Oxidative Stress Impairs Mitochondrial Function in Healthy and Asthmatic Primary Bronchial Epithelial Cells

Faezeh Fathi Aghdam
MSc

A thesis submitted for the degree of Doctor of Philosophy

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School of Medicine and Public Health
Faculty of Health
University of Newcastle, Australia
**Statement of originality**

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List of abbreviations:

AEC: Airway epithelial cell
AHR: airway hyper-responsiveness
APCs: antigen presenting cells
Apaf-1: apoptotic protease activating factor 1
ATCC: American type culture collection
ATP: adenosine triphosphate
BAL: bronchoalveolar lavage
BAK: BCL-2 antagonist killer
BAX: BCL-2 associated x protein
BCL-2: B-cell lymphoma
BEGM: Bronchial Epithelium Growth Medium
BHT: butylated hydroxytoluene
BSA: Bovin serum albumin
CAC: carnitine/acylcarnitine carrier
CARD: caspase recruitment domain
Cardiff: card adaptor inducing IFN-β
CAT: catalase
CBP: CREB binding protein
COPD: chronic obstructive pulmonary disease
CPT2: carnitine-palmitoyl-transferase 2
CS: Cigarette smoke
CSE: Cigarette smoke extract
DCs: dendritic cells
DMEM: Dulbecco’s Modified Eagle Medium
DMSO: dimethylsulfoxide
DNA: Deoxyribonucleic acid
DTT: Dithiothreitol
ECP: eosinophil cationic protein
EDTA: Ethylenediaminetetraacetic acid
ELISA: enzyme linked immunosorbent assay
EPO: Eosinophile peroxidise
ETC: electron transport chain
FADD: fas-associated protein with death domain
FCS/MEM: foetal calf serum/minimum essential medium
FEV: forced expiratory volumes
FVC: forced vital capacity
GCL: glutamate cysteine ligase
GM-CSF: granulocyte-monocyte colony stimulation factor
GSH: glutathione
GSHPx: glutathione peroxidase
H₂O₂: Hydrogen peroxide
HAV: hepatitis A
HCV: hepatitis C
HDL: high density lipoprotein
HSP: heat shock protein
ICAM-1: intercellular adhesion molecule-1
IFIH1: interferon-induced helicase C domain 1
IFN-γ: interferon-gamma
IL: Interleukin
IL-6: interleukin-6
IL-8: interleukin-8
IP-10: interferon-gamma inducible protein-10
IPS-1: beta promoter stimulator1
IRES: internal ribosomal entry site
IRF3: interferon response factor 3
LDH: lactate dehydrogenase
LDL: low density lipoprotein
MAVS: mitochondrial antiviral signalling protein
MDA5: melanoma differentiation associated gene 5
MOMP: mitochondrial outer membrane permeabilization
MPO: myeloperoxidase
MPT: Mitochondrial permeability transition
mtTFA: mitochondrial transcription factor A
mtTB1: mitochondrial transcription factor B1
mtTB2: mitochondrial transcription factor B2
NF-κB: nuclear factor-κB
NHS: national health survey
NO: nitric oxide
NS: Non-structural
pBECs: Primary bronchial epithelial cells
PCR: Polymerase chain reaction
PGDF: platelet-derived growth factor
PRR: pathogen recognition receptor
RANTES: regulated on activation normal T cell expressed and secreted
RIG-I: retinoic acid-inducible gene
RIP1: receptor interacting protein1
RNS: reactive nitrogen species
ROS: reactive oxygen species
RSV: respiratory syncytial virus
RT-PCR: reverse transcription polymerase chain reaction
RV: rhinovirus
SEM: standard error of mean
SOD: superoxide dismutase
TBK1: tank binding kinase1
TCID50: tissue culture infective dose 50%
TGF-β: Transforming growth factor-β
TH1: T helper 1
TH2: T helper 2
THF: tetrahydrofuran
TLR: toll-like receptor
TNF-α: tumour necrosis factor-α
TRAF: TNF receptor associated factor
UV: Ultraviolet
VEGF: vascular endothelial growth factor
VISA: virus-induced signalling adaptor
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Publications arising from this thesis:

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2. The effects of oxidative stress on mitochondrial function and inflammation can be reversed using lycopene and carnitine (Manuscript in preparation).
Synopsis

Asthma is associated with increased reactive oxidant species (ROS) in the airways which can lead to oxidative stress. A combination of several factors, such as viral infection, exposure to tobacco smoke or allergens may contribute to asthma exacerbations in the absence of effective type I and type III IFN responses. Rhinovirus infections (RVs) are associated with the majority of acute asthma exacerbations in children and adults. Mitochondria play a significant role in antiviral defence, via induction of innate immune responses through melanoma differentiation-associated gene 5 (MDA5) and mitochondrial antiviral signalling protein (MAVS) which is important for the downstream activation of type I and type III IFNs in epithelial cells. Our aims were to compare mitochondrial function and antiviral responses to RV infection in pBECs in asthmatics and healthy controls (HC) and to see whether the oxidative damage to the mitochondria can be reversed using lycopene and carnitine.

Exposure of pBECs to CSE/H₂O₂ resulted in mitochondrial damage; with impaired mitochondrial membrane integrity and the co-localisation of MAVS, MDA5 and mitochondria, increased expression of mTFs, release of cytochrome-c and ATP from mitochondria which was greater in asthmatic pBECs. RV+CSE/H₂O₂ further increased mTFs and ATP release which again was exaggerated in asthmatics compared to HC. CSE and H₂O₂ had no effect on MAVS cleavage or the protein expression of MDA5, pIRF3, TBK1 and IKKε in both groups. Asthmatics demonstrated an impaired IFN response with, reduced CXCL-10 and IFN- λ and an increase in CXCL-8 and IL-6 release compared to HC. Also, CSE and H₂O₂ led to increased RV replication which was greater in asthmatics compared to HC. Lycopene and carnitine restored mitochondrial membrane integrity and the co-localisation between MAVS, MDA5 and mitochondria. They decreased inflammation by a marked reduction in the release of CXCL-8 and IL-6. In addition, they reduced RV replication, but did not restore CXCL-10 and IFN- λ responses.
Chapter 1

Introduction
1.1 Asthma

1.1.1 Background

Asthma was first recognized in ancient Egypt and its term is derived from a Greek word which means short-drawn breath [1]. The Global Initiative for Asthma (GINA) defines asthma as a chronic inflammatory disorder of the airways in which many inflammatory cells and cellular components play a role. Asthma results in airway constriction, inflammation and bronchial hyper-responsiveness and remains a potential threat to life in adults and children [2]. Asthma exacerbations, which involve a deterioration of respiratory symptoms, are an important contributor to asthma mortality, medication usage and hospitalizations [3]. Asthma exacerbations can be triggered by cold dry air, exercise or common cold viruses [4]. It was estimated, in 2009 that 300 million people in the world suffer from asthma, which caused 250,000 deaths; this is expected to increase to 400 million affected in the next 20 years.

1.1.2 Asthma demographics:

1.1.2.1 Age and gender:

In Australia, the National Health Survey (NHS, 2011-12) reported that 10.2% of Australians (or around 2.3 million people) had asthma. As a whole, males and females had similar rates of asthma (9.5% of males and 10.9% of females), however, rates of asthma show a different pattern among age groups. Male children aged 0-14 years, had a higher rate of asthma (11.4%) than did females (7.2%). However, from 15 years, asthma was more common in women than men [5, 6].
1.1.2.2 Geographical Location:

The prevalence of asthma is increasing worldwide [7, 8]. Geographically, asthma prevalence is higher in western countries [9]. It has been also demonstrated that English-speaking countries have the highest rate of asthma as well as highest prevalence of “atopy” or allergic asthma [9, 10]. In Australia, Tasmania has the highest and NSW has the lowest prevalence of asthma [6, 11].

1.1.2.3 Family size and asthma:

It has been also shown that family size or birth order is important in the risk of asthma development in childhood. Reports have shown that children with no or few siblings are more susceptible to the risk of asthma [12, 13]. In addition, children who were born from asthmatic or allergic parents are more susceptible to the risk of asthma than the children of healthy parents [5] and in twins, genetics is of importance in the prevalence of asthma [14].

1.1.3 Aetiology of asthma:

Genetics and environmental triggers play a significant role in the prevalence of asthma [15]. The function of most of these genes, including ADAM33, PHF11, DPP10, GRPA and SPINK5, are not clear, but many of them have significant roles in the immune response and are involved in inflammation [16]. For example the ADAM33 gene, which is located on chromosome 20 and is expressed specifically in lung and muscle cells, has a significant relationship to asthma [17, 18]. Moreover, a recent report revealed that a single nucleotide polymorphism in genetic loci on chromosome 17 may be related to asthma which is especially seen in children [19]. Studies have shown that the interaction of environmental exposures and susceptibility genes is important in the development of asthma [15].
Environmental factors such as cigarette smoke, allergen exposure, pollen, animal skin, air pollution, chemical sprays, high ozone levels, reactive oxygen species [20], antibiotic usage in the first years of life [21], stress [22] and respiratory infections such as Rhinovirus, Chlamydia pneumoniae and Bordetella pertussis are involved in asthma exacerbation [23, 24]. These stimuli trigger the innate and acquired immune pathways, leading to the recruitment and activation of inflammatory cells such as neutrophils and eosinophils respectively [25, 26].

### 1.1.4 Eosinophilic and neutrophilic asthma:

Eosinophilic asthma, is associated with allergies, an elevated level of serum IgE, eosinophils in the airways, as well as increased eosinophils in the blood [27]. On the other hand, non-eosinophilic asthma, which is more frequently observed in severe asthma, involves an increased level of neutrophils in the airways [25]. Neutrophils, which are polymorphonuclear leukocytes, play an important role for defence against external triggers such as bacterial infections [28]. Research has identified that they can release mediators including proteases, such as myeloperoxidase (MPO), collagenases, and elastases, and cytokines which can recruit and accumulate more neutrophils in the airways, such as TNF-α and CXCL-8 [28-30]. Studies suggest that asthma can also occur in the absence of airway inflammatory cells such as eosinophils, neutrophils, or lymphocytes. In some cases, patients consume high doses of corticosteroids but they still show symptoms. This suggests that other cells such as mast cells, epithelial cells, or smooth muscle cells may lead to their symptoms [31]. Research has also shown that patients with non-eosinophilic asthma are more resistant to corticosteroids than patients with eosinophilic asthma [25, 32].
1.1.5 Evaluation of asthma:

1.1.5.1 Clinical diagnostic of asthma:

There are several tests which can help to clinically diagnose asthma, which one of which is spirometry.

1.1.5.1.1 Spirometry:

Spirometry is a simple physiological test that measures inhaled or exhaled volumes of air over time. It measures the forced vital capacity (FVC) and FEV₁. The FVC is the volume of air which is forcefully exhaled from the lungs during an expiration, while the forced expiratory volume in one second (FEV₁) is the volume delivered in the first second of expiration [33]. What is airflow obstruction? How is this defined? Airflow obstruction which, is the blockage of respiration in the upper or lower respiratory airways, is measured as the FEV₁/FVC ratio. In obstructive lung diseases such as asthma, the FEV₁ may be reduced due to airflow obstruction, thus FEV₁/FVC ratio will be reduced. According to the GINA guidelines, FEV₁/FVC ratio is normally >0.75-0.80 in adults and lower values suggest airflow obstruction [34].

1.1.6 Treatment of asthma:

Despite various therapeutic strategies, a cure for asthma is not available [35]. Thus, prevention and management of symptoms is very important [36]. Asthma drugs are divided into two groups: bronchodilators such as β- adrenergic agonists and theophyllines, which can dilate smooth muscle of the bronchial airways and secondly, Prophylactic or anti-inflammatory agents such as corticosteroids and sodium cromoglycate, which can inhibit the release of mediators from inflammatory cells such as mast cells [37].
1.2 Oxidative stress:

Increased oxidative stress characterises asthma exacerbations. Endogenous or exogenous sources can generate an excessive amount of reactive oxygen species (ROS). ROS, such as the superoxide (O$_2^-$) and hydroxyl (OH$^-$) ions and reactive nitrogen species (RNS), such as the nitric oxide radical (NO$^·$), peroxinitrite (ONOO$^-$) and the nitrogen dioxide radical (NO$_2^·$), are highly reactive molecules which have unpaired electrons and tend to interact with other molecules in different tissues to gain a stable configuration of electrons. ROS are produced in eukaryotic organisms through the respiration processes in the mitochondria. They have a significant role in cell signalling and homeostasis, but several environmental triggers like UV light, air pollution, ozone or infection can increase the ROS level dramatically and lead to oxidative stress. Oxidative stress can damage proteins, lipids, nucleic acids and carbohydrates [38]. They can cause DNA damage by oxidation, methylation, depurination, deamination, point mutation or strand breaks [39]. Their attack on proteins includes damage to DNA polymerases or DNA repair enzymes [39] Furthermore, they can interact with fatty acids in cellular membranes, including the mitochondrial membrane. This can cause mitochondrial damage and impaired mitochondrial function. All organisms have cellular defences against reactive oxygen species which are known as antioxidants. However, an imbalance between reactive oxygen species and antioxidants leads to oxidative stress which characterises many respiratory diseases such as asthma [26, 40].

1.2.1 Oxidative stress and asthma:

Oxidative stress is considered to be involved in the development of asthma and chronic obstructive pulmonary disease (COPD) [41]. A combination of several factors, such as viral
infection, exposure to allergens or increased oxidative stress may contribute to asthma exacerbations in the absence of an effective immune response [42].

Activation of airway inflammatory cells can cause oxidative stress via endogenous production of ROS. The most potent generators of these oxidants are eosinophils and neutrophils which are the most abundant cells in asthma and COPD [41, 43].

ROS, such as hydrogen peroxide (H$_2$O$_2$) which is produced from superoxide, can cause cell injury in asthmatics by the enzymatic function of peroxidases including eosinophil peroxidase (EPO) and myeloperoxidase (MPO). MPO can be found abundantly in neutrophils and during the respiratory burst forms hypochlorous acid (HOCl) from the reaction of H$_2$O$_2$ with chloride. EPO is present in eosinophils and catalyses the reaction of H$_2$O$_2$ with bromide forming hypobromous acid (HOBr) [41]. Furthermore, H$_2$O$_2$ is released from various inflammatory cells such as eosinophils, neutrophils and macrophages and can affect airway smooth muscle and increase mucin secretions. This can narrow respiratory airways and make breathing difficult [44].

Studies have revealed that the release of superoxide from airway macrophages is also increased in asthmatics compared to controls [45]. Various cytokines, such as tumour necrosis factor alpha (TNF$\alpha$), fibroblast growth factor-2, angiotensin II and thrombin may elevate ROS level by activating oxidases and ultimately lead to inflammation in the lung [46]. Thus, airway and intravascular inflammatory cells can both lead to increased oxidative stress in asthma and COPD [44].

Nitric oxide (NO) is another significant target of ROS in asthma. Research has described a dysregulation of NO in asthma, with an increased level of exhaled NO in asthmatics, that is suppressed by corticosteroid therapy [47]. Nitric oxide is a free radical produced by nitric oxide synthase (NOS) activity. There are three kinds of NOS in the lung. NOS I /nNOS (neuronal NOS); NOS II / iNOS (the inducible isoform) and NOS III/eNOS (endothelial
NOS). nNOS generates nitric oxide in nervous tissue and skeletal muscle and is involved in cell communication [48].

eNOS, which is found in the endothelium, is involved in blood vessel production and regulates vascular function [49]. iNOS, which is found on many inflammatory cells, plays a significant role in signalling pathways in bronchial smooth muscle and is only expressed during inflammation [50]. It is responsible for the elevated level of exhaled NO in asthma [51]. The level of iNOS may increase in an oxidative environment, and then may react with superoxide and generate peroxynitrite, which leads to cell toxicity. It has been shown that the elevated level of NO which reacts with oxidants may reduce the available NO, in sites where there is a need for NO mediated signalling, especially in respiratory smooth muscle and the vasculature resulting in the loss of bronchorelaxation [52].

