ANTI-VIRAL RESPONSE OF BRONCHIAL EPITHELIAL CELLS IN ADULTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

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Abstract

Chronic obstructive pulmonary disease (COPD) is the cause of a significant disease burden within Australia with the risk of dying from COPD increasing significantly with age over 50 years. Viral exacerbations of COPD decrease lung function and are a major cause of patient hospitalisation. Treatments for COPD are based on steroids and beta-agonist, which have long-term adverse side effects, particularly for patients with comorbidities such as heart disease. Immuno-modulatory drugs that fight viral infections within the airway epithelium may be a viable alternative. Therefore we aim to investigate if airway epithelial cells from patients with COPD are defective in the principal antiviral defence pathways of interferon production and signalling, and apoptosis. This study will focus on responses to human metapneumovirus (hMPV) and respiratory syncytial virus (RSV), both common viruses identified in COPD exacerbations.

Submerged and air-liquid interface (ALI) cultures of primary bronchial epithelial cells (BECs) from 5 COPD donors and 5 non-COPD (healthy) donors were infected with four viruses (2 hPMV strains and 2 RSV strains) at high or low dose of infection. Virus replication was assessed by shed virus plaque assays and RT-qPCR. In response to infection with hMPV and RSV, there was no association between COPD and elevated susceptibility in the submerged high MOI and low MOI. The ALI model was consistent with the submerged models in that COPD was not associated with significantly elevated virus production. Although it may be associated with elevated susceptibility to hMPV as demonstrated by infectious hMPV release early post-infection.

Type I and III interferon production was quantified by ELISA and RT-qPCR. IFN signalling was investigated by RT-qPCR of IFN-stimulated genes (ISGs). There was a trend for COPD to be associated with reduced IFN-β and –λ protein production in response to hMPV, although this did not correlate with reduced expression of IFN mRNA or downstream ISGs. However the low dose infection and ALI model demonstrated an interesting disease-related effect in that COPD was related to a shift in the dominant IFN type produced by BECs from IFN-β and –λ in response to both hMPV and RSV. This predominance of IFN-β produced by BECs from COPD subjects was not observed in the high dose model.

COPD was associated with elevated cell death according to the lactate dehydrogenase (LDH) release, although this was not due to elevated caspase-driven apoptosis.

This thesis demonstrated that there are disease-related differences in the IFN response, and cell death of BECs when infected with hMPV and RSV that are evidently dependent on the model used. However these differences are not directly associated with any increase in susceptibility to infection. It also provides evidence that these responses are dependent on the infecting virus. This project highlighted that further investigations in to the IFN-β response particularly in submerged low MOI and ALI cultured BECs are essential. The study presented several trends that suggest using a larger number of subjects may provide more significant results, hence a need to utilise clinical samples in the future.

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature

QUT Verified Signature

Date

26/04/2018

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Keywords

Apoptosis, bronchial epithelial cells, chronic obstructive pulmonary disease, human metapneumovirus, respiratory syncytial virus, interferon, virus exacerbations.

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Ab	Antibody
AEC	Airway epithelial cell
ALI	Air liquid interface
Apaf-1	Apoptotic protease activating factor 1
ATCC	American type culture collection
B-ALI	Bronchial – air liquid interface
BEC	Bronchial epithelial cells
BEGM	Bronchial epithelial growth medium
BSA	Bovine serum albumin
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
COPD	Chronic obstructive pulmonary disease
DHBE	Diseased human bronchial epithelial
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
F	Fusion protein
FasL	Fas ligand
FBS	Foetal bovine serum
G	Glycoprotein
H_2SO_4	Sulfuric acid
HBSS	Hanks' Balanced Salt Solution
HC	Hydrocortisone
Hep-2a	Human Epithelioma-2a
HF	Heart failure
HF	Heart failure
hMPV	Human metapneumovirus
HRP	Horseradish peroxidase
HSP	Heat shock protein
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
IP-10	Interferon gamma-induced protein
IRF-7	Interferon regulatory factor 7
L	Large polymerase
LB	Luria broth
LDH	Lactate dehydrogenase
LLC-MK2	Lilly Laboratories Cell - Monkey Kidney 2
M	Matrix
MEM	Minimal essential medium
MLKL	Mixed lineage kinase domain like protein
MOI	Multiplicity of infection
Mx-1	Myxovirus (influenza) resistance 1
N	Nucleoprotein
NaI	Sodium iodide
NEC	Nasal epithelial cells
NHBE	Normal human bronchial epithelial
NLR	NOD (nucleotide-binding oligomerisation domain) -like receptor
NLRP	Nucleotide-binding oligomerisation domain-like receptor protein
NS	Non-structural
O'MEM	Optimum Eagle's Minimum Essential Media

OAS-1	2'-5'-Oligoadenylate Synthetase 1
P	Phosphoprotein
p.i.	Post infection
P/S	Penicillin, Streptopmycin
PBS	Phosphate buffered saline
pfu	Plaque forming units
PINK1	PTEN-induced putative kinase 1
PRR	Pattern recognition receptor
RIP1/3	Receptor-interacting serine/threonine-protein 1/3
RLR	RIG-I (retinoic acid-inducible gene I)-like receptor
RSV	Respiratory syncytial virus
RT	Room temperature
RV	Rhinovirus
SH	Small hydrophobic
TAE	Tris-acetate-EDTA
TEER	Trans-epithelial electrical resistance
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TNS	Trypsin neutralising solution
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand

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1.0 Introduction

1.1 COPD Overview

Chronic obstructive pulmonary disease (COPD) is a major health concern affecting between 10-15% of adults aged 40 years or over, and a leading cause of morbidity and mortality in Australia and worldwide (1, 2). In 2015 is was estimated that 3 million deaths globally (5% of total deaths that year) were due to COPD, and in 2014 COPD was the fifth leading cause of death in Australia (3, 4). Symptoms of COPD include persistent airflow obstruction and difficulty breathing due to enhanced chronic inflammation. This airflow obstruction is exacerbated by air pollution, smoking, allergens and viruses. Comorbidities contribute to the severity of disease (5).

1.2 Aetiology of COPD

Risk factors for the development and progression of diminished lung function leading to a COPD diagnosis include genetic susceptibility, and early life exposure and/or continuous exposure to harmful environmental toxins, primarily cigarette smoke (6). In the 1950s, tobacco smoking was recognised as the most significant causative risk factor of COPD, therefore research was focused on this aspect of COPD until the early 2000's (7). Since then, there have been a number of studies that suggest there are several other important risk factors for COPD including air pollutants (other than tobacco smoke), chronic asthma, a history of pulmonary tuberculosis, and recurrent lower respiratory tract infections during childhood (7).

COPD often presents as a combination of clinical features such as chronic bronchitis, small airway destruction, and enlargement or disorganisation of the alveoli leading to alveolar tissue loss (emphysema) (8, 9). COPD is associated with chronic inflammation of the airways with distinct pathophysiological characteristics differing from those associated with asthma (10, 11). The inflammatory response in asthmatics involves predominantly eosinophils, mast cells and CD4 T lymphocytes, whereas in COPD neutrophils, macrophages and CD8 T lymphocytes are the predominant cell types (11, 12). Bronchoconstriction triggered by smooth muscle activation and basal membrane thickening causes airway obstruction in asthmatic patients (6, 12). However the reduced airway capacity in COPD patients is primarily caused by bronchial epithelial cell (BEC)

damage due to external toxins such as cigarette smoke (6, 13). These differences in inflammation and pathophysiology between asthma and COPD may also indicate differences in the mechanisms of antiviral defence within the airway epithelium. This requires resolution through experimental examination of viral defence mechanisms in COPD.

1.3 Current Treatment of COPD

Current treatment options for COPD are based on severity of disease and target the symptoms associated with exacerbations (14). There are several pharmaceutical options available, primarily inhaled short-acting anticholinergics and β-agonists, or long-acting forms as the disease progresses (14). The most common medication prescribed to COPD patients is tiotropium, a long-acting anticholinergic, due to its proven effectiveness in the control of symptoms and its prevention of exacerbations (15). Systemic corticosteroid use is well established in the treatment of COPD exacerbations, but extensive use and high-doses are associated with several adverse side effects (16).

Although there are somewhat successful therapies available, due to the frequent co-morbidities of patients with COPD these treatments often cause severe adverse reactions. Several studies report a prevalence of chronic heart failure (HF) in 25-50% of all COPD patients (17-19). Following an exacerbation of COPD, cardiovascular events have been attributed to 26% of mortality (19). The use of β -agonists therefore poses a great risk to COPD patients with HF, and these medications are not favourable in the long-term particularly for patients with HF already using β -antagonists (17). The use of β -antagonists is associated with improved survival after a heart attack in COPD patients (20); however the contra-indication with β-agonists can cause heart attacks (17), thus alternative drug therapies for COPD are required.

1.4 Viral Exacerbations of COPD

The progression of COPD, the frequency of exacerbations requiring hospitalisation, and the commonality of co-morbidities such as heart disease associated with COPD place enormous strains on the healthcare economy with an estimated $98.2 billion attributed to COPD in 2008 within Australia alone (21, 22). Therefore in treating COPD, priority should be given to preventing exacerbations, slowing disease progression and addressing the risk of co-morbidities (23). Viral infections are a major cause of COPD exacerbation

and the main reason for hospitalisation (6), thus contributing to a poor quality of life and substantial health costs (24). In individuals without chronic airway disease, respiratory viral infection by seasonal influenza, rhinovirus (RV), respiratory syncytial virus (RSV), and human metapneumovirus (hMPV) are usually self-limiting, restricted to the upper airways, and with few and fast resolving symptoms (25, 26). In patients with asthma or COPD, the same viral infections are more severe, more likely to spread to the lower airways, and cause exacerbation of the disease (27-29). Influenza, RV, and RSV are the most prevalent viruses identified in COPD exacerbations (27, 30-32). HMPV is emerging as a common exacerbator of COPD, although a global neglect to test for this virus has delayed this recognition (16, 32, 33). Not all patients diagnosed with COPD suffer from virus-associated exacerbations, and it isn't clear why some experience them frequently and others do not (24, 34). It is thought that the more advanced a patients disease state is, the more likely they are to experience acute exacerbations caused by viral infection (24).

1.4.1 HMPV

1.4.1.1 Virus Structure and Replication

HMPV is a common respiratory pathogen belonging to the *Pneumovirinae* subfamily of the *Paramyxoviridae* family (35). It is an enveloped single-stranded negative-sense RNA virus (36, 37) with a genome between 13,280 and 13,378 bp long containing 8 genes encoding for 9 viral proteins (38). The lipid membrane envelope contains the matrix (M) protein and three glycoproteins located on the transmembrane surface; the fusion (F), the attachment (G) and the small hydrophobic (SH) glycoproteins (36, 38). The nucleoprotein (N), phosphoprotein (P) and large polymerase protein (L) are located inside the envelope, along with the M2-1 and M2-2 proteins (36, 38). The order of the genes in the genome is shown in figure 1 (36).

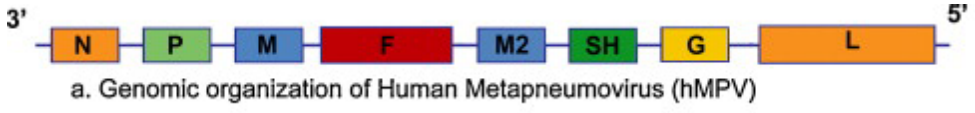

Fig. 1: hMPV genome. 8 genes encode 9 proteins (M2-1 and M2-2 individually transcribed from the M2 gene). 3' to 5'; Nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), M2**,** small hydrophobic (SH), glycosylated (G) and large (L) polymerase (36).

Viral replication of hMPV is initiated by the attachment of the virus to the host membrane, thought to be facilitated by the G protein attaching to heparin sulphate receptors (39). The F protein on the viral envelope fuses to the host cell membrane and the nucleocapsid enters the host cell cytoplasm and replicates (40). The exact process of hMPV replication is unclear, as the majority of knowledge is assumed from the studies of other closely related paramyxoviruses particularly RSV (38). It is inferred that the genome assembles with the P, N, L and M2 proteins, migrates towards the host cell membrane and buds from the cell with the G, F and SH glycoproteins on the viral membrane surface (36). Based on the variability in the sequence of the G and F proteins two groups (A and B) and 2 sub-groups (A1, A2, B1 and B2) of hMPV have been classified through genetic analysis of hMPV isolates (41).

1.4.1.2 Association with COPD

HMPV is a recently discovered pathogen first isolated in 2001 from children in the Netherlands presenting with respiratory illness similar to RSV (42). HPMV has primarily been associated with acute respiratory tract infection in children, but is becoming recognised as a significant cause of respiratory disease in the elderly, with outbreaks in aged-care facilities common (26). Infections caused by hMPV are most often restricted to the upper airways in otherwise healthy individuals, and are mild and self-limiting. However patients suffering from diseases such as COPD and asthma are immunocompromised, so these infections persist, spread to the lower respiratory tract and can cause fatal complications (26, 43). The association between hMPV infection and COPD exacerbations is becoming increasingly recognised in cases where hMPV pathology testing is routine (16, 27).

1.4.1.3 Treatments

There are currently no therapies other than supportive care available for the prevention or treatment of infection with hMPV. The development of effective antivirals for hMPV is limited due to the lack of understanding of the pathogenesis of the virus and the inflammatory response of the host (44). Several antiviral strategies have been trialled including ribavirin, monoclonal antibodies, and small molecules that inhibit fusion of the virus to the cell (45-47). Ribavirin is effective against many other viruses and has shown efficacy against hMPV. However this antiviral can cause severe adverse side effect, thus

is not ideal (48). Monoclonal antibody development has been limited. The most promising therapeutic candidates target the F protein and elicit neutralising antibodies but have not been developed beyond mouse models (48-51).

A safe and viable vaccine has not yet been developed for hMPV infection. Several vaccine candidates have been explored using rodent and non-human primate models, however there have been limited tests performed in humans (52). Of these potential vaccine methods there have been poor results from inactivated virus vaccines, but promising results from viral protein based and live attenuated vaccines (50, 53). Subunit vaccines have been shown to promote short-term protective responses to primary infection in non-human primates (49). Live attenuated viruses vaccines show some promise for infants but the balance of attenuation and level of immunogenicity has been difficult to obtain (52). More detailed knowledge of the viral pathogenesis is required to continue and enhance the development of effective treatments and vaccines against hMPV (54).

1.4.2 RSV

1.4.2.1 Virus Structure and Replication

RSV is a globally ubiquitous respiratory pathogen also belonging to the *Pneumovirinae* subfamily of the *Paramyxoviridae* family (35). Like hMPV it is an enveloped single-stranded negative-sense RNA virus with a genome of 15.2kb (36, 37, 55). The genome is similar to that of hMPV (figure. 2), in that it contains the same 8 genes, although in a slightly different order, as well as 2 extra genes encoding for the non-structural (NS) proteins NS1 and NS2 (36, 55). The 10 genes encode for 11 proteins as the M2-1 and M2-2 proteins are transcribed and translated from separate open reading frames of the M2 gene (55).

RSV has a lipid membrane envelope containing three glycoproteins located on the transmembrane surface; the F, G and the SH glycoproteins (36, 56). RSV gains entry to the host cell by G protein attachment to proteoglycans and F protein fusion to specific receptors such as TLRs, ICAM-1, and EGFR (56-59). The nucleocapsid is released in the cytoplasm following the fusion of the viral particle to the host membrane, and the genome is transcribed and replicated (60). The nucleoprotein, phosphoprotein and polymerase together form a complex that transcribes the ten capped, methylated and polyadenylated mRNAs, and host-cell machinery is used to translate the proteins (60, 61). M2-1 prevents destabilisation of the transcription complex and it is thought that M2-2 regulates the

synthesis of genomic RNA, balancing the rate of transcription and replication (62). Once the genome is replicated and the nucleopcapsid is synthesised it migrates towards the host cell membrane where it is assembled into virus particles coated with glycoproteins and buds from the membrane (60). The 2 non-structural proteins, NS1 and NS2 are responsible for blocking the synthesis and signal transduction of type I and III IFN in response to viral infection (63).

Fig.2: RSV genome. 10 genes encode 11 proteins (M2-1 and M2-2 transcribed together). There are two non-structural genes (NS-1 and NS-2) and the genes are in a different order to hMPV (26)

1.4.2.2 Association with COPD

RSV has long been associated with bronchiolitis in infants and young children, and also in the elderly and immunocompromised (64). The first point of infection is the upper respiratory tract, although infection can spread to the lower respiratory tract particularly in patients with chronic respiratory diseases including COPD (65). Although RV and influenza are the most common exacerbators of COPD, RSV is also very important; one study suggested it may account for up to 15% of all exacerbations (66). Patients suffering from COPD are at significant risk for RSV infection which causes severe decline in lung function frequently leading to hospitalisation (67-69). The viral and host factors that contribute to the increased risk of COPD patients is unclear, possibilities include the host susceptibility to RSV based on behaviour or genetics, innate, humoral and adaptive immunity and viral factors (67, 70). There remains many research opportunities to identify ways of alleviating the damaging effects of RSV infection of COPD patients (71).

1.4.2.3 Treatments

Much like hMPV there are no available vaccines for RSV, and most studies to date have focused on children rather than adults (72). Passive immunisation is available for infants using Palivizumab, which is a prophylactic monoclonal antibody targeting the fusion (F) protein on the surface of RSV (73). This is often administered to high risk patients such as pre-term infants and does reduce the frequency of hospitalisation (74). Palivizumab has recently been shown to reduce the RSV load in tracheal aspirates from infected

children (75, 76). An encouraging alternative target for therapy is the G protein. Targeting the G protein can prevent modulation of the host immune response and suppression of TLR signalling by RSV (77). The potential for a vaccine for RSV is closer now with the development of live recombinant viruses (78, 79). The most promising strategy currently is the vaccination of pregnant women which is proving effective for infants (80, 81). However, the development of vaccines and treatments targeting adult populations is less advanced. Treatment strategies for COPD patients are likely to be dependent on the identification of physical and immune defects which predispose patients to viral-induced exacerbations (71). One study performed a dose comparison of palivizumab administered to children and adults infected with COPD (82). In this study adults showed similar bioavailability of palivizumab but a slower absorption compared to children (82). Further research on the use of this therapy in adults should be performed to determine its value in COPD exacerbations.

1.4.3 Other Respiratory Viruses Associated with COPD

Most research regarding viral exacerbations of COPD has been focused on influenza and RV. Human RVs are responsible for more than 50 % of upper respiratory tract infections (25), and throughout Europe and Australia, RV and influenza are the two most commonly identified respiratory viruses in exacerbations of COPD (27, 66, 83, 84). These infections are often asymptomatic or cause mild symptoms in an otherwise healthy people (17). Influenza can infect both the upper and lower airways and causes a fever, headache, and general malaise in healthy people (85, 86). In patients suffering from COPD or asthma, these viruses often spread to the lower respiratory tract, causing exacerbation of disease (86).

1.5 The Airway Epithelium

1.5.1 Barrier Function

The first point of contact between respiratory viruses and infected individuals is the airway epithelium. The airway epithelium provides a physical and immunological barrier for defence against environmental pollutants, allergens, and pathogens such as respiratory viruses (87). The pseudostratified epithelium consists of 3 cell types; ciliated, non-ciliated and mucous-secretory (88). More than 50% of all airway epithelial cells (AECs) are ciliated and originate from undifferentiated basal cells (88). Ciliated AECs

have the ability to differentiate into goblet cells which produce mucous to trap and removes foreign particles (89). Overproduction of mucous can result in airway blockage and impaired mucous clearance, which is often seen in chronic airways conditions (88, 90).

The impermeable barrier of the airway epithelium is maintained by the formation of tight junctions between cells (91). Tight junctions inhibit the flow of water and other solutes and thus the invasion of environmental insults into the parenchyma of the airway tissue. Tight junctions also establish cell polarity by separating the apical and basal cell surface (92, 93). COPD patients are known to have reduced tight junction formation due primarily to cigarette smoke exposure (94). In addition, during exacerbation, lung inflammation elevates neutrophil recruitment to epithelial cells, leading to the release of pro-inflammatory cytokines which disrupt tight junctions further (95).

