activation markers, CD11b, CD64, NE and MPO in this chapter I also showed I found a similar deficit in upregulation in response to transepithelial migration of cord blood neutrophils in comparison to adult. The roles of these activation markers I have previously discussed in **Chapter 4**. Where I observed upregulation in our adult model, the same was not observed in my cord blood neutrophil experiments. Halfhide et al measured neutrophil activation markers in neonates with RSV bronchiolitis and found neutrophils retrieved from airways of infants with RSV bronchiolitis showed greater expression of CD11b than neutrophils from the blood, or the airways of healthy age matched control children(75). Similar magnitudes of increase in CD11b to Halfhide's study was seen in my cord blood data presented in this chapter, but these magnitudes of increase were not comparable to the differences seen in my adult neutrophil data. It could be hypothesised that similar processes *in vivo* and in our *in vitro* model are leading to these comparable findings.

In my analysis of the apical supernatants after adult and cord blood neutrophil transepithelial migration, secreted NE concentration was significantly greater in cord blood migration supernatants in comparison to adult. This is important to interpret in the context of neutrophil numbers, having shown greater numbers of cord blood neutrophils are found apically to RSV infected AECs after 1 hours transepithelial migration assay in comparison to adult. It could be that this is simply explained by the presence of greater numbers of cord blood neutrophils in releasing NE into the apical supernatant in comparison to adult. However, this is unlikely to be the full picture as NE concentration in the apical supernatant is roughly double in cord blood compared to adults, but numbers of cord blood apical and adhered neutrophils are much greater than double that of adults. It could be that each individual cord neutrophil is producing less NE than each individual adult neutrophil, but due to greater numbers the overall concentration is significantly more. Neutrophils in neonates have been previously shown to exhibit lower levels of degranulation and release of NE and other azurophilic

contents which supports this theory(159,272,273). Another possibility is that cord blood neutrophils adhered to the RSV infected AECs produce different amounts of NE in comparison to those present apically but not adhered, therefore adhered and apical neutrophils present contribute differentially to NE concentration. Unfortunately, it was not possible to test this in the scope of my thesis. However, the increased concentration of NE measured apically to AECs in my cord blood experiments in comparison to adult is highly clinically relevant. Neutrophil granule products including NE specifically have been implicated in damage to neonatal airway tissues during acute respiratory distress syndrome in neonates(274,275). It could be suggested a greater concentration of granule products such as NE present in airways of neonates with RSV Bronchiolitis may contribute to inflammation, airway damage and severe clinical symptoms.

Examining the apoptosis of neutrophils on the basolateral side of AECs, there was no significant difference in viability or apoptosis of cord blood neutrophils recovered from Mock, RSV Sup or RSV AECs in comparison to each other or non-AEC controls. However the same was not true of adult neutrophils, which showed significantly greater proportions of apoptosis when recovered from the basolateral side of RSV infected AECs in comparison to Mock, RSV Sup and no AEC neutrophils. It has been previously published that neonatal neutrophils are constitutively slower to undergo apoptosis in comparison to adults, due to lower expression of CASPASE 3(172), which could potentially explain the low levels of apoptosis in cord blood neutrophils recovered from the basolateral sides of AECs in this study in comparison to adult neutrophils, although this was a single time point and temporal analysis was not undertaken. It could also be the case that cord blood neutrophils are less responsive than adults to potential secreted mediators produced by RSV infected cells which induce apoptosis. Future differential analysis of secreted mediators released basolaterally and apically may shed light on this hypothesis.

Comparing apoptosis in neutrophils adhered to RSV infected AECs, cord blood neutrophils showed much greater proportions apoptotic in comparison to adults. In cord blood neutrophils, those adhered to RSV SUP AECs showed very similar levels of apoptosis to those adhered to RSV infected AECs which was not the case with adult neutrophils where far greater

proportions were apoptotic when adhered to RSV SUP AECs and very few were apoptotic when adhered to RSV infected AECs. Apoptosis of neutrophils adhered to mock infected AECs was comparable in adult and cord blood neutrophils. This difference is highly suggestive of a difference in response to specifically virally infected cells between adult and cord blood neutrophils as this is the only difference in experimental conditions between RSV SUP and RSV infected AECs. In adult neutrophils, this exposure seems to increase viability of neutrophils and decrease apoptosis in contrast to those exposed to RSV Sup cultures, but in cord blood experiments no difference was found.

In this chapter, I demonstrated that apical cord blood neutrophils showed greater proportions apoptotic in comparison to adult neutrophils in Mock AECs, RSV SUP AECs and RSV infected AECs. This is the closest correlate neutrophil population present in our model to those found in airway lumen of infants with RSV bronchiolitis, sampled by BAL or NPA. In previous analysis of clinical NPA samples recovered from infants with RSV bronchiolitis, a similar proportion of apoptotic neutrophils were present in NPA samples from infants with RSV bronchiolitis in comparison to what I observed in cord blood neutrophils recovered apically to RSV infected AECs(225). Not only was the proportion of apoptotic cord blood neutrophils found apically to RSV infected AECs greater than that of adults, significantly greater numbers of cord blood neutrophils were found apically to RSV infected AECs in comparison to adult. It is possible that sheer numbers of apoptotic neutrophils may overwhelm the ability of tissue resident phagocytes to clear them, driving secondary necrosis and driving further inflammation as has been documented in adult lung diseases(276–278).

A limitation of the experiments in this chapter were, unlike in my adult neutrophil experiments, it is not possible to use an AEC culture autologous to the cord blood neutrophil donor. However, this limitation is in part mitigated by the data shown in **Chapter 2** comparing autologous vs allogenic neutrophil-AEC pairing and the effect of this on neutrophil adherence and CD11b expression. In these experiments, I did not see a difference between adherence or CD11b expression in donors which were matched vs those which were not. The second limitation of these experiments were the differences in wait times between processing of cord blood samples and healthy volunteers. With the healthy volunteers it was possible to isolate

neutrophils immediately, whereas with cord blood samples I needed to transport the blood from the hospital to the lab which took about 20 minutes. It is possible this lead time may have influenced the recorded neutrophil responses, however everything possible was done to ensure efficient transfer of samples.

6.7 Summary

Data presented here demonstrates key differences in migration, apoptosis and activation in response to RSV infected AECs, between adult and cord blood derived neutrophils. In addition, observations seen in this study are mirrored in clinical studies of infants with RSV bronchiolitis, fostering support for the use of this *in vitro* transepithelial migration model for further study into interactions between neutrophils and RSV infected AECs.

Chapter 7 General Discussion

RSV bronchiolitis is a disease characterised by inflammation and neutrophil infiltration of the airways(79). The neutrophil mediated immune response to RSV has been understudied, partly because of the difficulties of undertaking clinical studies in small, acutely ill infants and due to the commonly held belief that neutrophils are mainly phagocytes of macroscopic pathogens and are not key to antiviral defence. However, recent developments in neutrophil research have now shown that neutrophils have roles in both promoting and suppressing inflammation, as well as the ability to regulate the immune response of other cells(279). In this thesis, the neutrophilic infiltration of RSV infected AECs has been modelled using a novel *in vitro* approach with transepithelial migration, and this model was used to explore the interaction and functional response of neutrophils to RSV infected AECs.

The overall aim of the work described in this thesis was to explore the interaction between neutrophils and RSV infected airway epithelial cells (AECs) and the effect of this interaction on neutrophil function. To do this, I needed to establish an *in vitro* model of the interaction between neutrophils and RSV infected AECs. The development of this model, using ultra purified neutrophils, ciliated human primary AECs grown at air liquid interface, and laboratory grown GFP RSV is described in **Chapter 2** and has been previously published by my research group (185). In previous literature, most studies of RSV infection of AECs have been conducted using immortalised cell lines such as A549s, which although highly reproducible, are not good physiological surrogates for human airway epithelium(201). Indeed, in the development of our model, our group first used A549 cells to study neutrophil migration(201). Using primary human epithelium grown at air liquid interface enables differentiation to occur including ciliation and the polarisation of an AEC layer, allowing the possibility to migrate neutrophils across infected AECs in a physiological basolateral to apical direction(280). This high-level differentiation and the potential to match airway donor to neutrophil donor, is a great step forward in improving translational models of clinical disease and the work in this thesis is strengthened by this. Likewise, primary human neutrophils are not easy to manipulate in a laboratory setting, due to their short lifespan *in vitro* and incompatibility with culture, storage

or resurrection. Due to this, it is important to isolate neutrophils on the day of an experiment, which poses logistical and ethical difficulties in identifying volunteers, accounting for illnesses and conditions which may affect the neutrophils and coordinating a convenient time between the researcher and volunteer donor. For this reason, many previous translational studies into RSV infection have used immortalised immune cell lines such as HL-60s, THP-1s or other phagocytes such as macrophages in place of neutrophils as they are much more amenable to culture(281–283). In this thesis, the examination of primary human neutrophils, and not a cell line increases the validity of this study, better modelling the neutrophil-RSV infected AEC interaction and allowing better translation of results back to clinical disease.

The first specific aim was to explore the profile of neutrophil movement across RSV infected AECs. In **Chapter 3**, I modified the transepithelial migration assay for microscopy to be able to image neutrophil transepithelial migration in my *in vitro* model as a 3D time lapse. Data in this chapter showed neutrophils migrating through RSV infected AECs in comparison to mock infected AECs show different patterns of adherence to AECs, speed and displacement while moving across AECs. It also illustrated the disappearance of RSV infected AECs during neutrophil transepithelial migration correlated with reduction in viral titre.

Using this technique, it was possible to compare the chemotactic and migratory behaviour of neutrophils exposed to RSV infected AECs and mock infected AECs, which showed that neutrophils moving across mock infected AECs move faster and further than neutrophils moving across RSV infected AECs. This was contrary to what I had expected to find, given that I found that neutrophils move faster and further towards the RSV infected AEC supernatants, in which I demonstrated greater concentrations of IL-8 and IP-10, in the absence of AECs. This led me to hypothesise that increased interaction of migrating neutrophils with RSV infected AECs, mediated either by permanent mechanical interaction between neutrophils facilitating adherence and AECs or transient interaction between neutrophils and RSV infected AECs allowing ‘patrolling’ movement along the apical side in response to local concentration gradients of secreted chemoattractants.

The second specific aim was to examine the effect of RSV infection of AECs on neutrophil apoptosis. In **Chapter 4**, I examined neutrophils at different positions within the

transepithelial migration model and determined what proportion were viable, apoptotic and dead. I also examined the effect of RSV alone, RSV proteins and RSV infected AEC supernatants on neutrophil apoptosis. Data presented in this chapter showed RSV and RSV G protein reduce neutrophil apoptosis, as do supernatants recovered from both mock and RSV infected ciliated epithelial cultures. It also showed neutrophils incubated with RSV infected ciliated AECs demonstrate greater apoptosis compared to mock controls and this is the same for neutrophils which migrate through RSV infected ciliated AECs and dissociate. Finally, this chapter showed that significantly more neutrophils remaining adherent to the RSV infected AECs were viable and fewer were apoptotic than those adherent to mock infected AECs.

Previous studies into neutrophil apoptosis during RSV infection have differed significantly in their methodologies and in their conclusions. Notably, Wang *et al* showed neutrophil apoptosis was increased by RSV infection by measuring apoptosis in the airways and peripheral blood of infants with RSV bronchiolitis in comparison to peripheral blood neutrophils of age matched healthy control children(224). In contrast to this, a separate study by Jones *et al* examined the effect of incubation with nasal lavage fluid (NLF) from RSV bronchiolitis patients on the apoptosis of healthy adult neutrophils *in vitro*(212), showing less apoptosis and greater viability in neutrophils incubated with NLF from infants with RSV bronchiolitis in comparison to NLF from healthy control children. Both studies measured apoptosis using annexin V and propidium iodide co-staining as was the case in my studies. Using this sophisticated *in vitro* transepithelial migration model, I aimed to investigate neutrophil apoptosis in more detail, in order to unpick these discrepancies. In this thesis I showed that neutrophil apoptosis is reduced by incubation with supernatants from RSV infected AECs, in agreement with what Jones *et al* described. I also found neutrophils that migrated through RSV infected AECs and dissociated showed increased levels of apoptosis that, if translated to a clinical scenario, is in keeping with what Wang *et al* described. Unlike these previous studies which investigated different snapshots of neutrophils multifaceted interaction with RSV infected AECs, my studies enabled a fuller picture of neutrophil apoptosis at different locations and stages of neutrophil interaction with RSV infected AECs. These findings combined with previous literature lead me to hypothesise that neutrophils which

transmigrate through RSV infected AECs and stay in close associated to the AECs are initially stimulated to stay viable by soluble factors released by the AECs, then once their function has been fulfilled and they dissociate from the AECs and begin to apoptose.