While endogenous ROS sources play an important role in asthma exacerbations, exogenous factors such as air pollution, ozone, viral infection or cigarette smoke (CS) can further increase the burden of ROS/RNS [53]. There are more than 4,000 chemicals in cigarette smoke, such as nitrosamines, carbon monoxide and metal oxides, thus one puff of cigarette smoke can produce billions of ROS, such as superoxide and hydroxyl radicals [54]. These free radicals react with antioxidants and they are able to damage airway epithelial cell membranes but they are not able to enter the cells. On the other hand, phenolic compounds, aldehydes, and polycyclic aromatic hydrocarbons, which are the lipophilic components of CS can diffuse through the plasma membranes and enter the cells [55]. Once they enter the cells, they cause mitochondrial dysfunction by disturbance of the electron transport chain (ETC) and leakage of free electrons from the ETC. These single electrons react with oxygen and produce superoxide which is known as a potent free radical. They can also activate the NF-κB pathway and lead to the activation of various pro inflammatory genes such as IL-1, IL-6, CXCL-8 and TNF-α [56].
Cigarette smoke may cause alveolar cell apoptosis by detrimental effects on pneumocytes or by affecting the epithelial cells and reducing vascular endothelial growth factor (VEGF) production [57]. They can also stimulate alveolar macrophages to further release ROS in the airways attracting neutrophils and other inflammatory cells into the lungs of asthma and COPD patients [41]. CSE can have detrimental effects on the lungs by activation of proteases, mucus secretion, airway smooth muscle contraction, airway hyperresponsiveness, and transcription of multiple inflammatory genes [58-61]. It has been shown that macrophages release more H₂O₂ in healthy smokers than non-smokers. In terms of asthma, it has been shown that smoking asthmatic patients have higher concentrations of exhaled H₂O₂ than non-smoking asthmatic patients while acute exposure to cigarette smoke can release more ROS in the airways and lead to a further elevation in exhaled H₂O₂ concentration in asthmatic patients [41].

Once ROS are generated, they attack membrane lipids and cause lipid peroxidation. Oxidative damage to lipids leads to the production of isoprostanes, malondialdehyde, and 4-hydroxy-2-nonenal (4-HNE)[41]. These markers have been widely used in the assessment of oxidative stress [41, 60, 62]. However, among these products 8-isoprostane is the most reliable marker and the best well-known isomer which reflects oxidative stress within cells [41, 63].
1.3 Inflammation

1.3.1 Human immune system:

The immune system encompasses both innate and adaptive responses [64, 65]. Innate immune responses are triggered by pathogens which successfully enter the host cells. These responses are non-specific and don’t produce long-lasting immunity against pathogens. Innate immune defences include surface barriers such as skin, mucous membranes and phagocytic cells [66]. The epithelium has an important role in encountering environmental exposures such as allergens, virus infection or pollution. It reacts with irritants and acts as a barrier against environmental triggers [67, 68]. The epithelium is not only a barrier against environmental triggers, but can also produce a range of mediators that lead to inflammatory and remodelling responses in the lungs of asthmatics [69]. Studies have described a defective response of the epithelium to environmental stimuli. This prevents normal cellular repair responses, thus, epithelial damage is a significant feature of asthma [69]. Epidermal growth factor (EGF) which is involved in epithelial cell proliferation is a significant factor in bronchial epithelial repair. Studies have mentioned to an increased expression of EGF receptors in asthma, however, no effective repair response in epithelium can be observed in asthma [69]. In this case, the increased expression of adhesion molecule CD44 can present the EGF more efficiently to its receptor; furthermore, chemokines and growth factors which are recruited to the damaged epithelium may regulate the repair response [70]. There are also several other growth factors like transforming growth factors TGF-β which are released from the epithelium and inflammatory cells and can play a key role in repair response. The TGF-β family are mostly involved in collagen production [71]. The epithelium also is able to produce a large number of other pro-inflammatory proteins which most of these cytokines are anti-apoptotic cells and they can protect eosinophils from programmed cell death, for example, granulocyte macrophage colony-stimulating factor (GM-CSF) is the main cytokine
which is mostly generated in the bronchial epithelium and can prolong the survival of eosinophils in the asthmatic airways [72]. It has been reported that although the main sources of GM-CSFs are bronchial epithelium cells but they can be also generated from macrophages, T cells, mast cells and myofibroblasts [72, 73]. In addition to airway epithelial damage, there is a remodelling response in asthma which may be due to abnormal interaction of myofibroblasts and the epithelium, which both play an important role in early lung development [74]. One hypothesis states that these changes occur in early life before the clinical symptoms can be observed and the symptoms may be visible when the epithelium is unable to reconstitute itself, in response to stimuli such as allergens, air pollution or viruses [75].

On the other hand, the adaptive immune response produces a stronger immune response as well as immunological memory. Antigen recognition by adaptive immune responses leads to the activation of a cascade of events and activation of cells including lymphocytes which protect host cell against the foreign invaders. Memory cells are involved in adaptive immune responses, which detect and remember specific antigens, thus when a pathogen invades a cell more than once, memory cells can eliminate it very quickly. Lymphocytes are divided into B-cells and T-cells. B-cells are involved in humoral immune responses but T-cells are involved in cell mediated immune responses [64, 66, 76].

In response to stimuli such as allergens, air pollution or infectious agents, T cells differentiate into effector cells, which are T helper 1 (Th1) or T helper 2 (Th2) type. Activated Th2 cells provoke immune responses against extracellular infections [77]. Th1 cells release interferon-gamma (IFN-γ) and TNF-β, while Th2 cells, which have critical roles in airway inflammation, produce CXCL4, CXCL5, IL6, CXCL9, CXCL10, CXCL13 and granulocyte macrophage-colony stimulating factor (GM-CSF). GM-CSF has an important role in
stimulating the growth of eosinophils and also activation of antigen presenting cells (APCs) [78].

1.3.2 Inflammatory responses:

Inflammatory responses are triggered by infectious agents or tissue damage leading to the release of inflammatory mediators. Cytokines are extracellular signalling proteins which are involved in cell-to-cell communication. They lead to a wide variety of functions including growth and cell differentiation, chemotaxis and release of other cytokines or mediators [73]. Some of the main inflammatory mediators are IL-6, CXCL-8, CXCL-10 and nuclear factor κB (NF-κB).

1.3.2.1 IL-6:

IL-6 is an interleukin which acts as a pro-inflammatory and anti-inflammatory cytokine [79]. It is secreted by various cell types such as T-cells, macrophages, fibroblasts and endothelial cells [80]. IL-6 is produced in response to viral or bacterial infection or tissue injury and participates in host defence. IL-6 secretion is stimulated by TNF-α, interleukin-1(IL-1) and platelet-derived growth factor (PDGF), also it can stimulate its own synthesis [81]. Constitutive production of IL-6 may have detrimental effects on the host cells. Thus dysregulation of IL-6 may contribute to many diseases such as rheumatoid arthritis and myeloma [81].

1.3.2.2 CXCL-8:

CXCL-8 is known as a potent neutrophil chemotactic factor which is important for neutrophil accumulation at the site of inflammation. Neutrophil accumulation in the lung may lead to acute inflammation in asthmatics [82, 83]. CXCL-8 is considered as a “second-order”
mediator which is less potent than IL-6 and it is secreted by monocytes, neutrophils, fibroblast and epithelial cells in response to cytokines such as IL-1, IL-3, TNF-α and IFN-γ and it is more specialized in causing inflammation and repair [80].

1.3.2.3 Interferon-gamma induced protein-10 (IP-10/CXCL-10):

CXCL-10 is a chemokine which is released by a wide variety of cells, particularly epithelial cells [417] in response to IFN-γ [394]. CXCL-10 is a selective chemoattractant of natural killer cells, activated T cells [418] and monocytes [394]. The expression of CXCL-10 is associated with lymphocyte numbers [419]. CXCL-10 expression is reportedly high in the airway of asthmatics [420] and people suffering from COPD [418]. In vitro experiments show that rhinovirus (RV) infection can increase CXCL-10 expression and this is associated with viral titre [376]. However, viral-replication-independent release of CXCL-10 has also been reported previously [421].

1.3.2.4 NF-κB:

NF-κB is a nuclear protein transcription factor which was discovered in 1986 [84]. NF-κB is a heterodimeric transcription factor that regulates many genes associated with inflammation and cancer. This heterodimeric complex which is formed from the Rel family of proteins is composed of p65 and p50 subunits [85, 86]. NF-κB is activated by different stimuli such as TNF-α, IL-1, lipopolysaccharide (LPS), viruses, UV light, and oxidative stress. Under normal conditions, NF-κB is found in the cytoplasm in an inactive form associated with inhibitor IκBα proteins. This association prevents its nuclear accumulation and transcription of inflammatory genes. A wide variety of stimuli lead to the activation of IκB kinases and phosphorylation of IκBα. 26S proteasome degrades phosphorylated IκBα, thereby allowing NF-κB to translocate into the nucleus. In the nucleus NF-κB attaches to DNA in the promoter
regions of target genes [85, 86]. NF-κB is required for the transcription of pro-inflammatory mediators such as enzymes, cytokines, adhesion molecules and chemokines including CXCL8 and IL6 [87-91]. Researchers have revealed that NF-κB has a major role in asthma pathogenesis. Activated NF-κB has been detected in the airways of asthmatics and it has been shown that exposure to environmental factors like allergens, ozone, or viral infections, results in NF-κB activation and will exacerbate asthma; also, corticosteroids, which are the mainstay of asthma pharmacotherapy, block NF-κB transcription and inhibit its activation [90]. CXCL10 is an extracellular inhibitor of NF-κB [92].

1.3.2.5 Tumour necrosis factor-α (TNF-α):

TNF-α is a cytokine that is involved in inflammation and acute phase reactions. It can be produced by many cell types such as macrophages, lymphocytes, mast cells and endothelial cells [80]. It plays a significant role in inflammatory activities. TNF-α mRNA expression is dependent on other cytokines such as IL-6 and IL-1 and leads to the infiltration of inflammatory cells to the site of infection [93].

1.3.3 Asthma and Inflammation:

Airway inflammation is important in the pathology of asthma and exacerbations of asthma [94, 95]. It is widely believed that infiltration of Th2 cells, eosinophils and mast cells into the airways will cause inflammation and lead to the development of allergic asthma [77]. In atopic asthma, which is triggered by allergies, IL-4 and IL-13 have significant roles in the expression of cell adhesion molecules on the endothelium [96]. Furthermore, chemokines recruit inflammatory cells and stimulate the production of IgE by B cells, which will cause bronchial hyper-reactivity [97].
It has been shown that the epithelium is not only a barrier against environmental triggers, but can also produce a range of mediators that lead to inflammatory and remodelling responses in the lungs of asthmatics [69]. Cytokines which are produced by Th2 cells and inflammatory cells have detrimental effects on the airway epithelium, subepithelial (myo-)fibroblasts and smooth muscle cells in the lung, which can cause severe structural abnormalities [77]. Studies have shown that inflammatory cells such as eosinophils, neutrophils, mast cells, T lymphocytes and macrophages are elevated in the bronchoalveolar lavage (BAL) of the airways of asthmatics [95, 98]. These cells can produce inflammatory mediators which can damage the airways and lead to chronic airway inflammation [73, 99]. One of the most abundant inflammatory cells are eosinophils which may be found in the submucosal and epithelial layers [94]. It has been shown that activated eosinophils can cause tissue damage in the airways as well as airway inflammation in 50% of asthma cases [25, 94, 100]. Eosinophils trigger epithelial cells to release cytokines which result in bronchial hyperresponsiveness and inflammation in asthma [73]. Mast cells are another group of inflammatory cells which are associated with asthma severity. It has been shown that mast cells are increased in the BAL as well as within airway smooth muscles in asthma [98, 99, 101]. Mast cells play an important role in the development of airway hyperresponsiveness (AHR). It is established that there is a correlation between the number of mast cells and the mediators released by mast cells such as histamine, tryptase and leukotrienes, in BAL and AHR [98]. Macrophages also play a significant role in airway inflammation. They are able to migrate to the site of infection for phagocytosis and clearance of cellular debris. In addition, they secrete inflammatory products such as leukotrienes, cyclooxygenase and reactive oxygen intermediates [102]. Thus, inflammatory cells can produce ROS which can have detrimental effects on airway epithelial cells [103].
1.4 Rhinovirus infection and asthma exacerbations:

Exacerbation of asthma may be triggered by viral infections. Rhinoviruses are among the most significant exogenous factors which may lead to an increased production of H₂O₂ and oxidative stress and exacerbate asthma [41, 104, 105]. It has been shown that RV can exert severe lower-airway dysfunction in asthmatics [106]. Clinical experiments illustrate that asthmatics have more severe and prolonged lower respiratory tract symptoms of RV infection compared to healthy people [107]. Research has shown that RVs can cause asthma exacerbations in up to 90% of young children [108]. RVs can induce the secretion of pro-inflammatory mediators and cytokines such as IL-1, IL-6, CXCL-8 and TNF-α. This leads to the recruitment of inflammatory cells such as neutrophils and eosinophils, which release neutrophil elastase or histamine which can cause airway epithelial cell damage, airway narrowing and worsen airway hyperresponsiveness [109].

Human rhinovirus (HRV), a member of Picornaviridae family, is a non-enveloped virus and it has a positive sense single strand RNA with a genome length of 7.2 to 8.5 kb [110]. More than 100 serotypes of RV have been determined in the genus Rhinovirus. Previously they were classified into 2 groups: HRVA with 75 serotypes and HRVB with 25 serotypes. Real time polymerase chain reaction (RT-PCR) experiments have identified a new group of rhinoviruses, group C. The HRV-C viruses have been shown to be associated with respiratory symptoms, especially wheezing and bronchiolitis [111]. Subspecies of HRVC are HRVA2, HRVNY, HRVQPM, and HRVX [112]. HRVs can also be divided into two groups based on the receptor by which they enter the epithelial cells of the respiratory tract. Major group RVs, which include 90% of the RVs (such as RV14, RV16, RV39 and RV43) bind to intracellular adhesion molecule-1 (ICAM-1) receptors on the airway epithelial cell (AEC) surface, whereas the minor group RVs, bind to low density lipoprotein receptor family (LDL-R)
RV-87 or EV-68 is one exception. Being an enterovirus, it binds to the decay accelerating factor also known as CD55 [114]. Primary bronchial epithelial cells (pBECs) of asthmatics are more susceptible to RV infections as they are not able to release effective type I interferon responses [115]. Moreover, studies have demonstrated a greater diversity of pBEC responses in minor group RV strains as compared with major group RV strains. Taking into account the possible role of minor group RV strains, which are likely more relevant in asthma in causing significant clinical symptoms, there is a need for more consideration of these strains in future studies of asthma and COPD [115].

1.4.1 Rhinovirus entry and infection:

The point of HRV entry and initial site of infection is the upper respiratory tract [116]. After binding to the relevant receptor the virus enters the cytoplasm of airway epithelial cell (AEC) by pore formation and injects its genome into the host cell [117]. The RV uses its positive strand RNA genome as a template to produce a negative-sense RNA strand. RNA replicase which exists in the nucleoprotein core catalyzes this process. The negative-sense strand is subsequently used as a template to produce positive-sense RNA strands (Fig1.4.1.1), so the virus replicates as rapidly as possible. Single strand (ss)RNA converts to a double strand (ds)RNA, which is the main form of viral RNA genome inside the cell and produces progeny viruses [117]. In RV, in contrast to other viruses, there is a viral protein (VPg) instead of a cap protein at the 5´ end of the RNA which initiates the viral RNA replication process. Furthermore, the internal ribosomal entry site (IRES) is also found at the 5´ end of the RNA. This can cause the attachment of viral RNA to the ribosomal subunits of the host translational machinery, to translate viral mRNAs, so they utilize the machinery and metabolism of a host cell for their reproduction. In this regard, the virus directs the host for manufacturing the viral
proteins which are required for its replication. Studies have shown that in positive-strand RNA viruses such as RV, there is a unique strategy for protein translation. In these viruses, the result of mRNA translation is a large polypeptide, which is later cleaved at definite sites by proteolytic enzymes and can provide smaller viral proteins. These are structural proteins required for capsid formation and non-structural (NS) proteins such as proteases and RNA polymerases. These proteins include early proteins which are enzymes used in genome replication, late proteins which construct the capsid and lytic proteins which are enzymes required for viral egression from the cell [118]. These viral components and enzymes then begin to assemble into a new virus. After the assembly of viral proteins and RNA genomes, the virions lyse the cells and egression of new virions from the infected cells lead to the spread of infection to the neighbouring cells [117] (Figure 1.4.1.1).