1.5.2 Interferon Response

The airway epithelium in not only a physical barrier, but also an immunological barrier. Infection with respiratory viruses triggers an innate immune response, activating pro-inflammatory cytokines, chemokines and a specific antiviral type I (β) and III (λ) IFN response (96-98). Pattern recognition receptors (PRRs) within the apical cells recognise viruses and activate the innate immune response (99). There are 3 types of PRRs involved in virus recognition; toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) like receptors (RLRs) and nucleotide oligomerisation domain (NOD)-like receptors (NLRs) (96). TLR and RLR activation results in the production of type I and type III IFNs (100). NLRs are involved in the regulation of inflammation and cell death pathways via caspase-1 activation (discussed more later) (100). The regulation of type I and III IFN is orchestrated primarily by interferon regulator factor 7 (IRF-7) (101). IRF-7 transcription and activation is amplified in response to elevated IFN-β and -λ which in turn promotes the induction of more IFN-β and -λ in a positive feed-back loop (101, 102). Secreted IFNs then activate a signalling pathway to transcribe IFN stimulated genes (ISGs) which encode hundreds of antiviral factors that specifically fight viral infection by targeting viral RNA for destruction or otherwise inhibiting viral function such as replication and assembly (102, 103). For example Mx1 has a critical role in antiviral activity against RNA viruses, specifically interfering with viral replication (103), while OAS1 cleaves viral RNA (104). The antiviral response is critical for protection of the airways and rapid clearance

of viruses. Inflammatory cytokines are also induced and released from epithelial cells to trigger a cellular immune response to infection (105).

1.5.3 Immune Defects in the Airway Epithelium

AECs are the first line of defence against viruses, and defects in the antiviral response of the epithelium lead to elevated viral load and increased potential for inflammation and damage. Most research concerning antiviral defects in the airway epithelium has been in the asthma field.

It is well known that asthmatics are more susceptible to lower respiratory viral infection due to defects in IFN-driven innate immunity, mostly within circulating immune cells (106, 107). In particular plasmacytoid dendritic cells from asthmatics produce significantly less IFN-α in response to viral infections (108, 109). The mechanism of viral susceptibility in the epithelium is not as clear (110). Most previous studies in which airway epithelial cells from asthma patients have been cultured and exposed to viruses have used RV as the infecting virus. Some of these studies suggest a defect in IFN-β and -λ production (106), however studies by our group and others do not support this as an overall reason for susceptibility to all viruses (110-112). The consensus in the literature currently concerning viral susceptibility of the asthmatic airway epithelium is that IFN defects are correlated only with severe asthma (106) and not mild to moderate asthma (112).

Some studies of RV in COPD have shown that defective immunity, particularly an impaired IFN-β response may be responsible for increased susceptibility to respiratory viruses. Mallia *et al.* demonstrated elevated viral titre and copy number in BECs from COPD patients post RV infection compared to healthy subjects and also elevated expression of intercellular adhesion molecule-1 (ICAM-1), which is a cellular receptor required for attachment of rhinovirus (113-115). However other RV experimental infection studies in humans have identified an increase in the induction of IFN-λ mRNA and protein secretion in COPD airway epithelial cells compared to healthy cells (116, 117). These reports highlight specific molecular changes and pathways that potentially underlie viral exacerbations of COPD, although there is conflicting evidence for an impaired antiviral response.

There is significantly less published research concerning the responses of adult airway epithelial cells, either healthy or from COPD patients, to RSV and hMPV infections. We have found that nasal epithelial cells (NECs) from mild asthmatic adults are more susceptible to experimental infection by hMPV than epithelial cells from healthy volunteers (118). However they were not more susceptible to infection by RV or RSV (118). Interestingly susceptibility was not due to deficient IFN production, rather susceptibility was associated with reduced apoptotic cell death (118). The only other study in which cultured BECs from COPD patients have been infected with hMPV was published during the course of this masters project (119). In this study BECs from COPD patients demonstrated elevated susceptibility to hMPV infection compared to healthy BECs, although also demonstrated elevated IFN-β production and downstream IP-10 and ISG56 expression. This suggests, similarly to our asthma study, that susceptibility to hMPV is not due to IFN deficiency.

In addition to IFN-driven antiviral immunity, AECs clear viral infections by programmed cell death pathways such as apoptosis (105). In our previous study using NECs from mild asthmatic adults, we found that caspase-driven apoptosis was reduced in response to hMPV infection (118). We also found elevated expression of the anti-apoptotic heat shock protein 70 (HSP70) in the same cells (118). HSP70 is a known inhibitor of the formation and function of the apoptosome necessary for caspase-activation and subsequent apoptosis, and was upregulated in NECs infected with hMPV (120). This suggests that cell death is critical to defence against viruses and that defects in this mechanism lead to higher susceptibility. From a clinical perspective these findings also suggest that asthmatics are susceptible to hMPV infection and that hMPV may be responsible for more viral exacerbations than is currently thought. The same may be true for COPD in that hMPV may also play a more important role in COPD exacerbation than currently thought, as the identification of hMPV in exacerbation increases (119).

1.6 Cell Death Pathways

1.6.1 Apoptosis

Apoptosis is a well-known mechanism of programmed cell death and an important part of antiviral defence in preventing viral replication and spread (105, 121). Apoptosis involves cell shrinkage and pyknotic condensing of the chromatin and organelles of a cell (122). This initial phase is followed by membrane blebbing and the formation of

apoptotic bodies (123). Eventually the bodies are phagocytosed by macrophages and other phagocytic cells (123). Apoptosis is a favourable mechanism for clearing virus-infected cells as it does not induce an inflammatory response (124, 125).

Apoptosis is triggered via several mechanisms including both intrinsic and extrinsic stimuli (121). Cysteine aspartyl proteases (caspases) are integral to the activation of both apoptotic pathways (121). The extrinsic pathway relies on the binding of ligands such as tumour necrosis factor (TNF), Fas ligands (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) to the cell membrane (126). TNF and FasL receptors are expressed on the surface of airway epithelial cells (AECs) and once bound to ligands form a signalling complex containing Fas-associated death domain which activates pro-caspase-8 (127). Cleavage of caspase-8 initiates apoptosis by cleavage and activation of executioner caspases 3, 6 and 7 (121, 128). AECs have shown upregulated TRAIL expression when infected with RSV, suggesting an association with apoptosis via the TRAIL pathway in RSV infected AECs (129). The intrinsic pathway is triggered by intracellular stimuli, such as RNA viruses, that promote the release of pro-apoptotic mitochondrial proteins (123). Upon release of these proteins, the caspase-dependent mitochondrial pathway is activated with cytochrome c binding to and activating apoptotic protease activating factor (Apaf-1) and pro-caspase-9 (130, 131). The binding of these proteins forms an 'apoptosome' which cleaves and activates caspase-9 which in turn cleaves and activates executioner caspases-3, and -7 (123, 132).

Apoptosis is important in the antiviral response of many cell types, and for this reason reduced apoptosis can severely impact viral clearance. The inability to contain and clear viruses results in elevated replication and cell-cell spread and eventually lead to prolonged infection and inflammation (124). This masters project is the first to investigate caspase-driven apoptotic responses in BECs from COPD patients when infected with RSV or hMPV. Interestingly, it is believed that cigarette smoke induces elevated apoptosis in the bronchial epithelium in COPD and contributes significantly to pathogenesis (133, 134).

1.6.2 Pyroptosis

Pyroptosis is a more recently identified pathway of programmed cell death triggered by several stimuli including microbial pathogens (135). The mechanisms of pyroptosis are distinct from apoptosis and the process is characterised by caspase-1 dependence (136).

NLRs on the host cell membrane can recognise signals induced by virus infection leading to the assembly of an inflammasome, a multimeric protein comprised of e.g. NLRP3, apoptosis-associated speck-like protein (ASC) and pro-caspase-1 (137). Activation of the inflammasome results in cleavage and subsequent activation of caspase-1 (138). Active capsase-1 then activates and induces the secretion of pro-inflammatory cytokines such as interleukin (IL)-1β and IL-18 (136).

Inflammation is a major part of COPD and it is speculated that inflammasomes play a crucial role in the COPD pathogenesis (139). Several studies have demonstrated an increase in receptors and key mediators of inflammasome activation in AECs response to cigarette smoke and in lung tissues of COPD patients (140-142).

Studies using healthy AECs infected with RV have shown an elevated release of pro-inflammatory cytokines IL-1β and IL-18 (143, 144). COPD may be associated with pro-inflammatory pyroptotic cell death, which is ineffective in clearing viral infections. However there are no studies using RSV or hMPV to experimentally infect BECs from COPD patients compared to healthy individuals to investigate the hypothesis.

1.7 Rationale for the Project

The aetiologies of asthma and COPD share some similarities, but are also different in some aspects, which raises the question of whether there are similar mechanisms of viral susceptibility in COPD as in asthma. More is known concerning the mechanism of viral susceptibility in asthma than in COPD. *In vitro* infections of AECs, either bronchial or nasal, using several viruses, (influenza, RV, RSV and hMPV) have identified defects in anti-viral defence associated with asthma (110, 145). Research concerning anti-viral defence is limited for COPD patients, although it is considered to be significant in the progression of disease. There is some limited evidence that BECs from patients with COPD are defective in the production of IFN (115). There are no direct treatments for viral exacerbation of COPD, current treatment being focused on reducing inflammation and opening the airways. The identification of defective mechanisms of anti-viral defence may lead to the identification of potential targets to prevent or treat viral exacerbations of COPD in patients.

The focus of this research project is to investigate anti-viral responses of BECs from subjects with COPD compared to healthy control subjects within the same age range. BECs have been sourced from a commercial supplier (Lonza), cultured as submerged monolayers and as well-differentiated cultures at air-liquid interface (ALI), and experimentally infected with both laboratory and clinical isolates of hMPV and RSV, as under-studied exacerbators of COPD. The antiviral responses investigated include type I and III IFN production and caspase-driven cell death.

1.8 Hypothesis

- COPD will be associated with increased susceptibility to hMPV and RSV infection
- BECs from COPD donors will have either an impaired IFN-driven antiviral response or an impaired caspase-driven cell death response to infection with hMPV and RSV
- BECs from COPD donors will respond differently to infection with hMPV and RSV.

1.9 Aims of the Thesis

- Identify if cultured BECs from COPD donors are more susceptible to viral infections compared to BECs from healthy donors within the same age group.
- Investigate if BECs from COPD donors have impaired anti-viral defence mechanisms that may contribute to increased susceptibility to infection. The focus will be on IFN driven responses and apoptosis.
- Investigate differences in BEC antiviral response based on the infecting virus.

2.0 Materials and Methods

2.1 Subjects

BECs were purchased from a commercial supplier (Lonza, Maryland, US). BECs from five COPD (DHBE-COPD) subjects and five healthy (NHBE) subjects were selected with the characteristics listed in table 1 below. Cells from all five subjects were established as submerged cultures. However cells from four subjects were well-differentiated at an air-liquid interface (ALI).

Table 1: Subject Characteristics

	Healthy	**COPD**
Number	5 (ALI – 4)	5 (ALI – 4)
Mean Age	59.3y (range: 54-66)	60.3 (range: 53-66)
Gender (% male)	80	100
Smokers (% yes)	0	100

2.2 Cell Culture

2.2.1 Cell Resuscitation & Expansion

Cryopreserved NHBE and DHBE-COPD (approx. 1 million cells/vial) shipped at passage one from Lonza were thawed quickly in a 37°C water bath and seeded at a density of 3,500 cells/cm^2 in bronchial epithelial growth media (BEGM) Complete (Lonza) with hydrocortisone (HC). The number of tissue culture flasks required was calculated using the seeding efficacy provided by Lonza for each subject and the vessels surface area. The cells were maintained by changing the media every two days until they reached 80% confluency and cryopreserved at passage two using the protocol below.

2.2.2 Cell Cryopreservation

Cells were washed with Hanks' balanced salt solution (HBSS) (7ml for T-75, 4ml for T-25) and detached with Trypsin/Ethylenediaminetetraacetic acid (EDTA) (6ml for T-75, 2ml for T-25; Lonza) at 37°C for 3-5min. Trypsin Neutralising Solution (TNS) was added (10ml for T-75, 4ml for T-25; Lonza) and cells pelleted at 200 x *g* for 5min at 22°C. The supernatant was discarded and the cells resuspended in 5ml BEGM Complete. Twenty µl

was used for cell counting using a haemocytometer. Cells were then aliquoted into vials for cryopreservation in liquid nitrogen. Vials contained 250,000, 500,000 or 1 million cells in 500µl of cryopreservation-media (80% BEGM/10%DMSO/10%FBS).

2.2.3 Culture of Submerged Monolayers

For experimental infection, culture plates were coated with collagen (0.03mg/ml) and incubated at 37°C for 30min prior to seeding cells. Vials of passage two cells were thawed in a 37°C water bath and added to 5ml of BEGM Complete with HC, and centrifuged at 192 x *g* for 5min at room temperature (RT) to remove DMSO and FBS. The cells were resuspended in required volume of BEGM Complete with HC (Table 2), the collagen was removed and cells seeded according to experimental and assay requirement (Table 2). The media was changed every 2 days until cells reached 50% confluency at which time the media was replace with BEGM without HC. Cells were cultured for a further 24h or until 75% confluent at which time they were infected.

Table 2: Submerged Monolayer Culture - Volumes Used for Culture Plates

Plate	Volume media	Experiment
12-well	2ml	MOI 2: shed virus, IFN and RT-qPCR
24-well	1ml	MOI 0.1: shed virus and IFN
96-well	200µl	MOI 2: Caspase and LDH Assays

2.2.4 Differentiation of Cells at ALI

Costar® trans-well inserts (6.5mm insert, 0.4µm membrane) were coated with collagen (0.03mg/ml; Roche) and incubated at 37°C for 30min prior to seeding cells. Vials of passage two cells were thawed in a 37°C water bath, added to 5ml of B-ALI Growth Media (Lonza), and centrifuged at 192 x *g* for 5min at 22°C. The cells were resuspended in required volume of B-ALI Growth Media (100µl/well), collagen removed from inserts prior to seeding onto the apical surface, with 500µl/well of growth media placed below the membrane (basal surface) in the 24-well cell culture plate. The apical and basal growth media was changed every 2 days until cells reached 100% confluency. At this

time, the apical media was removed to provide an "air-lifted" environment and basal media was replaced with B-ALI Differentiation media with HC and inducer (Lonza). The basal media was changed every 2 days until cells were approximately 80% ciliated and ready for infection. Ciliation was observed using a Nikon Eclipse TS100 microscope.

2.3 Virus Methodologies

Table 3: Virus Strain and Source

Virus	Strain	Source
hMPV	CAN 87-93	Dr. Guy Bovin (CHUQ, Quebec, Canada)
	AUS 001	QPID QLD
RSV	A2	ATCC
	RS4	Prof. Paul Young (UQ)

2.3.1 Virus Propagation

Two strains of both hMPV and RSV were used for this study to establish consistency of antiviral response based on virus species. HMPV-CAN 97-83 was gifted to our laboratory by Dr. Guy Bovin (CHUQ, Quebec, Canada) and hMPV-AUS-001 was obtained from the Queensland Paediatric Infectious Disease research group (QPID, Brisbane). RSV-A2 was obtained from the American Type Culture Collection (ATCC) and RSV-RS4 was gifted by Prof. Paul Young (UQ). Both CAN 97-83 and A2 represent highly passaged viruses that are used globally by many research groups. AUS-001 and RS4 were isolated from children admitted to the Royal Children's Hospital, Brisbane, and have been passaged less than 10 times from collection. The hMPV stocks were generated in rhesus monkey kidney epithelial cells (LLC-MK2; ATCC), the RSV stocks in human epithelial type 2a cells (Hep-2a; ATCC). HMPV infected LLC-MK2 cells were cultured in OptiMEM/5% FBS/1% penicillin-streptomycin (P-S) (Life Technologies) and incubated at 32°C with 5% CO_2. RSV infected Hep-2a cells were grown in the same media but at 37°C with 5% CO_2. Cells and supernatant were harvested 6-10 days post-infection as determined by observation of optimal cytopathic effect, and vortexed for 30sec in 50ml tubes to enhance virus detachment from cell membranes. The supernatants were then clarified by centrifugation at 2,360 x *g* for 10min at 4°C (Beckman Coulter, Allegra X-22R centrifuge). Clarified

supernatants were loaded onto sucrose cushions (30%/60% (w/v)) and centrifuged for at 120,000 x *g* for 2.5h at 4°C (Sorvall, Discovery SE centrifuge). The virus interface was removed from the sucrose cushion, diluted 1 in 10 with O'MEM, and the virus pelleted at 12,096 x *g* for 3h at 4°C (Beckman Coulter, Avanti J30I centrifuge). The supernatant was discarded and the final pellet was resuspended in O'MEM and aliquoted into vials for cryopreservation at -80°C. A small volume was then thawed for viral titration by immunoplaque assay.

2.3.2 Virus Titration by Immuno-plaque Assay

Plaque assays were used to quantify hMPV and RSV titres for stock viruses and also to quantify shed infectious virus in experimental infections of primary cells. LLC-MK2 (for hMPV) and Hep-2a cells (for RSV) were seeded into 24-well plates. A 10-fold serial dilution (10^{-1} to 10^{-7}) of virus stock or cell supernatant from experimental infections was performed using O'MEM. Cells were inoculated with 150µl (duplicates for experimental infections and triplicated for stock titration) of diluted virus preparation per well at 32°C (hMPV) or 37°C (RSV) for 2h. Methylcellulose (0.8% in O'MEM/2% FBS/1% P-S) was then added to the plates, and incubated for 10 days (32°C, hMPV), or 7 days (37°C, RSV). Cells were then fixed in ice-cold 60% methanol/40% acetone for ten min and blocked in PBS/5% skim milk powder for 30min at 37°C. The blocking buffer was then discarded and replaced with 150µl/well of mouse anti-hMPV F protein antibody (Merck/Millipore, clone 8) diluted to 1ug/ml (blocking buffer) or a goat anti-RSV polyclonal antibody (Virostat) diluted to 1ug/ml (blocking buffer). After 1h incubation at 37°C cells were washed in PBS three times and 150µl/well of anti-mouse (hMPV; Life Technologies) or anti-goat (RSV; Life Technologies) HRP conjugated antibody diluted to 1ug/ml (blocking buffer) was added. Cells were incubated again for 1h at 37°C and washed three times with PBS. 200µl of peroxidase solution with metal enhancer (Sigma-Aldrich) was added to each well, cells were incubated at RT for ten min and then the reaction was stopped by adding water. HMPV and RSV positive stained plaques were counted and the average number of plaques was multiplied by the dilution factor of the well and divided by the volume of inoculation (150µl) to give a result of plaque forming units per ml (pfu/ml).

2.4 Experimental Infection of Cells with hMPV or RSV

2.4.1 Submerged Cell Monolayers

Once cells reach a confluency of 75% in BEGM complete with no HC they were infected with CAN 97-83, AUS-001, A2 or RS4 strains at a low (0.1) or a high (2) multiplicity of infection (MOI) or exposed to virus-free culture media as a negative control. MOIs have been optimised previously to allow maximal antiviral response with minimal cell death. The volume of virus used to inoculate cells was calculated using the equation below. Cells were incubated with the virus for 2h at 37°C, then washed three times with 1X PBS and the media replaced with steroid-free BEGM.

$$\boldsymbol{virus\ per\ well}\ \ (\boldsymbol{ml}) = \frac{\boldsymbol{number\ cells\ per\ well\ x\ confluence}\ (\%)\ \boldsymbol{x\ MOI}}{\boldsymbol{virus\ titre}\ (\boldsymbol{pfu/ml})}$$

2.4.2 ALI Cell Cultures

The basal cell culture medium (B-ALI Differentiation media with HC and inducer) was removed and replaced with steroid-free B-ALI Differentiation media with inducer 24h prior to infection with hMPV or RSV. Cells at ALI were infected with CAN 97-83, AUS-001, A2 or RS4 strains at a high (3) MOI (optimised in previous unpublished studies using BECs, SPANN lab) or exposed to virus-free culture media as a negative control. Cells were incubated with the virus for 4h at 37°C, and then washed three times with HBSS (Lonza) and the basal media replaced with steroid-free B-ALI Differentiation media with inducer.

2.5 Cell Sampling

2.5.1 Submerged Cell Monolayers

Cell culture supernatants were collected from 12-well plates infected at MOI 2 24h post-infection (p.i.). They were clarified by centrifugation at 13,800 x *g* for 5min, and the supernatant snap frozen and stored at -80°. The cells in the plate were lysed in 400µl of TRIzol (Life Technologies) for 5min, then scraped from the plate and added to the cell pellet from the clarified supernatant and incubated for a further 2min. Cell lysates were snap frozen on dry ice and stored at -80°. For cells cultured in 96-well plates for cell death assays, 50µl of supernatant was removed for LDH assays 24h p.i. and the cells then used

for live-cell caspase activation assays. For cells infected at MOI 0.1, supernatants were removed, clarified as above, snap frozen and stored at -80° on day 0 (directly post-infection), days 1, 3 and 5 p.i. The cells remaining in each well on each day of sampling were lysed in 200µl of TRIzol as above, added to the cell pellet, snap frozen and stored at -80°C.