The third specific aim was to examine the effect of RSV infection of AECs on neutrophil activation. In **Chapter 5**, I examined neutrophils at different positions within the transepithelial migration model and determined their expression density of cell membrane based (CD11b and CD64) and secretory markers (NE and MPO) of neutrophil activation. Specific functions of these markers are discussed in **Chapter 4**. Data in this chapter showed that CD11b, an integrin mediating attachment to AECs, is expressed in greater amounts as neutrophils migrate through RSV infected AECs in a stepwise manner from basolateral, to adherent to apical neutrophils to a greater extent than seen in the mock. It also showed NE, a destructive enzyme made by neutrophils, is expressed in greater amounts as neutrophils migrate through RSV infected AECs in a stepwise manner from basolateral, to adherent to apical neutrophils but this stepwise increase was not seen in the mock. Whereas, MPO, another granular enzyme produced by neutrophils, and CD64, an Fc receptor upregulated in neutrophils in response to infection, were increased on neutrophils found adherent or apically to RSV infected cultures in comparison to those found basolaterally. Previous clinical studies investigating activation markers of neutrophils from infants with severe RSV bronchiolitis showed that greater expression of CD11b and MPO is found on neutrophils recovered in BAL in comparison to those isolated from peripheral circulation(75). Translating this into my *in vitro* model, I also found that neutrophils, which had migrated across to the apical side of RSV, infected AECs showed greater expression of CD11b and MPO in comparison to those which had not. This strengthens the hypothesis that migration *per se* is mediating the expression of neutrophil activation markers, or that neutrophils with greater expression of activation markers were more likely to migrate. An observation that I could not explain was the low level of expression of CD64 and MPO in neutrophils adherent to mock infected AECs which had the supernatant from RSV infected AECs on the apical side. CD64 is an Fc receptor present on neutrophils which is upregulated to allow greater detection of opsonised pathogen, and MPO is a secreted effector enzyme released during degranulation or retained as part of the

lysosome(49,248). I speculate this could be due to exposure of mock infected AECs to the supernatant from RSV infected AECs causes an unanticipated change in the secretory profile of uninfected AECs, mediating the release of a different pattern of inflammatory markers, however further research is needed to fully investigate this.

The final specific aim was to compare the difference in function of neutrophils from adults and neonates during RSV infection. In **Chapter 6**, I conducted neutrophil transepithelial migration assays with neutrophils derived from umbilical cord blood of healthy infants born at term, in comparison to neutrophils from healthy adults. Data in this chapter showed much greater numbers of cord blood neutrophils migrate through RSV infected AECs in comparison to adult neutrophils and that a greater proportion of cord blood neutrophils adherent to and dissociated from RSV infected AECs were apoptotic in comparison to adult neutrophils. This chapter also demonstrated that cord blood derived neutrophils showed greater baseline activation levels than adult neutrophils and in addition; where adult neutrophils were able to upregulate their activation markers in response to transepithelial migration cord blood neutrophils did not. Comparing neutrophil activation markers in cord blood neutrophils to adults, cord blood neutrophils demonstrated consistently greater in expression markers in comparison to adults, however where I observed upregulation in our adult model in response to migration or infection, the same was seldom observed in my cord blood neutrophil experiments. As discussed earlier in this section, Halfhide et al measured neutrophil activation markers in neonates with RSV bronchiolitis and found neutrophils retrieved from airways of infants with RSV bronchiolitis showed greater expression of CD11b than neutrophils from the blood, or the airways of healthy age matched control children(75). Similar magnitudes of increase as shown by Halfhide in CD11b was seen in my cord blood data, but these magnitudes of increase were not comparable to the differences seen in my adult neutrophil data. It could be hypothesised that similar mechanisms are at play in our two studies which future translational studies may shed more light on.

In this study, I also observed that a greater proportion of neutrophils adherent to or migrated across RSV infected AECs are apoptotic in cord blood neutrophil experiments in comparison to adult. This result was of particular interest due to conflicting opinions in the literature over

the effect of RSV infection on neutrophil survival *in vivo* and *in vitro*(224,225). It has been previously published that neonatal neutrophils are constitutively slower to undergo apoptosis in comparison to adults, due to lower expression of CASPASE 3(172), which was the case in neutrophils recovered from the basolateral sides of AECs in this study. It is important to interpret this greater proportion of apoptotic neutrophils in the context of the numbers of cord blood neutrophils retrieved from the apical side of RSV infected AECs. From these data it is likely that not only were there more apoptotic cord blood neutrophils on the apical side of RSV infected cultures, but also more viable neutrophils as the population as a whole is larger.

Overall, the overwhelming majority of neutrophils found from cord blood transepithelial migration experiments in comparison to adults are found apically. Whatever purpose they served, if any, on the apical side of AECs is likely executed quickly and then the neutrophils became apoptotic. This potentially demonstrates a protective mechanism as by becoming apoptotic they can be recycled and scavenged quickly(276,277). However high numbers of apoptotic neutrophils present may overwhelm the ability of airway resident scavenger phagocytes to clear them, a key part of the characteristic inflammation free resolution of apoptosis, may be overwhelmed, driving secondary necrosis and continued inflammation(278).

In the context of the data presented in this translational *in vitro* study, I would like to revisit the question posed at the start of my research of whether neutrophils are sufficient and necessary for removing RSV and resolving infection or whether their presence is enhancing inflammation causing damage to AECs.

On one hand, neutrophil numbers, activation state and apoptosis may regulate what is deemed an effective response to RSV and indeed may facilitate viral killing and subsequent resolution of inflammation. In **Chapter 3**, I showed an antiviral effect of neutrophils as neutrophil transepithelial migration was correlated with disappearance of RSV infected AECs and reduction in infective viral titre, indicating neutrophils are aiding the removal of RSV. This is the first time this interaction has been imaged, and implicated neutrophils adhered to the AECs were the effector population of this effect. In **Chapter 3**, I showed greater proportions of adult neutrophils adhere to RSV infected AECs in comparison to mock infected AECs, placing

greater numbers of neutrophils in a location to exert this effect, and in **Chapter 4** I demonstrated almost all neutrophils adhered to RSV infected AECs were viable and hypothetically able to execute their antiviral functions. In **Chapter 4**, I showed upregulation of neutrophil activation markers with migration through RSV infected AECs, increasing neutrophil capacity to respond to virus. Building on this, in **Chapter 6** I showed cord blood neutrophils, a more physiologically relevant group in comparison to the age of patients affected by RSV disease, showed constitutively greater expression of all expression markers, which could be interpreted that neonatal neutrophils are 'better prepared' to fight RSV infection.

On the other hand, the possibility of an 'overly exuberant' immune response may drive continued inflammation and cause collateral damage to host tissue. Neutrophil derived chemokines and granular products could well be responsible for increased damage to the airway (236). As previously stated, In **Chapter 5** I showed greater numbers of adult neutrophils remain adherent to RSV infected AECs. Comparing this to cord blood neutrophils in **Chapter 6**, even greater numbers of neutrophils were counted adherent to RSV infected AECs, meaning even greater concentration of neutrophil products are present in close proximity to AECs. Given that Herbert et al demonstrated in this model that reducing neutrophil binding to AECs using LFA-1 blocker reduced damage caused to the AECs, it is not inappropriate to correlate greater levels of adherence to increased damage to AECs (185). In addition, in **Chapter 6** I showed significantly greater numbers of cord blood neutrophils migrate across RSV infected AECs in comparison to adult neutrophils, implying greater amounts of neutrophil products are present apically. Further to this, I also showed in **Chapter 6** that a greater proportion of this large population of apical cord blood neutrophils are apoptosing in comparison to adult, raising the possibility that their number may overwhelm the ability of scavenger phagocytes to remove the products of apoptosis. This process may be contributing to increased inflammation and, if true *in vivo*, may contribute to clinical severity. In addition to inflammation impairing efficient gas exchange, large numbers of neutrophils infiltrating into the airway, as suggested may be the case in my *in vitro* work, could potentially contribute to plugging of bronchioles alongside mucus, causing airway obstruction as has been previously

described (51). I also showed in **Chapter 6** that although cord blood neutrophils at baseline express greater levels of activation markers, in response to migration through AECs their expression did not increase to the same magnitudes achieved in adult neutrophils. It could be that cord blood neutrophils are already close to maximally activated at rest, which may be necessary for homeostasis, and therefore lack the physiological reserve to upregulate their expression in response to stimulus. This may lead to less efficient clearance of virally infected cells and prolonging of infection and therefore inflammation.

These studies tentatively suggest that neutrophils are beneficial for clearance of RSV infection in adults, but physiological differences between adult and cord blood neutrophil response to RSV infected AECs may predispose to a more severe disease picture in neonates. It is important to note here that the proportion of children who experience severe bronchiolitis in response to RSV is very small, and most children experience a mild self-limiting illness. This leads me to hypothesise that young age is not sufficient to cause a severe disease and speculate that a second insult may exacerbate the already predisposed inflammation in the lung and cause a severe clinical illness. This insult may not be the same in all children, it is well documented that prematurity is a risk factor for severe RSV disease (284). This study did not use cord blood from preterm births, but this could be an avenue for future investigation. The insult could be a sub-clinical deficiency in cellular markers such as TLR4, Halfhide et al demonstrates in her study of term and preterm neonates that term neonates with RSV showed deficiency of TLR4 (75). Interestingly this was not seen on the preterm infants' neutrophils, indicating a different pathology determining severe disease. Clinically, understanding the mechanisms or causes of second insult better could improve our treatment of infants with severe RSV bronchiolitis and increase our ability to predict which children will experience severe disease in order to target prophylaxis.

Neutrophils are no longer regarded as simple phagocytes and their function, or indeed dysfunction are considered to have key roles in pathogenesis of infectious, inflammatory disease and even cancers. Future treatments in diseases where neutrophilic processes are implicated, may comprise neutrophil targeted therapies incorporating translational research into neutrophil function such as that presented in this thesis.

7.1 Study Limitations

Any *in vitro* system is an imperfect replica of an *in vivo* scenario, and it is important to recognise the limitations of this study for the context of wider interpretation. As discussed earlier in this thesis, RSV used in these experiments was a GFP tagged strain grown in Hep-2 cells. Despite best use of the infrastructure at UCL available in ultrafiltration for virus purification, it is a possibility that cytokines produced by the Hep-2 cells may remain in the viral preparation. This is a known problem in similar studies of slow growing viruses such as RSV. The presence of these mean that any change in measured neutrophil function as a result of RSV preparations alone could also be attributable to potential Hep-2 derived cytokines, although the profiles of specific cytokine release (IL-8, IL-6, CCL-2 and CL-5) in response to RSV infection are largely similar between Hep-2 cells and primary AECs as previously shown(285). However, these experiments are not interpreted in isolation but in comparison to neutrophil measurements in response to RSV infection of primary human AECs and the virus-free supernatant produced by these. In my transepithelial migration model using cord blood it was not possible to match airway donor to neutrophil donor for obvious reasons, and occasionally in my adult experiments the same was true due to on the day illness, donors leaving their job at UC, timetabling clashes etc. To quantify the effect of this, I performed a comparison of neutrophil response dependent on matching AEC donor to neutrophil and this is available in **Section 2.6.5**; It showed no significant difference in neutrophil CD11b expression or number of neutrophils adherent to AECs, however there is still a possibility this difference could have factored into the results I reported.

The model itself did not contain other immune cells such as macrophages which may influence the behaviour and responses of neutrophils *in vivo* during RSV infection. In addition, as discussed in **Chapter 1** this study used nasal brushings to grow ciliated AECs whereas RSV causes a severe bronchiolitis in infants, so a bronchially derived brushing sample may be a more valid reflection of the clinically relevant *in vivo* scenario. In this study nasal AECs allowed several modifications could be made of the basis of this study to better represent the *in vivo* airway environment and advance the scope of this work which is discussed further below.

7.2 Future work

I have thoroughly enjoyed working on this project, and there are so many things I would like to do to investigate it further as well as things that with hindsight I would do differently. In future studies it may be possible to perform studies of neutrophil expression on an mRNA level to gain better understanding of the transcriptional response to RSV infected AECs and the mechanisms underpinning them. The opportunity through this to explore subsetting of neutrophils using single cell RNA sequencing would be particularly interesting and may inform the direction of future research.

It would have been valuable to have run multiplex chemokine and cytokine analysis on supernatants from our RSV infected AECs, in tandem with some BAL from infants with RSV Bronchiolitis to validate these our model and therefore other findings in this thesis. This experiment would also help in attributing source of inflammatory mediators to the infected airway or due to infiltrating immune cells.

To further refine the *in vitro* transepithelial migration model, several steps could improve its physiological relevance and answer questions raised in this thesis. One approach could be finding some mechanism for varying the numbers of neutrophils permitted to migrate across AECs to investigate to what degree neutrophilia is sufficient and necessary to clear virus, or whether indeed too many neutrophils migrate through and contribute to excessive airway damage. Improving the model, the model could be optimised using a paediatric, or even better, infant AECs and neutrophils to better represent the age demographic of RSV bronchiolitis patients. Specifically for microscopy, improving the model could include optimising a flow system for neutrophils on the basolateral side of cultures for a more physiological model of transepithelial migration. It could also be interesting to include a third cell population, vascular endothelial cells, in a future iteration of the transepithelial migration model, to even better model the physiological migration of neutrophils from vasculature into the airway lumen.