*Figure 1.4.1.1. Structure and replication cycle of Rhinovirus*
1.4.2 Antiviral responses to rhinovirus infection and asthma exacerbations:

In respiratory diseases such as asthma, there is an impaired immune response to environmental triggers such as RV viruses [3, 115]. Immune responses which are induced by these viruses are of importance in symptom pathogenesis [119]. Several cells play an important role in asthma exacerbation. Firstly, the airway epithelial cells, which are the host cells for RV replication [119]. The susceptibility of epithelial cells to RV infection is associated with the density of viral receptors on the cell membrane. TNF-α which is a cytokine secreted by macrophages in response to RV infection, induces the expression of ICAM-1, similarly IFN-γ is secreted by lymphocytes in response to RV infection and can induce the expression of ICAM-1 cells on the surface of a variety of cells. Increased expression of ICAM-1 can attract inflammatory molecules to the site of infection and initiate an antiviral immune response [120]. Furthermore, the high expression of ICAM-1 receptors may make these cells susceptible to RV infection [121]. Therefore, viruses damage epithelial cells and enhance inflammatory infiltration to the airways [122]. Thus, epithelial cells which are considered as a host for RV infection, become active, causing inflammation through secretion of cytokines and inflammatory mediators such as IL-1β, CXCL-8, IL6, TNF-α and regulated on activation normal T cell expressed and secreted (RANTES) [89, 123-129]. These cells enhance airway inflammation and potentiate asthma. RANTES can recruit eosinophils and memory T cells to the site of infection. Similarly, CXCL8 attracts neutrophils and activated eosinophils leading to airway obstruction and inflammation in asthma[130, 131]. Furthermore, viral respiratory infections may lead to asthma exacerbation through activation of NF-κB [132]. Moreover, it has been revealed that the endogenous epithelial protective factors, such as heat shock protein (HSP) 27, labile Zn and superoxide dismutase are reduced in asthmatics in comparison with the healthy individuals, so the epithelium may be more susceptible to oxidative damage and apoptosis [133]. Viral infection in AECs leads
to IFN-β production which promotes apoptosis of infected cells and inhibits virus replication. In contrast, impaired IFN-β production and cell apoptosis leads to greater viral replication, which can cause cell necrosis and release of inflammatory mediators [3, 115].

Viral replication leads to secretion of pro-inflammatory compounds known as chemokines and cytokines, which can activate inflammatory mediators during immune responses [134]. Inflammatory mediators such as CXCL-8 and the pro-inflammatory cytokine IL6 have been detected in nasal samples of asthmatics during infection with rhinovirus [135, 136]. Similarly, an increased level of IL6, CXCL-8, and RANTES, which is secreted by cytotoxic T lymphocytes and acts as a potent chemoattractant for T cells and monocytes, have been identified in the sputum of asthmatic patients after rhinovirus infection [137]. Furthermore some reports have demonstrated the presence of eosinophil granular proteins including eosinophil cationic protein (ECP), in the nasal secretions of children who have RV infection with wheezing symptoms, and also in the sputum of asthmatics who have experimental infection with RV type 16 [109]. Hence, RV is able to provoke the immune system to produce pro-inflammatory cytokines and mediators [138], which can obstruct the airways and cause airway hyper-responsiveness, inflammation and ultimately lead to clinical exacerbations [139].

1.4.3 Pattern recognition receptors in viral detection and innate immune defence:

Pattern recognition receptors (PRR), like toll-like receptors (TLRs) and the RNA helicases MDA5 (melanoma differentiation associated gene 5) and RIG-I (retinoic acid-inducible gene), detect viral nucleic acids. They have been shown to detect viral dsRNA and single stranded RNA [140, 141]. Although they are known to detect viral nucleic acids, they act specifically to distinguish particular viruses.
MDA5 which is known as interferon-induced helicase C domain (IFIH1) detects picornaviruses (such as RV) [142], whereas RIG-I and TLR3 detect negative sense ssRNA viruses (such as respiratory syncytial virus (RSV) and influenza virus.

TLRs such as TLR3, 7, 8, and 9 are located within intracellular endosomes of airway epithelial cells (AECs), dendritic cells (DCs), macrophages and lymphocytes [143, 144]. They can activate protein kinases such as IkB kinase complex (IKKα, IKKβ, and NEMO/IKKγ) and the IKK-related kinases (TBK1 and IKKe). The IKK complex phosphorylates the NF-κB inhibitor (IkB), allowing NF-κB to enter the nucleus [145, 146]. On the other hand, TBK1 and IKKe phosphorylate IRF3 or IRF7 at specific serine sites in its C-terminal regulatory domain. IRF3 plays a crucial role for host defence against viral and microbial infection. IRF3 is normally found in the cytoplasm in an inactive state. However, its phosphorylation leads to its dimerization and association with the histone acetyltransferase nuclear proteins CREB binding protein (CBP) and p300, causing IRF3 to translocate into the nucleus. The IRF3–CBP complex induces the activation of type I interferon (IFN) α and β, and chemokine genes such as RANTES and CXCL-10 [147].

1.4.3.1 MDA5:

MDA5 is a cytoplasmic protein which is triggered by picornaviruses or the presence of synthetic RNA poly(I:C) [148]. MDA5 shows a close homology to the RIG-I receptors. MDA5 and RIG-I, the two homologous cytoplasmic receptors consist of a helicase domain at the C terminal and a caspase recruitment domain (CARD) at the N terminal. The helicase domain possesses ATPase activity which binds to dsRNA and then transmits signals through the CARD domain, which subsequently activates interferon response factor 3 (IRF-3), interferon response factor 7 (IRF-7) and NF-κB [149]. There are 23 and 35% amino acid identities in the N-terminal CARD and C-terminal helicase domain, respectively [150].
MDA5 is involved in the regulation of the growth and differentiation of melanoma cells [150, 151]. The CARD domain of MDA5 is responsible for initiating downstream signalling cascades and activation of type I IFN genes [152]. It has been shown that MDA5 and RIG-I transmit similar signals leading to the activation of IRF3, IRF7 and NF-κB [150].

1.4.3.2 MAVS:

RIG-I and MDA5 associate with an adaptor protein which is called MAVS (also known as beta promoter stimulator protein 1 (IPS-1), virus-induced signalling adaptor (VISA) or card adaptor inducing IFN-β (Cardiff)). MAVS is a single 70KDa integral protein which is located on the outer membrane of mitochondria. MAVS has a crucial role in defence against virus infection, through the recognition and inhibition of viral replication in the cytosol of different cells and it is required for the downstream activation of tank binding kinase 1(TBK1), IKKɛ, IRF3, NF-κB and antiviral type I IFNs [153] (Figure 1.4.3.1). It consists of an N-terminal CARD-like domain, a C-terminal transmembrane domain and a proline-rich region [154]. The CARD-like domain leads to interferon induction, so it is essential for the signalling process. Similarly the C-terminal transmembrane domain attaches to the mitochondrial outer membrane [154]. The interaction of the proline rich region with several signalling molecules like TNF receptor associated factor (TRAF)-6, TRAF-2, TRAF-3, receptor interacting protein 1 (RIP1) and Fas-associated protein with death domain (FADD), indicates the role of MAVS in TLR3-mediated pathways, in addition to RIG-I/MDA5-mediated signalling [155].

In addition to its significant role in IFN induction, MAVS also acts as a proapoptotic molecule which leads to the activation of caspases and may cause mitochondrial membrane disruption [156]. The function of MAVS is dependent on its mitochondrial localization, thus apoptosis terminates the interferon production by the cleavage and release of MAVS from the
outer mitochondrial membrane to the cytoplasm. It has been shown that several viruses such as hepatitis A (HAV) and hepatitis C virus (HCV) cleave MAVS from the outer mitochondrial membrane and by using this strategy they can escape from the host immune response [157]. The functional role of MAVS suggests that mitochondria may be implicated in antiviral immune responses through induction of apoptosis or initiating downstream signalling pathways [158](Figure 1.4.3.1).

*Figure 1.4.3.1. The role of MAVS in initiating interferon responses*
1.5 Mitochondria:

Mitochondria produce most of the cell’s energy in the form of adenosine-5’-triphosphate (ATP). The numbers of these vital organelles vary by the tissue type and the organism. Some cells may have only one mitochondrion, whereas, other cells can have several thousand mitochondria [159]. Mitochondria are known as one of the most important sources of endogenous ROS and RNS, because they metabolise cellular oxygen through the electron transport chain (ETC) via oxidative phosphorylation. In this case, some ROS will be released into surrounding cells. This causes oxidative damage to various cellular components, including the mitochondria themselves.

1.5.1 Structure

Mitochondria are composed of two phospholipid membranes (outer and inner membranes), the intermembrane space (the space between the outer and inner membranes), the matrix (space within the inner membrane), and the cristae (formed by infolding of the inner membrane) [160, 161] (Figure1.5.1).
1.5.2 Outer membrane

The outer mitochondrial membrane surrounds the organelle. It is composed of proteins and phospholipids and contains large integral proteins called porins. These porins act as pores to allow molecules with 5000 daltons or less in molecular weight to easily pass across the mitochondrial membrane [162, 163]. As such, the outer membrane is permeable to small molecules such as ions and sugars, however, large proteins must have special membrane transporters to transfer across the outer membrane [164]. Mitochondrial outer membrane permeabilization (MOMP) causes mitochondrial disruption by activating caspases which are involved in apoptosis and cell death [163]. Mitochondrial outer membrane oxidative damage can lead to mitochondrial disruption with leakage of proteins of the intermembrane space into the cytosol [163]. Potentially, mitochondrial outer membrane oxidative damage may affect MAVS which is located on the outer membrane of mitochondria.
1.5.3 Intermembrane space:

The space between the outer membrane and the inner membrane is called the intermembrane space or peri-mitochondrial space [164]. When a cell undergoes apoptosis, this can result in an opening of the transition pore (MPT) on the inner membrane of the mitochondria. This can lead to the release of numerous proteins which reside in the intermembrane space, such as cytochrome c, which can activate caspases leading to apoptosis. Cytochrome c is located in the intermembrane space and it is loosely attached to the inner membrane of the mitochondrion [163]. Cytochrome c is encoded by the CYCS gene and it is highly active in the oxidative phosphorylation pathway by transferring electrons from cytochrome c reductase to cytochrome c oxidase [165]. This process leads to ATP generation which is an essential compound for energy-dependent processes.

1.5.4 Inner membrane:

Unlike the outer membrane, the inner membrane is impermeable to all molecules, thus protein transporters are required to transfer molecules across the matrix [164]. The inner mitochondrial membrane contains 1/5 of the total protein in a mitochondrion and these proteins are involved in oxidative phosphorylation, ATP synthase, carrier proteins which regulate the passage of metabolites into and out of the matrix, protein import machinery and mitochondria fusion and fission [164]. Furthermore, the inner membrane contains high concentration of cardiolipin. This phospholipid can be only found in bacterial plasma membranes and mitochondrial inner membranes [166]. One of the most important functions of cardiolipin is anchoring cytochrome c to the inner membrane. This attachment is tight and irreversible, which makes cytochrome c functional in the ETC [167].
1.5.5 Cristae:

The inner mitochondrial membrane has a larger surface area than the outer membrane, due to infoldings of this membrane into the matrix, called cristae. This expanded surface area is important for ATP production. Thus, the cells which require more energy in the form of ATP, such as muscle cells, contain more cristae [168].

1.5.6 Matrix:

The matrix is the space enclosed by the inner membrane. About 2/3 of the total protein in a mitochondrion are located in the matrix [164]. Enzymes, mitochondrial ribosomes, tRNA, and the mitochondrial DNA genome are some examples of these important proteins which are involved in ATP production.

1.5.7 Mitochondrial genome:

Mitochondria have originated from endosymbiosis with bacteria, so the mitochondrial ribosomes are similar to those from bacteria in size and structure. Like bacteria they contain bacterial 70S ribosome with 16srRNA instead of 80S cytoplasmic ribosomes and 18srRNA [169]. They have also their own DNA (mtDNA), transcription factors and the machinery to synthetise their own RNA and proteins [170].

The mitochondrial transcription factors TFAM, TB1 and TB2 have important roles in mtDNA transcription. They regulate the mtDNA copy numbers and this is important for maintaining ATP production [171]. It has been shown that the mammalian RNA polymerase can bind the promoter DNA and initiate the transcription process by the assistance of mitochondrial transcription factor A (mtTFA), mitochondrial transcription factor B1 (mtTFB1) and mitochondrial transcription factor B2 (mtTFB2) [172]. TFB1 is not only a mitochondrial transcription factor but also acts as an rRNA methyltransferase. These dual-
function proteins have rRNA methyltransferas function which they can dimethylate two adenosine bases near the 3’ end of the small subunit rRNA during ribosome biogenesis and also they support mitochondrial transcription [173]. TFB2 has rRNA methyltransferase activity as well but its activity is less than TFB1 [172]. TFB2 is defined as a specialized transcription factor in mammalian mitochondria, since it has been demonstrated as a much more active transcription factor than TFB1 [174]. The levels of TFA directly regulate the activity of both TFB1 and TFB2 mitochondrial transcription factors. It has been shown that mTFA tends to bind to oxidative DNA damage, so it seems that mTFA is not only essential for mitochondrial gene expression but it is also important for mtDNA maintenance and repair [174]. It has been shown that mtDNA in lung tissue is more sensitive to oxidative stress in comparison with the DNA in other tissues [175].

1.5.8 Electron transport chain (ETC):

In the mitochondrial electron transport chain, electrons are transferred from electron donors to electron acceptors via a series of redox reactions. These reactions are coupled to the proton gradient (H+ ions) across the inner membrane. This electrochemical proton gradient leads to ATP production. Three proton complexes are involved in ATP production: I, III, and IV. Complex I (NADH coenzyme Q reductase) accepts electrons from nicotinamide adenine dinucleotide (NADH), and transfers them to coenzyme Q (ubiquinone), which also receives electrons from complex II (succinate dehydrogenase). Ubiquinone transfers electrons to complex III (cytochrome bc1 complex). Then the electrons are delivered to cytochrome c (cyt c). Cytochrome c transfers electrons to complex IV (cytochrome c oxidase). This complex transfers the electrons to molecular oxygen(O2) leading to the production of two molecules of water [176]. At the same time, four protons are removed from the mitochondrial matrix (although only two are translocated across the membrane), contributing to the proton gradient
Some ROS which are produced in mitochondria will be released into surrounding cells. This causes oxidative damage to various cellular components, including the mitochondria themselves.

**Figure 1.5.2. Mitochondrial ETC, adapted from Wikipedia**

1.5.9 **Effects of ROS on ETC:**

ROS, whether produced from endogenous or exogenous sources, can damage the mitochondria [177] and impair their activity resulting in a lack of ATP production and subsequent cell necrosis or apoptosis [178]. Both chronic and acute exposure of cells to ROS can affect cellular proteins, lipids, nucleic acids, the iron-sulphur (Fe-S) centers of the ETC at complexes I, II and III, as well as inactivation of the aconitase enzyme in the citric acid cycle which can lead to loss of ATP generation [179]. The iron-sulphur centers of the ETC are very sensitive to oxidative stress. The interaction of iron with hydrogen peroxide forms the highly reactive cytotoxic radical, OH⁻ [180]. Mitochondrial dysfunction can also cause the generation of RNS, by activation of nitric oxide synthetase, which leads to the production of superoxide and nitric oxide. Subsequently the reaction of nitric oxide with superoxide will
form peroxynitrite (ONOO−), a highly reactive radical, which can prevent the activation of cytochrome c oxidase and lead to further generation of superoxide and peroxynitrite. There are 13 subunits of the ETC, three of which have catalytic activity and are encoded by the mitochondria [181]. The third subunit which is particularly affected by oxidative stress is necessary for stabilization of the whole complex [182]. It has been shown that free radicals such as peroxynitrite can damage the heme and copper centers of cytochrome c oxidase in mitochondria and affect their activity in oxidative pathways [183]. Furthermore, oxidative stress leads to an increased number of mitochondria, ATP levels and mitochondrial abnormalities such as reduced cytochrome c oxidase activity and mRNA expression which have been shown in the lungs of asthmatics [184]. Also, morphological changes of mitochondria including the loss of cristae and mitochondrial swelling have been reported in an asthmatic mouse model [184]. The pre-existing mitochondrial dysfunction is involved in the inflammation of the airways of allergic asthmatic patients [185].