2.5.2 ALI Cell Cultures

Apical washes and the basal media were collected from each culture on day 0 (D0; to establish baseline quantification), D1 and then every 2-3 days p.i. For apical sampling 100µl of HBSS was added to the apical surface of cells on the trans-well membrane and incubated for 10min. The trans-epithelial electrical resistance (TEER) was measured using an EVOM^2 Epithelial Voltohmmeter (World Precision Instruments) to establish tight junction connectivity. After 10min, HBSS was collected for shed virus quantification. The basal media (500µl) was also collected for IFN secretion analysis on the same days. Apical washes and basal media were snap frozen and stored at -80°C. Sampling ceased when cells either died or lost ciliation.

2.6 Shed Virus Quantification

Cell culture supernatants from both MOI 2 and MOI 0.1 infection experiments, and apical washes from ALI cultures were used to quantify shed virus. Refer to section 2.3.2 Virus Titration by Immune-plaque Assay for the complete protocol.

2.7 RT-qPCR

2.7.1. Total RNA Extraction

Total RNA was extracted from cell lysates using TRIzol/chloroform phase separation and isopropanol precipitation. Cells lysed using TRIzol as previously described were thawed and chloroform (50µl per 200µl TRIzol) added. The samples were shaken vigorously and incubated at RT for 5min. Phase separation occurred by centrifugation at 13,800 x *g* for 30min at 4°C (Thermo Scientific, Heraeus, Fresco 17centrifuge). The top (aqueous) phase was transferred to a fresh 1.5ml tube and the RNA precipitated by the addition of an equal volume of isopropanol. The samples were vortexed and incubated for 30min at RT and the precipitated RNA was pelleted by centrifuging at 13,800 x *g* for 30min at 4°C. The supernatant was removed and the pellet washed in 500µl of 75% ethanol followed by a centrifugation at 13,800 x *g* for 15min at 4°C. The supernatant was removed and the RNA

pellet was air-dried and resuspended in 13µl of RNase-free water (Ambion). One µl of the RNA was used to quantify the RNA and check the quality using a Nanodrop (Thermo Scientific).

When necessary, i.e. a 260/280 ratio outside 1.8-2.2 or 260/230 ratio <1.5, the RNA was re-precipitated with 0.1 volume of 3M sodium acetate and 2.5 volume of absolute ethanol. After 30min on ice, the precipitated RNA was collected by centrifugation at 13,800 x *g* for 30min at 4°C. The supernatant was removed and the pellet washed with 75% ethanol. The RNA was collected by centrifuging at 13,800 x *g* for 15min at 4°C and the pellet was air-dried and resuspended in 13µl RNase-free water. One µl of the RNA was used to quantify the RNA and check the quality using a Nanodrop (Thermo Scientific).

2.7.2 cDNA Preparation

Two methods of reverse transcription were used to prepare cDNA. One method used random primers to generate cDNA and the second used Oligo (dT) primers to generate cDNA from primarily mRNA. Preparing cDNA with Oligo(dT) primers was used for viral transcript quantification, to avoid identification of genomic RNA potentially from the initial inoculum. Preparing cDNA with random primers increases the chance of detecting rare targets or targets present in low quantities, therefore this method was appropriate for IFN and IFN-stimulating gene targets.

For cDNA prepared using random primer 300ng RNA in 10µl RNase-free water was mixed with 10µl of the High Capacity master mix (Applied Biosystems) according to the manufacturers instruction (Table 4). cDNA was synthesised using the thermocycler (PTC-200; MJ Research) and the manufacturers recommended cycle conditions (Table 5).

Table 4: RT Master Mix (High Capacity, Applied Biosystems)

Reagent	Volume (µl)
10X RT buffer	2
25X dNTP (100mM)	0.8
10X RT random primers	2
Multiscribe™ reverse transcriptase	1
RNase inhibitor	1
Nuclease free-water	3.2

Table 5: High Capacity cDNA Synthesis cycle conditions

Temperature (°C)	Time (min)
25	10
37	120
85	5

To prepare cDNA to quantify viral transcription, 300ng RNA in 6µl RNase-free water was reverse transcribed to cDNA using Oligo $(dT)_{20}$ (Life Technologies). Reverse transcription reagents were added to the RNA according to the manufacturer's instructions (Table 6) and cDNA synthesised using a thermocycler and cycling conditions as recommended by the manufacturer (Table 6).

Table 6: SuperScript™ cDNA Synthesis (Life Technologies)

Reagent	Volume (µl)
Oligo$(dT)_{20}$ primers	1
Annealing buffer	1
65°C for 5min	
4°C for 1min	
2X First-Strand Reaction Mix	10
SuperScript™ III Enzyme Mix	2
50°C for 50min	
85°C for 5min	
4°C for 5min	

2.7.3 Plasmid Preparation as Standards for qPCR

Viral gene transcripts were quantified by qPCR relative to β-actin transcripts using cDNA and 10-fold dilutions of recombinant DNA plasmid standards containing the N gene of hMPV or RSV, or the β-actin gene. Plasmids were commercially produced for a previous project (146). Glycerol stocks of transformed *E.coli* containing the plasmids were grown in 100ml of Luria Broth (LB) plus ampicillin for selection (100µg/ml) at 37°C for 24h. The plasmids were extracted using Qiagen Plasmid Midi Kit according to the

manufacturer's instruction. Briefly, the bacterial cells were pelleted by centrifuging at 2,360 x *g* for 15min at 4°C (Beckman Coulter, Allegra X-22R centrifuge) then resuspended in 4ml buffer P1. Cells were then lysed in 4ml buffer P2 and the DNA was precipitated in 4ml buffer N3. The supernatant was cleared by centrifuging at 20,000 x *g* for 30min at 4°C. The plasmid DNA was eluted in 50µl of water using QIAprep spin column.

The plasmids were linearised by restriction enzyme digestion according to table 7 for 4h at 37°C, and then electrophoresed through a 1% agarose gel. The DNA band of interest was identified under long wave UV light and cut from the gel using a scalpel. The gel piece was melted and the DNA purified using the GENECLEAN® kit (MP Biomedicals) according to the manufacturer's instructions. The gel was weighed, three volumes of NaI solution were added and the gel melted at 55°C for 5min. One µl of GLASSMILK® was added per 100µl of NaI/DNA solution and incubated at RT for 5min. The bound DNA and GLASSMILK® was pelleted by centrifuging at 14,000 x *g* for 15sec, and the supernatant was discarded. The DNA/GLASSMILK® was washed twice with 500µl of NEW Wash buffer. DNA was eluted from the GLASSMILK® in 20µl RNAse-free water and centrifuged at 14,000 x *g* for 30sec. The DNA in the supernatant was removed and placed into a clean 1.5ml tube, and the concentration was measured using Nanodrop (Thermo Scientific) then stored at -20°C.

Table 7: Plasmid Linearisation

Plasmid Gene	**Actin**	**RSV N**	**hMPV N**
Enzyme	ApaL1 (20 units)	Xba1 (40 units)	EcoRV (40 units)
Buffer	Buffer 4 (10%)	Buffer 2 (10%)	Buffer 3 (10%)
BSA	1%	1%	1%
DNA	1µg	1µg	1µg
H_20	Up to 100µl	Up to 100µl	Up to 100µl
Heat Inactivation (post-linearisation)	N/A	65°C, 20min	80°C, 20min

2.6.4 qPCR Assay

Rotor-Gene® SYBR® Green (Qiagen) was used to amplify cDNA for qPCR. The qPCR mix was prepared according to table 8 with the primers in table 9. The reaction was run on QIAGEN Rotor-Gene-Q according to cycling conditions in table 10.

Table 8: qPCR Assay Mastermix

Reagent	Volume (µl)
2X SYBR master mix	10
Forward primer (10mM)	1
Reverse primer (10mM)	1
Water	6
cDNA or plasmid DNA	2

Table 9: Target Genes and Primers for qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
B-actin	TAC GCC AAC ACA GTG CTG TCT	TCT GCA TCC TGT CGG CAA T
hMPV	CAG AGA GAG TAC AGC AGA TTC TAA	TTC TCT ACT CCG TGT ATG TCT AAC
IFN-λ	CTC ACG CGA GAC CTC AAA TA	TAA GGA AGG AGT AGG GCT CA
IFN-β	CTC TGG CAC AAC AGG TAG TAG	GGA AAG AGC TGT AGT GGA GGA G
Mx1	GGA TTG GAA CCA TAG CTC TAC C	CCT CCC AGA GGA GTA GGA TTA T
IRF7	CCA CTG TTT AGG TTT CGC TTT C	AGT CAC AGG TGT TGA ACC AG
OAS1	TGT GTG TCC AAG GTG GTA AAG	TGA GAG GAC TGA GGA AGA CAA

Table 10: qPCR Cycling Conditions

Cycle Step	Temperature	Time
1. Activation	95°C	5min
2a. Denaturation	95°C	5min
2b. Extension	60°C	10min
Step 2: 40 X repeats		
3. Melt Curve Analysis	Increase from 60-95°C	1°C increments every 5sec

For cellular gene expression, fold expression over uninfected controls were calculated relative to β-actin using the $2^{-\Delta\Delta Ct}$ equation. Absolute quantitation of hMPV or RSV N gene transcript was calculated using a standard curve prepared from a 10-fold dilution series of DNA plasmids containing viral N gene or β-actin gene as outlined above. The standard curves ranged from gene copy number of 200 to 20,000,000.

2.8 Cytokine Assays

2.8.1 IFN-λ ELISA (Invitrogen)

Supernatants from both the MOI 2 and MOI 0.1 infection experiments were used to quantify secreted IFN-λ (IL-29). Basal media from the ALI cultures were also used to quantify secreted IFN-λ. The ELISA assay was performed according to the manufacturer's (Thermo-Fisher/Invitrogen) instructions. High binding, 96-well Costar® assay plates (Corning) were coated with 100µl/well of capture antibody (anti-IFN-λ) diluted in coating buffer at 4°C overnight, then washed 3 times in 200µl/well of wash buffer (1 x PBS with 0.05% Tween-20). The plates were then blocked with 200µl/well of assay diluent at RT for 1h. The wells were washed once and 100µl/well of supplied IFN-λ standards or thawed supernatant samples were added and incubated at 4°C overnight. The wells were washed 3 times with PBS-Tween-20 (0.05% Tween-20) and 100µl/well of biotin-conjugated antibody (anti-IFN-λ) was added and incubated at RT for 30min. Wells were washed 3 times with PBS-Tween-20 (0.05% Tween-20) and 100µl/well of avidin-HRP solution was added and incubated at RT for 30min. The wells were washed 5 times with PBS-Tween-20 (0.05% Tween-20) and 100µl/well of trimethylbenzidine (TMB) substrate solution was added and incubated at RT for 15min. The reaction was stopped with 50µl/well of H_2SO_4 (1M) to each well. The absorbance was read at 450nm with a corrected wavelength of 570nm using a Clariostar Omega reader. Sample concentrations were calculated using the standard curve.

2.8.2 IFN-β AlphaLISA (Perkin Elmer)

Supernatants from both the MOI 2 and MOI 0.1 infection experiments were used to quantify secreted IFN-β. Basal media from the ALI cultures were also used to quantify secreted IFN-β. The ELISA assay was performed according to the manufacturer's (Perkin Elmer) instructions. Two µl of standard IFN-β protein provided by the manufacturer or thawed supernatant was added to a 384 well AlphaPlate™ and immediately 4µl of 5X anti-

analyte acceptor beads was added to each well. The plate was incubated at RT for 30min. Four µl of 5X biotinylated antibody anti-analyte was then added to each well containing bound sample and acceptor bead, and incubated at RT for 60min. Ten µl of 2X SA-donor beads was added to each well now containing bound sample, acceptor bead and antibody, and incubated at RT in the dark for 30min. The AlphaLISA signal was read (excitation at 680nm, emission at 615nm) using CLARIOstar Omega plate reader. Sample concentrations were calculated using the standard curve.

2.9 Cell Death Assays

2.9.1 LDH Assay (Promega)

Supernatants from the MOI 2 infection of BECs in 96-well plates was used to quantify secreted lactate dehydrogenase (LDH). At each sampling time point 50µl/well of cell sample supernatant or blank control was added to a black walled 96 well plate. Then 50µl of CytoTox-ONE Reagent was added to each well, mixed for 30sec and incubated at RT for 10min. Twenty five µl of Stop Solution was added to each well and the plate was mixed for 10sec. The fluorescence was read with an excitation wavelength of 560nm and emission wavelength of 590nm using a CLARIOstar Omega reader. The sample results were calculated as fold induction of infected samples compared to uninfected.

2.9.2 Caspase Activation Assays (Promega)

Cells were cultured in 96-well plates and infected as per methods described above. At each sampling time-point 50µl of Caspase-Glo reagent (1, 3/7, 8 or 9) was added to each sample or blank (cell culture media) well. Caspase-Glo reagents 1, 8 and 9 were supplemented with MG-132 Inhibitor (0.3%) according to the manufacturer's instructions. Plates were placed on a rocker for 1h at RT then the contents of each well transferred to a white-walled 96 well plate and the luminescence read using CLARIOstar Omega reader. The results were calculated as fold induction of infected samples compared to uninfected.

2.10 Statistical Analysis

Grouped analysis of the data was performed using t-tests in GraphPad Prism (version 7) to identify statistical differences between disease states and/or viruses. Where p values less than 0.05 were identified, they were considered statistically significant.

2.11 Biosafety precautions

The correct PPE was worn throughout the duration of this project. The virus and cells were only handled in a biosafety cabinet.

3.0 Results – High Dose (MOI 2)

3.1 Viral Replication and Shedding are not Altered Based on Disease State

In this study BECs were infected at both high and low doses. A MOI of 2 represents an infection in which most cells are infected with the initial inoculum and therefore responding simultaneously at 24h post infection (p.i.). This was established prior to commencement of the project (Spann Lab). A MOI of 0.1 represents an infection in which only approx. 10% of the cells are initially infected and the kinetics of infection within a population of cells can be investigated over time; in this case 5 days p.i. We will consider the response to a high dose infection first.

The susceptibility of BECs to infection and viral replication was investigated using both plaque assays to quantify the amount of live infectious virus shed by the cells and RT-qPCR for expression of hMPV or RSV N mRNA as representative of viral transcription. COPD was not associated with elevated susceptibility to either hMPV or RSV, as measured by viral transcription (Fig. 3a) or infectious virus production (Fig. 3b). This was observed for both strains of hMPV and RSV used.

Although there was no elevated susceptibility of BECs to infection based on disease state, there was a trend towards elevated transcription of hMPV compared to RSV for both healthy and COPD BECs (Fig. 3a). This was statistically significant for RS4, as the median expression of mRNA was 13.4 copies (healthy) and 14.2 copies (COPD) per 10,000 copies of β-actin, which was 3.5 $\log_{10}$ lower than the medians for the same healthy and COPD BECs infected with AUS-001 for example. This difference was less evident for shed infectious virus, as there was a trend for all BECs to shed 1 $\log_{10}$ less RS4 (healthy median = 483.36pfu/ml, COPD median = 883.4pfu/ml), than RSV-A2 or either hMPV strains CAN 97-83 and AUS-001. These data suggest that RS4 replicates with reduced efficacy in all BECs compared to the other three viruses.

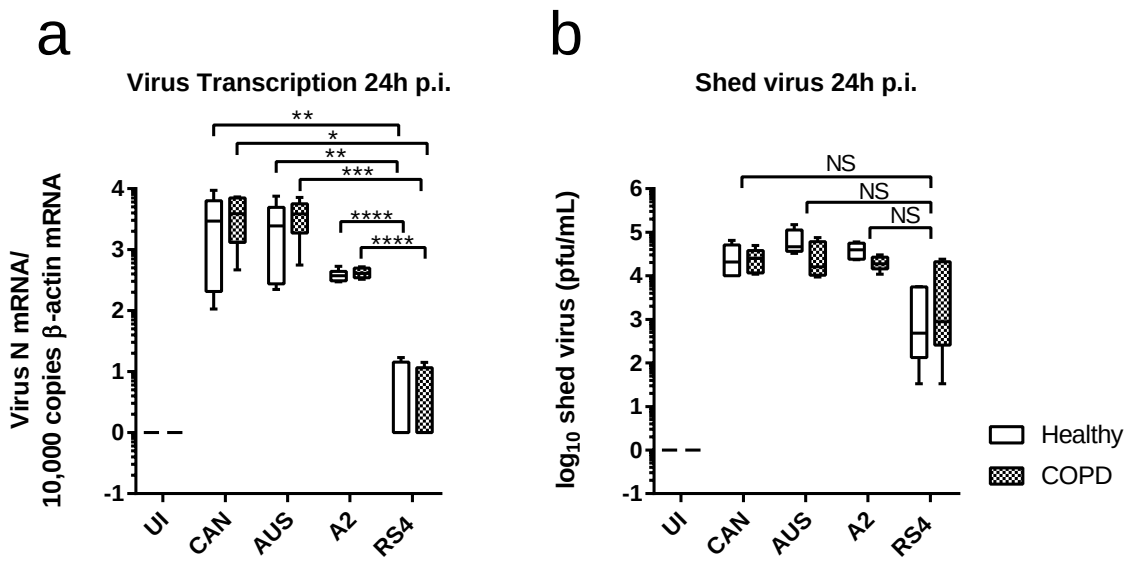

Fig. 3a: Viral Replication and Shedding: hMPV and RSV N mRNA copy number quantified by RT-qPCR using total RNA from BECs infected at a MOI of 2pfu/cell CAN 97-83, AUS-001, A2 or RS4. A standard curve was created using control plasmids for hMPV N, RSV N and β-actin to quantify copy number. **Fig. 3b:** BECs infected with CAN, AUS, A2 or RS4 at MOI 2. Shed virus was quantified by plaque assay. The box represents the 5th and 95th percentile, the bar represents the median and the whiskers represent the minimum and maximum. Healthy n=5, COPD n = 5. Statistical significance between RS4 and other 3 viruses indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

3.2 Interferon Response is not Altered based on Disease State

3.2.1 IFN-λ Production

In these same cells, IFN-λ (IL-29) mRNA expression was quantified (Fig. 4a). In response to CAN 97-83 and AUS-001 there was a trend towards increased IFN-λ gene expression in BECs from COPD subjects compared to healthy subjects. However significance was not reached due to a large range of responses amongst the COPD subjects. This trend for elevated IFN-λ mRNA expression in BECs from COPD subjects was not observed in response to A2. A2 was a poor inducer of IFN-λ in BECs from COPD subjects compared to RS4 and hMPV, with a median fold induction compared to uninfected cells of 36.2 for A2 compared to 645 for RS4 897.6 for CAN 91-83 and 760.1 for AUS-001.

This trend towards elevated IFN-λ mRNA expression in BECs from COPD subjects when infected with hMPV was not observed when secreted IFN-λ was quantified (Fig. 4b). In fact there was a trend towards less IFN-λ protein production associated with COPD and

in response to hMPV infection, as the median was approximately 1 log_{10} lower in these cells compared to healthy BECs. Unlike the pattern demonstrated by mRNA expression, protein secretion was not different in response to hMPV compared to RSV. However large variation amongst subjects makes conclusive observations difficult.