7.3 Final conclusions

Evidence presented in this thesis characterises the interaction between neutrophils and RSV infected airway epithelial cells using a novel *in vitro* model of transepithelial migration. For the first time, neutrophil transepithelial migration during RSV infection has been imaged and quantified, where neutrophils appeared to destroy RSV infected cells. In addition, neutrophil apoptosis and activation during transepithelial migration has been explored, specifically highlighting unexpected differences in neutrophil viability and activation marker expression in adherence to RSV infected AECs. Finally, for the first time, the responses of adult and cord blood derived neutrophils have been compared in a novel *in vitro* model, illustrating key differences in migration, apoptosis and activation when exposed to RSV infected AECs.

Appendix

Appendix 1

Table 7-1 A table to show medias used in experiments, their composition and preparation details.

Media	Composition
F-media	<p>10% Complete DMEM and F12 medium (Gibco) at a 3:1 ratio</p> <p>Y-27632 (5 μM) (ROCK inhibitor) (Cambridge Bioscience, Cambridge, UK)</p> <p>Hydrocortisone (25 ng/ml) (Sigma-Aldrich),</p> <p>Epidermal growth factor (0.125 ng/ml) (Sino Biological)</p> <p>Insulin (5 μg/ml) (Sigma-Aldrich),</p> <p>Cholera toxin (0.1 nM) (Sigma-Aldrich),</p> <p>Amphotericin B (250 ng/ml) (ThermoFisher Scientific)</p> <p>Gentamicin (10 μg/ml) (Gibco)</p> <p>Filter sterilise with 0.2μm filter</p>
10% Complete DMEM	<p>To make 500ml</p> <p>450ml DMEM (Gibco 41966)</p> <p>50ml FBS (Fetal Bovine Serum) (Gibco 26170)</p> <p>5ml Penicillin/Streptomycin (Gibco 15070)</p>
ALI Media	<p>To make 100ml</p> <p>50ml complete promocell</p> <p>50ml DMEM</p> <p>1ml Penicillin/Streptomycin</p> <p>1μl Retinoic Acid</p> <p>Filter sterilise with 0.2μm filter</p>
Complete Promocell	<p>500ml Airway Epithelial Cell Basal Medium (C-21260)</p> <p>Supplement Pack – Airway Epithelial Cell Growth Medium (C-39160)</p>
J2F Growth Medium	<p>DMEM (Gibco) Supplemented with</p> <p>8% v/v Bovine Serum (BS) (Gibco) and</p> <p>1% v/v 100x penicillin-streptomycin (Gibco).</p>

Robosept buffer	To make 50ml 49ml Sterile PBS 1ml FBS 200ul EDTA
Collection media	M199 media (Gibco) supplemented 20mM HEPES 1mM Penicillin/Streptomycin (Gibco) 1mM Amphotericin B (Invitrogen)
FACS buffer	To make 500ml 50ml 10% BSA in PBS () 5ml 0.5M EDTA 445ml PBS Filter sterilise with 0.2um filter
FACS fixing reagent	FACS buffer supplemented with 1% PFA
Annexin Binding Buffer	To make 20ml 1ml Annexin binding buffer concentrate 19ml sterile PBS
BHI	To make 400ml 14.8g of BHI granules 400ml ddH ₂ O (distilled water) Ensure granules thoroughly dissolved and autoclave.
Blood Agar	To make 200ml 8g of blood agar base powder in 200ml of ddH ₂ O (distilled water) Ensure granules thoroughly dissolved and autoclave. Once autoclaved and cooled, add 10ml of defibrinated sheep blood.

Appendix 2



Patient Information Sheet

You will be given a copy of this information sheet.

Title of Project: Respiratory Infections: The clinical and immunological role of the neutrophil

This study has been approved by the UCL Research Ethics Committee (Project ID Number): 4735/002

Names Dr Claire Smith, Prof Rosalind Smyth, Prof Chris O'Callaghan
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You are being invited to take part in a research study for the University College London. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

We have a major interest in working out how to prevent and treat chest infections and how to reduce damage to the airways seen in diseases such as asthma and cystic fibrosis. If somebody has a common cold it is more likely that they will suffer a chest infection. The aim of our research is to work out why this is and to try to prevent chest infections.

Why have I been chosen?

You have been asked to take part in the study because you are currently healthy.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to complete and sign a consent form. You are still free to withdraw at any time and without giving a reason.

What happens if I agree to take part?

We will collect cells by gently brushing the inside of your nose a very small brush. We insert the brush into one of your nostrils and brush gently against the side. The brushing takes 2–3 seconds and causes only minor discomfort.

We would also like to take about 50ml, (approximately 3 tablespoons) of blood from your arm. This is so we can collect your immune cells and see how they interact with your cells that we have grown in culture. This may occur on the same day as the nasal brushing or may occur about 8 weeks after your nasal brush sample was collected. If you agree we will contact you via email to invite you to back to ICH so we can collect this sample. You are under no obligation to provide this sample and you can withdraw your consent at any time.

We may also ask you to come and visit us so we can measure your nasal and exhaled nasal oxide and temperature. These are very simple tests. To measure nasal nitric oxide you will just be asked to hold your breath while a probe is held against one of your nostrils to pull air slowly out of it. To measure nasal and exhaled temperature you will just breathe out into a simple temperature measuring device.



We would also like you to keep us informed of your respiratory health by letting us know when you have developed a respiratory infection by informing us of any cold or flu like symptoms. This will be done by email and you can opt out anytime. This is because we have found that the cells from people from certain ethnic groups or with nasal and chest problems can react differently to infection. Knowing what problems you have will therefore, help us to interpret our results.

What will happen with my samples?

1. Nasal brushing

We will grow your cells over a period of several weeks. We will then be able to expose the cells to viruses determine how infection can damage the cells and how we can prevent and treat the infections. We may also attempt to immortalize your cells which will allow them to grow in culture indefinitely. Your immortalized cells could potentially be shared with other scientists for research purposes.

2. Blood sample

We will collect specific immune cells from your blood sample and see how your immune cells interact with your cells that we have grown in culture. This will tell us how the immune cells make the infection better or worse.

Are there any risks?

1. Nasal brushing

Some subjects feel minor discomfort when the nasal brush is inserted. There is a small risk of a minor nose bleed and if you do get a nose bleed the nose bleed can be easily stopped by compressing the nose, there are no lasting injuries.

2. Blood sample

Some subjects feel discomfort and sometimes pain but measures will be taken to minimise discomfort such as limiting our sampling to one blood sample per subject. You must not agree to take part if you have any clotting disorder.

At the site of blood collection you may experience the following:

- Occasionally pain and bruising at the site from where blood was drawn,
- Light-headedness,
- Rarely fainting.

What arrangements are there for ensuring my anonymity and confidentiality?

Your sample will be anonymised and your name will only appear on the consent form which will be kept locked in a filing cabinet. Only the research team will have access to the consent forms.

Your identity as well as any other type of information regarding you will be treated as strictly confidential. Data on your age, whether you have asthma, hayfever or rhinitis and your ethnic background will be anonymised and will be treated with the usual degree of confidentiality in accordance with the Data Protection Act 1998.

Please note that results obtained from this study may be published in scientific journals and presented at both national and international conferences, meetings, seminars and talks as appropriate.



Informed Consent Form

Please complete this form after you have read the Information Sheet and/or listened to an explanation about the research.

Title of Project: Respiratory Infections: The clinical and immunological role of the neutrophil

This study has been approved by the UCL Research Ethics Committee (Project ID Number): 4735/002

Thank you for your interest in taking part in this research. Before you agree to take part, the person organising the research must explain the project to you. If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you to decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

Participant's Statement

I _____

- have read the notes written above and the Information Sheet, and understand what the study involves.
- am aware that deciding not to participate in this study will not disadvantage me in any way.
- consent to the processing of my personal information for the purposes of this research study.
- understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the Data Protection Act 1998.
- agree that the results from this study may be published in scientific journals and presented at both national and international conferences, meetings, seminars and talks as appropriate.
- understand that I must not take part if I have if I have symptoms of an upper respiratory tract infection or a clot clotting disorder as described in the information sheet.
- understand that I am gifting my sample to UCL and will have no rights to it thereafter.
- understand that I cannot withdraw my sample and any data generated from my sample once my sample has been processed.
- understand that by providing my email address I may be contacted by the researchers to provide further samples and/or data, but that I am under no obligation to provide this.
- agree that the research project named above has been explained to me to my satisfaction and I agree to take part in this study.

Signed: _____

Date: _____

Email address: _____

Appendix 3

Version no. 2.0
Date: 28th April 2016
Ethics: 14/LO/0863
Participant ID:

Fetal Medicine Unit
Elizabeth Garrett Anderson Wing
University College Hospital
235 Euston Road
London
NW1 2BU

Direct line: 020 3447 9872
Fax: 020 7387 9984
Switchboard: 020 3456 7890

PARTICIPANT INFORMATION SHEET – Labour Ward

Study title: Amniotic Fluid, placental and fetal stem cells at birth
Researchers: Dr Anna David, Consultant in Fetal Medicine
Dr Paul Winyard, Consultant in Paediatric Nephrology
Mr Paolo de Coppi, Consultant Paediatric Surgeon

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and ask us if there is anything that is not clear.

What is the purpose of the study? We would like to find out whether we can grow stem cells from amniotic fluid, placental tissue and fetal fluid, and whether proteins found in the fluid can be used to indicate long term outcome for problems such as kidney disease.

Stem cells have the remarkable potential to develop into many different cell types in the body. Serving as a repair system for the body, they can theoretically divide without limit to replenish other cells as long as the person or animal is still alive. When a stem cell divides, each new cell has the potential to either remain a stem cell or become another type of cell with a more specialised function, such as a muscle cell, a red blood cell, or a brain cell for example. Adults have stem cells that are commonly collected from the bone marrow. Recent studies have found stem cells in the amniotic fluid and the placenta. Stem cells are more abundant in the fetus than the adult. They may be better able to divide, grow and develop into different cell types.

We would like to study **amniotic fluid, placental and fetal stem cells** to find out about their characteristics, how they grow and what tissues they can turn into. We will grow them in the laboratory to see if they can repair damaged tissues such as muscle and bone, and analyse the proteins in the amniotic fluid to look for crucial growth factors and chemicals which tell us about the baby's condition. We are keen to check whether genes that are missing in genetic diseases can be introduced into stem cells. We also want to monitor how cells behave in the body and some cells may be introduced into animals to do this. These animal studies have received ethical approval and are in accordance with relevant legislation. In the future it might be possible to use corrected stem cells to treat people with genetic diseases. For example, a stem cell from a patient with thalassaemia, a genetic disease that causes severe anaemia, could have a gene inserted to correct the anaemia. Introduction of the corrected stem cell into the affected patient might then cure the disease.

We are hoping that our research will show that these stem cells are a potential **treatment of diseases in newborn babies**. Our early data has shown that amniotic fluid stem cells might be useful to treat necrotizing enterocolitis, a serious gut disease that affects up to 1 in 10 premature neonates. These stem cells may also be useful for repairing congenital structural problems in babies such as hernias. For these reasons we would also like to store some cells and tissues for future ethically approved clinical trials in a special cell biobank. To ensure that any cells that are biobanked for potential use as a treatment are free from infection, we would like to collect a sample of your blood (20mls or equivalent to 4 teaspoons) to test for infections

such as HIV, hepatitis and toxoplasma. If you would prefer not to biobank the samples, we would still like to study them in our research but we would not use them for future therapy, and you would not be asked to give a blood sample.

We are also performing research to improve our understanding of the placenta, and how it works in health and disease. The placenta is a complex organ, which provides the growing baby with all the oxygen and nutrients it needs in the womb. Despite it being essential to a healthy pregnancy, it is the organ we know least about. We are developing novel imaging methods at UCLH to analyse the placenta after it is delivered. This will help us to better understand the placenta, how it functions, and how it changes in different diseases. The methods we develop may be used clinically in the future to help healthcare professionals make decisions to manage pregnancies better.

Why have I been invited to participate? We are asking pregnant woman who attend UCLH to deliver their baby whether they would take part in this study.

Do I have to take part? There is no obligation to take part and your decision will not have any affect on your future medical care. It is up to you to decide whether you would like to be involved. We will give you this information sheet to look at and keep, then ask if you are happy to sign a consent form. You are still free to withdraw at any time without giving a reason even if you decide to take part.