1.5.10 Apoptosis:

Regulation of apoptosis is one of the critical roles of the mitochondria, which entails high levels of energy in the form of ATP [186]. Apoptosis is the process of programmed cell death in which apoptotic bodies are engulfed and damaged cells quickly removed before they release pro-inflammatory mediators. Necrosis may occur, in the absence of apoptosis, which is a form of cell death that may cause severe damage by releasing lactate dehydrogenase (LDH) and leads to airway injury and inflammation [187].

Increased ROS/RNS in the mitochondria cause mitochondrial damage and lead to caspase-depandant apoptosis. Caspases are cystein proteases which contain thiol groups and play a critical role in cell apoptosis.
Caspases can be activated through two main pathways: the extrinsic (death receptor mediated) and intrinsic (mitochondrial mediated) pathways that may result in cellular apoptosis [188]. Activation of the extrinsic pathway, involves the interaction of the Fas ligand with the cell surface death receptor that leads to the accumulation of Fas associated death domain protein (FADD), resulting in the activation of pro-caspase-8. This results in the dimerization and activation of caspase 8 which then can cleave and activate caspase 3 and caspase 7 leading to apoptosis or cell death.

The intrinsic pathway which is triggered by stimuli such as DNA damage, activates B-cell lymphoma-2(BCL-2) homology 3 (BH3)-only proteins leading to BCL-2 antagonist killer (BAK) and BCL-2 associated X protein (BAX) activation. BAX and BAK are the most important pro-apoptotic BCL-2 family proteins which can cause mitochondrial outer membrane permeabilization (MOMP) by producing pores on the outer membrane of mitochondria. Following MOMP, release of mitochondrial proteins leads to caspase activation and apoptosis. One of these proteins is cytochrome c which binds to apoptotic protease activating factor 1 (Apaf-1) inducing its oligomerization and forms apoptosome complex which results in the activation of caspase-9 (Figure 1.5.3.1) [188]. Subsequently, Caspase 9 cleaves and activates caspase 3 and caspase 7 leading to apoptosis.

Figure 1.5.3.1. Apoptotic pathways
1.6 Antioxidants in cellular defence:

Antioxidants are important in cellular defence. It has been shown that in respiratory diseases, ROS overwhelm host antioxidant defences, leading to the tissue damage [43]. Antioxidants include endogenous metabolites (glutathione (GSH), N-acetylcysteine, heme oxygenase), exogenous antioxidants (vitamin C, E, carotenoids and flavonoids) and enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx). Furthermore, there are various low molecular weight antioxidants in the lung such as specific antioxidant enzymes, metal binding proteins and mucus glycoproteins [189-191]. GSHPx plays an important role in antioxidant defence. It is a seleno-protein which consists of four subunits, with each subunit containing a selenium atom [192]. GSHPx and CAT convert hydrogen peroxide to water and protect the cells from oxidative damage [193]. SOD converts superoxide to hydrogen peroxide. It exists in three forms, based on the metal cofactor: Cu/Zn superoxide, Fe/Mn superoxide and Ni superoxide dismutase. Amongst the exogenous antioxidants, vitamin C or ascorbic acid is the most potent water-soluble antioxidant. It is mainly found in fruits and vegetables and it is considered as an electron donor, thus by donating two electrons, it prevents the oxidation of other compounds [194]. It has been demonstrated that levels of non-enzymatic antioxidants like vitamin C, vitamin E, lycopene, lutein, α-carotene and β-carotene are lower in asthmatics in comparison with controls [195]. Furthermore, changes in the activities of enzymatic antioxidants, including SOD and GSHPx have been shown to be modified in asthma compared to controls [196]. Studies have revealed a decrease in total antioxidant capacity of plasma in smokers and patients with asthma and COPD [197]. Furthermore, GSH and glutamate cysteine ligase (GCL), are decreased in macrophages and in the airways of smokers compared to non-smokers [198]. However, the exact mechanism of antioxidant biomarkers in asthma or COPD and their association with disease severity is poorly understood [41].
As discussed earlier, asthma and COPD are associated with an elevated oxidant burden. The use of antioxidants as therapeutic strategies to improve the endogenous antioxidant system may help to overcome the existing imbalance between ROS and antioxidants [41, 199]. This approach may alleviate the inflammatory responses in asthma and COPD [199]. Here, I will present in details the specific antioxidants which were used in this thesis, lycopene and L-carnitine.

1.6.1 Carotenoids:

Carotenoids are natural fat-soluble compounds which are found abundantly in fruits and vegetables such as tomatoes, carrots, pumpkins and broccoli [200, 201]. The yellow, orange and red colour pigments of fruits, vegetables and plant leaves are due to these important molecules [202, 203]. Humans and some animals are not able to synthesis carotenoids and they must be consumed in the diet [143]. They are a subclass of phytochemicals called terpene [204, 205]. There are more than 600 carotenoids which have been detected and characterized from natural sources [206]. However, only 5 are present in significant concentrations in human plasma: α and β-carotene, lutein, lycopene and β-cryptoxanthin which are shown in figure 1.6.1.1.1 [207, 208]. They exist in both cis and trans isomers, however the trans isomer is the most stable and prevalent form of carotenoids [209].

They are extremely hydrophobic compounds with poor solubility in water which makes them difficult for in vitro studies. however, they can be dissolved in tetrahydrofuran (THF) at more than 4mg/ml [200, 210]. These hydrophobic compounds accumulate in hydrophobic parts of the cells, such as the inner core of membranes and lipid globules [209].
1.6.1.1 Classification of carotenoids:

Carotenoids are divided in two groups: the group which contains oxygen called xanthophylls such as lutein, zeaxanthin, α-cryptoxanthin and β-cryptoxanthin and a second group is known as carotenes, which are pure hydrocarbons with no oxygen including α-carotene, β-carotene and lycopene [202]. Extreme heat, light, acids and bases have destructive effects on carotenoids [205]. Furthermore, free radicals such as hydroxyl radicals can disrupt the structure of carotenoids [202, 211].

*Figure 1.6.1.1.1. Structure of some common carotenoids:*

- α-carotene:

- β-carotene:

- Lycopene:
Lutein:

β-Cryptoxanthin:

1.6.1.2 Bioavailability of carotenoids:

Bioavailability of carotenoids is mainly based on measurement of carotenoids in serum or plasma after ingestion [212]. The physiological level of lycopene in human blood and tissue varies with intake and tissue type [213]. Plasma lycopene concentration is from 0.14µM/L [214] to 0.25µM/L [215]. That is equivalent to 0.8 to 1.34µg/ml. Recent studies have shown that lycopene from tomato paste is more bioavailable than fresh tomatoes [216]. Furthermore, the cis isomeric form is more abundant than trans [217]. It has been shown that lycopene can be absorbed more when it is consumed with β-carotene [218]. Carotenoids are resistant to heat and they are not destroyed during food processing [219, 220]. When carotenoids are dissolved in oil they can be absorbed more readily [212]. Thus, intake of dietary fat may have a positive effect on carotenoids bioavailability [212].
Also intake of alcohol can reduce the levels of carotenoids in serum[212, 221]. Furthermore, it has been shown that carotenoids levels are higher in women than men [212, 222].

1.6.1.3 Potential toxic effects of carotenoids:

Studies have shown that intake of fruits and vegetables with high levels of carotenoids decreases the risk of lung cancer [223, 224], however, trials of the intake of β-carotene supplements in heavy smokers show an increased risk of lung cancer. Although, the exact mechanism is unclear, it has been suggested that the interaction between ROS of cigarette smoke causes β-carotene oxidation leading to the production of toxic β-carotene metabolites [225, 226].

1.6.1.4 Biological functions of carotenoids:

1.6.1.4.1 Pro-vitamin A activity:

Several carotenoids can be metabolised in the body to produce vitamin A (< 10% of carotenoid isomers) [227]. Of particular note, β-carotene in the presence of oxygen can be converted to two equivalent retinal molecules. Each of these retinal molecules, then react to produce retinol (vitamin A) [228].

1.6.1.4.2 Antioxidant activity of carotenoids:

In recent years there has been growing interest in the antioxidant properties of carotenoids [229]. The conjugated double bonds in the polyene skeleton of carotenoids make them potentially useful for antioxidant activities. To act as a suitable antioxidant these molecules need to reach an appropriate concentration in the tissues [202].
A number of epidemiological studies have shown that the high intake of carotenoids in the daily diet is of importance in the prevention of several degenerative disorders such as cancer and cardiovascular diseases [230]. The antioxidant properties of carotenoids are associated with their ability to scavenge reactive oxygen species such as singlet molecular oxygen (O$_2$) and peroxyl radicals [230, 231]. For example, β-carotene reacts with peroxyl radicals which are generated through lipid peroxidation and inhibits the reaction which if left unchecked, could result in membrane damage [230]. Thus, they are thought to play a significant role in the protection of cells and tissues from oxidative damage [230, 232].

The antioxidant activity of carotenoids is as follows: lycopene > α-carotene > β-cryptoxanthin > zeaxanthin = β-carotene > lutein [233]. Thus, lycopene has the highest antioxidant activity among the carotenoids.

1.6.1.4.3 Antioxidant property of lycopene:

Lycopene is a lipophilic compound that is not soluble in water, thus it is mostly accumulated within cell membranes and lipoprotein compounds. Lycopene is a red pigment which is found predominantly in tomato products. Furthermore, red fruits, watermelon, apricots and pink grapefruit also contain high amounts of lycopene [234-236]. Lycopene is considered to be the most potent singlet oxygen quencher as well as the most potent scavenger of ROS among the carotenoids [237-240], furthermore, lycopene can inactivate hydrogen peroxide and nitrogen dioxide [237-239]. Recent studies have shown that lycopene is more active than β-carotene in protecting lymphocytes against NO$_2$ radicals and protecting cells from oxidative damage [239, 241]. Lycopene also acts as an anticarcinogen by increasing the expression of connexin43 and up-regulating gap junction communication [242-244].
Epidemiological studies have shown that lycopene intake is associated with reduced risk of several disease such as cancer and cardiovascular diseases [234].

1.6.2 Carnitine:

Carnitine (β-hydroxy-γ-N-trimethylaminobutyric acid) is obtained from foods [245] and is also synthesized endogenously in the liver, kidney and brain from, lysine and methionine, by the function of 4-butyrobetaine hydroxylase [246, 247]. In humans, 75% of carnitine is absorbed from dietary sources and 25% is obtained from endogenous biosynthesis [248]. The biologically active stereoisomer of carnitine is L-carnitine and its bioavailability is dependent on the dietary sources [249]. Carnitine is easily dissolved in water and acts as a carrier of long-chain fatty acids into the mitochondria for the production of metabolic energy [247].

1.6.2.1 Food sources of carnitine:

Carnitine is mainly found in red meat and dairy products, however grains, fruits and vegetables also contain small amounts of carnitine [250, 251]. The status of carnitine in humans varies by body composition, gender and overall diet [250].

Table 1.6.2.1.1 provides carnitine content from some selected foods, grains, fruits and vegetables.
Table 1.6.2.1.1. Carnitine content of some selected animal products, grains, fruits and vegetables[250]:

<table>
<thead>
<tr>
<th>Foods (animal products, grains, fruits and vegetables)</th>
<th>Total carnitine/100g food (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steak, prepared</td>
<td>525</td>
</tr>
<tr>
<td>Ground Beef, prepared</td>
<td>300</td>
</tr>
<tr>
<td>Chicken prepared</td>
<td>60</td>
</tr>
<tr>
<td>Egg, chicken, prepared</td>
<td>5</td>
</tr>
<tr>
<td>Cornflakes</td>
<td>59</td>
</tr>
<tr>
<td>Rice</td>
<td>44</td>
</tr>
<tr>
<td>Bread</td>
<td>10.9</td>
</tr>
<tr>
<td>Wheat seed</td>
<td>2.47</td>
</tr>
<tr>
<td>Pears</td>
<td>17</td>
</tr>
<tr>
<td>Peaches</td>
<td>10</td>
</tr>
<tr>
<td>Pineapple</td>
<td>6.5</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>18</td>
</tr>
<tr>
<td>Asparagus</td>
<td>8</td>
</tr>
<tr>
<td>Avocado</td>
<td>4.9</td>
</tr>
</tbody>
</table>

1.6.2.2 Bioavailability of carnitine:

Intake of carnitine by humans ranges from <1 to ∼15μmol/kg body wt/day, with the higher intakes (6-15μmol carnitine/kg body wt/day) observed in people who regularly consume red meat [252]. Studies have shown that vegetarians acquire less carnitine, usually <1μmol/kg body wt/day [252].
Carnitine concentrations are higher in tissues than body fluids. It has been shown that the carnitine levels are 50 times more in skeletal and cardiac muscle than in plasma[253]. Reports have evaluated that after oral doses of 1–6g, carnitine bioavailability will be in a range of 5–18% [254-256]. The majority of non-absorbed carnitine is degraded in the large intestine by bacterial flora in the gastrointestinal tract [253].

1.6.2.3 Carnitine biosynthesis and metabolism:

In the cytosol, fatty acids are transferred from acyl-CoAs to carnitine by the action of the carnitine-palmitoyl-transferase 1 (CPT1) which is found on the outer membrane of mitochondria [257-259].

\[
\text{Carnitine} + \text{acyl-CoA} \rightleftharpoons \text{acylcarnitine} + \text{CoA}
\]

Then these acylcarnitines pass through the outer membrane and translocate into the inner membrane of the mitochondria by the action of carnitine/acylcarnitine carrier (CAC) [257]. Then in the matrix, the formed fatty acyls are transferred from carnitine to matrix CoA by carnitine-palmitoyl-transferase 2 (CPT2). These mitochondrial acyl-CoAs are then oxidized by \(\beta\)-oxidation enzymes to produce ATP or metabolic energy [260]. L-carnitine is then transported to other tissues especially fat tissues which use fatty acids as their primary energy such as skeletal and cardiac muscle [247].
1.6.2.4 Antioxidant property of carnitine:

Carnitine has been shown to reduce oxidative stress in cells. In some studies, where it has been shown to scavenge free radicals, such as the superoxide anion and hydrogen peroxide [248, 261]. Also it protects mitochondrial enzymes such as succinate dehydrogenase and preserves glutathione peroxidase, catalase and superoxide dismutase activity [248]. Studies have shown that serum carnitine levels are reduced in children with respiratory tract infections as well as children with moderate asthma during exacerbations of asthma, compared to healthy children [262-264]. Viral respiratory infections are considered as important triggers of asthma, thus children with low levels of carnitine may be more susceptible to asthma [262]. Lack of carnitine leads to the accumulation of long chain fatty acids in the cytosol which the toxic accumulation of saturated and monounsaturated of fatty acids may contribute to airway inflammation [262, 265].

1.7 Hypothesis and Aims:

The hypothesis of this thesis is that oxidative stress impairs mitochondrial function as well as type I and type III IFN responses to RV infection and this can be corrected using antioxidant supplementation.

The aims of this thesis are:

Aim1: To determine if exposure of epithelial cells to oxidative stress, in the form of H$_2$O$_2$ and CSE results in impaired mitochondrial function and antiviral responses in primary bronchial epithelial cells (pBECs).
Aim 2: To compare the effects of oxidative stress on mitochondrial function and antiviral responses in pBECs of asthmatics versus non-asthmatics.