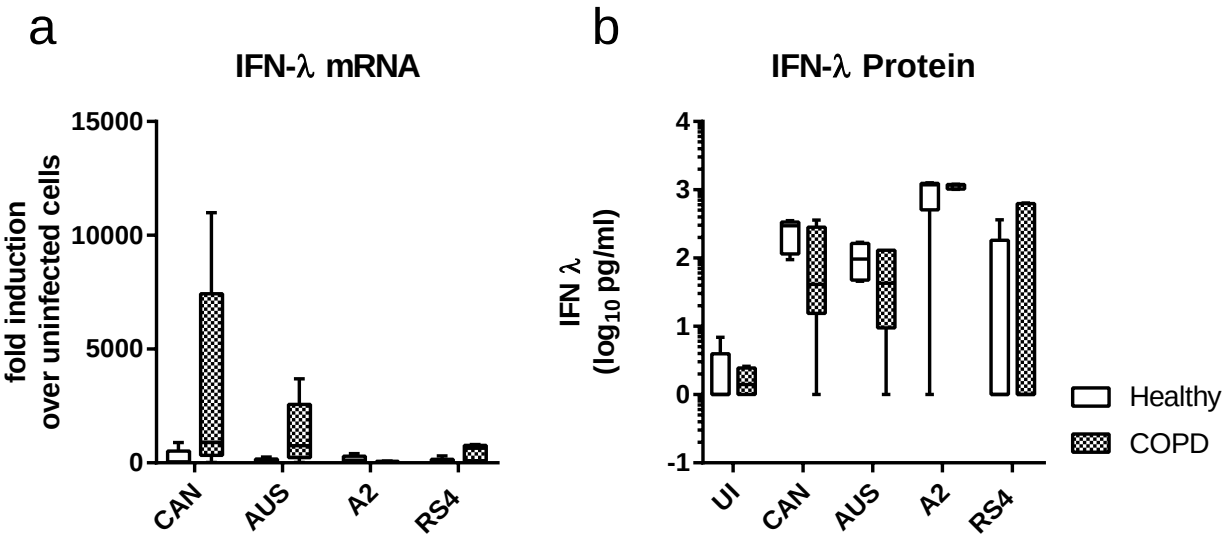

Fig. 4a: IFN-λ Production: BECs infected with CAN 97-83, AUS-001, A2, and RS4 at MOI 2. Fold induction of the IFN-λ gene was quantified by RT-qPCR from total RNA of cell lysates 24h post-infection. Fold induction calculated using $2^{-\Delta\Delta Ct}$ relative to β-actin expression. **4b:** BECs infected with CAN 97-83, AUS-001, A2, and RS4 at MOI 2. Secreted IFN-λ was quantified in cell supernatant at 24h post-infection by ELISA. The box represents the 5th and 95th percentile, the bar represents the median and the whiskers represent the minimum and maximum. Healthy n=5, COPD n = 5. Unpaired t-tests were performed, but no statistical significance was observed.

3.2.2 IFN-β Production

A similar pattern was observed for the expression of IFN-β mRNA as for the expression of the IFN-λ mRNA in response to infection with all four virus strains (Fig. 5a). Both strains of hMPV induced more IFN-β mRNA in BECs from COPD subjects compared to healthy subjects, although large variation in individual COPD subject responses made statistical significance difficult to achieve. CAN 97-83 induced a 10-fold increase and AUS-001 induced a 38-fold increase in median IFN-β mRNA expression in BECs from COPD subjects compared to healthy subjects (Fig. 5a). As seen with IFN-λ, RSV was a poor inducer compared to hMPV and there was no difference in mRNA expression based on disease state in response to either RS4 or A2. The outlying subject expressing high amounts of IFN-β mRNA in response to hMPV is the same subject that expressed high

amounts of IFN-λ mRNA (Fig. 4a). Again these data indicate a difference in response based on the infecting virus rather than disease status.

IFN-β secretion was also quantified from the cell supernatants. As observed for IFN-λ, the pattern of secreted protein did not reflect the pattern of mRNA expression (Fig. 5b). In response to CAN 97-83 there was significantly elevated protein secreted from healthy BECs compared to COPD (healthy median = 124.0pg/ml, COPD median = 1.0pg/ml), and a similar trend in response to AUS-001 although this trend did not reach statistical significance (healthy median = 8.0pg/ml, COPD median = 1.0pg/ml). In response to RSV there was no difference in IFN-β production based on disease state, showing a similar pattern to the induction of gene expression.

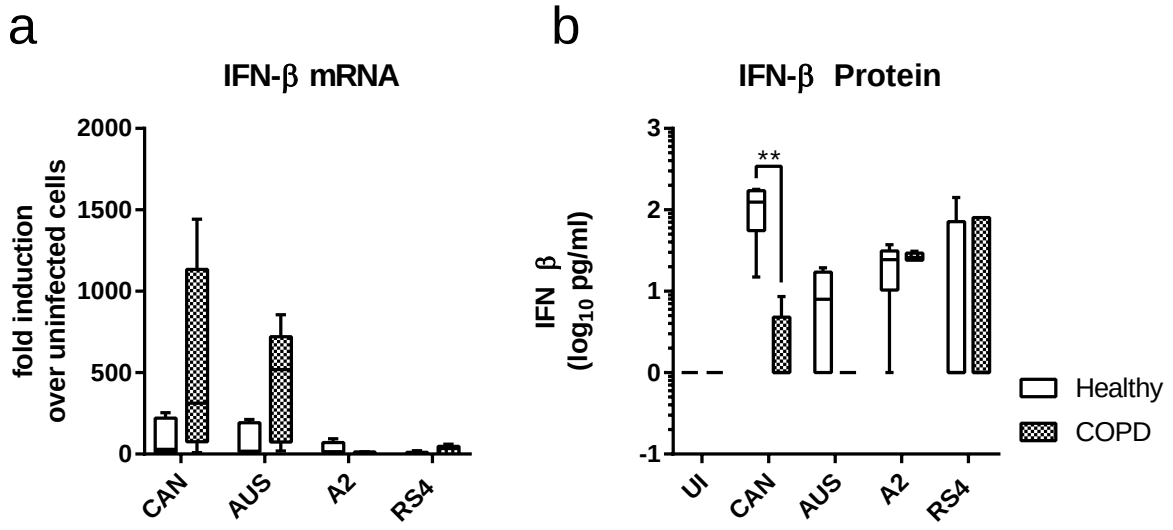

Fig. 5a: IFN-β Production: BECs infected with CAN 97-83, AUS-001, A2, and RS4 at MOI 2. Fold induction of the IFN-β gene was quantified by RT-qPCR from total RNA of cell lysates 24h post-infection. Fold induction calculated using $2^{-\Delta\Delta Ct}$ relative to β-actin expression. **5b:** BECs infected with CAN 97-83, AUS-001, A2, and RS4 at MOI 2. Secreted IFN-β was quantified by AlphaLISA using cell supernatant at 24h post-infection. The box represents the 5th and 95th percentile, the bar represents the median and the whiskers represent the minimum and maximum. Healthy n=5, COPD n = 5. ** denotes $p<0.01$ for COPD group compared to the healthy group

3.2.3 IFN Signalling

Released IFN-β and -λ induce the expression of genes that encode for antiviral factors. IRF7 plays a crucial role in amplification of IFN-β and λ response, while OAS1 and Mx1 are interferon stimulated genes (ISGs) which have antiviral activity against RNA viruses (105). These downstream signalling genes were quantified to investigate if IFN signalling differed with disease status or the infecting virus.

In response to hMPV and RSV isolates there was no significant difference in gene expression of Mx1, OAS1 or IRF7 based on disease state (Fig. 6). There was a trend for elevated expression of Mx1 (Fig. 6a) and IRF7 (Fig. 6b) in BECs from COPD subjects in response to hMPV isolates compared to healthy cells. There was also a trend towards elevated induction of OAS1 expression in BECs from COPD subjects in response to RS4 but not RSV-A2 (Fig. 6c). However overall these data indicate no significant difference in downstream IFN signalling based on disease status.

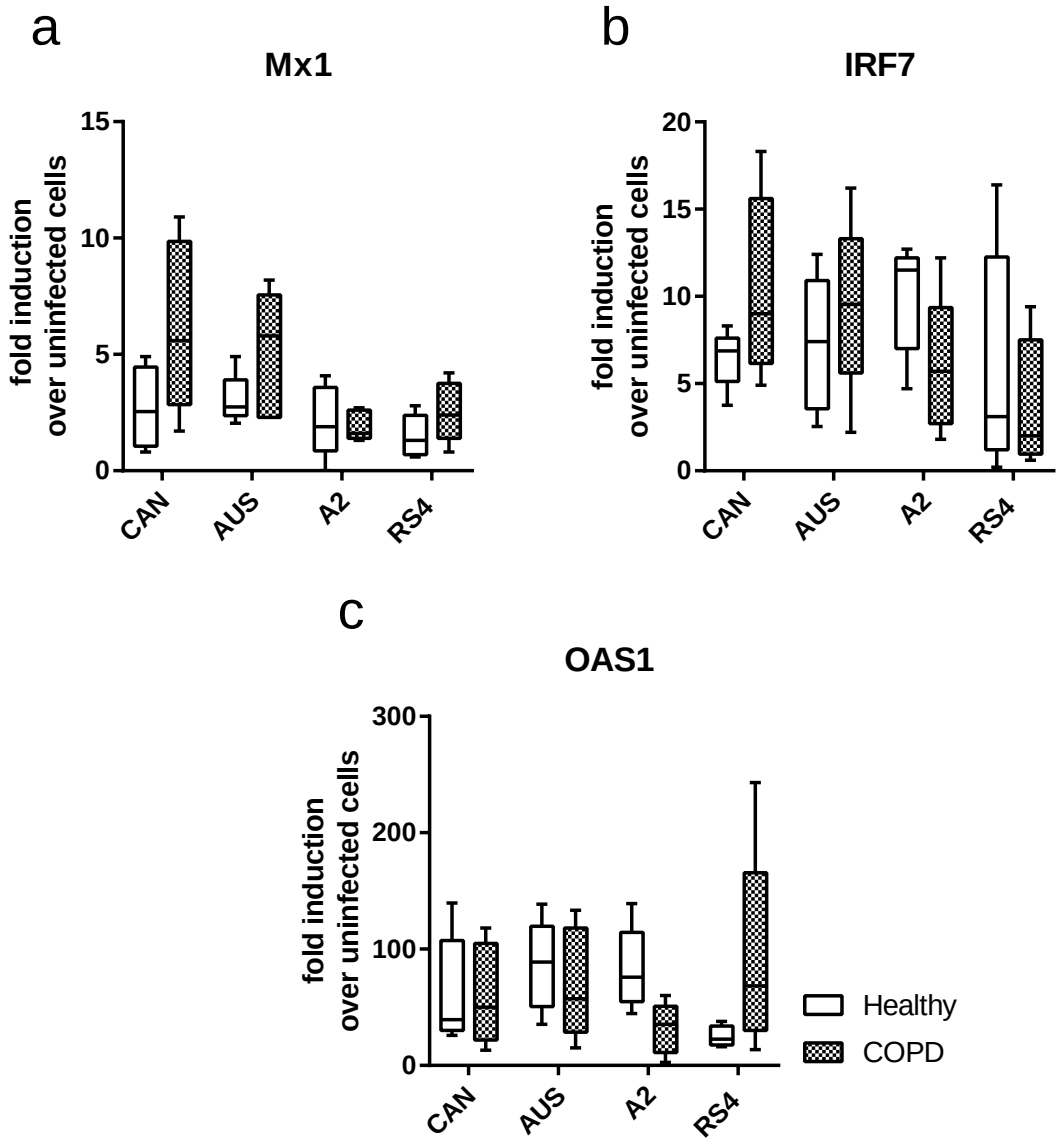

Fig. 6: IFN Signalling: BECs infected with CAN 97-83, AUS-001, A2, and RS4 at MOI 2. Fold induction of IFN downstream genes Mx1 **(a)**, IRF7 **(b)**, and OAS1 **(c)** genes was quantified by RT-qPCR from total RNA of cell lysates 24h post-infection. Fold induction calculated using $2^{-\Delta\Delta Ct}$ relative to β-actin expression. The box represents the 5th and 95th percentile, the bar represents the median and the whiskers represent the minimum and maximum. Healthy n=5, COPD n = 5. Unpaired t-tests were performed, but no statistical significance was observed.

3.3 Overall Conclusion

These data show that COPD was not associated with elevated susceptibility to infection by hMPV or RSV. There was a trend for COPD to be associated with reduced IFN-β and –λ protein production in response to hMPV, although this did not lead to reduced expression of downstream ISGs.

4.0 Results – Low Dose (MOI 0.1)

4.1 Viral Shedding is Elevated from Healthy BECs Compared to COPD in Response to hMPV but not RSV

In addition to MOI 2 infection, a lower dose of virus was applied to cultures at the same time as the MOI 2. The kinetics of infection within each population of cells was then investigated over 5 days to identify susceptibility to infection in relation to spread and viral release over time.

The kinetics of hMPV and RSV shed from BECs over 5 days were very similar for both healthy and COPD subjects. After vigorous washing the amount of shed virus detected in all cells in response to all viruses was higher than expected at day 0. However, new shed virus was detected from day 1 p.i. In response to hMPV infection, there was a trend for more virus to be shed from healthy BECs than from COPD subjects (Fig. 7a & b). For all subjects and treatment groups (except RS4 in healthy subjects), infection peaked at 3 days p.i. In response to CAN 97-83 virus shed from healthy BEC was 1 $\log_{10}$ higher than from COPD subjects at both 3 (healthy mean = 37601.9pfu/ml, COPD mean = 5240.46pfu/ml) and 5 days p.i, (healthy mean = 26674.7pfu/ml, COPD mean = 3757.06pfu/ml), although statistical significance was not reached (Fig. 7a). The same trend reached statistical significance in response to AUS-001 infection, with a 1 $\log_{10}$ difference between shed virus from healthy BECs (29723.9pfu/ml) and those from COPD subjects (2920.35pfu/ml) at day 5 p.i (Fig. 7b).

The pattern of shed virus was different in response to RSV. There was no clear difference in infectious A2 shed from BECs from healthy or COPD subjects (Fig. 7c). There was a 1 log10 difference between RS4 shed from healthy BECS (mean = 7082pfu/ml) compared to BECs from COPD subjects (mean = 706.902pfu/ml) at day 5 p.i. although this was not statistically different (Fig. 7d). Interestingly, there was again a trend towards reduced production of infectious RS4 compared to hMPV, as observed at MOI 2. These data correspond to those generated at a MOI 2 in that COPD is not related to elevated viral load or susceptibility to infection.

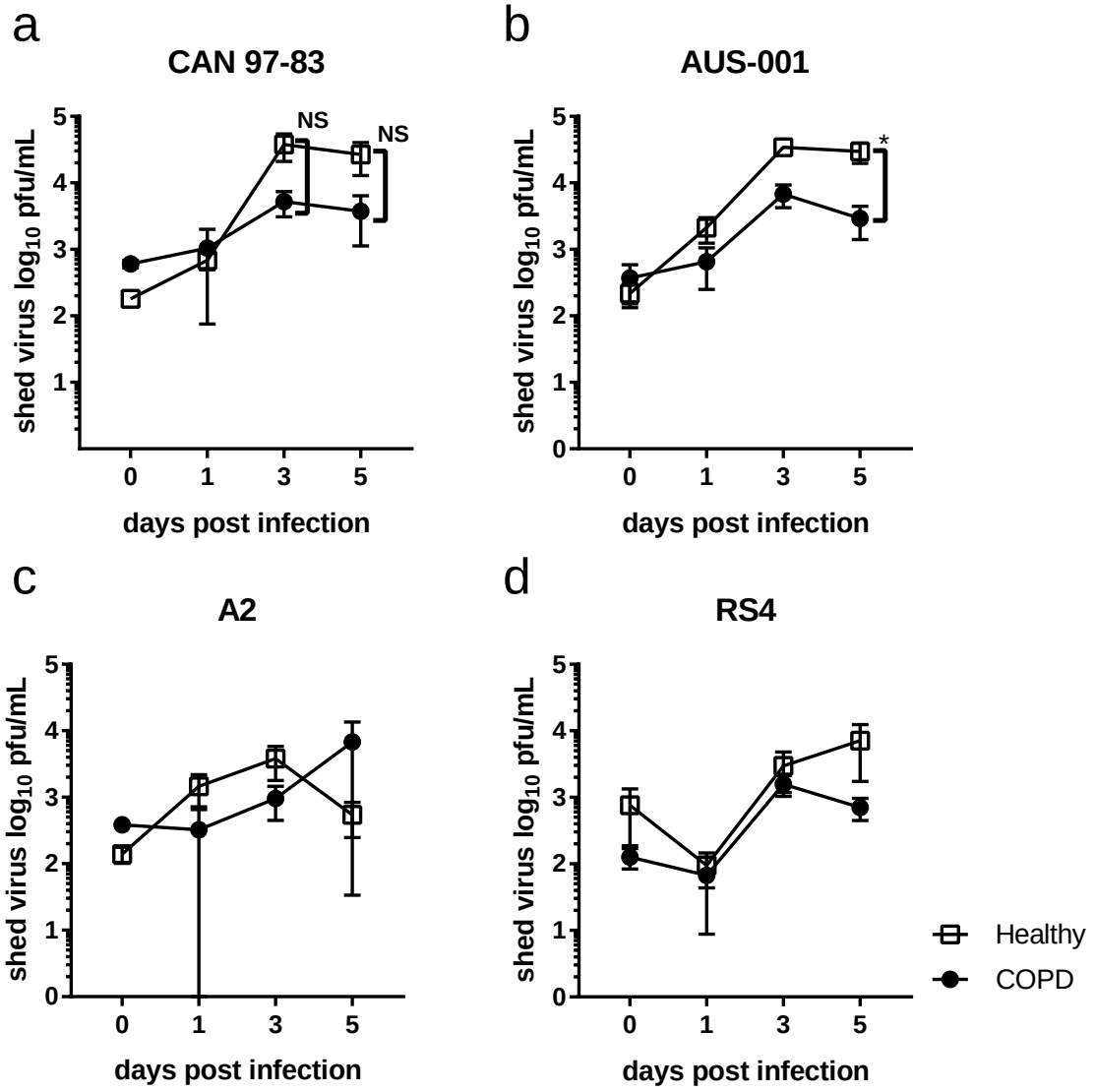

Fig. 7: Viral Shedding: BECs infected with CAN 97-83 **(a)**, AUS-001 **(b)**, A2 **(c)** or RS4 **(d)** at MOI 0.1. Shed virus was quantified by plaque assay. The symbols represent the mean ± SEM. Healthy n=5, COPD n = 5. * denotes $p<0.05$ for COPD group compared to the healthy group.

4.2 Interferon Response

4.2.1 IFN-λ Production is Elevated in Healthy BECs in Response to A2

Secreted IFN-λ protein was quantified in the same cell supernatants as were used to quantify shed virus. The kinetics were similar to those of shed virus for both hMPV and RSV BECs from healthy and COPD subjects in that there was a trend for elevated IFN-λ production from healthy BECs compared to COPD in response to all viruses (Fig. 8). At day 5 p.i. CAN 97-83 and AUS-001 induced means of 358.66pg/ml and 340.26pg/ml

respectively from healthy BECs and 159.94pg/ml and 89.7pg/ml respectively from COPD BECs (Fig. 8a & 8b). The mean amount of IFN-λ induced in healthy BECs by A2 infection (mean = 626.3pg/ml) was significantly more than BECs from COPD subjects (mean = 262.44pg/ml) at day 3 and continued to be higher in healthy BECs at day 5 p.i. (Fig. 8c). There was no difference in IFN-λ produced by BECs from healthy and COPD subjects in response to RS4 (Fig. 8d). There is no evidence from these data that BECs from COPD patients are less able to produce IFN-λ in response to infection by RSV or hMPV as reduced IFN-λ corresponded to reduced shed virus. However they do highlight differences in response amongst viruses.

CAN 97-83, A2 and RS4 showed a trend towards increasing IFN-λ production from day 0 to day 5 p.i. whereas there was only a small reduction in IFN-λ induced by AUS-001 on day 5 p.i. compared to day 3 p.i. in both healthy and COPD BECs (Fig. 8b). Data for IFN-λ on day 3 p.i. was extracted and compared to further identify virus-specific differences (Fig. 9). A2 was the greatest inducer of IFN-λ in healthy BECs, with a mean induction of 626.3pg/ml on day 3 p.i. compared to 284.92pg/ml for CAN-97-83 and 425.32pg/ml for AUS 001 in the same cells (Fig. 9). As for shed virus, the amount of IFN-λ induced by RS4 was much lower at days 3 and 5 p.i. compared to the other 3 viruses, and statistically significantly lower than A2. This correlated to lower shed virus for RS4 (Fig. 7).