What will happen to me if I take part? The placenta, umbilical cord and amniotic membranes that deliver after your baby is born are checked by your midwife and then usually discarded. We would like to collect samples of the amniotic membrane, placental cells or whole placenta, the umbilical cord blood and umbilical cord from your placenta after it has been checked. If your baby is born by Caesarean section, the amniotic fluid is usually collected during surgery and discarded at the end of the operation. We will collect a sample of amniotic fluid if you deliver your baby by Caesarean section for research and biobanking. We will only take samples from your placenta, umbilical cord or the amniotic fluid for research and for biobanking purposes if otherwise discarded. We are asking for your permission to store and use these tissues or cells for ethically approved research studies and clinical trials. If you agree to biobank your samples we will collect a sample of your blood (20mls or equivalent to 4 teaspoons) from your arm to test for infections. By gifting the samples to the Principal Investigator, you will give up all rights over the samples. If you agree to the cells being stored in the biobank for future therapeutic use, **you retain the right to withdraw them from the biobank after which they will be destroyed.** After seven years, the cells may become unsuitable for future therapeutic use but they can still continue to be used for research purposes.

We will assign to the samples and the information we collect about you a unique identifying number so that the information becomes anonymous to the researchers. **You do not have to do anything different during your tests or during the rest of your pregnancy if you take part in this study.**

What tests will be done on the amniotic fluid, placental and fetal cells? We will study the chemicals and proteins in the amniotic fluid, how the cells grow and develop, what cell types they become and whether corrective genes can be introduced into them. For some studies the sample may leave the UK for analysis in other countries, but your personal details will not be revealed. If you agree to biobank the cells we will store them for future ethically approved research and clinical studies, and you will retain the right at any time over the next 30 years, to request these cells or tissues are removed from the biobank and then destroyed.

For the imaging studies the whole placenta will be taken to the lab where we will visualise the placenta using different imaging modalities.

Will you require access to my medical records? We may need to access your or your baby's medical records, and may collect limited clinical information about you and your baby, such as pregnancy complications and outcome. These will all be kept confidential.

What are known risks of the study? There are no additional risks to your health or the health of your baby from taking part in the research because there are no extra procedures. All of the samples are being collected as part of your normal clinical tests, and we will just use the extra material which would normally be thrown away. Collection of a small blood sample has momentary discomfort and occasionally results in bruising to your arm.

What are the possible benefits of taking part? There will be no immediate benefits to your pregnancy, but this research may help us to treat patients with congenital diseases or structural abnormalities better in the future.

What if something goes wrong? As there is no extra intervention being performed other than taking some of your blood, we do not expect any risks. If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns of this study, the normal National Health Service complaints mechanisms should be available to you.

Will my taking part in this study be kept confidential? All information collected about you during the research will be kept strictly confidential and samples will be anonymised to the individual researchers. Any information about you, which leaves the hospital will have your name, hospital or NHS number and addressed removed so that you cannot be recognised from it. All data will be kept safe and secure in accordance with the Data Protection Act 1998 and will be collected, stored and handled by the researchers listed at UCL.

Who is organising and funding the research? The research is organised by the Tissue Engineering Laboratory and the Nephro-Urology Unit, both at UCL Institute of Child Health and Great Ormond Street Hospital and the UCL Institute for Women's Health. It is funded by the Royal Society, UCLH Charities, Kids Kidney Research, Sparks, Wellcome Trust and the European Union. Imaging research is funded by the Wellcome Trust and Engineering and Physical Sciences Research Council.

What will happen to the results of the research study? The results will be analysed, presented in scientific meetings and published in peer reviewed journals. Your identity will not be revealed in any report or publication. You may obtain a copy a copy of the results from Dr Anna David at the Institute for Women's Health at UCL (a.david@ucl.ac.uk)

The Bloomsbury Research Ethics Committee has reviewed this study and given its approval.

Contact for further information:
Dr Eleni Antoniadou 07708 666945
Dr Anna David 07852 220 375
Dr Rosalind Pratt 07958 044337

Thank you for taking part in this study!

Version no. 2.0
 Date: 12th May 2016
 Ethics: 14/LO/0863
 Participant ID:

Fetal Medicine Unit
 Elizabeth Garrett Anderson Wing
 University College Hospital
 235 Euston Road
 London
 NW1 2BU

Direct line: 020 3447 9872
 Fax: 020 7387 9984
 Switchboard: 020 3456 7890

CONSENT FORM – Labour Ward

Study title: Amniotic Fluid, placental and fetal stem cells at birth
Researchers: Dr Anna David, Consultant in Fetal Medicine
 Dr Paul Winyard, Consultant in Paediatric Nephrology
 Mr Paolo de Coppi, Consultant Paediatric Surgeon

	Please initial box
1. I confirm that I have read and understood the information sheet dated 28 April 2016 version 2.0 for the above study and have had the opportunity to ask questions	
2. I confirm that I have had sufficient time to consider whether or not I want to be included in the study	
3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected	
4. I understand that sections of any of my <u>and my child's</u> medical notes may be looked at by the researchers/ sponsor organisation where it is relevant to my taking part in research. I give permission for these individuals to have access to my records	
5. I agree to take part in the above study	
6. I agree to gift to the researchers samples of my amniotic fluid, amniotic membrane, umbilical cord blood, umbilical cord, placenta and any intellectual property	
7. I consent to have my samples stored in a cell Biobank and consent to have a blood test to screen for infection	
8. I consent to storage and use of these cells for any ethically (and future ethically) approved medical research or clinical trial	
9. I agree to samples being sent to laboratories working on the research	

Name of participant	Date	Signature
Name of person taking consent	Date	Signature
Researcher to be contacted (if there are any problems)	Phone Number.	Email address

1 form for patient; 1 to be kept as part of the study documentation; 1 to be kept with hospital notes

Appendix 4

This is a macro written in ImageJ script for batch processing multichannel images to separate their constituent colour channels and save them as a new file.

```
dir = getDirectory("Choose a Directory to PROCESS");
dir2 = getDirectory("Choose a Directory for SAVING");

setBatchMode(true);
list = getFileList(dir);
count = 0;
countFiles(dir);
n = 0;
processFiles(dir);
//print(count+" files processed");

function countFiles(dir) {
  list = getFileList(dir);
  for (i=0; i<list.length; i++) {
    if (endsWith(list[i], "."))
      countFiles(""+dir+list[i]);
    else
      count++;
  }
}

function processFiles(dir) {
  list = getFileList(dir);
  for (i=0; i<list.length; i++) {
    if (endsWith(list[i], "."))
      processFiles(""+dir+list[i]);
    else {
      showProgress(n++, count);
      path = dir+list[i];
      processFile(path);
    }
  }
}

function processFile(path) {
  if (endsWith(path, ".nd2")) {
    //open(path);
    //run("Bio-Formats Windowless Importer", "open=[path]");
    //run("Bio-Formats Importer",
"open=[path] autoscale color_mode=Default rois_import=[ROI manager]
view=Hyperstack stack_order=XYCZT");
    run("Bio-Formats Importer", "open=[path] autoscale color_mode=Default rois_import=[ROI
manager] view=Hyperstack stack_order=XYCZT");
    t=getTitle();
```



```
run("Split Channels");
selectWindow("C1-" + t);
saveAs("BRIGHTFIELD" + t + ".Tiff", dir2);
run("Close");
```

```
selectWindow("C2-" + t);
saveAs("GFP" + t + ".Tiff", dir2);
run("Close");
```

```
selectWindow("C3-" + t);
saveAs("NEUT" + t + ".Tiff", dir2);
run("Close");
```

```
    }
}
```

Appendix 5

This is a macro for counting neutrophils adherent in images of fixed and stained AEC cultures.

The macro is written in ImageJ script.

```
dir = getDirectory("Choose a Directory to PROCESS");
dir2 = getDirectory("Choose a Directory for SAVING");

setBatchMode(true);
list = getFileList(dir);
count = 0;
countFiles(dir);
n = 0;
processFiles(dir);
//print(count+" files processed");

function countFiles(dir) {
    list = getFileList(dir);
    for (i=0; i<list.length; i++) {
        if (endsWith(list[i], "/"))
            countFiles(""+dir+list[i]);
        else
            count++;
    }
}

function processFiles(dir) {
    list = getFileList(dir);
    for (i=0; i<list.length; i++) {
        if (endsWith(list[i], "/"))
            processFiles(""+dir+list[i]);
        else {
            showProgress(n++, count);
            path = dir+list[i];
            processFile(path);
        }
    }
}

function processFile(path) {
    if (endsWith(path, ".lsm")) {
        //open(path);
        //run("Bio-Formats Windowless Importer", "open=[path]");
        run("Bio-Formats Importer", "open=[path] autoscale color_mode=Default rois_import=[ROI
manager] view=Hyperstack stack_order=XYCZT");
        t=getTitle();
        run("Split Channels");
    }
}
```

```

selectWindow("C1-" + t);
//saveAs("Tiff", dir2);
close();
selectWindow("C2-" + t);
//saveAs("Tiff", dir2);
close();
selectWindow("C3-" + t);
//saveAs("Tiff", dir2);
//Make Maximum intensity projection
run("Z Project...", "projection=[Max Intensity]");
//Subtract background
//setTool("line");
makeLine(16, 16, 1932, 1940);
run("Subtract Background...", "rolling=20");
// Set Threshold
run("Threshold...");
//waitForUser("set the threshold and press OK, cancel will quit the macro")
run("Convert to Mask");
run("Watershed");
rename(t);
//saveAs("Tiff", dir2);
// Count Neutrophils
run("Analyze Particles...", "size=10.00-Infinity display clear summarize");
selectWindow("Results");
saveAs("Results", dir2 + t + ".csv");
close();

//COUNT DAPI
//t=getTitle();
//selectWindow("C1-" + t);
//saveAs("Tiff", dir2);
//Make Maximum intensity projection
//run("Z Project...", "projection=[Max Intensity]");
//Subtract background
//setTool("line");
//makeLine(16, 16, 1932, 1940);
//run("Subtract Background...", "rolling=20");
// Set Threshold
//run("Threshold...");
//waitForUser("set the threshold and press OK, cancel will quit the macro")
//run("Convert to Mask");
//run("Watershed");
//rename(t);
//saveAs("Tiff", dir2);
// Count Neutrophils
//run("Analyze Particles...", "size=10.00-Infinity display clear summarize");
//selectWindow("Results");

```

```
//saveAs("Results BLUE", dir2 + t + ".csv");  
//close();  
}  
}  
selectWindow("Summary");  
saveAs("Results", dir2 + "Counts list" + ".csv");
```

Appendix 6

This is a macro for ImageJ which determines 3D coordinates of neutrophils within an multidimensional image stack. After this there is an R script which generates spatial statistics and measures then plots distance from each neutrophil to its nearest neighbours.

IMAGE J CODE

```
dir = getDirectory("Choose a Directory to PROCESS");
```

```
dir2 = getDirectory("Directory for SAVING");
```

```
setBatchMode(true);
```

```
list = getFileList(dir);
```

```
count = 0;
```

```
countFiles(dir);
```

```
n = 0;
```

```
processFiles(dir);
```

```
//print(count+" files processed");
```

```
function countFiles(dir) {
```

```
list = getFileList(dir);
```

```
for (i=0; i<list.length; i++) {
```

```
if (endsWith(list[i], "/"))
```

```
countFiles(""+dir+list[i]);
```

```
else
```

```
count++;
```

```
}
```

```
}
```

```
function processFiles(dir) {  
    list = getFileList(dir);  
    for (i=0; i<list.length; i++) {  
        if (endsWith(list[i], "/"))  
            processFiles(""+dir+list[i]);  
        else {  
            showProgress(n++, count);  
            path = dir+list[i];  
            processFile(path);  
        }  
    }  
}
```

```
function processFile(path) {  
    if (endsWith(path, ".ids")) {  
        //open(path);  
        //run("Bio-Formats Windowless Importer", "open=[path]");  
        //run("Bio-Formats Importer",  
"open=[path] autoscale color_mode=Default rois_import=[ROI manager]  
view=Hyperstack stack_order=XYCZT");  
        run("Bio-Formats Importer", "open=[path] autoscale color_mode=Default rois_import=[ROI  
manager] view=Hyperstack stack_order=XYCZT");  
        t=getTitle();  
run("Split Channels");
```

```
selectWindow("C4-" + t);
run("Close");
```

```
selectWindow("C1-" + t);
run("Close");
```

```
selectWindow("C2-" + t);
run("Close");
```

```
// SELECT NEUT STACK AND FIND 3D OBJECTS
```

```
selectWindow("C3-" + t);
saveAs("Tiff", dir2+"NEUT.tif");
```

```
run("3D Objects Counter", "threshold=1718 slice=4 min.=100
max.=5062500 exclude_objects_on_edges objects centroids centres_of_masses statistics
summary");
```

```
selectWindow("Statistics for NEUT.tif");
```

```
saveAs("Results", dir2 + t + ".csv");
run("Close");
```

```
}
```

```
}
```

```
//selectWindow("Summary");
```

```
//saveAs("Results", dir2 + "Counts list" + ".csv");
```