Aim 3: To determine if oxidative damage to mitochondria and antiviral signalling pathways can be reversed using antioxidants (lycopene and L-carnitine).
Chapter 2

General Materials and Methods
2.1. Tissue Culture

2.1.1 Immortalised cell lines:

2.1.1.1 BEAS-2B

BEAS-2B cell lines (American Type Culture Collection (ATCC), USA) were obtained from normal human bronchial epithelium (non-cancerous individuals), which were infected with an adenovirus 12-SV40 virus. BEGM complete media were used for the maintenance of the cells and then they were incubated at 37°C in a humidified atmosphere of 5%CO₂. Cells were grown in T175 flasks until 70-80% confluence. To passage the BEAS-2B cells flasks were rinsed with PBS, a dilution of 1:10 of trypsin EDTA (Invitrogen) and PBS were added, and cells were incubated at 37°C in a humidified atmosphere of 5%CO₂ for 15-20 minutes. Once cells detached, 2.1ml FCS was added to inactivate the trypsin. Cells were centrifuged at 1100rpm for 10 minutes. The supernatants were removed and the cells were re-suspended in 5ml BEGM complete media. Cells were counted using a hemocytometer, and 3 x 10⁵ cells was seeded in each well of a 12 well tissue culture plate and the rest of the cells were placed in a separate flask for subsequent cell culture. BEAS-2B cells were used to optimise all the experiments in which we needed to identify the appropriate doses of oxidative stress or the harvesting time-point. Then, following optimisation, all the experiments were performed again with appropriate doses of oxidative stress and specific time-points on primary bronchial epithelial cells (pBECs) of healthy and asthmatic patients.

2.1.1.2 RD-ICAM

RD-ICAM cell line is a transformed cell line enriched with ICAM-1 receptor expression. Major group of human rhinoviruses use ICAM-1 receptors which are on the airway epithelial cell (AEC) surface for binding and entering the cells.
RD-ICAM cell lines in Dulbecco’s Modified Eagles Medium (DMEM) (Invitrogen) containing 1% FCS were used for making rhinovirus stock and TCID50 assay.

2.1.2 Media for tissue culture

- BEGM (Bronchial Epithelial Growth Medium) complete media: BEBM (Basal Epithelial Bronchial Medium) (Lonza) supplemented with BEGM Singlequots (Insulin, Bovine pituitary extract, Human epidermal growth factor, Epinephrine, Transferrin, Triiodothyronine (T3), Retinoic acid) and hydrocortisone at 1μl/ml of complete BEGM, 2ml penicillin/streptomycin solution per 100ml BEGM, 1ml amphotericin B solution per 100ml BEGM.

- BEBM minimal media: BEBM (bronchial epithelium basal medium) (no growth factors) (Lonza) containing 1xITS+1 (insulin, transferring and sodium selenite) liquid media supplement (sigma).

- DMEM (Dulbecco’s Modified Eagles Medium) (Thermo scientific): 1% penicillin-streptomycin, 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5g/litre sodium bicarbonate were added to DMEM base media and then 5% foetal bovine serum (FBS) was added per 100ml of DMEM media.
2.1.3 Primary Bronchial epithelial cells (pBECs)

2.1.3.1 pBECs collection from healthy and asthmatic subjects

Bronchoscopy is a recognised investigation and is being performed for clinical indications by experienced personnel in the endoscopy suite at John Hunter Hospital. Healthy volunteers with no history of smoking and no evidence of airflow obstruction as well as subjects with moderate to severe asthma were recruited for this study. They were questioned about the previous severity of cold symptoms. At the time of recruitment no subjects had symptoms of an acute respiratory tract infection for the preceding 4 weeks. Table 2.1.3.1.1 shows GINA classification of asthma severity. A clinical history, examination and spirometry were performed on all individuals. All subjects underwent fibreoptic bronchoscopy in accordance with standard guidelines. These procedures were performed by specialist respiratory physicians, who have extensive experience in research bronchoscopy. pBECs were obtained using a single sheathed nylon cytology brush applied under direct vision. Approximately 5-10 brushings were taken from second to third generation bronchi. All volunteers gave written informed consent.
Table 2.1.3.1.1 GINA classification of asthma severity

<table>
<thead>
<tr>
<th>severity</th>
<th>Symptoms/Day</th>
<th>Symptoms/Night</th>
<th>PEF or FEV1</th>
<th>PEF variability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermittent</td>
<td>&lt; 1 time a week</td>
<td>&lt;= 2 times a month</td>
<td>&gt;= 80%</td>
<td>&lt; 20%</td>
</tr>
<tr>
<td></td>
<td>Asymptomatic and normal PEF between attacks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild Persistent</td>
<td>&gt; 1 time a week but &lt; 1 time a day</td>
<td>&gt; 2 times a month</td>
<td>&gt;= 80%</td>
<td>20-30%</td>
</tr>
<tr>
<td></td>
<td>Attacks may affect activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate Persistent</td>
<td>Daily</td>
<td>&gt; 1 time a week</td>
<td>60%-80%</td>
<td>&gt; 30%</td>
</tr>
<tr>
<td></td>
<td>Attacks affect activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe Persistent</td>
<td>Continuous</td>
<td>Frequent</td>
<td>&lt;= 60%</td>
<td>&gt; 30%</td>
</tr>
<tr>
<td></td>
<td>Limited physical activity</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.1.3.2 Characterization of the pBECs of healthy and asthmatic subjects

pBECs of 4 asthmatics and 4 healthy volunteer were used to see the effects of oxidative stress on mitochondrial function and immune responses and pBECs of 5 asthmatics and 5 healthy volunteers were used to identify the effects of antioxidants on mitochondrial function and immune responses. Table 2.1.3.1 shows the characterization of the healthy and asthmatic volunteers which were recruited in this project. The healthy controls were non-smokers with no history of heart or lung disease and normal lung function. The asthmatics were non-smokers with moderate to severe persistent asthma and had a consistent evidence of bronchial hyperresponsiveness. Allergy skin tests used a panel of common aeroallergens and were considered positive if the wheal response was >3 mm than the negative control. Lung function was assessed by spirometry and bronchial hyperresponsiveness by histamine challenge. Asthma was diagnosed in atopic individuals with a consistent history and evidence of bronchial hyperresponsiveness (defined by a PC<sub>20</sub> histamine < 8 mg/ml) and was categorized in accordance with the GINA guidelines [266].
<table>
<thead>
<tr>
<th>Study No.</th>
<th>Sex</th>
<th>Age</th>
<th>Category</th>
<th>Gina stage</th>
<th>Smoking history</th>
<th>Atopy</th>
<th>Spirometry</th>
<th>Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS139</td>
<td>F</td>
<td>70</td>
<td>Asthma</td>
<td>Severe persistent</td>
<td>Former smoker</td>
<td>No</td>
<td>65%</td>
<td>FEV1/FVC ratio: 1.75, FVC: 59.7 Seretide, Spiriva, Somac</td>
</tr>
<tr>
<td>AS140</td>
<td>F</td>
<td>61</td>
<td>Asthma</td>
<td>Moderate persistent</td>
<td>Never smoked</td>
<td>No</td>
<td>101%</td>
<td>FEV1/FVC ratio: 3.47 Not determined Seretide, Salbutamol, Thyroxine</td>
</tr>
<tr>
<td>AS065</td>
<td>F</td>
<td>61</td>
<td>Asthma</td>
<td>Severe persistent</td>
<td>Never smoked</td>
<td>No</td>
<td>48%</td>
<td>FEV1/FVC ratio: 2.2 Not determined Seretide, Salbutamol</td>
</tr>
<tr>
<td>AS142</td>
<td>M</td>
<td>57</td>
<td>Asthma</td>
<td>Moderate persistent</td>
<td>Never smoked</td>
<td>Yes</td>
<td>71%</td>
<td>FEV1/FVC ratio: 3.52, FVC: 72 Seretide, Salbutamol,</td>
</tr>
<tr>
<td>AS094</td>
<td>M</td>
<td>68</td>
<td>Asthma</td>
<td>Severe persistent</td>
<td>Never smoked</td>
<td>Yes</td>
<td>50%</td>
<td>FEV1/FVC ratio: 2.95, FVC: 50 Seretide, Tiotropium, Salbutamol</td>
</tr>
<tr>
<td>AS095</td>
<td>M</td>
<td>57</td>
<td>Asthma</td>
<td>Severe persistent</td>
<td>Never smoked</td>
<td>Yes</td>
<td>79%</td>
<td>FEV1/FVC ratio: 5.48, FVC: 64 Seretide, Salbutamol, Singulair, Acimax</td>
</tr>
<tr>
<td>AS155</td>
<td>F</td>
<td>36</td>
<td>Asthma</td>
<td>Moderate persistent</td>
<td>Never smoked</td>
<td>Yes</td>
<td>96%</td>
<td>FEV1/FVC ratio: 4.39, FVC: 75 Seretide, Salbutamol</td>
</tr>
<tr>
<td>AS102</td>
<td>F</td>
<td>60</td>
<td>Asthma</td>
<td>Severe persistent</td>
<td>Never smoked</td>
<td>Yes</td>
<td>56%</td>
<td>FEV1/FVC ratio: 2.03, FVC: 59 Seretide, Tiotropium, Pepsidine,</td>
</tr>
<tr>
<td>AS119</td>
<td>F</td>
<td>48</td>
<td>Asthma</td>
<td>Severe persistent</td>
<td>Never smoked</td>
<td>No</td>
<td>61%</td>
<td>FEV1/FVC ratio: 2.28, FVC: 65 Avamys, Symbicort, Tiotropium</td>
</tr>
<tr>
<td>HC114</td>
<td>F</td>
<td>64</td>
<td>Healthy nonsmoker</td>
<td>Not determined</td>
<td>Never smoked</td>
<td>Yes</td>
<td>79%</td>
<td>FEV1/FVC ratio: 2.92, FVC: 70 Not determined</td>
</tr>
<tr>
<td>HC083</td>
<td>F</td>
<td>45</td>
<td>Healthy nonsmoker</td>
<td>Not determined</td>
<td>Never smoked</td>
<td>No</td>
<td>79%</td>
<td>FEV1/FVC ratio: 2.92, FVC: 70 Not determined</td>
</tr>
<tr>
<td>HC085</td>
<td>M</td>
<td>48</td>
<td>Healthy nonsmoker</td>
<td>Not determined</td>
<td>Never smoked</td>
<td>Yes</td>
<td>112%</td>
<td>FEV1/FVC ratio: 5.45, FVC: 84 Not determined</td>
</tr>
<tr>
<td>HC118</td>
<td>F</td>
<td>55</td>
<td>Healthy nonsmoker</td>
<td>Not determined</td>
<td>Never smoked</td>
<td>No</td>
<td>79%</td>
<td>FEV1/FVC ratio: 2.92, FVC: 70 Not determined</td>
</tr>
<tr>
<td>HC094</td>
<td>F</td>
<td>61</td>
<td>Healthy nonsmoker</td>
<td>Not determined</td>
<td>Never smoked</td>
<td>No</td>
<td>79%</td>
<td>FEV1/FVC ratio: 2.92, FVC: 70 Not determined</td>
</tr>
<tr>
<td>HC095</td>
<td>F</td>
<td>60</td>
<td>Healthy nonsmoker</td>
<td>Not determined</td>
<td>Never smoked</td>
<td>No</td>
<td>79%</td>
<td>FEV1/FVC ratio: 2.92, FVC: 70 Not determined</td>
</tr>
<tr>
<td>HC097</td>
<td>F</td>
<td>69</td>
<td>Healthy nonsmoker</td>
<td>Not determined</td>
<td>Never smoked</td>
<td>No</td>
<td>79%</td>
<td>FEV1/FVC ratio: 2.92, FVC: 70 Not determined</td>
</tr>
<tr>
<td>HC096</td>
<td>M</td>
<td>65</td>
<td>Healthy nonsmoker</td>
<td>Not determined</td>
<td>Never smoked</td>
<td>No</td>
<td>79%</td>
<td>FEV1/FVC ratio: 2.92, FVC: 70 Not determined</td>
</tr>
<tr>
<td>HC099</td>
<td>F</td>
<td>48</td>
<td>Healthy nonsmoker</td>
<td>Not determined</td>
<td>Never smoked</td>
<td>No</td>
<td>79%</td>
<td>FEV1/FVC ratio: 2.92, FVC: 70 Not determined</td>
</tr>
</tbody>
</table>
2.1.3.3 pBEC culture

Bronchial brushing performed into a tube of DMEM media by submerging the brush and shaking vigorously by hand for approx. 10 seconds. pBECs were then centrifuged at 1100rpm for 10 minutes at room temperature. DMEM media were then removed and replaced with 3ml complete BEGM media containing extra antibiotics (2ml penicillin/streptomycin solution and 1ml amphotericin B solution per 100ml BEGM). The cell pellet was gently homogenised. Cells were transferred to T25 flasks and 5ml of complete BEGM media containing extra antibiotics were added to the flasks. The media were replaced after 24 hours. Cells took 5-7 days to become confluent after which they were expanded into T75 flasks [133, 267-269]. At passage two, pBECs were seeded onto 24 well tissue culture plate until 80% confluent before exposure to RV1B.
2.2. Preparing RV1B stock and TCID50 assay:

RV1B which was originated from clinical samples in 2005 and had already sequenced to confirm its identity was used for making a 200ml of RV1B stock. The previous stock of serotype RV1B was cultured using the susceptible cell line RD-ICAM-1 in DMEM containing 1% FCS. Cells were maintained at 37°C in a humidified incubator containing 5% CO₂. Significant cytopathological effects (CPE) of RV1B in RD-ICAM-1 cells were confirmed by microscope, then the cell culture supernatant was collected and centrifuged for 10min at 2000rpm. The clarified supernatant stock was then aliquoted and stored at -80°C until use. The titre of virus stock was determined by Tissue culture infectious dose at 50% (TCID50/ml).

2.2.1 Viral titration assay:

TCID50/ml experiments were performed using confluent RD-ICAM-1 cells. Cells were seeded in 96-well tissue culture plates (NUNC, Roskilde, Denmark). Culture samples containing virus were serially diluted in 1% DMEM from 10⁻¹-10⁻⁸ dilutions in a separate 96-well tissue culture plate. The spent media of RD-ICAM cells was removed and the cells were infected with 100µl of appropriate dilutions which were prepared. Plates were incubated at 33°C/5% CO₂ for 5 days. Infected wells were scored for CPE; >50% CPE demonstrated by inverted microscopy were considered as positive results. Results were used to determine the TCID₅₀/ml using the Karber Method as below:
\[ \text{Log (TCID}_{50}) = a - D (\Sigma p - 0.5) \]

**Where**
- \( a \) = dilution index for last dilution where all wells are infected
- \( D \) = log of dilution factor (i.e., for a dilution factor of 100: \( \log 100 = 2 \))
- \( \Sigma p \) = sum of \( p \) between \( x \) and \( y \)

**Where**
- \( p \) = proportion of well infected
- \( X \) = greatest dilution at which all are infected
- \( Y \) = lowest dilution at which none are infected
The Karber formula provides and estimates the dilution factor of the original sample required to cause infection in 50% of test wells. The titre was then graphed using Graphpad software (Graphpad Prism 6, CA, USA).