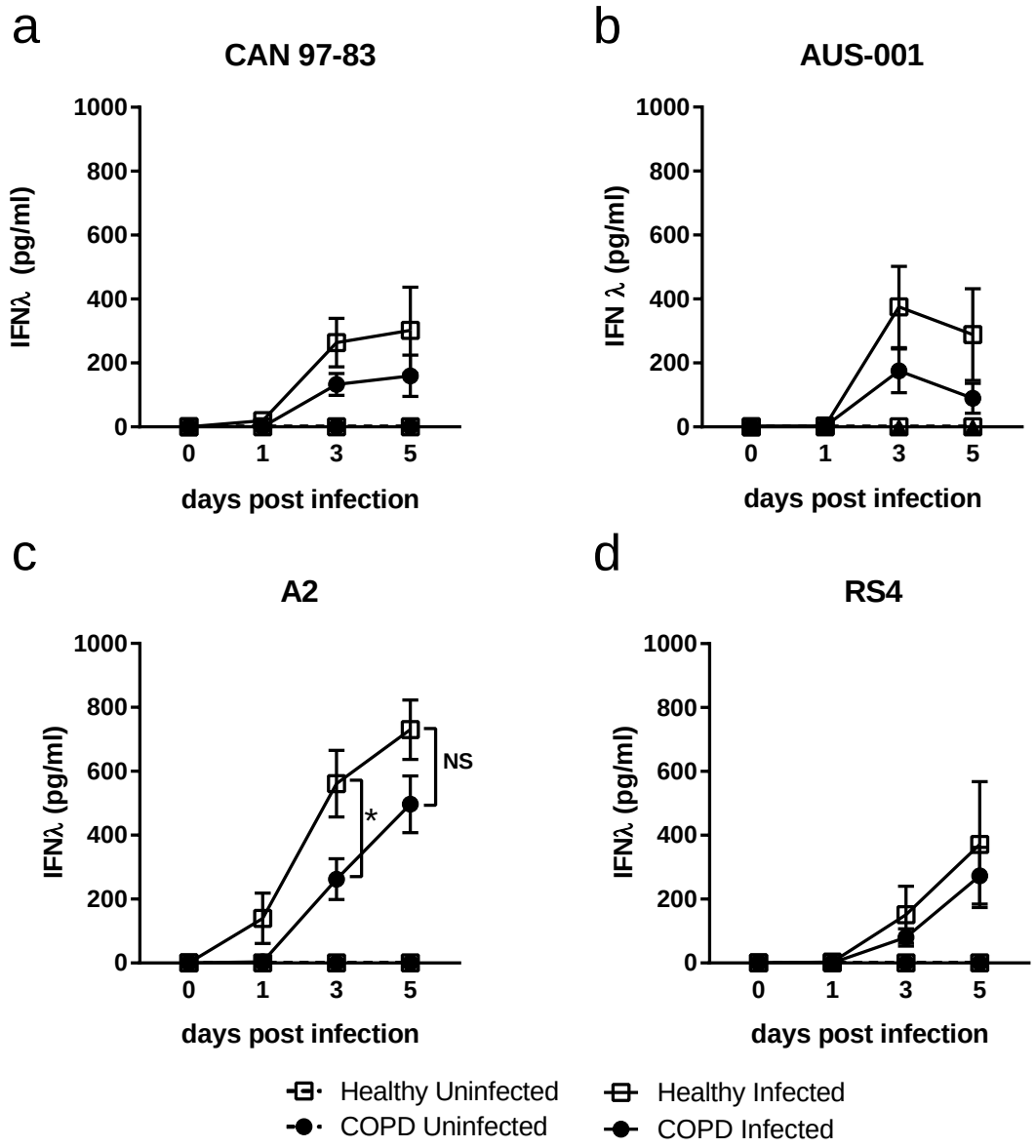

Fig 8: IFN-λ Production: BECs infected with CAN 97-83 **(a)**, AUS-001 **(b)**, A2 **(c)** or RS4 **(d)** at MOI 0.1. Secreted IFN-λ was quantified in cell supernatant at day 0, 1, 3, and 5 post-infection by ELISA. The symbols represent the mean ± SEM. Healthy n=5, COPD n = 5. Unpaired t-tests shows statistical significance, as * denotes $p<0.05$ for COPD group compared to the healthy group.

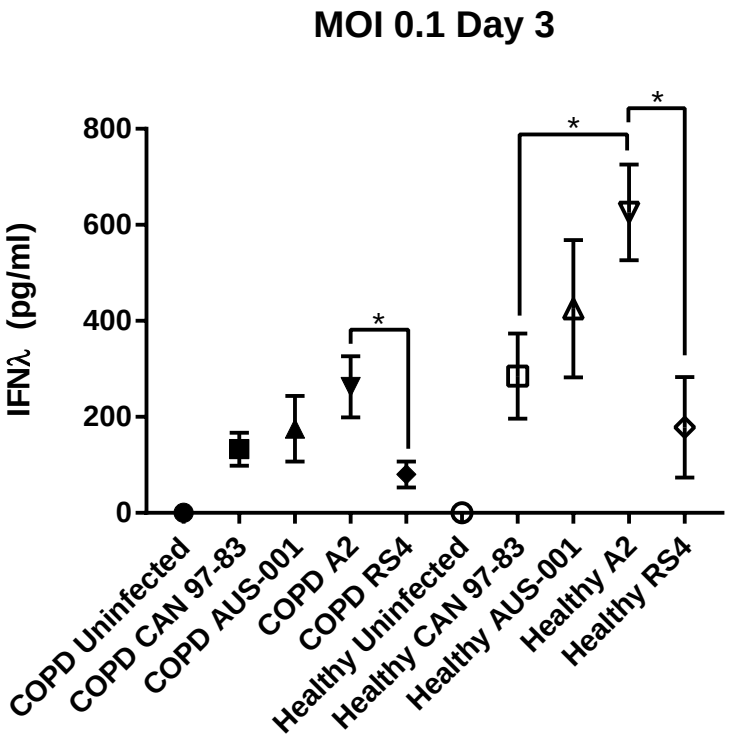

Fig 9: IFN-λ secreted day 3 p.i. for all infection groups: BECs infected with CAN 97-83, AUS-001, A2, or RS4 at MOI 0.1. Secreted IFN-λ was quantified by ELISA in cell culture supernatant at day 3 post-infection. Healthy n=5, COPD n = 5. An unpaired t-test showed statistical significance between viruses as indicated by * $p<0.05$.

4.2.2 IFN-β Production is Elevated in COPD BECs in Response to hMPV and RSV

IFN-β protein secretion was also quantified in the same cell supernatants and demonstrated very different kinetics to both IFN-λ protein secretion and virus shedding. Firstly in response to all viruses, BECs from COPD subjects produced significantly more IFN-β protein than BECs from healthy subjects from days 1 to 5 (Fig. 10). Healthy BECs produced very little IFN-β protein over the time-course with a maximum mean of 60.6pg/ml at day 5 in response to A2 (Fig. 10c). In fact IFN-β production was only significantly higher compared to uninfected cells for CAN 97-83 and AUS-001 viruses at days 3 and 5 p.i. For BECs from COPD subjects, A2 induced the most IFN-β (mean = 628 pg/ml) the earliest at day 3 p.i. (Fig. 10c) followed by CAN 97-83 with a peak mean = 551 pg/ml at day 5 p.i (Fig. 10a). Although, the difference in IFN-β produced by BECs from COPD and healthy subjects in response to AUS-001 did not reach significance over the 5 days due to some variability in cell response, a peak mean of 409pg/ml was produced on

day 3 p.i. in BECs from COPD subjects. As for IFN-λ production, RS4 induced the lowest mean peak of IFN-β production of 349pg/ml on day 3 p.i. However this was still significantly greater at all times than the IFN-β produced by healthy BECs (Fig. 10d). As for IFN-λ, data for day 3 p.i. was extracted and compared to further identify virus-specific differences (Fig. 11). There were no obvious differences in the production of IFN-β from healthy cells based on the infecting virus, as all induced low amounts. In fact A2 and RS4 did not induce IFN-β above the uninfected baseline. A2 was the earliest and most effective inducer of IFN-β from BECs from COPD subjects. Again, RS4 was a poor inducer compared to A2.

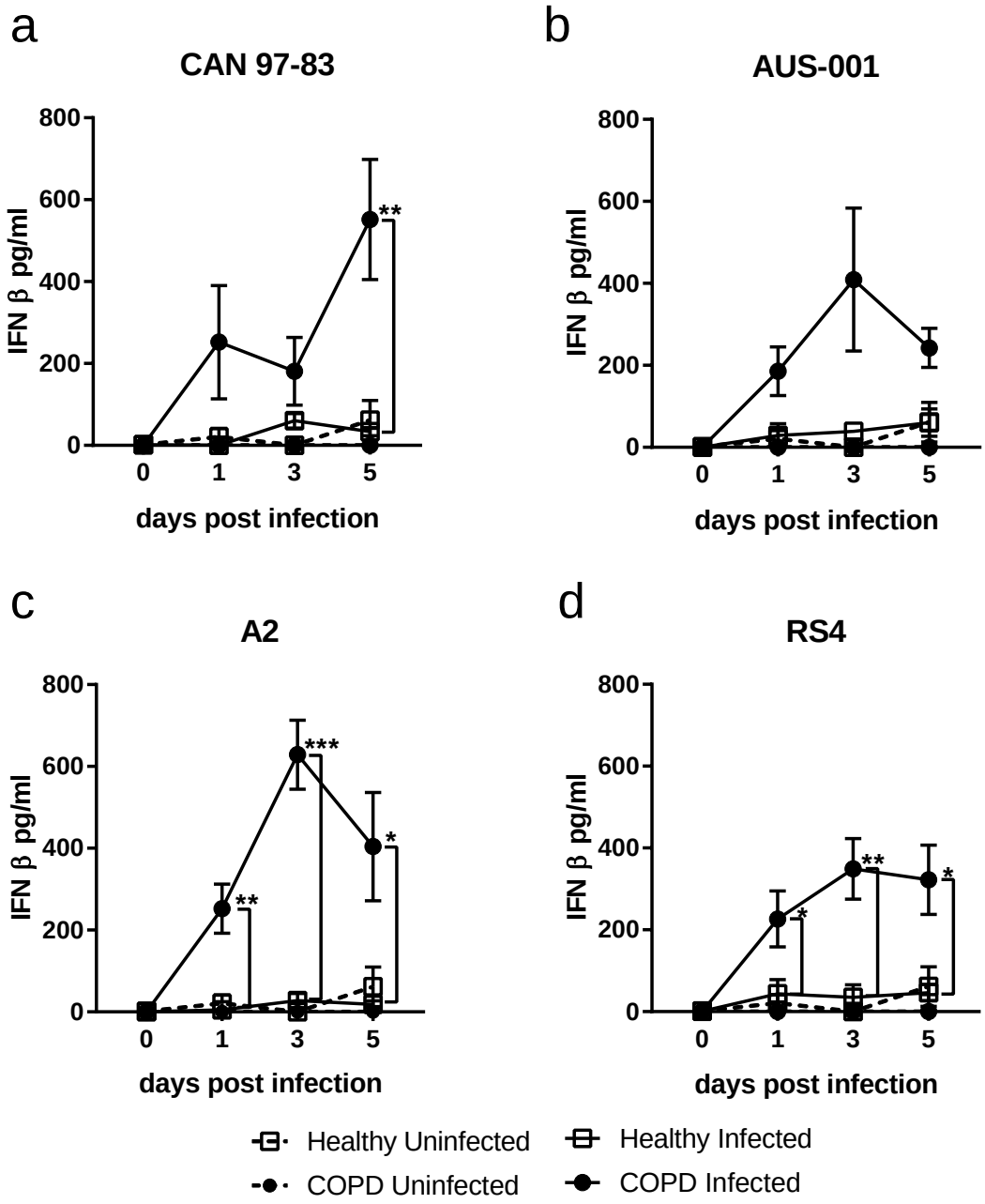

Fig 10: IFN-β Production: BECs infected with CAN 97-83 **(a)**, AUS-001 **(b)**, A2 **(c)** or RS4 **(d)** at MOI 0.1. Secreted IFN-β was quantified in cell supernatant at day 0, 1, 3, and 5 post-infection by AlphaLISA. The symbols represent the mean ± SEM. Healthy n=5, COPD n = 5. * denotes $p<0.05$, ** denotes $p<0.01$ and *** denotes $p<0.001$ for COPD group compared to the healthy group.

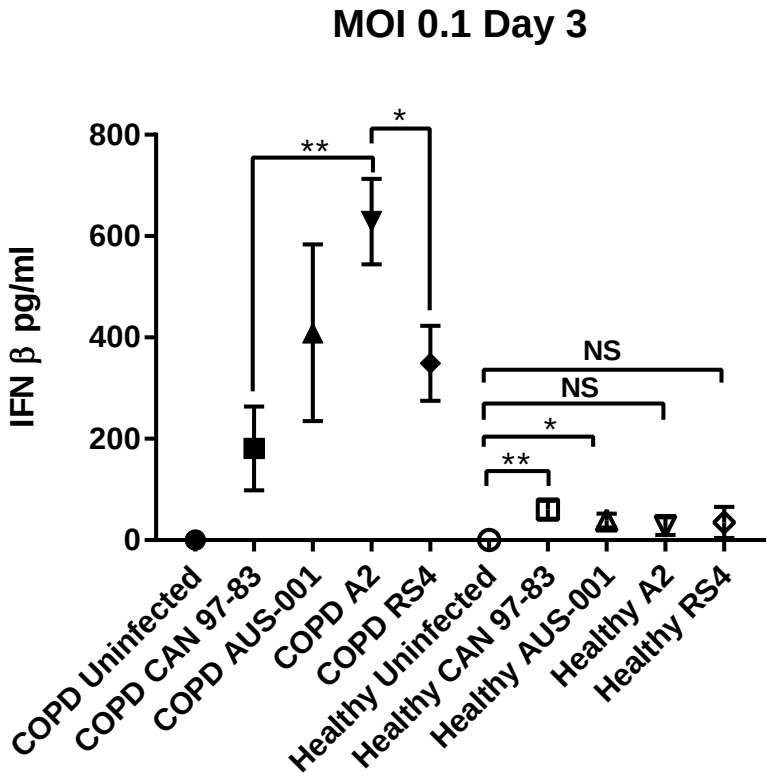

Fig 11: IFN-β secreted day 3 p.i. for all infection groups: BECs infected with CAN 97-83, AUS-001, A2, or RS4 at MOI 0.1. Secreted IFN-β was quantified by AlphaLISA in cell culture supernatant at day 3 post-infection. Healthy n=5, COPD n = 5. An unpaired t-test showed statistical significance between viruses as indicated by * $p<0.05$ and ** $p<0.01$.

4.3 Overall Conclusion

These data correlate to the high MOI data in that COPD was not associated with elevated susceptibility to infection. However the low dose infection model demonstrated an interesting disease-related effect in that COPD is associated with a shift in the dominant IFN type produced by BECs from IFN-λ for healthy cells to IFN-β for diseased cells. This predominance of IFN-β produced by BECs from COPD subjects was not observed when infected at a MOI 2, which indicates the importance of the amount of initial infection in driving the IFN response, and the importance of the infection model chosen.

5.0 Results - Air-liquid Interface Cultures

The data generated using submerged cultures suggested that COPD was not associated with increased susceptibility to infection or viral load. However data for IFN responses was not consistent between infection models. At low dose, COPD was associated with a switch from IFN-λ to -β as the dominant IFN type produced by BECs. In light of this we utilised a model of well differentiated, ciliated cells in addition to the submerged monolayers. The TEER values were measured to establish tight junctions had been formed prior to infection. The TEER was measured over time for several subjects after infection, however these results were deemed unreliable so the data was not included.

5.1 Viral Shedding is Delayed in Response to A2 and RS4

5.1.1 Infection Kinetics – hMPV

ALI cultures were prepared using cells from four subjects from the same donor pool as the submerged culture study, and infected with all four virus isolated. Apical wash samples were collected every 2-3 days for 27 days and shed virus was quantified from these samples (Fig. 12).

There was no statistical difference in virus shed from BECs from healthy and COPD subjects in response to CAN 97-83 for the entire 27 days of investigation. However there was a trend for peak shedding at day 1 p.i. for BECs from COPD subjects and day 3 p.i. for BECs from healthy subjects. In response to infection with AUS-001, there was a distinct peak of virus shed from BECs from COPD subjects on day 1 p.i. with a mean of 504,025 pfu/ml. However there was large variability on the amount of shed virus amongst the four COPD subjects, which negated statistical significance. The same peak of shedding was not seen in healthy BECs, which reached a maximum mean of shedding of 15,625.8 pfu/ml on day 3 p.i. Following this initial peak shedding, the amount of infectious virus released from BECs was the same regardless of disease status. Interestingly, it was more difficult to remove the AUS-001 inoculum from COPD cell cultures than from healthy cell cultures, resulting in high mean virus (30,134.9 pfu/ml) on the apical surface of BECs from healthy subjects (238,558 pfu/ml) compared to COPD subjects at day 0.

At day 14 for the AUS-001 infection and day 21 for the CAN 97-83 infection, shed virus was no longer detected from cultures from COPD subjects due to cell death as observed

microscopically. Healthy cultures survived and produced infectious virus for longer; until day 18 for AUS-001 infection and day 24 for CAN 97-83 infection. These data suggest that BECs from COPD subjects may be more susceptible to hMPV infection and may die earlier post-infection than healthy BECs.

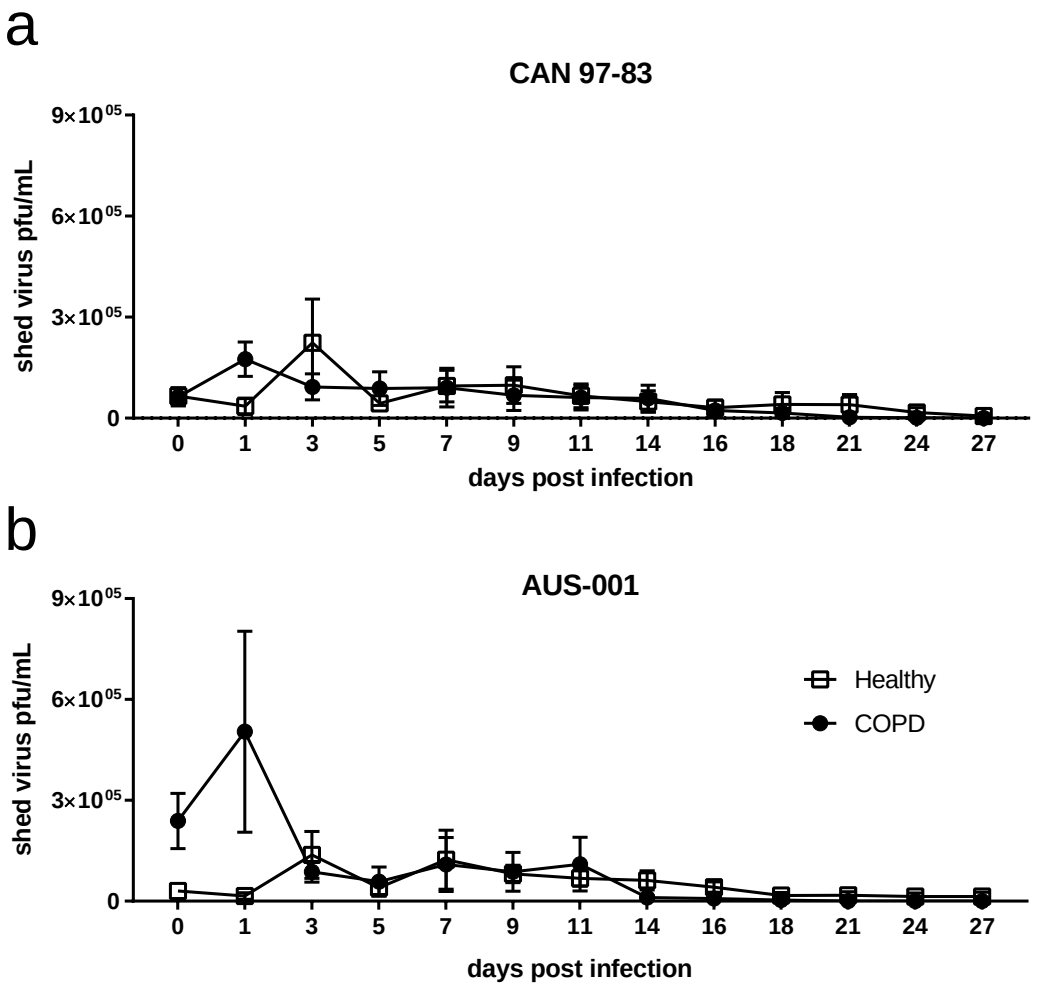

Fig. 12: Virus Shedding-hMPV: Air-liquid interface cultures infected with CAN 97-83 **(a)** and AUS-001 **(b)** at MOI 3, apical surface washed 3 times per week. Shed virus was quantified by plaque assay. The symbols represent the mean ± SEM. Healthy n=4, COPD n = 4. Unpaired t-tests were performed, but no statistical significance was observed.

5.1.2 Infection Kinetics - RSV

The kinetics of RSV shed from ALI cultures of both healthy and COPD BECs was different to that observed for hMPV (Fig. 13). Healthy BECs demonstrated elevated shed A2 and RS4 at all time-points over the 27 days compared to COPD BECs however this did not reach significance at any time due to large variability amongst subjects within each disease status group. These data support the data from submerged cultures that COPD is not associated with elevated susceptibility to infection.