R CODE

```
#EJR 08072020
```

```
library(distances)
library(spatstat)
library(neighbr)
library(knitr)
library(readr)
```

```
#setwd("~/Dropbox/R directory/Profiling neutrophil movement")
```

```
points <- read_csv("Data/ab063001 m72 m+n 1h 10x-5.ids.csv")
view(points)
```

```

plot(points)
plot(points$X,points$Y,point$Z)

# Euclidean distances needs a matrix to work off (made a data frame of just x y and z variables as
data points)
my_data_points <- data.frame(c(points$X), c(points$Y), c(points$Z))
write.csv(my_data_points,"Result batch/my_data_points.csv", row.names = FALSE)
# Euclidean distances as a string of values using my new data matrix.
my_distances2 <- distances(my_data_points)
write.csv(my_distances2,"Result batch/my_distances2.csv", row.names = FALSE)
# Using my distances matrix it can calculate which points are the 5 nearest neighbours, which is
good for graphing but I'd like the actual distances.
nearest_neighbor_search(my_distances2, 5)
#NEW APPROACH WITH NEW PACKAGE

# CALCULATES EACH POINTS DISTANCE TO IT'S NEAREST NEIGHBOUR
d <- nndist(my_data_points)
# CALCULATES EACH POINTS DISTANCES TO ITS 5 NEAREST NEIGHBOURS AND GIVES YOU A NICE
TABLE
d1to5 <- nndist(my_data_points, k=1:5)
write.csv(d1to5,"Result batch/d1to5.csv", row.names = FALSE)
# Calculate mean distances for each cell
meandist<-rowMeans(d1to5, na.rm = FALSE, dims = 1)
write.csv(meandist,"Result batch/meandist.csv", row.names = FALSE)

# draw histogram with density line
hist(meandist*-1, xlab = 'mean distance to closest neutrophil', ylim=c(0, 0.03), xlim=c(0, 350), main =
'Density plot of mean distance', prob = TRUE)
lines(density(meandist*-1))
# save JPEG of histogram
jpeg('Result batch/histogram.jpg')
hist(meandist*-1, xlab = 'mean distance to closest neutrophil', ylim=c(0, 0.03), xlim=c(0, 350), main =
'Density plot of mean distance', prob = TRUE)
lines(density(meandist*-1))
dev.off()

```


Appendix 7

This is an R script which generates descriptive statistics of neutrophil activation data and fits a linear model of mixed effects analysis. This code was written for the project by Professor Mario Cortina-Borja. After the scripts are the readouts for analysis performed in **Chapter 5**.

```
#### MCB 20.05.20; 08.06.20; 10.06.20; 11.06.20; 15.06.20; 19.06.20 13.07.20; 14.07.20; 15.07.20
#### ### EJR 150720 200720 22072020
summarySE <- function(data=NULL, measurevar, groupvars=NULL, na.rm=FALSE,
                      conf.interval=.95, .drop=TRUE)
{
# Summarizes data.
## Gives count, mean, standard deviation, standard error of the mean, and confidence interval
## (default 95%).
## data: a data frame.
## measurevar: the name of a column that contains the variable to be summarized
## groupvars: a vector containing names of columns that contain grouping variables
## na.rm: a boolean that indicates whether to ignore NA's
## conf.interval: the percent range of the confidence interval (default is 95%)
#### from http://www.cookbook-r.com/Manipulating\_data/Summarizing\_data/
#library(plyr)

# New version of length which can handle NA's: if na.rm==T, don't count them
length2 <- function (x, na.rm=FALSE) {
  if (na.rm) sum(!is.na(x))
  else length(x)
}

# This does the summary. For each group's data frame, return a vector with
# N, mean, and sd
datac <- ddply(data, groupvars, .drop=.drop,
              .fun = function(xx, col) {
                c(N = length2(xx[[col]], na.rm=na.rm),
                  mean = mean (xx[[col]], na.rm=na.rm),
                  sd = sd (xx[[col]], na.rm=na.rm)
                )
              },
              measurevar
)

# Rename the "mean" column
datac <- rename(datac, c("mean" = measurevar))

datac$se <- datac$sd / sqrt(datac$N) # Calculate standard error of the mean

# Confidence interval multiplier for standard error
# Calculate t-statistic for confidence interval:
```

```

# e.g., if conf.interval is .95, use .975 (above/below), and use df=N-1
ciMult <- qt(conf.interval/2 + .5, datac$N-1)
datac$ci <- datac$se * ciMult

return(datac)
}

#### MCB 20.05.20; 08.06.20; 10.06.20; 11.06.20; 15.06.20; 19.06.20 13.07.20; 14.07.20; 15.07.20
#### ### EJR 150720 200720 22072020

library(tidyverse)
library(plyr)
library(scales)
library(readxl)
library(nlme)
library(wesanderson)
library(knitr)
library(purrrlyr)
library(ggplot2)

##### functions #####
## data_summary
# adapted from http://www.sthda.com/english/wiki/ggplot2-error-bars-quick-start-guide-r-software-and-data-visualization
#++++++
# Function to calculate the mean and the standard deviation
# for each group
#++++++
# data : a data frame
# varname : the name of a column containing the variable
#to be summarized
# groupnames : vector of column names to be used as
# grouping variables
data_summary <- function(data, varname, groupnames){
  require(plyr)
  summary_func <- function(x, col){
    c(mean = mean(x[[col]], na.rm=TRUE),
      se = sd(x[[col]], na.rm=TRUE)/sqrt(length(x)))
  }
  data_sum <- ddply(data, groupnames, .fun=summary_func,
    varname)
  data_sum <- rename(data_sum, c("mean" = varname))
  return(data_sum)
}

data_MeanSD <- function(data, varname, groupnames){
  require(plyr)

```

```

summary_func <- function(x, col){
  c(mean = mean(x[[col]], na.rm=TRUE),
    sd = sd(x[[col]], na.rm=TRUE))
}
data_sum<-ddply(data, groupnames, .fun=summary_func,
  varname)
data_sum <- rename(data_sum, c("mean" = varname))
return(data_sum)
}

##### colours #####
colors3<- c("dodgerblue2", "darkorange2", "forestgreen") # migration colours

colors3<- c("olivedrab1", "olivedrab3", "olivedrab") # FOLD CHANGE NO AEC RSV colours
colors4<- c("lightpink", "olivedrab1", "olivedrab3", "olivedrab") # NO AEC RSV colours

colors4<- c("darkorange1", "darkorange3", "purple2", "purple4") # FOLD CHANGE No AEC FG colours
colors5<- c("lightpink", "darkorange1", "darkorange3", "purple2", "purple4") # No AEC FG colours

colors4<- c("steelblue1", "steelblue4", "springgreen2", "springgreen4") # FOLD CHANGE No AEC
Supernatants
colors5<- c("lightpink", "steelblue1", "steelblue4", "springgreen2", "springgreen4") # No AEC
Supernatants

#colors3<- wes_palette('Darjeeling1',3)[1:3]
#colors4<- wes_palette('Darjeeling1',4)[1:4]
#colors5<- wes_palette('Darjeeling1',5)[1:5]

return.color<- function(logical.test,cond1,cond2)
{
  if(logical.test){return(cond1)}
  else{return(cond2)}
}

source('summarySE.R')

##### rsv, slides 11, 12, 13 #####

rsv<-
  read_excel("Neutrophil Activation database.xlsx",
    sheet = "RSV ALONE ADULT(N)")

aux<- rsv[rsv$slide=='s11' & rsv$Infection=='Media Alone',]
aux$slide<-'s12'
### assume the Media Alone measurements from slid1e 11 are common for s12

```

```

aux<- rbind(rsv, aux)
rsv<- aux %>% arrange(slide, Donor)

rsv$Donor<- as.factor(paste("D",rsv$Donor, sep=""))
rsv$Infection<- as.factor(rsv$Infection)
rsv$slide<- as.factor(rsv$slide)

#### fold change
### compute fold_change = (media alone - value) / media alone for each donor

fc<- NA

for(i in 3:5) ### indices for variables (CD11b, NE, MpO) in rsv data.frame
{
for(i.slide in c('s11', 's12', 's13')) ### freqs = 12, 15, 15
{
df.aux<- rsv[rsv$slide==i.slide, c(1:2,i)] ### rsv has only donor and infection
names(df.aux)[3]<-'Y'
df.aux$Donor<- as.factor(as.character(df.aux$Donor)) ## to account for different sets of donors
for(j in levels(df.aux$Donor))
{
aux<- df.aux[df.aux$Donor==j, ]
media_alone<- aux$Y[aux$Infection=='Media Alone']
fc<- c(fc, (aux$Y-media_alone)/media_alone)
do<- c(do, rep(j, dim(aux)[1] ))
}
}### end iteration over variables
}### end iteration over slides
fc<- fc[-1]

fc1<- as.data.frame(matrix(fc, ncol=3, byrow=FALSE))
names(fc1)<- paste(names(rsv)[3:5],"foldChange",sep="_")
#fc1<- cbind(fc1, do)

rsv<- cbind(rsv, fc1)

rsv.function<- function(slide.name, var.name,
                        y.min=NULL, y.max=NULL, y.delta=NULL,
                        plot.width=200, plot.height=200)
{
#### defaults of plot.width and plot.height give A4 size
print(paste(rep("=",40, sep=' '),quote=FALSE)
print(paste('rsv', slide.name, var.name, sep=' '), quote=FALSE)
print(paste(rep("=",40, sep=' '),quote=FALSE)

```

```

rsv.aux<- rsv %>% filter(slide==slide.name)
rsv.aux$Infection <- relevel(rsv.aux$Infection, 'Media Alone')

rsv.aux$Donor<- as.factor(as.character(rsv.aux$Donor))
rsv.aux$Infection<- as.factor(as.character(rsv.aux$Infection))

rsv.aux.summary <- rsv.aux %>%
  data_summary(varname=var.name, groupnames=c("Infection"))
names(rsv.aux.summary)[2]<-'mean'

fold.change.summary <- rsv.aux %>% filter(Infection!='Media Alone') %>%
  data_summary(varname=paste(var.name,"foldChange", sep="_"),
              groupnames=c("Infection"))
names(fold.change.summary)[2]<- 'mean'
print('summary', quote=FALSE)
rsv.aux.summary
print('fold change summary', quote=FALSE)
fold.change.summary

if(is.null(y.max))
{
  y.min<-0
  y.max<- 1.10*max(rsv.aux.summary$mean + rsv.aux.summary$se)
  y.delta<- round(y.max/10)
}

print(rsv.aux.summary)

colors.aux<- return.color(slide.name=='s11', colors4, colors5)

plot.rsv <- ggplot(rsv.aux.summary, aes(x=Infection, y=mean, fill=Infection)) +
  geom_bar(stat='identity', position=position_dodge()) +
  geom_errorbar(aes(ymin=mean-se, ymax=mean+se),
              width=0.2, position=position_dodge(0.9)) +
  scale_fill_manual(values=colors.aux) +
  scale_y_continuous(breaks=seq(y.min,y.max,by=y.delta),
                    label=comma) +
  ylab('mean fluorescence intensity (MFI)) +
  theme_classic() +
  theme(legend.position = 'none') +
  ggtitle(var.name) +
  # theme(legend.position = c(x.legend, y.legend)) +
  NULL

```

```

print(plot.rsv)
ggsave(filename=paste(paste('plotVar',slide.name, var.name, substring(date(),5,10),
      sep="_"),".jpg", sep=""),
      plot=plot.rsv, device="jpeg", units="mm", height=plot.height, width=plot.width, ##A4 size
      dpi=300 #### density of the plot in pixels per inch
      )

colors.aux<- return.color(slide.name=='s11', colors3, colors4)

plot.fold.change<-
ggplot(fold.change.summary, aes(x=Infection, y=mean, fill=Infection)) +
geom_bar(stat='identity', position=position_dodge()) +
geom_errorbar(aes(ymin=mean-se, ymax=mean+se), width=0.2, position=position_dodge(0.9)) +
scale_fill_manual(values=colors.aux) +
ylab('fold change MFI') +
ggtitle(paste('fold change', var.name, sep=' - ')) +
theme_classic() +
theme(legend.position = 'none') +
NULL

#if(!is.null(x.legend))
#plot.fold.change<-
#  plot.fold.change + theme(legend.position = c(x.legend, y.legend)) +
#  NULL

print(plot.fold.change)
ggsave(filename=paste(paste('plotFoldChange',slide.name, var.name, substring(date(),5,10),
      sep="_"),".jpg", sep=""),
      plot=plot.fold.change, device="jpeg", units="mm", height=plot.height, width=plot.width, ##A4
      size
      dpi=300 #### density of the plot in pixels per inch
      )

rsv.aux.df<- rsv.aux %>% select(Donor:Infection | contains(var.name))
names(rsv.aux.df)[3:4]<-c('Y', 'Y_foldChange')

rsv.aux.Y<- groupedData(Y ~ Infection | Donor, data=rsv.aux.df)

rsv.aux.foldChange<- groupedData(Y_foldChange ~ Infection | Donor,
      data=rsv.aux.df[rsv.aux.df$Infection!='Media Alone',])

lme1.a <- lme(Y ~ Infection, random=~1, data=rsv.aux.Y,
      na.action=na.omit)

lme2.a <- lme(Y_foldChange ~ Infection, random=~1, data=rsv.aux.foldChange,
      na.action=na.omit)