2.3. RV1B infection

Beas-2B: Beas-2B cells were seeded into a 12 well tissue culture plate in duplicate at a seeding concentration of $3 \times 10^5$ cells/well, then they were incubated overnight or until 80% confluence was reached, then virus was diluted in BEBM minimal media (1%ITS) and added to the cells in a volume of 400μl at multiplicity of infection (M.O.I.) of 1. MOI 1, in this thesis, was shown to induce appropriate immune responses without causing cytopathic effects. Thus an MOI of 1 we used in all experiments. Wells containing UV-inactivated virus were also included as a control. UV inactivated virus was used at the same quantity as live virus. The plate was placed at room temperature (RT) on the orbital shaker set at 120-150 rpm for one hour. Then the virus dilutions were removed and 2ml BEBM minimal media (1%ITS) were added into each well. The plate was incubated at 33°C/5%CO₂ and then the supernatants were harvested after 24+48hrs and stored at -80°C.

pBECs: pBECs were seeded into a 24 well tissue culture plate at a seeding concentration of $1 \times 10^5$cells/well, then they were incubated overnight or until 80% confluence was reached, then virus was diluted in BEBM minimal media (1%ITS) and added to the cells in a volume of 200μl at multiplicity of infection (M.O.I.) of 1. Wells containing UV-inactivated virus were also included as a control. UV inactivated virus was used at the same quantity as live virus. To make UV inactivated virus, 5ml RV was placed under
UV light for 5-6 hour. The plate was placed at RT on the orbital shaker set at 120-150 rpm for one hour for viral attachment to the receptors. Then the virus dilutions were removed and 1ml BEBM minimal media (1%ITS) were added into each well. The plate was incubated at 33°C/5%CO₂ and then the supernatants were harvested after 4+24hrs and stored at -80°C.

2.4 Preparation of CSE extract:

The whole filter off the cigarette was cut and inserted into the 10mm end of the oxygen tube, then the cigarette was taped in place. The 5mm end of the oxygen tube was attached to a 50ml syringe. A cannula was placed into the BEBM minimal media (10ml) in a 50ml falcon tube. In fume hood, the cigarette was lit. The plunger of the syringe was slowly drawn up, then the syringe was disconnected from the oxygen tube and it was connected to the cannula. The plunger was pressed down to force the CSE to be expelled through the bubbles created in the media. The syringe was again reconnected to the oxygen tube and the mentioned steps were repeated until the cigarette was reached 1cm from the filter. Then the media which was infused with CSE was taken (100%) and appropriate dilutions of CSE were created in BEBM minimal media.

2.5. Assessment of cell viability – Annexin V-PE

The viability of BEAS-2B cells and pBECs were measured using PE Annexin V Apoptosis Detection kit I (Becton Dickinson) according to manufacturer’s instruction. This allows the assessment of cells in terms of viability, apoptosis and necrosis.
In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is transferred from the inner to the outer side of the membrane. Annexin V is a phospholipid binding protein that has a high affinity for PS. Since exposure of PS in the outer membrane occurs in the early stages of apoptosis, so Annexin V can identify apoptosis at an earlier stage.

PE Annexin V staining detects viable cells from apoptotic and necrotic cells. Thus, in this method, PE Annexin V is used in conjunction with a vital dye 7-amino-actinomycin (7-AAD). Viable cells with intact membrane exclude 7-AAD, but 7-AAD accumulates within the dead and damaged cells. Therefore, viable cells are PE Annexin V and 7-AAD negative, cells that are in early apoptosis are PE Annexin V positive and 7-AAD negative and dead cells or the cells which are in late apoptosis are both PE Annexin and 7-AAD positive.

In this assay, cells were infected with RV1B for 1hr and then were treated with 1% CSE and 0.2mM H2O2. After 24hr the cells were trypsinized off from tissue culture plate as detailed in 2.1.1. Then they were inactivated with FCS and they were washed with PBS. Then they were centrifuged at 1,200rpm for 8min and the supernatants were discarded. The cell pellets were resuspended in 1X binding buffer. They were transferred to FACS tubes and a further 1X binding buffer was added to the tubes. The samples were centrifuged at 1,200rpm for 5min, supernatants were discarded and the cell pellets were resuspended in staining solution (1X Binding Buffer containing annexin V-PE stain and vital dye 7-amino-actinomycin (7-AAD)) and incubated at RT for 15min in dark. 1X Binding Buffer was added to each tube and analysed by FACSCanto II (Becton Dickinson) within 15mins. The results were graphed using Graphpad software (Graphpad Prism 6, CA, USA).
2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a sensitive and specific assay for the detection and quantitation of antigens or antibodies. 24hr post-infection supernatant was used to measure the secretion of CXCL10, CXCL8, IL6 and IFN-λ by DuoSet (R&D Systems) ELISA kits. Duoset ELISA Development kit contains the basic components required for the development of sandwich ELISAs which measures the amount of antigen between two layers of antibodies (capture and detection antibody). In this assay a 96-well plate was coated with diluted Capture Antibody, the plate was incubated overnight at RT, the wells were washed with wash buffer and then Reagent Diluent was added to each well. The plate was incubated for a minimum of 1 hour at RT, then the wells were washed again. Samples of appropriate dilution, standards and media were added and the plate was incubated for 2hrs at RT. The aspiration/wash was repeated, diluted Detection Antibody was added into each well, the plate was incubated for 2hrs at RT. The aspiration/wash was again repeated and working dilution (1:200) of streptavidine-HRP was added, prior to incubation for 20min at RT. The aspiration/wash was repeated again and then substrate solution was added to each well, prior to incubating the plate for 20min at RT. Stop solution was added to each well, then the OD of each well was determined immediately at 450nm. The detection limit of CXCL-8, IL-6, CXCL-10 and IFN-λ were 31.3pg/ml, 9.38pg/ml, 31.3pg/ml and 62.5pg/ml respectively. The FLUOSTAR OPTIMA (BMG Labtech Pty. Ltd. Offenburg, Germany) was used to determine the optical density of each well and the dose response graphed using Graphpad software (Graphpad Prism 6, CA, USA).
2.6.1 Cytochrome c

The released level of cytochrome c from mitochondria was performed using Human cytochrome c immunoassay Quantikine kit (Abcam) and according to the manufacturer’s instructions. This assay is based on the quantitative sandwich enzyme immunoassay technique.

pBECs were seeded in appropriate tissue culture plates as described in 2.1.2. The next day the cells were infected with RV1B for 1hr and then were treated with 1%CSE and 0.2mMH$_2$O$_2$ as were optimised in BEAS-2B cells. The cells were trypsinized after 4hr. They were inactivated with FCS and were washed with PBS as detailed in 2.1.1. The cell pellets were then re-suspended in 0.5ml Cell Lysis Buffer2. The tubes were incubated for 1 hour at RT with gentle mixing and they were centrifuged at 1000xg for 15 minutes. The supernatants were then stored at ≤-70°C.

A monoclonal antibody specific for cytochrome c has been pre-coated onto a microplate. Calibrator Diluent RD5P (1X) was added into each well. Then samples of appropriate dilution, standards and media were pipetted into the wells and the plate was incubated for 2hr at RT. The wells were washed 4 times with wash buffer to remove any unbound substances. Then cytochrome c conjugate was added to the wells and the plate was incubated at RT for 2 hours. The wells were washed again 4 times with wash buffer to remove any unbound antibody-enzyme reagent. Then substrate solution was added to the wells and the plate was incubated for 30minutes at RT in dark. Colour develops in proportion to the amount of cytochrome c bound in the initial step. The colour development was stopped by adding stop solution and the intensity of the colour was measured within 30min at 450nm and correction at 540/570. The results were graphed using Graphpad software (Graphpad Prism 6, CA, USA).
2.6.2 ATP level:

The released ATP from mitochondria was measured using a bioluminescent assay kit (Abcam) according to the manufacturer’s instructions. The assay utilizes the enzyme luciferase to catalyse the formation of light from ATP and luciferin, and the light can be measured using a luminometer.

4hr post-infection supernatants were used to measure ATP level. For each sample well to be measured, reaction mix was prepared by mixing ATP Monitoring Enzyme and Nucleotide Releasing Buffer. Reaction mix was added to the appropriate wells of a 96-well plate and the background luminescence (Data A) was determined by SpectraMaxM5 luminescent. Then samples were added into each well. The luminescence was determined in a SpectraMaxM5 luminometer after 2 min (Data B). To measure ADP levels in the cells, the samples were read again (Data C), and then ADP Converting Enzyme was added into each well. The samples were read again after 2 min in the luminometer (Data D).

Data Analysis

ADP/ATP Ratio is calculated as:

\[
\frac{\text{Data D} - \text{Data C}}{\text{Data B} - \text{Data A}}
\]
2.7 RNA analysis

2.7.1 RNA harvesting and extraction

pBECs were infected with RV1B for 1hr and then were treated with 1%CSE and 0.2mM H2O2. After 4hr, the cells were lysed in 350µl of RLT buffer (buffer RLT; Qiagen, Hilden, Germany) containing 300mM β-mercaptoethanol for five minutes at RT. The lysates were then collected for RNA extraction using the Rneasy mini-kit (Qiagen) and QIAcube machine according to manufacturer’s instructions. RNA concentration was determined using the NanoDrop 2000 spectrophotometer.

2.7.2 Reverse transcription of RNA to cDNA

RNA (200ng) was reverse transcribed to total cDNA using random primers and the SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA). 10µl of the RNA was mixed with a master mix containing 2µl of 10XRT buffer, 0.8µl of 25X dNTP mix (100mM), 2µl of 10X RT random primers, 1µl of Reverse transcriptase, 1µl of Rnase inhibitor and 3.2µl of Nuclease free H2O2. The samples were incubated at 25°C for 10min, followed by 37°C for 120min and then 85°C for 5min.

2.7.3 Real-time quantitative polymerase chain reaction (RT-qPCR)

In real-time PCR, which is also called quantitative PCR, the progress of the PCR reaction is detected as it occurs in real time. The amount of generated DNA is measured after each cycle via fluorescent dyes. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to reach the threshold. The increasing fluorescent signal is in a direct proportion to the quantity of the amplified target. However, Ct
values are inversely proportional to the amount of target in the sample. In this reaction, PCR products are quantitatively synthesized from cDNA samples using the TaqMan Gene Expression Master Mix. cDNA products from the reverse transcription step were used as template for RT-qPCR. Reaction was performed in duplicate using 1µl of the cDNA, 6.25µl of 2X master mix, 0.625µl of 20X Taqman gene expression and 4.625µl of Nuclease free water. PCR primers and probes for mitochondrial transcription factors (mTFA, mTB1 and mTB2) were obtained and quantified against a stable comparator mammalian eukaryotic 18S ribosomal RNA (18S rRNA) in duplex real-time PCR (Applied Biosystems 7500; Applied Biosystems, Foster City, CA, USA) and analysed as a fold change over the media control.

The cycles performed in the RT-qPCR reaction is described below.

*Table 2.7.3.1 RT-qPCR program*

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature(°C)</th>
<th>Time(minutes)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>15sec</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Raw data was normalized by dividing the mean value of the Ct (threshold cycle) value of 18S RNA and was expressed as an exponent of 2 (2^Ct) by the mean value of 2^Ct for the target gene. The fold induction over the medium control was then calculated by dividing the normalized target value by the normalized control value. The results were graphed using Graphpad software (Graphpad Prism 6, CA, USA).
2.8 Protein analysis

2.8.1 Harvesting of whole cell lysate

MAVS cleavage, MDA5, pIRF3, TBK1, IKKe and p65 expression were assessed by Western blotting. The cells were infected with RV1B for 1hr and then were treated with 1% CSE and 0.2mM H2O2. After 4hr the cells were lysed in RIPA buffer (in-house) for 15min at RT. Proteins released from cells were protected by protease inhibitor tablets (Roche Diagnostics). The lysates were sonicated on ice (2x 10 seconds burst set at 200W output with a 20KHz converter) using the Misonix ultrasonic processor XL-2000(Qsonica,Newtown,CT).

2.8.2 Total protein concentration quantification

To determine sample protein concentrations, a Pierce BCA protein assay was conducted (Thermo Scientific, Rockford, USA). Standards made up in RIPA buffer, then 10µl of standards and samples were loaded in duplicate in a 96 well plate. Buffer A and buffer B (Thermo Scientific, Rockford, USA) were mixed and 200µl of this mixture was added into each well. The plate was placed on a plate shaker and mixed at 300rpm for 30 seconds, and then incubated at 37°C for 30 minutes. The plate was then read at absorbance of 560nm in a microplate reader (BMG Labtech) using FLUOstar OPTIMA (BMG Labtech Pty. Ltd. Offenburg, Germany). The results of this analysis were used to calculate the volume of sample required for a concentration of 5µg protein.
2.8.3 Western blot

Western blot is a widely used technique which detects specific proteins from a complex mixture of proteins in cell lysates or tissue homogenates. It uses gel electrophoresis to separate denatured proteins by the length of the polypeptide. The proteins are then transferred to a nitrocellulose or PVDF membrane, then the membrane is blocked with 5% milk to prevent from non-specific binding of proteins to membrane. The membrane is then stained with primary and secondary antibodies specific to the target protein. The bound antibodies are then detected by developing the membrane and then it is visualised using Chemi-Doc MP Imaging System.

To do the western blot, samples were prepared with 2× reducing loading buffer at a concentration of 5µg protein as a loading control. Samples were reduced on a 100°C heating block for 10 minutes and then run on a Bio-Rad mini protean 12% Bis-tris gel in the Mini-Protean Tetra Cell electrophoresis tank (Bio-Rad) using the 1XSDS running buffer. Precision Plus Dual Colour (Bio-Rad) was used as a protein standard. Proteins were then transferred to a PVDF membrane through Western blot method.

Membranes were blocked in solution of 5% skim milk powder/TBS-T for one hour and probed with appropriate concentrations of primary antibodies for overnight at 4°C. The next day they were washed in TBS-T for 10 minutes, for a total of three washes and then incubated with appropriate concentrations of the secondary antibodies in TBS-T dilution and StrepTactin HRP-conjugate for one hour at RT. Membranes were then washed three times in TBS-T for as long as possible. The membranes were developed using Super Signal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, USA) for 2 minutes and visualised using Chemi-Doc MP Imaging System.
Then blots were stripped with mild stripping buffer (in-house) and washed in PBS and TBS-T, and blocked to re-probe with β-actin antibody as a loading control. β-actin is a housekeeping protein which is found in all cell types.

Photographs of the blots were analysed by measuring band intensity using the Image Lab 4.1 software. The fold change of targets was calculated from fold change of media. The results were graphed using Graphpad software (Graphpad Prism 6, CA, USA).

**Antibodies:** For detection of MAVS, rabbit polyclonal to human MAVS (Sapphire Bioscience) was used at 1/5000 dilution as primary antibody and goat polyclonal to rabbit IgG:HRP (Abcam) was used at 1/6000 dilution as secondary antibody.

For detection of MDA5, rabbit polyclonal to human MDA5 (Sapphire Bioscience) was used at 1/4000 dilution as primary antibody and goat anti-rabbit IgG: HRP (Abcam) was used at 1/5000 dilution as secondary antibody.

β-actin: β-actin polyclonal antibody (Sapphire Bioscience) was used at 1/10000 dilution as primary antibody and goat anti-rabbit IgG: HRP (Abcam) was used at 1/20000 dilution as secondary antibody.

pIRF3: Anti-IRF3 (phosphor S386) (Sapphire Bioscience) was used at 1/4000 dilution as primary antibody and goat polyclonal to rabbit IgG:HRP (Abcam) was used respectively as secondary antibody at 1/5000 dilution.

TBK1: TBK1 (cell signalling) was used at 1/4000 dilution as primary antibody and goat polyclonal to rabbit IgG:HRP (Abcam) was used respectively as secondary antibody at 1/5000 dilution.
IKKε: IKKε (cell signalling) was used at 1/4000 dilution as primary antibody and goat polyclonal to rabbit IgG:HRP (Abcam) was used as secondary antibody at 1/5000 dilution.

P65: P65 (Abcam) was used at 1/3000 dilution as primary antibody and goat polyclonal to rabbit IgG:HRP (Abcam) was used as secondary antibody at 1/4000 dilution.

2.9 Confocal laser scanning Microscopy (CLSM):

CLSM is a technique for obtaining high resolution images. It uses a laser beam with a precise wavelength selectivity and high illumination power. In confocal microscopy, the slide is scanned by a point light source and a point detector. The lights which come from the focal point are detected and the out of focus light is rejected. Using Olympus FV 1000, images are captured point by point with fluorescent probes, including XY and Z stacks and are recorded by a computer in a three dimensional format.