Interestingly, the kinetics of shed virus for RS4 and A2 were very different over the 27 days of investigation regardless of disease status. In healthy BECs, shed A2 was not detected until day 7 p.i with peak shedding on days 18 (mean 866,711 pfu/ml) and 24 p.i. (mean 822,617 pfu/ml). RS4, however was detected on day 1 p.i., with a peak mean of 948,375 pfu/ml detected on day 7 p.i. Virus release was sustained within half a $\log_{10}$ of this level until day 21 p.i., then dropped to 0 on day 24 p.i. due to cell death as observed microscopically.

In response to A2 COPD BECs produced a similar kinetic to healthy cells, with no detectable virus shed until day 16 p.i., 8 days later than for healthy BECs. Peak shedding occurred between days 16 and 18 (mean = 866711pfu/ml), with no detectable virus shed later than day 21 p.i. For BECs from COPD subjects infected with RS4, shed virus was detected at day 1 p.i., as for healthy BECs. However, there was no increase in shedding beyond this time, and no shed virus detected beyond day 18. These data highlight differences in infection kinetics amongst isolates and species of virus, although the kinetics of viral production based on disease status are consistent regardless of the infecting virus, in that susceptibility is not related to COPD status.

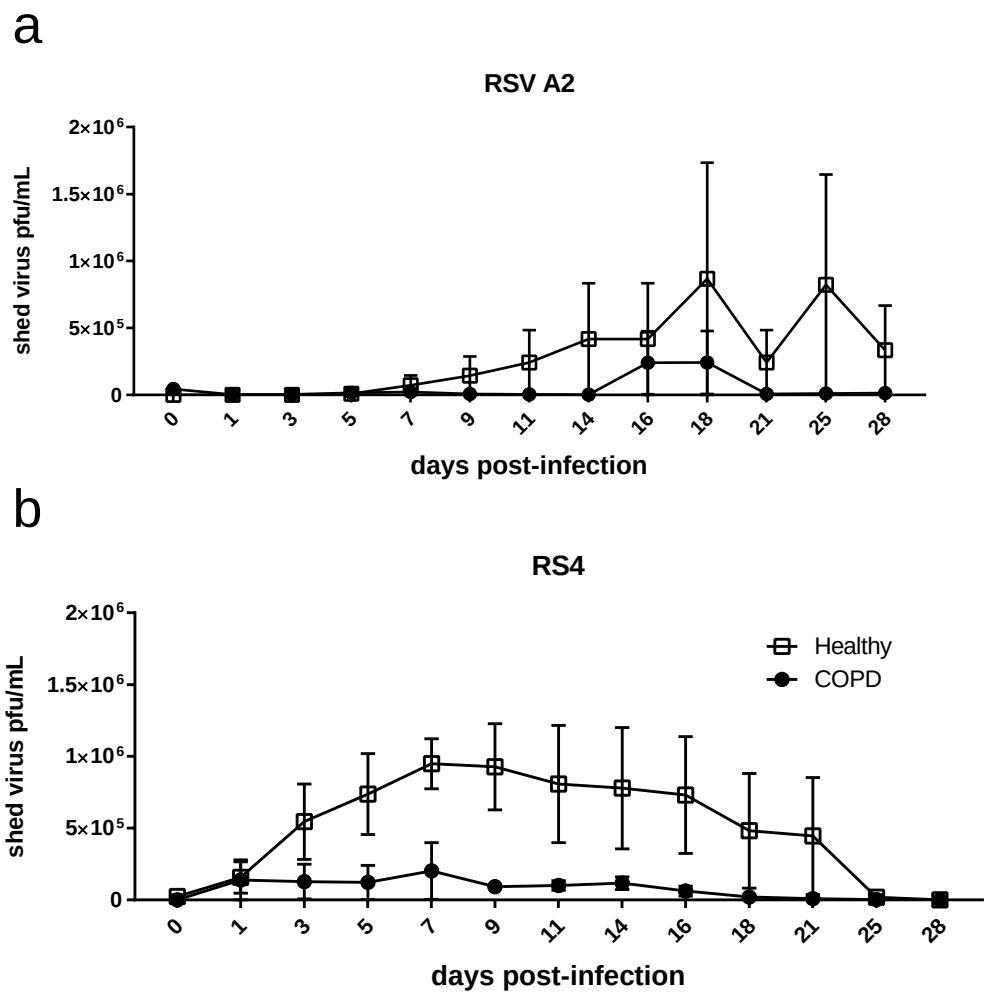

Fig. 13: Virus Shedding – RSV: Air-liquid interface cultures infected with RSV-A2 **(a)** and RS4 **(b)** at MOI 3, apical surface washed 3 times per week. Shed virus was quantified by plaque assay. The symbols represent the mean ± SEM. Healthy n=4, COPD n = 4. Unpaired t-tests were performed, but no statistical significance was observed.

5.2 Interferon Response

5.2.1 IFN-λ Production is not Altered Based on Disease State

Basal culture media was collected from the same ALI cultures as used for shed virus, and secreted IFN-λ protein was quantified (Fig. 14). In response to hMPV both healthy and COPD BECs produced less than 50pg/ml, excluding the one outlier. The kinetics of IFN-λ release were similar in response to both CAN 97-83 and AUS-001 with most protein production from healthy and COPD BECs between days 1 and 13 p.i. COPD was not associated with reduced IFN-λ production in response to either hMPV isolate. In response

to CAN 97-83, there was a small peak from healthy cells at day 7 p.i. (mean = 32.5 pg/ml) and a small peak from COPD cells at day 3 p.i. (mean = 26.1 pg/ml), but no statistical significance was reached based on disease status and no trend was identified. There was a trend for AUS-001 to induce more IFN-λ in healthy BECs, than CAN 97-83, with a peak mean of 52pg/ml on day 3 compared to a peak mean of 26.1pg/ml on day 7 p.i. for CAN 97-83. However this was not statistically different. IFN-λ was not detected above baseline for the assay beyond day 11 p.i. excluding the one outlier.

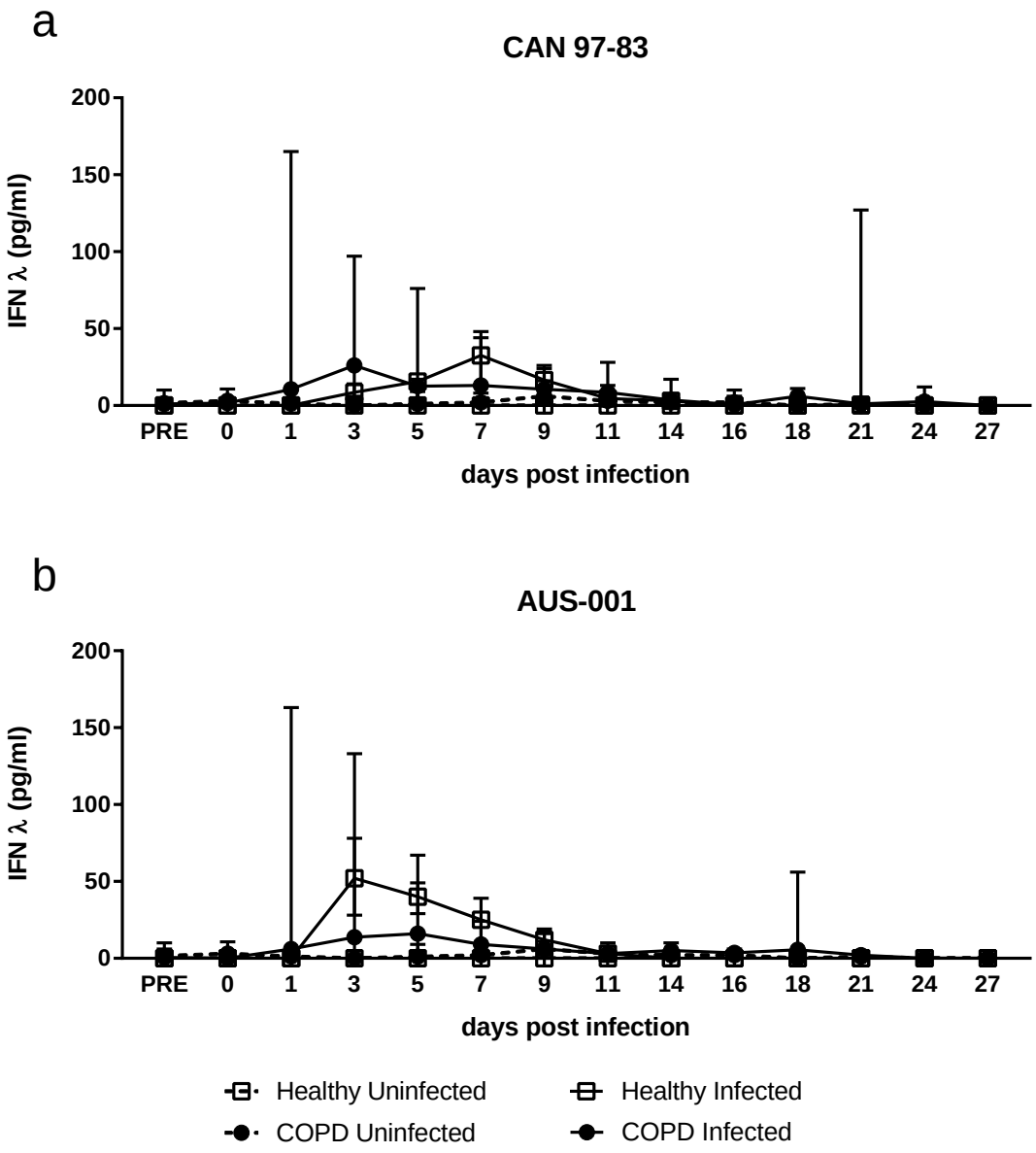

Fig. 14: IFN-λ Production - hMPV: Air-liquid interface cultures infected with CAN 97-83 **(a)** and AUS-001 **(b)** at MOI 3, basal media was sampled 3 times per week. Secreted IFN-λ was quantified by ELISA. The symbols represent the mean ± SEM. Healthy n=4, COPD n = 4. Unpaired t-tests were performed, but no statistical significance was observed.

In response to RSV, BECs from both healthy and COPD subjects produced less than 50pg/ml IFN-λ, excluding the one outlier (Fig. 15). There was also no difference in IFN-λ production between BECs from healthy and COPD donors. In response to A2, IFN-λ was detectable earlier in COPD cultures (day 1 p.i.) than in healthy cultures (day 7 p.i.). IFN-λ produced in response to RS4 was also detectable earlier in BECs from COPD subjects than from healthy subjects (day 1 compared to day 3 for healthy), and was detected for a longer time from COPD subjects (until day 15) compared to healthy subjects (until day 11).

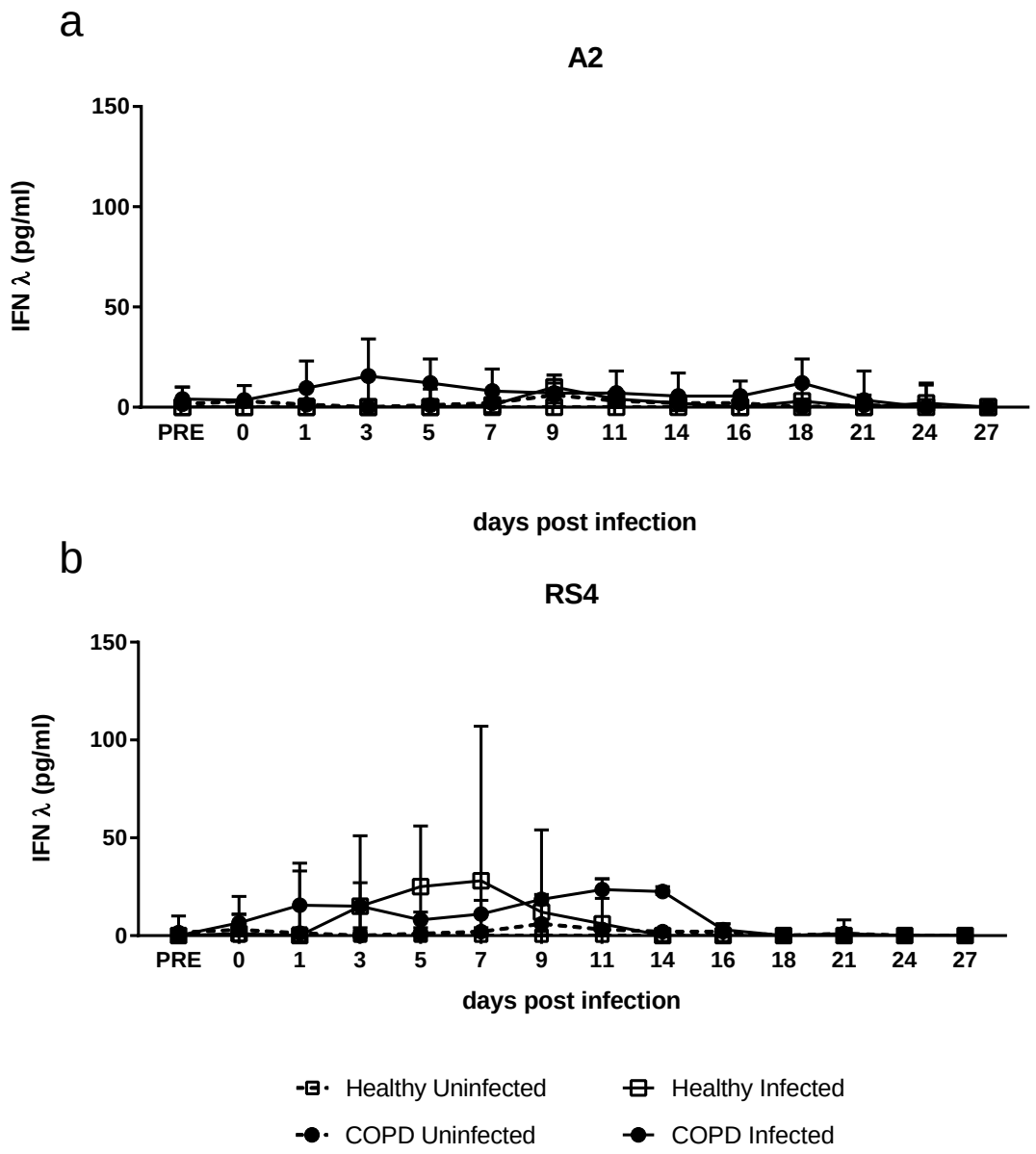

Fig. 15: IFN-λ Production – RSV: Air-liquid interface cultures infected with RSV-A2 **(a)** and RS4 **(b)** at MOI 3, basal media was sampled 3 times per week. Secreted IFN-λ was quantified by ELISA. The symbols represent the mean ± SEM. Healthy n=4, COPD n = 4. Unpaired t-tests were performed, but no statistical significance was observed.

5.2.2 IFN-β Production is Elevated in COPD BECs Compared to Healthy in Response to hMPV and RSV

The same basal media samples from the ALI cultures were used to quantify secreted IFN-β (Fig. 16). The kinetics of IFN-β production were very different to those of IFN-λ. Both isolates of hMPV induced significantly more IFN-β production by BECs from COPD

subjects than healthy subjects. Healthy BECs infected with CAN 97-83 produced IFN-β between days 3 – 11 p.i. with a peak mean of 204.7 pg/ml on day 7 p.i. In response to AUS-001, healthy BECs produced IFN-β for a similar time, although from day 1-9 p.i. with a lower mean peak of 69.9pg/ml compared to CAN 97-83. Interestingly, healthy BECs in response to both hMPV isolates demonstrated the suggestion of a second IFN production event from day 24 p.i., with IFN-β being detected after a period of several days of non-detection.

In response to CAN 97-83 infection, BECs from COPD subjects produced a mean peak of IFN-β production of 310.2pg/ml on day 14 p.i., which was statistically significantly greater than production from healthy BECs. The same kinetics were observed in response to AUS-001 infection, with IFN-β production reaching a peak of mean 277.8pg/ml slightly earlier on day 11 p.i. Again, this was statistically significantly greater than IFN-β production by healthy BECs at the same time p.i. However, unlike the healthy BECs, BECs from COPD subjects did not produce detectable IFN-β beyond day 21 p.i. As seen in the submerged MOI 0.1 model, there was a significant association between COPD and elevated IFN-β protein when infected with hMPV.

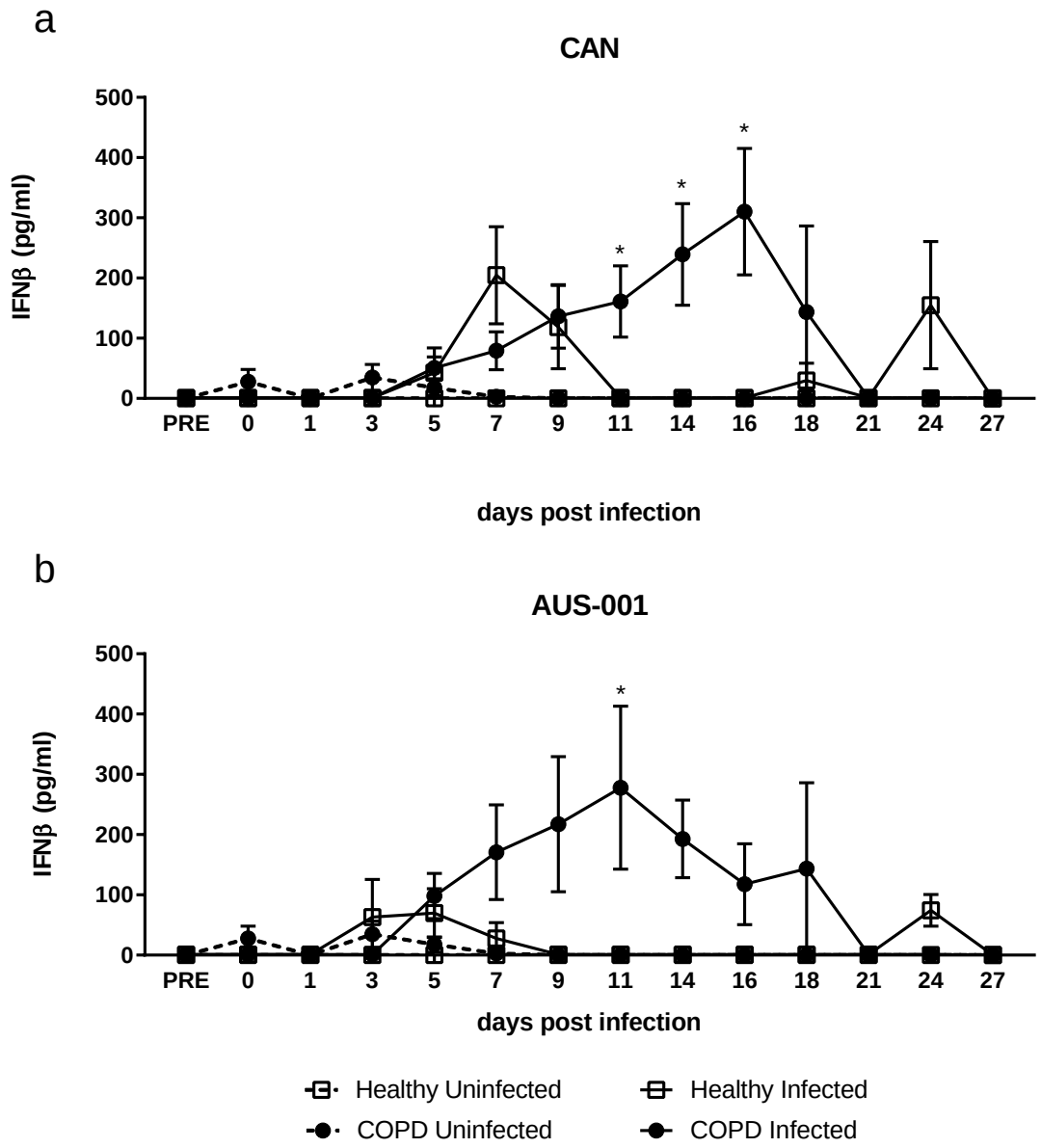

Fig 16: IFN-β Production – hMPV: Air-liquid interface cultures infected with CAN 97-83**(a)** and AUS-001 **(b)** at MOI 3, basal media was sampled 3 times per week. Secreted IFN-β was quantified by AlphaLISA. The symbols represent the mean ± SEM. Healthy n=4, COPD n = 4. Unpaired t-tests showed statistical significance, as * denotes $p<0.05$ for COPD group compared to healthy group.