```

```

print(summary(lme1.a))
print(summary(lme2.a))

sink(file=paste(paste('LME',slide.name, var.name, substring(date(),5,10),
                    sep="_"),".txt", sep=")) ### sends output to file

print(paste(rep("=",40, sep=' '),quote=FALSE)
print(paste('rsv', slide.name, var.name, sep=' '), quote=FALSE)
print(paste(rep("=",40, sep=' '),quote=FALSE)

print('summary', quote=FALSE)
rsv.aux.summary
print('fold change summary', quote=FALSE)
fold.change.summary

print(summary(lme1.a))
print(summary(lme2.a))
sink() ### returns control to console

invisible(NULL)

}
## end of rsv function

rsv.function(slide.name='s11', var.name='CD11b', y.min=0, y.max=260, y.delta=50,
            plot.width=200, plot.height=200) ## square graphs
rsv.function('s11', 'NE')
rsv.function('s11', 'MPO')

rsv.function('s12', 'CD11b')
rsv.function(slide.name='s12', var.name='NE')
rsv.function('s12', 'MPO')

rsv.function('s13', 'CD11b')
rsv.function(slide.name='s13', var.name='NE')
rsv.function(slide.name = 's13', var.name = 'MPO')

##### slides 8 and 9 - migration #####

migration <-
  read_excel("Neutrophil Activation database.xlsx",
            sheet = "Migration ADULT(N)-ADULT(CA)")

migration$CD64<- as.numeric(migration$CD64)
migration$Neut_population<- as.factor(migration$Neut_population)

```

```

migration$Donor<- as.factor(paste("D",migration$Donor, sep=""))
migration$Infection<-as.factor(migration$Infection)

names(migration)
#[1] "Donor"      "Date"      "Infection"  "Neut_population"
#[5] "CD11b"     "NE"       "MPO"       "CD64"

test<- migration$Neut_population
test<- relevel(test,'Basolateral')
migration$Neut_population<- test

test<- migration$Infection
test<- relevel(test, 'Mock')
migration$Infection<- test

migration<- groupedData(CD11b ~ Infection | Donor, data=migration)

migration$Donor<- as.factor(as.character(migration$Donor))

#### fold change

fc<-NA
for(i in 5:8) #### indices for variables in migration data.frame
{
  df.aux<- migration[, c(1:4,i)]
  names(df.aux)[5]<-'Y'
  for(j in levels(df.aux$Donor))
  {
    aux<- df.aux[df.aux$Donor==j, ]
    media_alone<- aux$Y[aux$Neut_population=='NO AEC']
    fc<- c(fc,(aux$Y-media_alone)/media_alone)
  }
}

fc<- fc[-1] ## remove initial NA
fc<- as.data.frame(matrix(fc, ncol=4, byrow=FALSE))
names(fc)<- paste(names(migration)[5:8],"foldChange",sep="_")

migration<- cbind(migration,fc)

### Slides 8 and 9 ####
migration.function<-
  function(slide.name, var.name, y.min=NULL, y.max=NULL, y.delta=NULL,

```



```

        x.legend=NULL, y.legend=NULL,
        plot.width=210, plot.height=297)
{
  ##### defaults of plot.width and plot.height give A4 size

print(paste(rep("=",40, sep=' '),quote=FALSE)
print(paste('migration', var.name, sep=' '), quote=FALSE)
print(paste(rep("=",40, sep=' '),quote=FALSE)

if(is.null(x.legend)) x.legend<-0.1
if(is.null(y.legend)) y.legend<-0.85
pos.var.name<- (1:12)[var.name==names(migration)]
s8_df<- migration[, c(1:4, pos.var.name)]

s8_summary<-
  migration %>% filter(Infection != 'Clean') %>%
  data_summary(varname=var.name, groupnames=c('Neut_population', 'Infection'))
names(s8_summary)[3]<-'mean'

if(is.null(y.max))
{
  y.min<-0
  y.max<- 1.10*max(s8_summary$mean + s8_summary$se)
  y.delta<- round(y.max/10)
}

s8_plot<-
s8_summary %>%
ggplot(aes(x=Neut_population, y=mean, fill=Infection)) +
geom_bar(stat="identity", color="black", position=position_dodge()) +
geom_errorbar(aes(ymin=mean, ymax=mean+se), width=.2,
              position=position_dodge(.9)) +
ggtitle(var.name) +
scale_fill_manual(values=colors3) +
labs(x='Neutrophil population',y='') +
scale_y_continuous(breaks=seq(y.min,y.max,by=y.delta), label=comma) +
theme_bw() +
theme(legend.position = c(x.legend, y.legend)) +
NULL

print(s8_plot)

ggsave(filename=paste(paste('plot',slide.name, var.name, substring(date()),5,10),
                      sep="_"),".jpg", sep=""),

```

```

    plot=s8_plot, device="jpeg", units="mm", height=plot.height, width=plot.width, ##A4 size
    dpi=300 ### density of the plot in pixels per inch
)

#### LME models
names(s8_df)[5]<-'Y'

s8_df<- groupedData(Y ~ Infection | Donor, data=s8_df)

lme1.a <- lme(Y ~ Infection, random=~1, data=s8_df, na.action=na.omit,
  subset=Infection!='Clean' & Neut_population!='NO AEC')

lme1.b <- lme(Y ~ Neut_population, random=~1, data=s8_df, na.action=na.omit,
  subset=Infection!='Clean' & Neut_population!='NO AEC')

lme1.c <- lme(Y ~ Infection + Neut_population, random=~1, data=s8_df, na.action=na.omit,
  subset=Infection!='Clean' & Neut_population!='NO AEC')

lme1.d <- lme(Y ~ Infection * Neut_population, random=~1, data=s8_df, na.action=na.omit,
  subset=Infection!='Clean' & Neut_population!='NO AEC')

sink(file=paste(paste('LME',slide.name, var.name, substring(date(),5,10),
  sep=" _"),".txt", sep=""))

print(paste(rep("=",40, sep=' '),quote=FALSE)
print(paste('migration', var.name, sep=' '), quote=FALSE)
print(paste(rep("=",40, sep=' '),quote=FALSE)

print('summary', quote=FALSE)
print(var.name)
print(s8_summary)

print('BIC', quote=FALSE)
print(BIC(lme1.a, lme1.b, lme1.c, lme1.d))

print(summary(lme1.a))
print(summary(lme1.b))
print(summary(lme1.c))
print(summary(lme1.d))

sink()
invisible(NULL)
}
### end of migration.function

```

```

migration.function(slide.name = "s8", var.name = "CD11b",
  y.min=0, y.max=32000, y.delta=4000,
  plot.width = 200, plot.height = 200)
migration.function(slide.name = "s9", var.name = "NE", y.min=0, y.max=400000, y.delta=50000,
  plot.width = 200, plot.height = 200)
migration.function(slide.name = "s9", var.name = "MPO", y.min=0, y.max=700000, y.delta=5000,
  plot.width = 200, plot.height = 200)
migration.function(slide.name = "s8", var.name = "CD64", y.min=0, y.max=700000, y.delta=5000,
  plot.width = 200, plot.height = 200)

```

```

migration.function(slide.name = "s8", var.name
="CD11b_foldChange", y.min=0, y.max=180, y.delta=40, x.legend=0.25,
  plot.width = 200, plot.height = 200)
migration.function(slide.name = "s9", var.name =
"NE_foldChange", y.min=0, y.max=160, y.delta=40, x.legend=0.25,
  plot.width = 200, plot.height = 200)
migration.function(slide.name = "s9", var.name =
"MPO_foldChange", y.min=0, y.max=40, y.delta=5, x.legend=0.25,
  plot.width = 200, plot.height = 200)
migration.function(slide.name = "s8", var.name
="CD64_foldChange", y.min=0, y.max=30, y.delta=5, x.legend=0.25,
  plot.width = 200, plot.height = 200)

```

#####

CD11b

[1] =====

[39] = =

[1] migration CD11b

[1] =====

[39] = =

[1] summary

[1] "CD11b"

	Neut_population	Infection	mean	se
1	Basolateral	Mock	524.1667	73.19686
2	Basolateral	M+R	682.3333	14.64108

3	Basolateral	RSV	1096.5000	66.52838
4	Adhered	Mock	4187.1667	1115.12459
5	Adhered	M+R	6379.6667	1148.02055
6	Adhered	RSV	11975.8333	424.95115
7	Migrated	Mock	9790.6667	1522.82206
8	Migrated	M+R	19514.6667	363.87238
9	Migrated	RSV	28587.8333	2724.77942

[1] BIC

df	BIC
----	-----

lme1.a 5 915.6157

lme1.b 5 887.5691

lme1.c 7 840.8404

lme1.d 11 760.0985

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC	BIC	logLik
-----	-----	--------

906.9274 915.6157 -448.4637

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 0.5827335 9545.191

Fixed effects: Y ~ Infection

	Value	Std.Error	DF	t-value	p-value
--	-------	-----------	----	---------	---------

(Intercept) 4834.000 2249.823 37 2.148613 0.0383

InfectionM+R 4024.889 3896.808 37 1.032868 0.3084

InfectionRSV 9052.722 3181.730 37 2.845220 0.0072

Correlation:

(Intr) InfM+R

InfectionM+R -0.577

InfectionRSV -0.707 0.408

Standardized Within-Group Residuals:

Min Q1 Med Q3 Max

-1.3577226 -0.4629556 -0.2492063 0.4041826 2.9565964

Number of Observations: 45

Number of Groups: 6

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC BIC logLik

878.8808 887.5691 -434.4404

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 0.4494581 6823.747

Fixed effects: Y ~ Neut_population

Value Std.Error DF t-value p-value

(Intercept) 784.733 1761.884 37 0.445394 0.6586

Neut_populationAdhered 6956.400 2491.680 37 2.791851 0.0082

Neut_populationMigrated 18469.600 2491.680 37 7.412508 0.0000

Correlation:

(Intr) Nt_ppA

Neut_populationAdhered -0.707

Neut_populationMigrated -0.707 0.500

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-2.298492779	-0.341767254	0.003761364	0.237650463	3.349137424

Number of Observations: 45

Number of Groups: 6

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC	BIC	logLik
829.0182	840.8404	-407.5091

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 0.3260524 5514.28

Fixed effects: Y ~ Infection + Neut_population

	Value	Std.Error	DF	t-value	p-value
(Intercept)	-3641.333	1743.768	35	-2.088198	0.0441
InfectionM+R	4024.889	2251.195	35	1.787890	0.0825
InfectionRSV	9052.722	1838.093	35	4.925061	0.0000
Neut_populationAdhered	6956.400	2013.530	35	3.454827	0.0015

Neut_populationMigrated 18469.600 2013.530 35 9.172745 0.0000

Correlation:

	(Intr)	InfM+R	InfRSV	Nt_ppA
InfectionM+R		-0.430		
InfectionRSV		-0.527	0.408	
Neut_populationAdhered		-0.577	0.000	0.000
Neut_populationMigrated		-0.577	0.000	0.000
				0.500

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-2.04165668	-0.66209712	0.01554751	0.61876518	3.30541993

Number of Observations: 45

Number of Groups: 6

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC	BIC	logLik
742.6798	760.0985	-360.3399

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 0.3738472 4426.72

Fixed effects: Y ~ Infection * Neut_population

	Value	Std.Error	DF	t-value	p-value
(Intercept)	524.167	1807.201	31	0.290043	0.7737

InfectionM+R	158.167	3130.163	31	0.050530	0.9600
InfectionRSV	572.333	2555.768	31	0.223938	0.8243
Neut_populationAdhered	3663.000	2555.768	31	1.433229	0.1618
Neut_populationMigrated	9266.500	2555.768	31	3.625721	0.0010
InfectionM+R:Neut_populationAdhered	2034.333	4426.720	31	0.459558	0.6490
InfectionRSV:Neut_populationAdhered	7216.333	3614.401	31	1.996550	0.0547
InfectionM+R:Neut_populationMigrated	9565.833	4426.720	31	2.160931	0.0385
InfectionRSV:Neut_populationMigrated	18224.833	3614.401	31	5.042283	0.0000

Correlation:

	(Intr)	InfM+R	InfRSV	Nt_ppA	Nt_ppM	IM+R:N_A
InfectionM+R		-0.577				
InfectionRSV		-0.707	0.408			
Neut_populationAdhered		-0.707	0.408	0.500		
Neut_populationMigrated		-0.707	0.408	0.500	0.500	
InfectionM+R:Neut_populationAdhered		0.408	-0.707	-0.289	-0.577	-0.289
InfectionRSV:Neut_populationAdhered		0.500	-0.289	-0.707	-0.707	-0.354
InfectionM+R:Neut_populationMigrated		0.408	-0.707	-0.289	-0.289	-0.577
InfectionRSV:Neut_populationMigrated		0.500	-0.289	-0.707	-0.354	-0.707