2.9.1 Preparation of slides

In order to determine the co-localisation of MDA5, MAVS and mitochondria, a fluorescent double-staining procedure was undertaken. pBECs were double-stained with MAVS and MDA5 antibodies and then they were stained with Mitotracker Red FM probe (Invitrogen).

pBECs were seeded in a 6 well plate on a coverslip. The next day, the cells were infected with RV1B for 1hr at RT and then were treated with 1% CSE and 0.2mM H\textsubscript{2}O\textsubscript{2}. After 4hr, the media was removed and the cells were fixed with 3.7% paraformaldehyde at 37 °C for 15 minutes and subsequently the cells were permeablized with Tritonx-100 at RT for 5min. Then after washing with PBS, the cells were blocked with 3ml blocking buffer (1% BSA in PBS) for 30 minutes at 37°C to minimize non-specific adsorption of
the antibodies to the coverslip. After washing with PBS, the cells were incubated with the mixture of diluted primary antibodies in blocking solution for 1 hour. Isotype controls were also added at the same concentration as other primary antibodies to the corresponding wells as control. For MAVS detection, Rabbit polyclonal to MAVS as primary antibody (Abcam) and MAVS isotype control (Abcam) were used in 1:200 dilution and for MDA5 detection Mouse polyclonal to MDA5 as primary Ab (Abcam) and mouse isotype control to MDA5 (Abcam) were used in 1:200 dilution.

After washing, the cells were incubated with secondary antibodies in blocking solution. AlexaFluor 405 goat anti rabbit IgG (H+L) was used for MAVS detection and applied using a 1:200 dilution and AlexaFluor 514 goat anti Mouse IgG (H+L) was used for MDA5 detection and applied using a 1:200 dilution. The same concentrations of secondary antibodies were also added to isotype control wells, they were then incubated at RT for 1hr. After washing they were stained with Mitotracker Red FM probe (Invitrogen) at 5000nM for 30min, then the cells were washed 3X with PBS and then the coverslips were prepared for confocal microscopy using Prolong Gold antifade reagent (Invitrogen).

Coverslips were removed from the wells; any excess liquid from the coverslips was removed by tapping the side of the coverslips to a clean wipe. Then antifade reagent was added onto a clean slide and then the coverslip was mounted onto the slide. The slides were left for 24hr on a flat surface in the dark to dry. For long term storage, the edges were sealed with nail polish and they were stored upright in a box at 4°C until ready for observation under a confocal microscope.

2.9.2 Imaging

Imaging of the cells was performed using Olympus FV 1000 CLSM. Images were digitally recorded and analysed by FV10-ASW (Olympus). Within the program, red
colour channel with the wavelength of 581nm was used for mitochondria, blue channel with the wavelength of 405nm for MAVS and the green channel with the wavelength of 514nm was used for MDA5. Slides were first examined at 10X magnification and then the images were recorded at 60X magnification. The images were then quantified by Image J, co-localisation plugins and graphed using Graphpad software (Graphpad Prism 6, CA, USA).

2.10 Observation of RV1B entry into the pBECs by confocal microscopy

2.10.1 Set Up

5M NaCl

NaCl purchased from Chem Supply, 2.922g dissolved in 10mL sterile water (BAXTER water for irrigation), sterile filtered with 0.22μM PES (Millipore). Keep at 4°C.

150mM Tris HCL pH 7.2

Dissolve 0.360g of Tris base (MERCK) in 10mL sterile water (BAXTER water for irrigation), adjust pH to 7.2 with 1M HCl. Bring volume to 20mL, sterile filter with 0.22μM PES (Millipore) and keep at 4°C.

20% (w/v) BSA/DPBS

Dissolve 2g BSA (Sigma Life Sciences) in 10mL of DPBS (Hyclone DPBS/modified, Thermo Scientific) and sterile filter, keep at 4°C.

0.1% (w/v) BSA/DPBS

Dissolve 0.01g BSA (Sigma Life Sciences) in 10mL DPBS (Hyclone DPBS/modified, Thermo Scientific) and sterile filter with 0.22μM PES (Millipore). Keep at 4°C.
50% (w/v) PEG-8000

Dissolve 5g of PEG-8000 (Sigma) in 10mL DPBS using the 37°C bead bath. Ensure it is fully dissolved and then sterile filter with 0.22µM PES (Millipore) and keep at 4°C.

0.1M NaHCO₃

Prepare a 1M sodium bicarbonate solution by adding 1mL deionized water to the vial of sodium bicarbonate provided in the kit. Pipette up and down until fully dissolved. This solution may be stored at 2-6°C for up to two weeks or frozen for long term storage. To make 0.1M solution, add 10uL of 1M NaHCO₃ to 90uL of DPBS.

2.10.2 RV1B Purification and labelling

In order to visualise the RV1B entry into the pBECs, 24ml of RV1B stock was centrifuged at 5250g at 4°C for 60 minutes. The clarified supernatant was transferred to new 50mL centrifuge tubes, then the pellet was discarded. The clarified virus was transferred into Amicon Ultra- 15 100K centrifugal filter devices. 12mL of sample was added into the tube and then it was centrifuged at 4000xg at 4°C for 10 minutes. Then this procedure was repeated again for 5 minutes until only 500uL to 1mL is left. The flow through was retrieved and it was spined through again. 10mL of PBS was added and it was resuspended gently with a 1mL pipette. It was re-centrifuged again until the solution is the clarity of PBS alone. This took 3 spins X 10 minutes. Then the concentrated virus was transferred into a 10mL centrifuge tube. 1 volume of 50% PEG-8000 was added to 4 volumes of concentrated virus- containing supernatant. This was initial volume of virus-containing
supernatant divided by 4 = the volume of 50% PEG-8000 required. Half this volume of 5M NaCl was added to the supernatant. 4% of this volume of 20% BSA was added to the supernatant. It was wrapped in parafilm and incubated at room temperature for 1.5 hours. Then it was centrifuged at 4000xg for 30 minutes at 4°C and then the pellet was resuspended completely in 1mL of DPBS and was transferred into 4mL tube.

2.10.3 Fluorescent Labelling

One vial of AlexaFluor 555 carboxysuccinimidyl ester was dissolved in 10uL of DMSO. Then this was added to 1mL of purified virus in the 4mL tube. It was mixed well with 100uL of 0.1M NaHCO₃ and hold at 4°C for 1 hr, then it was placed in the cold room on the rocker for one hour. Then 1mL of 150mM Tris-HCL pH 7.2 was added to quench the reaction and it was held another 1 hr at 4°C to completely quench, in the cold room on the rocker. The volume was brought to exactly 4mL with DPBS and was transferred to a 15mL conical centrifuge tube before proceeding.

Then 380uL 5M NaCl, 20uL 20% BSA and 600uL 50% PEG-8000 were added and well mixed, then incubated at 4°C for overnight. The next day, the sides of the tube were washed down to remove all residues so that they are in liquid, then it was centrifuged at 2000xg at 4°C for 30 minutes. The 0.1%BSA/DPBS was sterile filtered. Then all the fluid over the visibly coloured pellet was carefully removed and then resuspended in 200uL of 0.1% BSA/DPBS.
2.10.4 Pre-treatment, Infection and secondary treatment

Healthy pBECs were seeded in an 8well chamber slide at $1.5 \times 10^4$ cells/well. The next day, the cells were pre-treated with 2.5µg/ml lycopene and 0.5mM l-carnitine for 4hr at 37°C/5%CO₂ as described in 2.10. Then they were infected with RV1B for 1hr at RT. Then the supernatants were removed and the cells were treated with 1% CSE and 0.2mM H₂O₂ for 2hr at 33°C/5%CO₂. Then the cells were fixed with 4% paraformaldehyde by adding 500µl of fixative solution into each well and then incubated for 10min at RT. Then the wells were washed with PBS three times for 5min, then the slides were mounted with coverslips by adding 10µl/well of Prolong gold antifade mounting media with DAPI. The next day, the slides were sealed with nail polish and they were stored at 4°C until visualise under a confocal microscope as described in 2.9.2. The cells were stained in blue and the tagged RV1B was stained in red.

2.11. Preparing medium containing lycopene and l-carnitine

2.11.1 Preparing medium containing lycopene-THF:

The stock solution of lycopene/THF was prepared by dissolving 1mg of lycopene (Sigma) in 2mL of tetrahydrofuran (THF) (Sigma). The appropriate amount of this stock solution was added to BEBM minimal medium to achieve 0.5% THF/lycopene containing medium (2.5µg/ml). Then as a control, similar percentages of THF (with no lycopene) were added to BEBM minimal medium.
2.11.2 Preparing medium containing L-carnitine:

The stock solution of 0.1M L-carnitine was prepared by dissolving 0.11g L-carnitine to 5ml BEBM minimal medium. The appropriate amount of this stock solution was added to BEBM minimal medium to achieve 0.1mM, 0.5mM and 1mM L-carnitine in BEBM minimal medium.

2.12 Lycopene enrichment of cultured airway epithelial cells with lycopene and L-carnitine:

Confluent pBECs were enriched with 2.5µg/ml and 0.5mM lycopene and L-carnitine respectively for 4hr, then they were infected with RV1B for 1hr at RT. The infected cells were then treated with 1%CSE and 0.2mMH2O2. The cells and supernatants were then collected after 4hr and 24 hr.

2.13 Data analysis

All results of Beas-2B cells were obtained from three independent experiments and the results of pBECs were obtained from 4 asthmatics and 4 healthy subjects in chapter 4 and 5 asthmatics and 5 healthy subjects in chapter 5. The subjects in chapter 5 are different from the subjects of chapter 4.

The statistical analysis program Prism version 6 was used to analyse data. Statistical significance was assessed by either paired t-test or one-way analysis of variance (ANOVA) to compare mean values followed by Bonferroni’s multiple comparisons test. Two-way analysis of variance (ANOVA) was used to compare the healthy groups and the asthmatic groups. A significant result was determined as p<0.05. When data are not normally distributed (Beas-2B cells), were analysed using nonparametric equivalents and multiple comparisons first analysed by the Kruskal-Wallis test and then by individual testing if significant. The results
of RV1B replication, were presented as mean ± standard error of mean (SEM) for 9 healthy subjects (n=9) with two replicates for each subject and 9 asthmatic subjects (n=9) with two replicates for each subject, for all MOI 1 data.

A one-way and two-way Anova followed by Bonferroni’s multiple comparisons test were performed on mRNA relative fold change. Ct values of the samples were compared with media alone and the results were expressed as fold change. Densitometry in western blotting was performed and the values were expressed as protein/β-actine ratio for MAVS, MDA5, pIRF3, TBK1 and IKKε and were presented as fold induction from medium control. Differences between lycopene and carnitine supplemented and unsupplemented groups were analysed using one-way and two-way Anova followed by Bonferroni’s multiple comparisons test.

2.14 Ethics approval:

Project involving in human participants in this thesis was approved by University of Newcastle Ethics Committee (Approval Reference No: H-163-1205) and Hunter New England Area Research Ethics Committee (Approval Reference No: 05/08/10/3.09). All of the participants gave written informed consent to participate in the study.
Chapter 3

Oxidative stress impairs mitochondrial function and leads to deficient antiviral responses to rhinovirus in healthy primary bronchial epithelial cells
3.1 Introduction

Oxidative stress is implicated in the progression of many diseases processes. Oxidative stress describes the damage that occurs when the amount of ROS present, overwhelm the antioxidant defences of the host [61]. Both endogenous and exogenous sources can lead to oxidative stress. Endogenous production of ROS can occur when activated inflammatory cells respond with a ‘respiratory burst’, which involves the uptake of oxygen and subsequent release of ROS into surrounding cells [44]. Exogenous production of ROS can occur in response to exposures such as cigarette smoke, ozone, or ionising radiation [53]. ROS, such as the superoxide (O$_2^-$) and hydroxyl (OH') ions, are highly reactive molecules which have unpaired electrons and tend to interact with other molecules in different tissues to gain a stable configuration of electrons [38]. As a result, ROS can damage proteins, lipids, nucleic acids and carbohydrates [38]. Furthermore, ROS can interact with fatty acids in cellular membranes, including the mitochondrial membrane [270, 271]. This can cause mitochondrial damage and impaired mitochondrial function [272, 273]. It has been shown that mitochondria are not only the major sources of endogenous ROS but also play a significant role in antiviral defence, via induction of innate immune responses through the cytoplasmic protein melanoma differentiation-associated gene 5 (MDA5), mitochondrial antiviral signalling protein (MAVS), and downstream activation of type I and type III IFNs [153, 274, 275]. RNA helicases such as MDA5 and RIG-I are present in the cytosol and detect viral dsRNA and ssRNA. After the RV injects its genome by pore formation into the host cell, its positive strand RNA genome is used as a template to produce a negative-sense RNA strand and then it replicates as rapidly as possible. Single strand (ss)RNA converts to a double strand (ds)RNA to form progeny viruses. The 5´ end of the dsRNA produced during replication may be recognized by the host pattern recognition receptors such as MDA5. Once the attachment of viral RNA to MDA5 is complete, they
translocate to the mitochondrial surface and interact with the MAVS protein [117]. MAVS has a crucial role in defence against virus infection, through the recognition and inhibition of viral replication in the cytosol of different cells [276]. MAVS is also required for the downstream activation of TBK1, IKKe, IRF3 and antiviral type I IFNs [153]. The function of MAVS is dependent on its mitochondrial localization. Thus, cleavage or suppression of MAVS expression inhibits interferon production and increases viral replication [277].

The lungs are exposed to numerous environmental toxins, including particulate air pollution and tobacco smoke. Tobacco smoke is a potent source of exogenous oxidants; composed of 4,000 chemicals, such as nitrosamines, carbon monoxide and metal oxides, which produce ROS, such as superoxide and hydroxyl radicals. These free radicals react with antioxidants and they are able to damage airway epithelial cell membranes [278, 279]. Moreover, tobacco smoke components can accumulate in the mitochondria and damage electron transport chain (ETC) function, thereby affecting ATP production [280]. A number of studies have shown the combined effects of tobacco smoke and rhinovirus to immune responses. It has been shown that tobacco smoke can modulate the epithelial cell responses to RV infection [281, 282]. Tobacco smoke can increase the risk of wheezing and airway respiratory infections by affecting the host immune defence. Gaseous components of tobacco smoke such as nitrogen dioxide, ammonia, cyanides, aldehydes and ketones affect immune defence and mucociliary apparatus leading to reduced efficacy of host immune system to defend against bacterial or viral pathogens. This can lead to inflammation which can worsen the infection as the pathogens can further damage inflamed and narrowed airways [283].

Epithelial cells are the initial site of virus entry; as such their response is important for an effective innate immune response. It has been revealed that the endogenous epithelial protective factors, such as heat shock protein 27, labile Zn, superoxide dismutase are reduced in asthmatics in comparison with the healthy individuals, so the epithelium may be more
susceptible to oxidative damage and apoptosis [133]. Based on these observations, our aim was to assess whether exposure to oxidative stress in the form of CSE or H$_2$O$_2$ is associated with mitochondrial damage, impairs type I and type III IFN responses and increases RV-induced inflammation and RV replication in healthy pBECs.

**3.2. Materials and Methods:**

Beas-2B cell and pBEC viability were measured by Annexin V-PE (methods described in chapter 2). Using ELISA the concentration of CXCL10, CXCL8, IL6, IFN-λ and cytochrome c were measured (methods described in chapter 2). ATP levels were measured by bioluminescent assay (methods described in chapter 2). Mitochondrial membrane integrity, co-localisation of MAVS and MDA5 as well as RV1B entry were observed by confocal microscopy. Cleavage of MAVS, expression of MDA5, pIRF3, TBK1, IKKe and P65 were measured by western blotting (methods described in chapter 2). RV1B replication was measured by TCID50 (methods described in chapter 2). Gene expression of mitochondrial transcription factors was measured by Real-time PCR (methods described in chapter 2).