The kinetics of IFN-β production in response to RSV infection were also different to IFN-λ production. In response to A2, both healthy BECs and those from COPD donors reached similar mean peaks of production of 190.2pg/ml for healthy BECs and 239.2pg/ml for BECs from COPD subjects. However the peak for COPD was later at day 9 p.i., compared

to day 7 p.i. for healthy BECs, and detection was also sustained for longer in COPD cultures (20 days) compared to healthy cultures (8 days). In response to RS4, IFN-β production by COPD cultures was significantly greater than production by healthy cultures. Peak production by COPD cultures occurred on day 9 p.i with a mean of 626.8pg/ml. Detection was also sustained for 21 days. In healthy cultures, very little IFN-β was detected between days 5 and 11 p.i., with a peak of 50.3pg/ml on day 9 p.i. Interestingly, in response to both RSV isolates, there was a suggestion of a second IFN-β production event, as observed for IFN-λ in the same cultures, with detectable IFN-β produced on day 27 p.i. Overall the ALI culture data correlated to the submerged culture MOI 0.1 model in that COPD was associated with significantly elevated IFN-β production in response to RSV.

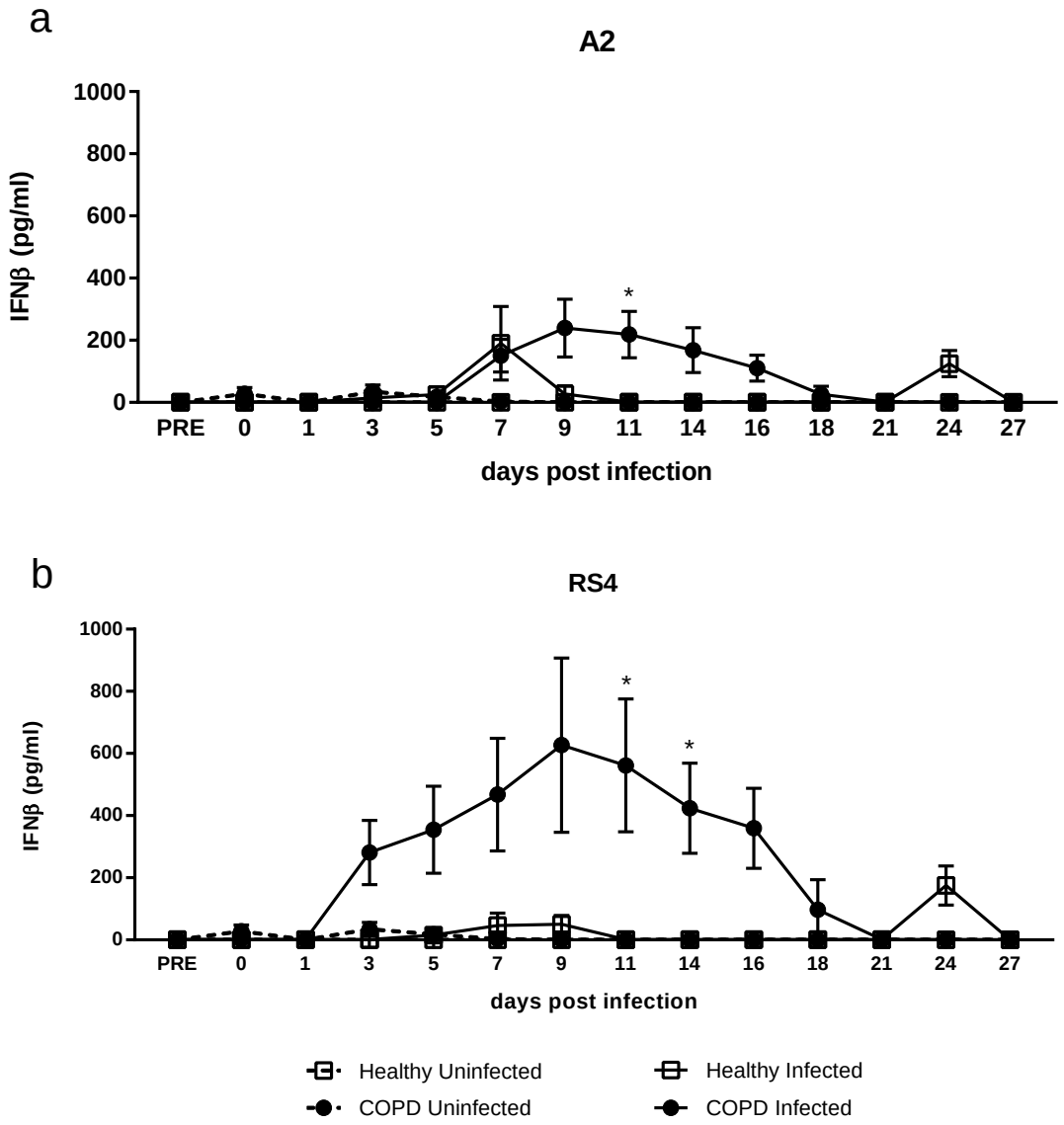

Fig 17: IFN-β Production – RSV: Air-liquid interface cultures infected with RSV-A2 **(a)** and RS4 **(b)** at MOI 3, basal media was sampled 3 times per week. Secreted IFN-β was quantified by AlphaLISA. The symbols represent the mean ± SEM. Healthy n=4, COPD n = 4. An unpaired t-test showed statistical significance, as* denotes $p<0.05$ for COPD group compared to healthy group.

5.3 Overall Conclusion

The ALI model was consistent with the submerged models in that COPD was not associated with significantly elevated virus production. Although it may be associated with elevated susceptibility hMPV as demonstrated by early hMPV infectious virus release post-infection. The ALI cultures confirm the findings of the submerged culture MOI 0.1 infection model, that COPD is associated with elevated IFN-β production in response to both hMPV and RSV.

6.0 Results - Cell Death Response

Submerged monolayers of BECs infected at a MOI of 2 were assayed for cell death as indicated by LDH release (Fig. 18) and caspase (Figs. 19-22) activation 24h p.i.

6.1 LDH Release is Elevated in COPD BECs Compared to Healthy in Response to AUS-001 and RS4

All four virus isolates induced elevated LDH release from BECs from COPD subjects compared to healthy subjects, although the difference was statistically significant for only AUS-001 and RS4 infections. Healthy BECs demonstrated lower LDH release, with no more than a four-fold increase over uninfected cells in response to any virus. In contrast BECs from COPD subjects demonstrated a four or more-fold increase in LDH release over uninfected cells for all four viruses. These data demonstrate a susceptibility to virus-induced cell death associated with COPD. There was not only an association based on disease, there was also a trend towards hMPV inducing more LDH release than RSV, suggesting hMPV may have a more pathogenic effect on BECs than RSV.

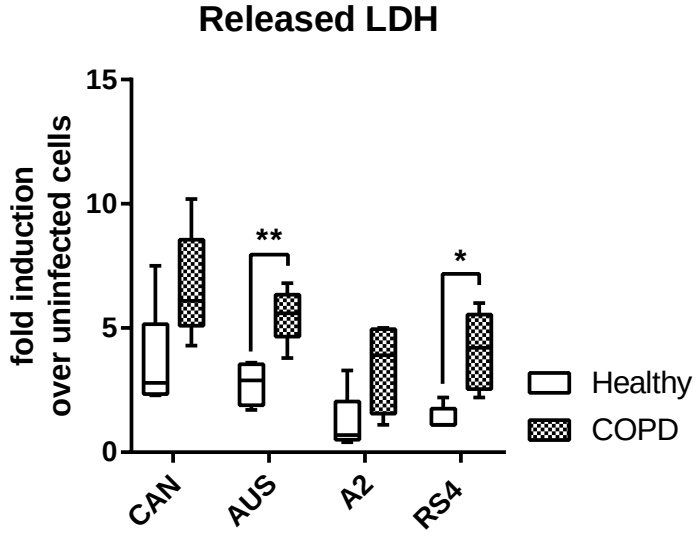

Fig. 18: Released LDH: BECs infected with CAN 97-83, AUS-001, A2, and RS4 at MOI 2. Fold induction over uninfected secreted LDH was quantified in cell supernatant at 24h post-infection by Cyto-Tox 1 homogenous membrane integrity assay. The box represents the 5th and 95th percentile, the bar represents the median and the whiskers represent the minimum and maximum. Healthy n=5, COPD n = 5. Unpaired t-tests showed statistical significance, as * denotes $p<0.05$ and ** denotes $p<0.01$ for COPD group compared to the healthy group.

6.2 Caspase Activation

Apoptosis is a key cell death pathway driven by caspases. Therefore we quantified the activation of key caspases in response to infection of submerged monolayer cultures with the four viruses at a MOI of 2. This was performed with the same cultures as used for the LDH release assay.

6.2.1 Caspase 3/7 Activation is not Altered Based on Disease State

There was no statistical association between elevated caspase-3/7 activation and disease state or infecting virus (Fig. 19). There was a trend towards CAN 97-83 inducing the most caspase-3/7 activation of all the viruses, with a median of 5-fold induction over uninfected cells for BECs from both healthy and COPD subjects compared to uninfected cells. RS4 was a poor inducer of caspase-3/7, with a median fold induction not above uninfected cells

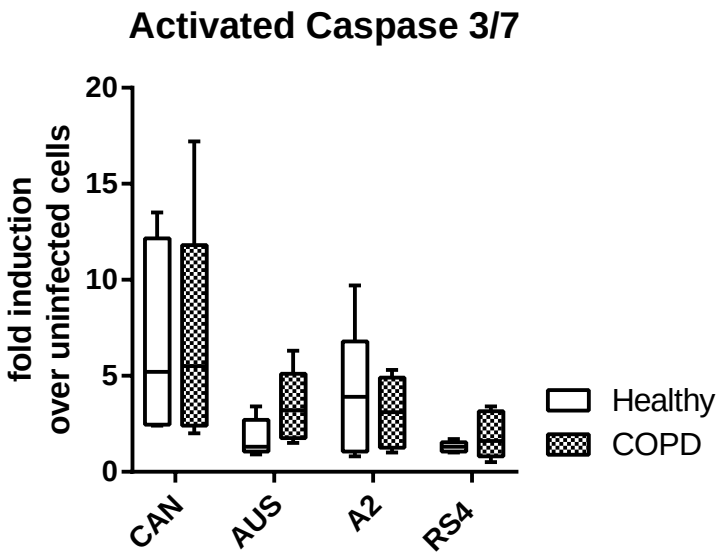

Fig. 19: Activated Caspase 3/7: BECs infected with CAN 97-83, AUS-001, A2, and RS4 at MOI 2. Fold induction over uninfected. Caspase-3/7 activation was quantified in cells at 24h p.i. by Caspase-Glo 3/7 assay. The box represents the 5th and 95th percentile, the bar represents the median and the whiskers represent the minimum and maximum. Healthy n=5, COPD n = 5. Unpaired t-tests were performed, but no statistical significance was observed.

6.2.2 Caspase 8 Activation is not Altered Based on Disease State

There was no difference in caspase-8 activation between BECs from healthy and COPD subjects in response to any of the virus isolates. As for caspase-3/7, there was a trend for CAN 97-83 to be the best inducer, although this was not statistically significant. Caspase-8 activation was poorly induced by AUS-001, A2 and RS4, all inducing less that a median 2-fold induction over uninfected cells.

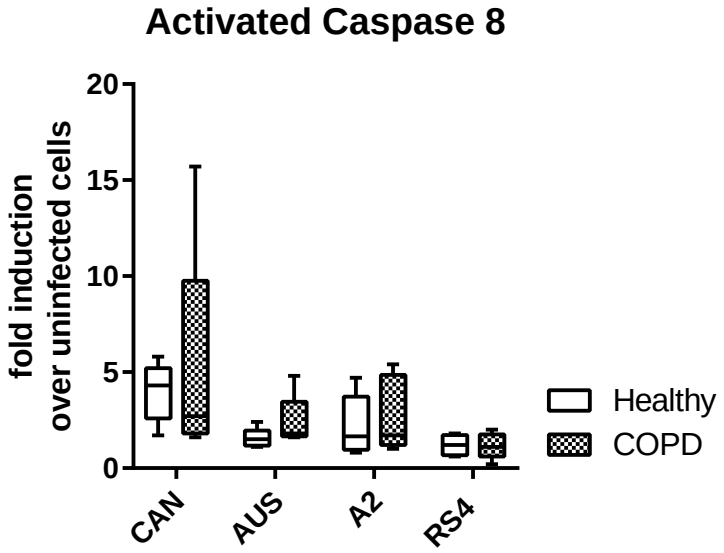

Fig. 20: Activated Caspase 8: BECs infected with CAN 97-83, AUS-001, A2, and RS4 at MOI 2. Fold induction over uninfected. Caspase-8 activation was quantified in cells at 24h p.i. by Caspase-Glo 8 assay. The box represents the 5th and 95th percentile, the bar represents the median and the whiskers represent the minimum and maximum. Healthy n=5, COPD n = 5. Unpaired t-tests were performed, but no statistical significance was observed.

6.2.3 Caspase 9 Activation is not Altered Based on Disease State

Similarly to caspase-8, there was no difference in caspase-9 activation between BECs from COPD and healthy subjects in response to any virus infection (Fig. 18). There was a trend however towards elevated caspase-9 activation compared to caspase-8 by all viruses particularly from COPD BECs, but this was not significant. Again there was a trend that CAN 97-83 induced greater capsase-9 activation in healthy and COPD BECs than the other 3 viruses. AUS-001 and RS4 induced a median 1 to 2-fold induction over uninfected, with one outlier inducing a 12-fold induction over uninfected cells. CAN 97-83 and RSV-

A2 induced a median 4-6 fold induction over uninfected. Together with the caspase-3/7 and -8 data, this suggests that hMPV and RSV induce cell death in BECs from COPD subjects via a mechanism other than apoptosis.

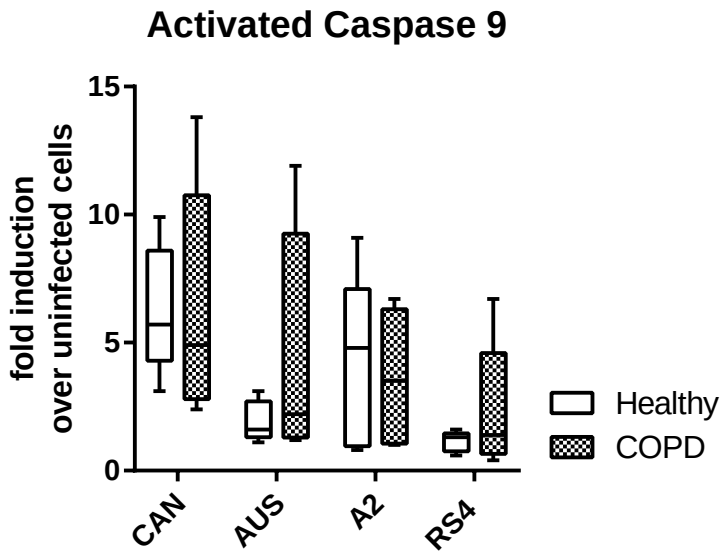

Fig. 21: Activated Caspase 9: BECs infected with CAN 97-83, AUS-001, A2, and RS4 at MOI 2. Fold induction over uninfected. Caspase-9 activation was quantified in cells at 24h p.i. by Caspase-Glo 9 assay. The box represents the 5th and 95th percentile, the bar represents the median and the whiskers represent the minimum and maximum. Healthy n=5, COPD n = 5. Unpaired t-tests were performed, but no statistical significance was observed.

6.2.4 Caspase 1 Activation is not Altered Based on Disease State

Caspase 1 activation is indicative of the non-apoptotic inflammatory cell death pathway of the inflammasome, which leads to pyroptotic death. However, caspase-1 activity was poorly induced by HMPV and RSV in BECs from healthy and COPD subjects (Fig. 20). The highest median induction over uninfected cells was 1.9-fold in response to CAN 97-83 and 2.3-fold in response to A2, both in healthy BECs. As a 2-fold increase in activation is unlikely to be biologically relevant, this indicates that the inflammasome or pyroptosis is not a principal cell death pathway induced in BECs in response to these viruses, and that cell death is via a different mechanism.

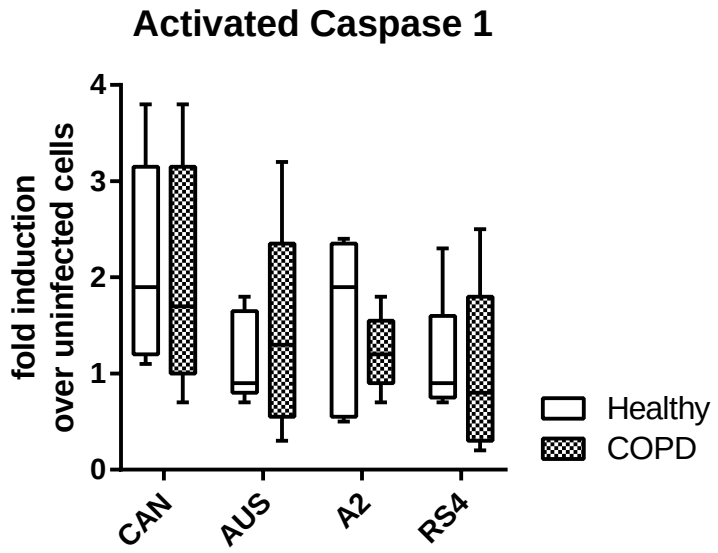

Fig. 22: Activated Caspase 1: BECs infected with CAN 97-83, AUS-001, A2, and RS4 at MOI 2. Fold induction over uninfected. Caspase-1 activation was quantified in cells at 24h p.i. by Caspase-Glo 1 assay. The box represents the 5th and 95th percentile, the bar represents the median and the whiskers represent the minimum and maximum. Healthy n=5, COPD n = 5. Unpaired t-tests were performed, but no statistical significance was observed.

6.3 Overall Conclusion

COPD was associated with elevated cell death in the MOI 2, submerged monolayer infection model, although this does not appear to be due to elevated caspase-driven apoptosis.

7.0 Summary and Discussion

7.1 Conclusion

COPD is associated with extensive airway remodelling and changes to innate immunity that are relevant to the pathogenesis of acute exacerbations (6, 10, 147). Exacerbations of COPD are most commonly caused by respiratory viral infections (148). However, most research on COPD has been focused on the role of inflammation in the pathogenesis of acute exacerbations, with limited research on the specific mechanisms of anti-viral defence associated with viral infection. This is an important aspect of exacerbations as COPD patients are highly susceptible to respiratory virus infections (117, 149). Similarly to research in the asthma field, the exact role of the antiviral response of the airway epithelium in COPD is not clear, as some research has identified innate immune defects associated with COPD (113, 149), while others have conversely identified elevated antiviral factors, such as IFN-λ associated with viral infections in COPD (106, 107). There is almost no research concerning the response of COPD patients to viruses other than RV and influenza, although many viruses cause acute exacerbations of COPD. We know from our lab's previous asthma research that viruses induce different responses in cultured primary AECs and that generalisations based on disease status cannot be made. Hence, the aims of this study were to identify if BECs from COPD donors were more susceptible to viral infections compared to BECs from healthy donors and to investigate if BECs from COPD donors have impaired anti-viral defence mechanisms that may contribute to increased susceptibility to infection, focusing on IFN driven responses and apoptosis. We used four different isolates of two paramyxoviruses to investigate potential differences in response based on the infecting virus rather than disease. We also used three different infection models; submerged monolayers infected at high and low dose, and ALI cultures, also to allow for robust identification of disease-related difference in cellular response to infection.