IRSV:N_A IM+R:N_M

InfectionM+R		
InfectionRSV		
Neut_populationAdhered		
Neut_populationMigrated		
InfectionM+R:Neut_populationAdhered		
InfectionRSV:Neut_populationAdhered		
InfectionM+R:Neut_populationMigrated	0.204	
InfectionRSV:Neut_populationMigrated	0.500	0.408

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-2.67756590	-0.29995875	-0.01886272	0.30752645	3.05421802

Number of Observations: 45

Number of Groups: 6

CD64

[1] =====

[39] ==

[1] migration CD64

[1] =====

[39] ==

[1] summary

[1] "CD64"

	Neut_population	Infection	mean	se
1	Basolateral	Mock	2014.333	174.65235
2	Basolateral	M+R	2279.000	50.20292
3	Basolateral	RSV	4429.667	355.10800
4	Adhered	Mock	45000.000	1216.56453
5	Adhered	M+R	4531.667	532.60995
6	Adhered	RSV	43103.000	4032.98172
7	Migrated	Mock	49969.000	8844.29445
8	Migrated	M+R	58513.333	6651.09792
9	Migrated	RSV	58599.000	8001.92605

[1] BIC

df	BIC
----	-----

lme1.a 5 583.3758

lme1.b 5 562.0855

lme1.c 7 525.4343

lme1.d 11 442.0645

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC BIC logLik

577.4856 583.3758 -283.7428

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 2.147922 28748.79

Fixed effects: Y ~ Infection

Value Std.Error DF t-value p-value

(Intercept) 32327.78 9582.929 22 3.373476 0.0027

InfectionM+R -10553.11 13552.308 22 -0.778695 0.4445

InfectionRSV 3049.44 13552.308 22 0.225013 0.8240

Correlation:

(Intr) InfM+R

InfectionM+R -0.707

InfectionRSV -0.707 0.500

Standardized Within-Group Residuals:

Min Q1 Med Q3 Max

-1.10168898 -0.68036493 -0.01032315 0.52522988 2.16010269

Number of Observations: 27

Number of Groups: 3

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC BIC logLik

556.1953 562.0855 -273.0976

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 3570.868 18222.91

Fixed effects: Y ~ Neut_population

Value Std.Error DF t-value p-value

(Intercept) 2907.67 6414.633 22 0.453286 0.6548

Neut_populationAdhered 27970.56 8590.361 22 3.256039 0.0036

Neut_populationMigrated 52786.11 8590.361 22 6.144807 0.0000

Correlation:

(Intr) Nt_ppA

Neut_populationAdhered -0.67

Neut_populationMigrated -0.67 0.50

Standardized Within-Group Residuals:

Min Q1 Med Q3 Max

-1.56587539 -0.60265758 0.02510944 0.55853850 1.78550063

Number of Observations: 27

Number of Groups: 3

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC BIC logLik

517.797 525.4343 -251.8985

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 3762.486 17872.53

Fixed effects: Y ~ Infection + Neut_population

	Value	Std.Error	DF	t-value	p-value
(Intercept)	5408.89	7991.995	20	0.676788	0.5063
InfectionM+R	-10553.11	8425.192	20	-1.252566	0.2248
InfectionRSV	3049.44	8425.192	20	0.361944	0.7212
Neut_populationAdhered	27970.56	8425.192	20	3.319872	0.0034
Neut_populationMigrated	52786.11	8425.192	20	6.265271	0.0000

Correlation:

	(Intr)	InfM+R	InfRSV	Nt_ppA
InfectionM+R		-0.527		
InfectionRSV		-0.527	0.500	
Neut_populationAdhered		-0.527	0.000	0.000
Neut_populationMigrated		-0.527	0.000	0.000 0.500

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-1.5651254	-0.5394754	-0.1433424	0.4639857	1.9261044

Number of Observations: 27

Number of Groups: 3

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC	BIC	logLik
432.2704	442.0645	-205.1352

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 4662.771 15848.08

Fixed effects: Y ~ Infection * Neut_population

	Value	Std.Error	DF	t-value	p-value
(Intercept)	2014.33	9537.697	16	0.211197	0.8354
InfectionM+R	264.67	12939.901	16	0.020454	0.9839
InfectionRSV	2415.33	12939.901	16	0.186658	0.8543
Neut_populationAdhered	42985.67	12939.901	16	3.321947	0.0043
Neut_populationMigrated	47954.67	12939.901	16	3.705953	0.0019
InfectionM+R:Neut_populationAdhered	-40733.00	18299.784	16	-2.225873	0.0407
InfectionRSV:Neut_populationAdhered	-4312.33	18299.784	16	-0.235649	0.8167
InfectionM+R:Neut_populationMigrated	8279.67	18299.784	16	0.452446	0.6570
InfectionRSV:Neut_populationMigrated	6214.67	18299.784	16	0.339603	0.7386

Correlation:

	(Intr)	InfM+R	InfRSV	Nt_ppA	Nt_ppM	IM+R:N_A
InfectionM+R		-0.678				
InfectionRSV		-0.678	0.500			
Neut_populationAdhered		-0.678	0.500	0.500		
Neut_populationMigrated		-0.678	0.500	0.500	0.500	
InfectionM+R:Neut_populationAdhered		0.480	-0.707	-0.354	-0.707	-0.354
InfectionRSV:Neut_populationAdhered		0.480	-0.354	-0.707	-0.707	-0.354
InfectionM+R:Neut_populationMigrated		0.480	-0.707	-0.354	-0.354	-0.707
InfectionRSV:Neut_populationMigrated		0.480	-0.354	-0.707	-0.354	-0.707

IRSV:N_A IM+R:N_M

InfectionM+R	
InfectionRSV	
Neut_populationAdhered	
Neut_populationMigrated	
InfectionM+R:Neut_populationAdhered	
InfectionRSV:Neut_populationAdhered	
InfectionM+R:Neut_populationMigrated	0.250
InfectionRSV:Neut_populationMigrated	0.500 0.500

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-1.30715541	-0.36795431	-0.08000141	0.21847672	2.19791941

Number of Observations: 27

Number of Groups: 3

MPO

[1] =====

[39] ==

[1] migration MPO

[1] =====

[39] ==

[1] summary

[1] "MPO"

	Neut_population	Infection	mean	se
1	Basolateral	Mock	5769.000	628.68999
2	Basolateral	M+R	4451.000	48.37958
3	Basolateral	RSV	9320.167	1202.73413
4	Adhered	Mock	45119.333	2341.14593
5	Adhered	M+R	4183.000	779.01043
6	Adhered	RSV	31333.333	7096.73657
7	Migrated	Mock	43472.333	7707.65169
8	Migrated	M+R	62852.000	7544.76451
9	Migrated	RSV	53559.667	7968.39667

[1] BIC

	df	BIC
lme1.a	5	1003.3247
lme1.b	5	976.6347
lme1.c	7	942.7937
lme1.d	11	860.5648

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

	AIC	BIC	logLik
	994.6363	1003.325	-492.3182

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 5.921494 27117.83

Fixed effects: Y ~ Infection

Value Std.Error DF t-value p-value

(Intercept) 31453.556 6391.734 37 4.920974 0.0000

InfectionM+R -7624.891 11070.807 37 -0.688739 0.4953

InfectionRSV -49.167 9039.276 37 -0.005439 0.9957

Correlation:

(Intr) InfM+R

InfectionM+R -0.577

InfectionRSV -0.707 0.408

Standardized Within-Group Residuals:

Min Q1 Med Q3 Max

-1.01204857 -0.80388694 -0.03416152 0.62650443 2.60664708

Number of Observations: 45

Number of Groups: 6

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC BIC logLik

967.9463 976.6347 -478.9732

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 4489.983 19310.93

Fixed effects: Y ~ Neut_population

Value Std.Error DF t-value p-value

(Intercept) 6631.89 5321.422 37 1.246263 0.2205

Neut_populationAdhered 24491.80 7051.354 37 3.473347 0.0013

Neut_populationMigrated 44457.33 7051.354 37 6.304794 0.0000

Correlation:

(Intr) Nt_ppA

Neut_populationAdhered -0.663

Neut_populationMigrated -0.663 0.500

Standardized Within-Group Residuals:

Min Q1 Med Q3 Max

-1.95255058 -0.67351457 -0.02738649 0.45365168 2.43688859

Number of Observations: 45

Number of Groups: 6

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC BIC logLik

930.9716 942.7937 -458.4858

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 6959.533 18906.66

Fixed effects: Y ~ Infection + Neut_population

	Value	Std.Error	DF	t-value	p-value
(Intercept)	8470.51	6619.570	35	1.279616	0.2091
InfectionM+R	-11129.50	8001.850	35	-1.390866	0.1730
InfectionRSV	-49.17	6302.221	35	-0.007801	0.9938
Neut_populationAdhered	24491.80	6903.738	35	3.547615	0.0011
Neut_populationMigrated	44457.33	6903.738	35	6.439604	0.0000

Correlation:

	(Intr)	InfM+R	InfRSV	Nt_ppA
InfectionM+R		-0.375		
InfectionRSV		-0.476	0.394	
Neut_populationAdhered		-0.521	0.000	0.000
Neut_populationMigrated		-0.521	0.000	0.500

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-2.00924908	-0.69455637	0.08334213	0.40458732	2.29734057

Number of Observations: 45

Number of Groups: 6

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC BIC logLik

843.1461 860.5648 -410.5731

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 7742.31 16758.35

Fixed effects: Y ~ Infection * Neut_population

	Value	Std.Error	DF	t-value	p-value
(Intercept)	5769.00	7536.418	31	0.765483	0.4498
InfectionM+R	-5706.58	12033.357	31	-0.474230	0.6387
InfectionRSV	3551.17	9675.436	31	0.367029	0.7161
Neut_populationAdhered	39350.33	9675.436	31	4.067035	0.0003
Neut_populationMigrated	37703.33	9675.436	31	3.896810	0.0005
InfectionM+R:Neut_populationAdhered	-39618.33	16758.347	31	-2.364096	0.0245
InfectionRSV:Neut_populationAdhered	-17337.17	13683.133	31	-1.267047	0.2146
InfectionM+R:Neut_populationMigrated	20697.67	16758.347	31	1.235066	0.2261
InfectionRSV:Neut_populationMigrated	6536.17	13683.133	31	0.477681	0.6362

Correlation:

	(Intr)	InfM+R	InfRSV	Nt_ppA	Nt_ppM	IM+R:N_A	
InfectionM+R		-0.516					
InfectionRSV		-0.642	0.402				
Neut_populationAdhered		-0.642	0.402	0.500			
Neut_populationMigrated		-0.642	0.402	0.500	0.500		
InfectionM+R:Neut_populationAdhered		0.371	-0.696	-0.289	-0.577	-0.289	
InfectionRSV:Neut_populationAdhered		0.454	-0.284	-0.707	-0.707	-0.354	0.408
InfectionM+R:Neut_populationMigrated		0.371	-0.696	-0.289	-0.289	-0.577	0.500
InfectionRSV:Neut_populationMigrated		0.454	-0.284	-0.707	-0.354	-0.707	0.204

IRSV:N_A IM+R:N_M

InfectionM+R

InfectionRSV

Neut_populationAdhered

Neut_populationMigrated

InfectionM+R:Neut_populationAdhered

InfectionRSV:Neut_populationAdhered

InfectionM+R:Neut_populationMigrated 0.204

InfectionRSV:Neut_populationMigrated 0.500 0.408

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-1.6657260	-0.6109943	0.0148542	0.3767587	2.5937677

Number of Observations: 45

Number of Groups: 6

NE

[1] =====

[39] ==

[1] migration NE

[1] =====

[39] ==

[1] summary

[1] "NE"

	Neut_population	Infection	mean	se
1	Basolateral	Mock	5289.500	942.82690

2	Basolateral	M+R	2557.667	15.89112
3	Basolateral	RSV	8152.833	1182.91684
4	Adhered	Mock	34324.000	2649.24077
5	Adhered	M+R	13006.000	1216.18039
6	Adhered	RSV	105434.667	21914.09576
7	Migrated	Mock	40764.167	5672.63496
8	Migrated	M+R	44021.000	2082.32264
9	Migrated	RSV	295000.667	80133.71133

[1] BIC

df	BIC
----	-----

lme1.a 5 1133.0240

lme1.b 5 1131.6510

lme1.c 7 1083.7454

lme1.d 11 988.5315

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC	BIC	logLik
-----	-----	--------

1124.336 1133.024 -557.1678

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 25700.46 125021.5

Fixed effects: Y ~ Infection

	Value	Std.Error	DF	t-value	p-value
--	-------	-----------	----	---------	---------

(Intercept) 26792.56 31280.02 37 0.8565389 0.3972

InfectionM+R 4335.63 51892.98 37 0.0835494 0.9339

InfectionRSV 109403.50 41673.83 37 2.6252325 0.0125

Correlation:

(Intr) InfM+R

InfectionM+R -0.535

InfectionRSV -0.666 0.402

Standardized Within-Group Residuals:

Min Q1 Med Q3 Max

-1.09152401 -0.64360229 -0.07925101 0.12370904 3.63379137

Number of Observations: 45

Number of Groups: 6

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC BIC logLik

1122.963 1131.651 -556.4813

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 42880.59 119889.6

Fixed effects: Y ~ Neut_population

Value Std.Error DF t-value p-value

(Intercept) 11324.59 35652.11 37 0.3176416 0.7525

Neut_populationAdhered 52616.20 43777.49 37 1.2019009 0.2370

Neut_populationMigrated 137221.67 43777.49 37 3.1345258 0.0034

Correlation:

(Intr) Nt_ppA

Neut_populationAdhered -0.614

Neut_populationMigrated -0.614 0.500

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-1.1493930	-0.6022878	-0.2027811	0.1582945	3.5427626

Number of Observations: 45

Number of Groups: 6

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC	BIC	logLik
1071.923	1083.745	-528.9616

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 36774.36 110762.3

Fixed effects: Y ~ Infection + Neut_population

	Value	Std.Error	DF	t-value	p-value
(Intercept)	-36486.73	38108.01	35	-0.957456	0.3449
InfectionM+R	15243.71	46694.53	35	0.326456	0.7460
InfectionRSV	109403.50	36920.76	35	2.963197	0.0054

Neut_populationAdhered 52616.20 40444.67 35 1.300943 0.2018

Neut_populationMigrated 137221.67 40444.67 35 3.392825 0.0017

Correlation:

(Intr) InfM+R InfRSV Nt_ppA

InfectionM+R -0.383

InfectionRSV -0.484 0.395

Neut_populationAdhered -0.531 0.000 0.000

Neut_populationMigrated -0.531 0.000 0.000 0.500

Standardized Within-Group Residuals:

Min Q1 Med Q3 Max

-1.3157465 -0.5140586 -0.0744544 0.3965219 3.3256472

Number of Observations: 45

Number of Groups: 6

Linear mixed-effects model fit by REML

Data: s8_df

Subset: Infection != "Clean" & Neut_population != "NO AEC"

AIC BIC logLik

971.1128 988.5315 -474.5564

Random effects:

Formula: ~1 | Donor

(Intercept) Residual

StdDev: 42628.85 99732.27

Fixed effects: Y ~ Infection * Neut_population

Value Std.Error DF t-value p-value

(Intercept)	5289.50	44278.94	31	0.1194586	0.9057
InfectionM+R	26397.31	71538.44	31	0.3689947	0.7146
InfectionRSV	2863.33	57580.45	31	0.0497275	0.9607
Neut_populationAdhered	29034.50	57580.45	31	0.5042423	0.6177
Neut_populationMigrated	35474.67	57580.45	31	0.6160887	0.5423
InfectionM+R:Neut_populationAdhered	-18586.17	99732.27	31	-0.1863606	0.8534
InfectionRSV:Neut_populationAdhered	68247.33	81431.06	31	0.8380995	0.4084
InfectionM+R:Neut_populationMigrated	5988.67	99732.27	31	0.0600474	0.9525
InfectionRSV:Neut_populationMigrated	251373.17	81431.06	31	3.0869446	0.0042

Correlation:

	(Intr)	InfM+R	InfRSV	Nt_ppA	Nt_ppM	IM+R:N_A
InfectionM+R		-0.523				
InfectionRSV		-0.650	0.402			
Neut_populationAdhered		-0.650	0.402	0.500		
Neut_populationMigrated		-0.650	0.402	0.500	0.500	
InfectionM+R:Neut_populationAdhered		0.375	-0.697	-0.289	-0.577	-0.289
InfectionRSV:Neut_populationAdhered		0.460	-0.285	-0.707	-0.707	-0.354
InfectionM+R:Neut_populationMigrated		0.375	-0.697	-0.289	-0.289	-0.577
InfectionRSV:Neut_populationMigrated		0.460	-0.285	-0.707	-0.354	-0.707

IRSV:N_A IM+R:N_M

InfectionM+R		
InfectionRSV		
Neut_populationAdhered		
Neut_populationMigrated		
InfectionM+R:Neut_populationAdhered		
InfectionRSV:Neut_populationAdhered		
InfectionM+R:Neut_populationMigrated	0.204	
InfectionRSV:Neut_populationMigrated	0.500	0.408

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-2.240997641	-0.281522026	0.008721961	0.250090013	2.765818201

Number of Observations: 45

Number of Groups: 6

Appendix 8

Legend of videos included on USB stick attached.

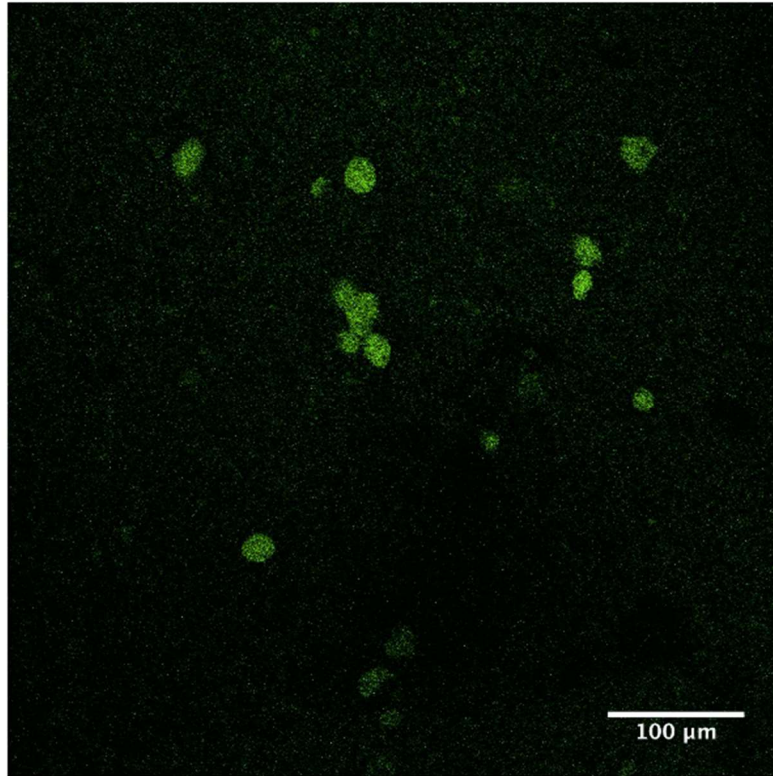
Video 1 – Healthy ciliated AEC culture imaged using high speed video microscopy to observe ciliary beating. Imaging performed as described in **Section 2.6.1.1**.

Video 2 – Fast time lapse of neutrophil cluster formation and nearest neighbour analysis over time as shown in **Figure 3-12**. Imaging was performed using a Zeiss Spinning disk microscope as described in **Section 2.7.2.2** and adherent neutrophil distribution analysis was performed as described in **Section 2.6.6**.

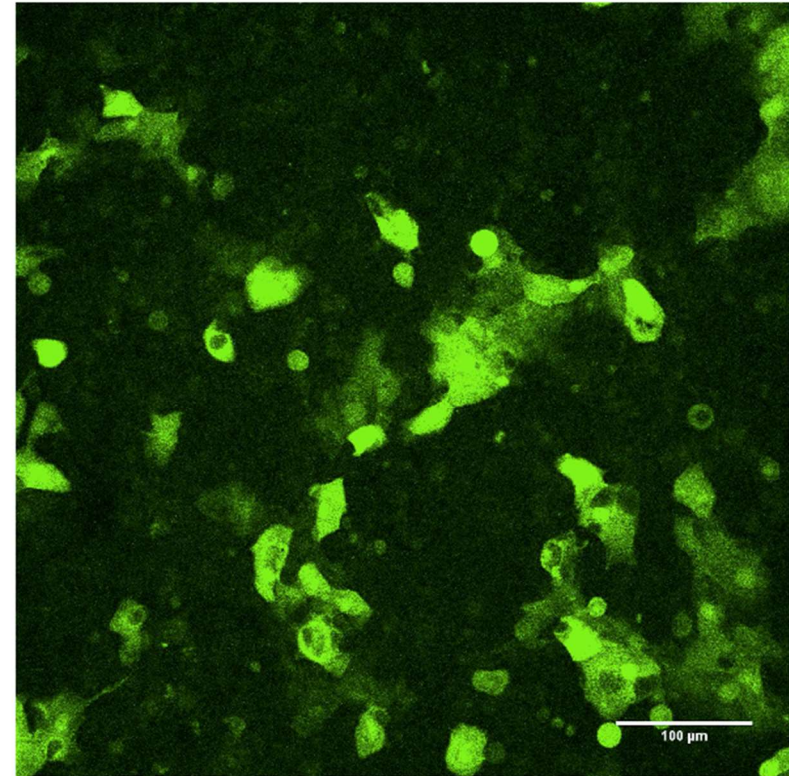
Video 3 - Healthy ciliated AEC culture, 72 hours post mock infection, imaged for 1 hour from the addition of neutrophils using an inverted Zeiss LSM 710 confocal microscope as described in **Section 2.7.2**.

Video 4 - Healthy ciliated AEC culture, 72 hours post RSV infection, imaged for 1 hour from the addition of neutrophils using an inverted Zeiss LSM 710 confocal microscope as described in **Section 2.7.2**.

Appendix 9



24h Infection



72h Infection

Figure 7-1 Representative images of ciliated adult AEC cultures infected with GFP RSV.

Cultures were infected for 24 hours (left) or 72 hours (right) and imaged as described in **Section 2.6.2**.

Appendix 10

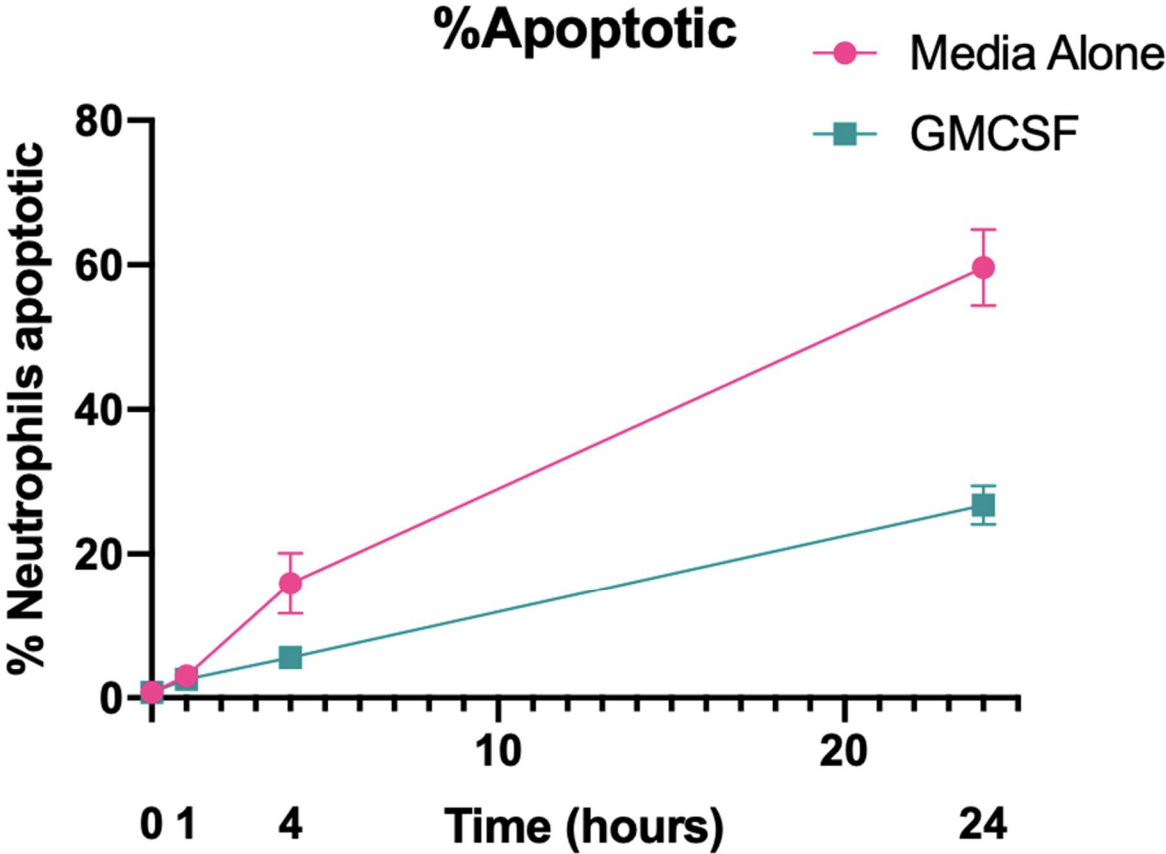


Figure 7-2 Apoptosis of naïve healthy adult neutrophils incubated with Media Alone (HBSS) or GMCSF for 0, 1, 4 and 24 hours.

Percentage of apoptotic cells measured at 0 hour, 1 hour, 4 hour and 24 hour time points after incubation with HBSS alone (pink) or HBSS containing 5ng/ml GMCSF (Teal). Apoptosis measured using Annexin V-FITC and Propidium iodide (PI) as described in **Section 2.7.4**. N=7.

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