7.2 Key Findings

7.2.1 Susceptibility to Infection Based on Disease Status

BECs from COPD subjects were not more susceptible to RSV or hMPV infection than healthy cells from age-matched subjects regardless of the infecting virus or the cell culture model. This contradicts a previous study from Kan-O *et. al.* (2017) that used hMPV to infect clinically sourced submerged BECs from healthy and COPD subjects at a MOI of 1. The study demonstrated an elevated viral titre ($TCID_{50}$) and elevated N gene expression in BECs from COPD subjects compared to healthy subjects, however this was identified at 48h and 72h p.i. (119). It may be that sampling later than 24h p.i at a MOI of 2 in our study would have identified a difference between COPD and healthy. However our low MOI model included several time-points over 5 days and did not demonstrate any increased virus shedding from COPD BECs. Another study from Hsu *et. al.* (2016) in which clinically-sourced BECs were infected with Influenza H5N1 also demonstrated an elevated viral load in COPD BECs compared to healthy cells via plaque assay 24h p.i. (150). However one study from Baines *et. al.* (2013) used clinically-sourced submerged BECs infected with RV-1B (MOI 20) and demonstrated no difference in viral load dependant on disease status based on a $TCID_{50}$ and qPCR assay 6h p.i. (116). One common difference between these studies and ours is the use of clinically sourced BECs as opposed to commercially purchased cells. However there are so few published studies investigating virus-susceptibility of BECs in COPD it is difficult to directly compare, especially when different viruses are used. It is a future aim to further investigate the antiviral response in COPD using clinically-sourced cells and in particular nasal epithelial cells (NECs). Kan-o *et. al.* (2017) also used a greater number of subjects (n = 9 COPD and 12 healthy) than we did. One of the limitations of this study was the low sample number, which can be addressed using clinically sourced cells which are cheaper to collect (once ethics approvals are in place), than purchasing commercial cells. We will further investigate the potential use on NECs for anti-viral response studies as they are easier to collect from patients and would allow for greater sample numbers.

Interestingly, the ALI culture model did demonstrate a trend towards elevated hMPV, particularly AUS-001, viral shedding from COPD BECs within the first 24h p.i. compared to healthy cells. There is one other study using ALI cultures of clinically-sourced COPD and healthy BECs, infected with RV-39 at an MOI of 1 (117), which demonstrated an

elevated virus replication by qPCR at both 24h and 48h p.i. With a larger number of subjects the results from our study may become significant; however the infecting virus again makes it impossible to directly compare the two studies. The lack of susceptibility of COPD BECs to RSV infection in any model is maybe not surprising, as we also demonstrated that adult asthma is not associated with elevated susceptibility to RSV using submerged cultures (146). Epidemiology suggests that RSV is identified in up to 15% COPD exacerbations, which is significantly less than RV or influenza virus (65). Our data also suggest that within the age range investigated here, RSV may not be more prevalent in association with COPD. However the lack of studies such as ours makes this conclusion difficult to resolve. The ALI cultures also demonstrated a delayed RSV shedding particularly RSV-A2, this may be due to mucous inhibiting the spread of infection to neighbouring cells. A study to removing the mucous prior to infection of the cells in this model may give varying results, and is something to consider in future studies of ALI cultures.

7.2.2 Differences in the IFN Response Based on Disease Status

The IFN response of BECs was investigated to identify a potential defective in the antiviral response. However, as we did not see any significant susceptibility to infection associated with COPD, this would suggest that there may not be any defect in the antiviral IFN response in COPD either. In the high MOI submerged cultures, there was a trend towards elevated IFN-λ and –β mRNA expression in response to hMPV infection but not RSV infection. However, at the protein production level, there was a converse trend towards lower IFN-λ and-β expression in the same cells. This suggests a potential defect in protein translation in COPD, although requires further investigation as there is nothing published concerning intrinsic defects in the translational machinery of AECs in COPD. The Kan-o study previously discussed also investigated the IFN response and demonstrated an elevated IFN-β mRNA expression by qPCR in association with COPD. (117). Baines et al (2013) also demonstrated elevated IFN-β mRNA expression and protein secretion via ELISA from COPD BECs in response to RV-1B. However, similar to our study, reduced IFN-β protein in response to Influenza H1N1 has been reported in association with COPD (116, 150). These conflicting results dependant on the infecting virus make comparisons

difficult and conclusions concerning the intrinsic antiviral response of AECs in COPD difficult. However they support the need for virus-specific investigation.

IFN-λ protein secretion from submerged BECs infected at a low MOI did demonstrate a trend to be reduced in comparison to healthy BECs. However, as virus shedding was also reduced in the same COPD cell cultures, this does not demonstrate any defect in IFN-λ production. In ALI cultures, IFN-λ production by all cultures was so low, that no defects associated with COPD were observed despite the early peak in hMPV production by COPD BECs. These data support the hypothesis that COPD is not associated with an intrinsic defect in IFN production by BECs, which correlates to a lack of elevated susceptibility to viral infection in this study. We have also demonstrated that AECS in adults with asthma (146) and children with wheeze (110) do not have any intrinsic defects in IFN response.

Rather than a lack of IFN response, this study demonstrated elevated IFN-β protein production in association with COPD and in response to all four viruses in both submerged BECs infected at a low MOI and BECs cultured at ALI. Due to the low number of subjects (n=4), this trend did not reach statistical significance in ALI cultures, although it did in submerged, low MOI infected BECs. A study by Hsu *et. al.* (2017) using clinical BECs cultured at ALI, infected with Influenza H3N2 and H5N1 at a MOI of 5 conversely demonstrated reduced IFN-β production from COPD BECs compared to healthy BECs (151). Other than the infecting virus being different, this study used a higher MOI and clinically-sourced BECs, which may partly explain the difference in observations. Schneider *et. al.* (2010) demonstrated similar results to Hsu *et. al.*in their study of clinical AECs cultured at ALI infected with RV-39 at MOI 1, with an IFN-β protein level below the limits of detection (ELISA), but a significantly elevated IFN-β mRNA expression from COPD and healthy BECs (117). This study also demonstrated elevated IFN-λ mRNA expression and protein secretion from COPD BECs compared to healthy cells (117), which is directly opposing our data, although does also demonstrated an elevated rather than reduced IFN response associated with COPD. In our study, the elevated IFN-β production may correspond to reduced virus shed in COPD in response to both strains of RSV infection, although there is no corresponding difference in hMPV shedding that may be directly explained by elevated IFN-β production. Therefore, in response to hMPV, these data may suggest that elevated IFN-β production in COPD is not effective against hMPV infection and is more effective against RSV infection. In order to confirm the preference

for IFN-β production over IFN-λ production in COPD, IFN gene expression will be quantified in these cells from both the ALI model and the submerged low MOI infection model in the future.

This study is the first in the world to infect ALI cultures with hMPV and quantify the kinetics of viral shedding and IFN response. Therefore the observation that IFN-β production is elevated in association with COPD is novel and requires further investigation.

Sykes et al (2012) hypothesised that a defective interferon induction in asthmatics AECs in cultures (152, 153) may be apparent only at low levels of viral infection and that this is a more effective model that high MOI. Our data using AECs from children with wheeze also showed benefits of this model (110), as does this masters study, as the low MOI model showed an elevated IFN-β from COPD BECs. Therefore this masters further demonstrates that the type of model used can influence the IFN response.

7.2.3 Differences in the Cell Death Response Based on Disease Status

COPD was associated with elevated cell death (LDH release), although death was not caspase-driven.

In addition to type I and III IFN-driven antiviral responses, programmed cell death is critical in control of viral spread within a population of cells. Based on our asthma data (146) we initially hypothesised that BECs from COPD subjects may have a defect in caspase-3/7activation and apoptotic cell death, which would lead to a higher viral load in these cells. As discussed, we did not see a higher viral load associated with COPD. Therefore, it isn't surprising that we did not see reduced caspase-3/7 activation as for the asthma model (146). In fact we did not see any difference in activation of the key caspases involved in apoptosis: caspases-3/7, -9 and -8. As these data suggested no difference in apoptosis we did not continue to investigate apoptosis so did not perform more detailed studies involving TRAIL expression or annexin V staining. Both assays are common indicators of apoptosis induced by viral infection (129, 154).

Some previous studies of apoptosis in COPD BECs suggest that there is elevated susceptibility to cell death via apoptosis, although these studies compared COPD BECs to BECs from non-COPD smokers, not to healthy, non-smoking subjects (155, 156). One study used influenza H3N2 at a MOI of 5 to infect clinical BECs and identified elevated

apoptosis at 6h p.i. using flow cytometry in response to infection, which lead to decreased viral replication (157). Our data, when considered with other published data suggest that different viruses induce different apoptotic responses in BECs from COPD subjects. Although we did not observed caspase activation differences, LDH release does suggests that BECs from COPD subjects died at a higher rate than healthy BECs. This may contribute to a trend towards lower viral load associated with COPD rather than high viral load. This observation also suggests that cell death pathways other than apoptosis are relevant in COPD and may contribute to pathogenesis.

Pyroptosis is an inflammatory cell death pathway induced by the inflammasome and known to be detrimental in asthma (136, 158, 159). This pathway was briefly investigated by quantifying activated caspase-1, which is a product of inflammasome activation (138, 160). In turn active caspase-1 stimulates the production of several inflammatory cytokines leading to pro-inflammatory programmed cell death, pyroptosis (136). In this study caspase-1 activation was poorly induced in both healthy and COPD BECs infected with the four virus isolates. No elevation from COPD BECs was observed suggesting no association based on disease state and no association with hMPV and RSV infection of BECs. However, pyroptosis will be further investigated by quantifying IL-1β production.

Cell death was not investigated using the ALI culture model in this study. However reduced RSV and hMPV shedding occurred earlier for COPD cultures than healthy cultures due to more rapid cell death as observed microscopically. Studies of cell death in ALI cultures are limited, particularly in response to viral infection. One study did investigate ALI cultured BECs infected with influenza and demonstrated elevated cell death and apoptosis in response to infection (157), although this was not in the context of COPD. It has been hypothesised that the inflammasome plays an important part in the pathogenesis of COPD in AECs; one study demonstrated an overexpression and release of IL-1β in the sputum and lung tissue of cigarette smoke-induced exacerbated COPD patients (161). Another study demonstrated a significant elevation in ASC specks in BAL fluid of mice with smoke-induced COPD compared to mice exposed to air (162). There is however a study that demonstrated no correlation between the NLRP3 inflammasome, caspase-1 and IL-1β and the progression of COPD (163). These studies did not use viruses

to investigate inflammasome induction in COPD but rather cigarette smoke. Data concerning viral responses is lacking.

This masters study did not extend to the investigation of different programmed cell death pathways in ALI cultures, as the main aim was to establish infection and IFN kinetics over an extended time in order to establish the peak of response post-infection in this model. Further investigations should be performed using the ALI model to look at cell death at critical times post-infection.

Another form of programmed inflammatory cell death that warrants investigation is necroptosis. Although there are no studies directly investigating necroptosis in the pathogenesis of COPD one study suggests that mitophagy-dependent necroptosis may play a role in cigarette-smoke exacerbations via elevated expression of PINK1, a key mitophagy regulator, and RIP1/3 kinase, as these were quantified in human COPD lung tissue (164). This needs to be further investigated by looking at the activation of the key markers of necroptosis, MLKL and RIP1/3 kinase in our cultures (165-167). It may be that this key inflammatory death pathway, which is critical in the pathogenesis of asthma is significant in the aetiology of virus-induced exacerbations of COPD.

7.2.4 Virus-Dependent Cellular Response

Some cellular responses to infection were dependent on the infecting virus, not disease status.

One of the aims of this study was to identify if there was consistency in cellular response amongst isolates of the same virus and also amongst members of the paramyxovirus family. It is important for clinical translation to establish if cellular responses associated with disease are consistent for different viral infection and therefore intrinsic to disease status.

The results of this study indicate that the antiviral response of BECs is dependent on the infecting virus, as susceptibility to infection, IFN response, and cell death differed in response to RSV and hMPV within both COPD and healthy groups in submerged cultures. We have observed this previously using NECs from children with wheeze and atopy, in that wheeze and atopy was associated with reduced IFN production in response to RSV but not hMPV (110). The importance of the isolate or strain to the cellular response of

primary BECS in submerged culture has also been demonstrated using an array of different RV isolates (168).

Viral differences were most evident in the present study using the ALI culture model; in that hMPV peak shedding was within the first 1-2 days, while RSV peak shedding was much later between days 18 and 24 for A2, and 7 and 9 for RS4. This is the first report of viral shedding and IFN production in the ALI model using hMPV as the infecting virus and the first published comparison between RSV and hMPV. However, Bartlett *et. al.* also demonstrated that RV shedding from healthy adult BECs in ALI culture peaked within the first 1-2 days post-infection (Bartlett N 2017, oral communication, November 3rd). Why RSV and hMPV would demonstrate such differences in shedding remains to be established. To support and confirm the shed virus data, viral transcription over the 27 days post-infection will be quantified in a follow-up study. This would identify the rate of infection and replication within the population over time. This is especially important for the hMPV infection as very little infectious virus was identified in any of the cultures beyond day 5 p.i.

The most variation in cellular response within either the RSV or hMPV isolates was observed between A2 and RS4. RS4 consistently replicated less well and induced significantly less of an IFN response in submerged cultures. Interestingly RS4 replicated and was shed earlier in ALI cultures than A2. This may be related to the clinical source and low passage *ex-vivo* of RS4 compared to the commercial source and high (unknown) passage number of the A2 isolate. Full-genome sequencing is required and may identify potential differences in protein sequence that relate to function. In particular the ALI cultures may express particular cell surface receptors favourable to attachment and fusion of a clinical isolate compared to a high passage laboratory isolate (A2) that may be lacking in submerged cultures.

One of the aims of this study was to map the kinetics of RSV and hMPV infection over time to inform further studies using the ALI model. In response to hMPV infection occurred early, indicating that sampling and investigation of BECs during the first 5 days will provide the maximum useful information. RSV infection occurred later and was not cleared as rapidly, therefore sampling and experimenting across the entire 27 day time-course performed in this study will be essential to provide accurate information.

7.3 Limitations

A limitation of this study was the use of commercial BECs from donors. This limited the number of subjects included in the study due to the cost of these primary cells. During exacerbations of COPD the inflammatory pathophysiology is within the lower respiratory tract and BECS are a relevant cell to study in this aspect (5, 34). However, previous studies in our lab using clinically-sourced nasal epithelial cells (NECs) instead of BECs suggest that these are an excellent model for the investigation of antiviral responses, as the nasal lining is the first point of infection of respiratory viruses, NECs are easy to collect and hence larger samples sizes are possible. It will be important to compare the use of NECs as a surrogate for BECs in future COPD studies. The timeline of the project was a limiting factor in gaining ethics to recruit patients and collect samples from a clinical cohort, so the use of NECs was not an option for this project.

Another limitation of the study was the number of donors included in the ALI cultures. Although for the first two models n=5 for both healthy and COPD groups, only n=4 was included in the ALI model. This was due to a lack of time to culture, infect and sample the final 2 donors within the time-frame of the project.

The inclusion of COPD non-smoker and healthy smoker groups would have been useful to confirm that findings are truly based on disease state rather than representing a biological effect of smoking. One reason for the exclusion of this in this masters was the lack of COPD non-smoker donors available, but it would be beneficial to expand the study to these groups in future research.

Future studies will focus on the cell death response to viral infection in COPD and importantly use clinically sourced AECs to identify if there truly is no elevated susceptibility to infection associated with COPD. We will also investigate further and confirm the elevated IFN-β response in association with COPD and the potential clinical implications of this.

9.0 Appendices

Table 11: General Reagents

Name	Composition	Supplier
1 X Phosphate Buffered Saline (PBS)	137mM sodium chloride, 2.7mM potassium chloride, 10mM phosphate buffer	Amresco

Table 12: Virus Methodologies

Name	Composition	Supplier
OptiMEM™ (O'MEM)	See manufacturers datasheet	Gibco - Life Technologies
Foetal Bovine Serum (FBS)		Gibco - Life Technologies
Penicillin & Streptomycin (P-S)	5,000 U/ml (100X)	Gibco - Life Technologies
Trypsin/EDTA 0.25%	Phenol Red	Gibco - Life Technologies
RSV Primary Ab	Goat anti-RSV	Virostat
RSV Secondary Ab	Rabbit anti-goat	Life Technologies
hMPV Primary Ab	Mouse anti-hMPV	Merck-Millipore
hMPV Secondary Ab	Rabbit anti-mouse	Invitrogen
Peroxidase Solution		Sigma

Table 13: Submerged Primary Cell Culture

Name	Composition	Supplier
Bronchial Epithelial Growth Medium (BEBM)	See manufacturer's datasheet	Lonza
Bronchial Epithelial Growth Medium (BEGM) single quots	Bovine Pit. Extract, 0.4%	Lonza
	Insulin, 0.1%	
	Hydrocortisone, 0.1%	
	Gentamycin, 0.1%	
	Retinoic Acid, 0.1%	
	Epinephrine, 0.1%	
	Transferrin, 0.1%	
	Triiodothyronine, 0.1%	
	Human Epidermal Growth, 0.1%	
Steroid-free complete BEGM	BEBM + single quot additives (excluding hydrocortisone)	Lonza
Complete BEGM	BEBM + single quot additives (including hydrocortisone)	Lonza
Trypsin (Infect)	2.5%	Gibco – Life Technologies
Trypsin/EDTA		Lonza
Trypsin Neutralising Solution (TNS)		Lonza
Hepes Buffered Saline Solution (HBSS)		Lonza
Trypan Blue 0.4%		Sigma

Table 14: Cell Death Assays

Name	Composition	Supplier
CytoTox-ONE Homogenous Membrane Integrity Assay	CytoTox-ONE Reagent, Stop Solution	Promega
Caspase-1	Caspase-Glo 1 Reagent, MG-132 Inhibitor	Promega
Caspase-3/7	Caspase-Glo 3/7	Promega
Caspase-8	Caspase-Glo 8 Reagent, MG-132 Inhibitor	Promega
Caspase-9	Caspase-Glo 9 Reagent, MG-132 Inhibitor	Promega

Table 15: ALI Primary Cell Culture

Name	Composition	Supplier
Bronchial-Air Liquid Interface (B-ALI) Growth Media	See manufacturer's datasheet	Lonza
B-ALI Differentiation Media	See manufacturer's datasheet	Lonza
B-ALI Differentiation Media – Single Quots	Bovine Pit. Extract, 0.4%	Lonza
	Insulin, 0.1%	
	Hydrocortisone, 0.1%	
	Gentamycin, 0.1%	
	Retinoic Acid, 0.1%	
	Epinephrine, 0.1%	
	Transferrin, 0.1%	
	Triiodothyronine, 0.1%	
	Human Epidermal Growth, 0.1%	
	Inducer	
Steroid-free B-ALI	BEBM + single quot additives (excluding hydrocortisone, including inducer)	Lonza

Table 16: Cytokine Assays

Name	Composition	Supplier
AlphaLISA (IFN-β)	AlphaLISA Anti-IFN-β Acceptor beads (5mg/ml), Streptavidin (SA)-coated Donor beads (5mg/ml), Biotinylated Antibody Anti-IFN-β (500 nM), AlphaLISA human IFN-β (0.3µg), 10X AlphaLISA Immunoassay Buffer	Perkin Elmer
ELISA (IFN-λ)	Capture Antibody, Detection Antibody, Standard, Coating Buffer Powder, 5X Assay Diluent, Detection enzyme (Avidin-HRP), Tetramethylbenzidine (TMB) substrate, Stop Solution (H_2SO_4)	Affymetrix Ebioscience

Table 17: RT-qPCR

Name	Composition	Supplier
TRIzol		Life Technologies
cDNA High Capacity Kit	10x RT Buffer, 10x RT Random Primers, 25x dNTP (100mM), Multiscribe™ Reverse Transcriptase, RNase Inhibitor	Applied Biosystems
cDNA SuperScript® III Kit	2X Reaction Mix, SuperScript® III Enzyme Mix, Oligo(dT)$_{20}$ 50 µM), Random hexamers (50 ng/µl), Annealing buffer	Life Technologies
Rotor-gene® SYBR®		Qiagen
Ampicillin		Sigma
Luria broth		Sigma
E.coli		Life Technologies
ApaL1	10,000 units/ml	New England BioLabs Inc.
Xbal	20,000 units/ml	New England BioLabs Inc.
EcoRV	20,000 units/ml	New England BioLabs Inc.
Buffer 2	50nM NaCl, 10mM Tris-HCL, 10mM $MgCl_2$, 1mM DTT, pH 7.9	New England BioLabs Inc.
Buffer 3	100nM NaCl, 50mM Tris-HCL, 10mM $MgCl_2$, 1mM DTT, pH 7.9	New England BioLabs Inc.
Buffer 4	50mM Potassium Acetate, 20mM Tris-acetate, 10mM Magnesium Acetate, 1mM DTT, pH 7.9	New England BioLabs Inc.
BSA		Sigma
Loading Dye (6x)	2.5% Ficoll®-400, 11mM EDTA, 3.3mM Tris-HCL, 0.017% SDS, 0.015% bromophenol blue, pH 8.0 at 25°C	New England BioLabs Inc.
DNA Ladder	500bp to 10,000bp	New England BioLabs Inc.
Tris Acetate EDTA (TAE) (1X)	40mM Tris (pH 7.6), 20mM acetic acid, 1mM EDTA	

10.0 References

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