Optimisation of the mitochondrial function experiments (mTFs, cytochrome c and ATP) were performed in Beas-2B cells at 4hr and 24hr time-points and then all these experiments were performed in Beas-2B cells three times separately (n=3) at 4hr to see the effects of oxidative stress on mitochondrial function as 24hr time-point seemed to be a late time-point for the effects of oxidative stress on mitochondrial function. The measurements of inflammatory response (CXCL8 and IL6) and antiviral responses (CXCL10 and IFN-λ) were performed at 24hr as the release of cytokines or antiviral responses occur at 24hr. The measurement of RV replication was measured at 24hr and 48hr. Measurement of the Beas-2B cells viability were performed at 24hr.
All the mitochondrial function experiments were then assessed again in pBECs of 4 different healthy subjects at 4hr. pBECs cells viability, CXCL-8, IL-6, CXCL-10, IFN-λ and RV replication were assessed at 24hr in pBECs.

3.3 Results

Determining the dose response in Beas-2B cells

Beas-2B cells were infected with RV1B at MOI of 0.01, 0.1, 1, 5 and 10 at 24hr and 48hr to choose the most effective MOI based on the dose responses by measuring CXCL10 and CXCL8. Figure 3.3.1a shows that MOI 1 was the highest with a drop off of CXCL10 with MOI 5 and 10. Figure 3.3.1b shows that MOI 1 was the optimal value in CXCL8 as well as for our future experiments. The concentration of RV1B at MOI 1 was 31.25µl and the RV1B concentration at MOI 5 and 10 which led to cell death was 156.25µl and 312.5µl respectively.
Figure 3.3.1 Beas-2B cell dose responses: a) CXCL10 and b) CXCL8; measured at 24 and 48 hours
Determining the viability of Beas-2B cells by annexin V-PE:

Beas-2B cells were infected with RV1B at an MOI of 1 and were then treated with H$_2$O$_2$ (0.1mM, 0.2mM) and CSE (0.5%, 1%). The concentrations of H$_2$O$_2$ and CSE were chosen based on their potential to induce immune responses, while maintaining cell viability in the Beas-2B cell line. Cell viability was measured by annexin V-PE after 24hr. Figure 3.3.2 shows that there are no significant changes in the viability of Beas-2B cells exposed to CSE/H$_2$O$_2$ compared to media. However, there are more necrotic cells in cells exposed to RV1B, RV+CSE and RV+H$_2$O$_2$ compared to media. We will choose higher doses of CSE and H$_2$O$_2$ for our experiments.

Figure 3.3.2. Beas-2B cell viability when exposed to oxidative stressors: a) CSE, RVIB and RV+CSE and b) H$_2$O$_2$, RVIB and RVIB+H$_2$O$_2$; measured at 24 hours
Determining the viability of healthy pBECs by annexin V-PE:
pBECs were infected with RV1B at an MOI of 1 and were then treated with H₂O₂ (0.2mM) and CSE (1%) and then the viability was measured by annexin V-PE after 24hr. The concentrations of H₂O₂ and CSE were chosen based on their potential to induce immune responses, while maintaining cell viability in the Beas-2B cell line. Figure 3.3.3 shows that there are no significant changes in the pBECs viability in all conditions compared to media.
Figure 3.3.3 pBEC viability to the oxidative stressors; RV, CSE and H$_2$O$_2$; measured at 24 hours

The effect of oxidative stress on mitochondrial transcription factors in Beas-2B cells

Beas-2B cells were infected with RV1B at an MOI of 1 and were then treated with CSE (1%, 0.5%) and H$_2$O$_2$ (0.2mM, 0.1mM). Figure 3.3.4(a-c) show that the mRNA expression levels of mTFA, mTB1 and mTB2 are increased cells exposed to the combination of RV1B and 1% CSE compared to media. However, there are no changes in other conditions compared to media. In figure 3.3.4(d-f), RV+0.2mM H$_2$O$_2$ increased the mRNA expression of TFA compared to media, while RV+0.2mM H$_2$O$_2$ and RV+0.1mM H$_2$O$_2$ both increased mRNA expression of TB1 and TB2 compared to media.
Figure 3.3.4 The effect of the oxidative stressors, a-c) RV, CSE and RV+CSE and d-f) RV, H\textsubscript{2}O\textsubscript{2} and RV+H\textsubscript{2}O\textsubscript{2}, on the mRNA expression of mitochondrial transcription factors, TFA, TB1 and TB2, in Beas-2B cells; measured at 4 hours.
The effect of oxidative stress on mitochondrial transcription factors in healthy pBECs

pBECs from healthy non-smokers were infected with RV1B at an MOI of 1 and were then treated with 1% CSE and 0.2mM H2O2. Figure 3.3.5(a-b) show that the combinations of RV+CSE and RV+H2O2 increased mRNA expression of mTFA and mTB1 compared to media, while in Figure 3.3.5c, the combination of RV+CSE led to an increased level of mRNA expression of TB2 compared to media.
Figure 3.3.5 The effect of the oxidative stressors, RV, CSE and H$_2$O$_2$, on the mRNA expression of mitochondrial transcription factors, a) TFA, b) TB1 and c) TB2, in healthy pBECs; measured at 4 hours

a)

![TFA diagram]

b)

![TB1 diagram]
The effect of oxidative stress on the release of cytochrome c in Beas-2B cells

Figure 3.3.6(a,b) show that while CSE alone and H₂O₂ alone did not induce the release of cytochrome c into the cytosol, RV, RV+CSE and RV+H₂O₂ resulted in the increased release of cytochrome c into the cytosol compared to media. Furthermore, RV+1%CSE and RV+H₂O₂ (0.2mM and 0.1mM) led to a further release of cytochrome c into the cytosol compared to RV alone.
Figure 3.3.6 The effect of the oxidative stressors, a) RV, CSE and RV+CSE and b) RV, $H_2O_2$ and RV+$H_2O_2$ on the release of cytochrome c from mitochondria in Beas-2B cells; measured at 4 hours.
The effect of oxidative stress on the release of cytochrome c in healthy pBECs

Figure 3.3.7 shows that while CSE alone and H$_2$O$_2$ alone didn’t induce the release of cytochrome c, RV, RV+CSE and RV+H$_2$O$_2$ resulted in a significant release of cytochrome c into the cytosol compared to media. Furthermore, RV+H$_2$O$_2$ led to a further release of cytochrome c compared to RV alone.

**Figure 3.3.7 The release of cytochrome c from mitochondria following exposure to RV, CSE and H$_2$O$_2$ in pBECs of healthy subjects; measured at 4 hours**

**ATP release from Beas-2B cells following exposure to oxidative stress**

Figure 3.3.8a) shows that 1%CSE alone, RV alone and RV+CSE induced the release of ATP from mitochondria into the cytosol. Figure 3.3.9b) shows that 0.2mM H$_2$O$_2$ alone and RV+0.2mM H$_2$O$_2$ resulted in the release of ATP from mitochondria.
Figure 3.3.8 The release of ATP from mitochondria in Beas-2B cells following exposure to a) RV, CSE and RV+CSE and b) RV, \(H_2O_2\) and RV+\(H_2O_2\), measured at 4 hours.
ATP release from healthy pBECs following exposure to oxidative stress

Figure 3.3.9 shows that CSE alone as well as RV+CSE and RV+H\textsubscript{2}O\textsubscript{2} induced the release of ATP from mitochondria into the cytosol. Furthermore, RV+CSE resulted in a further release of ATP compared to RV alone.

*Figure 3.3.9 The release of ATP from mitochondria in pBECs following exposure to the oxidative stressors, RV, CSE and H\textsubscript{2}O\textsubscript{2}; measured at 4 hours.*
MAVS cleavage and MDA5 expression in Beas-2B cells, exposed to oxidative stress

MAVS cleavage and MDA5 expression were determined in Beas-2B cells by western blot. Densitometry was measured and compared with β-actin control and expressed as a fold change compared to media. Figure 3.3.10a) shows that all conditions led to a partial cleavage of MAVS, however, CSE/H2O2 had no further effect on cleavage of MAVS or MDA-5 expression (Figure 3.3.10b).

Figure 3.3.10 a) MAVS cleavage and b) MDA5 expression in Beas-2B cells following exposure to the oxidative stressors, a) RV, CSE, H2O2, RV+CSE and RV+H2O2 and b) RV, CSE, H2O2, RV+CSE and RV+H2O2; measured at 4 hours.
b) 

<table>
<thead>
<tr>
<th>Condition</th>
<th>Media</th>
<th>0.5% CSE</th>
<th>1% CSE</th>
<th>RV-1B</th>
<th>RV-1B+0.5% CSE</th>
<th>RV-1B+1% CSE</th>
<th>UV</th>
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<tr>
<td>MDA5</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fold change from media</td>
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<td>1.00</td>
<td>1.00</td>
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<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**B-actin**

**MDA5**

- Media
- 0.5% CSE
- 1% CSE
- RV-1B
- RV-1B+0.5% CSE
- RV-1B+1% CSE
- UV

**Fold change from media**

- 0.1 mM H_2O_2
- 0.2 mM H_2O_2
- RV-1B
- RV-1B+0.1 mM H_2O_2
- RV-1B+0.2 mM H_2O_2
- UV
MAVS cleavage and MDA5 expression in pBECs following exposure to oxidative stress

MAVS cleavage and MDA5 expression were determined by western blot. Densitometry was measured and compared with β-actin control and expressed as a fold change compared to media. Figure 3.3.1a) shows that all conditions led to a partial cleavage of MAVS, however CSE, H2O2 exposure had no further effect on cleavage of MAVS or MDA5 expression (Figure 3.3.1b).

Figure 3.3.11a) MAVS cleavage and b) MDA5 expression in pBECs following exposure to the oxidative stressors, a) RV, CSE, H2O2, RV+CSE and RV+H2O2 and b) RV, CSE, H2O2, RV+CSE and RV+H2O2; measured at 4 hours.

a)
b) MDA5

Fold change from media

<table>
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<th>Condition</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>1% CSE</td>
<td>1.2</td>
</tr>
<tr>
<td>0.2 mM H₂O₂</td>
<td>1.1</td>
</tr>
<tr>
<td>RV1B</td>
<td>1.3</td>
</tr>
<tr>
<td>RV1B + 1% CSE</td>
<td>1.4</td>
</tr>
<tr>
<td>RV1B + 0.2 mM H₂O₂</td>
<td>1.5</td>
</tr>
<tr>
<td>UV</td>
<td>0.8</td>
</tr>
</tbody>
</table>

B-actin
Co-localisation of MDA5, MAVS and mitochondria in healthy pBECs by confocal microscopy

Co-localisation of MAVS and MDA5 were detected in healthy pBECs by confocal microscopy. Figure 3.3.12b) shows that there is more MAVS in the CSE compared to media. Also, figure 3.3.12 c) shows that there is no difference in MDA5 in all conditions compared to media. However, figure 3.3.12 d) shows that mitochondrial membrane integrity impaired in RV+CSE compared to media and figure e) shows that the co-localisation of MAVS, MDA5 and mitochondria decreased in the RV+CSE compared to media.
Figure 3.3.12 Co-localisation of MAVS, MDA5 and mitochondria in pBECs, following exposure to the oxidative stressors, RV, CSE and H$_2$O$_2$; measured at 4 hours. White arrows show the co-localisation of MAVS/MDA5 and mitochondria.
Mitochondrial membrane integrity

Colocalisation of MAVS, MDA5 and mitochondria
pIRF3, TBK1, IKKε and p65 expression in healthy pBECs following exposure to oxidative stress

4hr protein lysates were used to determine pIRF3, TBK1, IKKε and p65 expression from 3 different healthy subjects by western blot. Densitometry was measured and compared with β-actin control and expressed as a fold change compared to media. Figures 3.3.13 shows that CSE/H₂O₂ had no effect on pIRF3, TBK1, IKKε and p65 expression.

Figure 3.3.13 a) pIRF3, b) TBK1, c) IKKε, d) P65 expression following exposure to RV, CSE and H₂O₂ in healthy pBECs; measured at 4 hours

a)
b)

**TBK1**

**B-actin**

---

**TBK1**

**Fold change from media**

- Media
- 1% CSE
- RV1B+0.2m
- RV1B+1% CSE
- UV
c) 

IKKɛ 

B-actin 

Fold change from media
CXCL8 release from Beas-2B cells following exposure to oxidative stress

24hr supernatants were used to measure CXCL8 in Beas-2B cells. Figure 3.3.14 (a,b) show that CSE/H$_2$O$_2$ alone did not increase the level of CXCL8 compared to media. However, RV, RV+CSE and RV+H$_2$O$_2$ increased the release of CXCL8 compared to media in Beas-2B cells.
Figure 3.3.14 CXCL8 release in Beas-2B cells following exposure to a) RV, CSE and RV+CSE and b) RV, H₂O₂ and RV+H₂O₂; measured at 24 hours

a)

![Graph a)

b)

![Graph b)
**CXCL8 release from pBECs following exposure to oxidative stress**

24hr supernatants were used to measure CXCL8 in pBECs. Figure 3.3.15 shows that CSE/H$_2$O$_2$ alone did not increase the level of CXCL8 compared to media. However, RV, RV+CSE and RV+H$_2$O$_2$ increased the release of CXCL8 compared to media inducing inflammatory responses.

*Figure 3.3.15 CXCL8 release in pBECs following exposure to the oxidative stressors, RV, CSE and H$_2$O$_2$, measured at 24 hours*
IL6 release from pBECs following exposure to oxidative stress

24hr supernatants were used to measure IL6 in pBECs. Figure 3.3.16 shows that CSE/H₂O₂ and RV alone did not increase the level of IL6 compared to media. However, RV+CSE and RV+H₂O₂ increased the release of IL6 compared to media inducing inflammatory responses.

Figure 3.3.16 IL6 release in pBECs following exposure to the oxidative stressors, RV, CSE and H₂O₂, measured at 24 hours

CXCL10 response in Beas-2B cells following exposure to oxidative stress

Figure 3.3.17,a) shows that there is a marked reduction of CXCL10 in RV1B+1%CSE, RV1B+0.5%CSE and RV1B+0.2mM of H₂O₂ compared to RV1B alone. This suggests that CSE and higher doses of H₂O₂ result in the suppression of CXCL10 release compared to RV1B alone.
Figure 3.3.17 CXCL10 release in Beas-2B cells exposed to the oxidative stressors, a) RV, CSE and RV+CSE and b) RV, H$_2$O$_2$ and RV+H$_2$O$_2$; measured at 24 hours
CXCL10 response in pBECs following exposure to oxidative stress

Figure 3.3.18 shows that RV infection increased the release of CXCL10 compared to media. However, if the cells were infected with RV prior to CSE or H$_2$O$_2$ exposure, there was a marked reduction in CXCL-10 compared to RV alone. Thus, exposure to oxidative stress resulted in a specific suppression of the release of type I IFN stimulated protein (CXCL10).
Figure 3.3.18 CXCL10 release in pBECs exposed to the oxidative stressors, RV, CSE and H$_2$O$_2$; measured at 24 hours.

![CXCL10 concentration graph](image)

**IFN-λ response in pBECs following exposure to oxidative stress**

Figure 3.3.19 shows that RV infection increased the release of IFN-λ compared to media. However, if the cells were infected with RV prior to CSE exposure, there was a marked reduction in IFN-λ response compared to RV alone. Thus, exposure to oxidative stress resulted in a specific suppression of the release of type III IFN (IFN-λ1/3).
Figure 3.3.19 IFN-λ release in pBECs exposed to the oxidative stressors, RV, CSE and H₂O₂; measured at 24 hours

![IFN-λ release bar chart](image)

**RV1B replication in Beas-2B cells exposed to oxidative stress**

RV replication was measured by TCID50 in Beas-2B cells after 24 and 48hr. Figure 3.3.20 shows that there are no significant changes of RV replication in all conditions compared to RV alone.
Figure 3.3.20 RV1B replication in Beas-2B cells following exposure to the oxidative stressors, CSE and H$_2$O$_2$, measured at a) 24 hours and b) 48 hours

a)

![RV1B TCID50/ml 24hr graph](image)

b)

![RV1B TCID50/ml 48hr graph](image)
**RV1B replication in pBECs exposed to oxidative stress**

As there was no difference in RV replication in Beas-2B cells after 24hr and 48hr, so in the next step, RV replication was measured by TCID50 in pBECs of 4 different healthy subjects after 24hr. Figure 3.3.21 shows that RV+CSE led to an elevated level of viral replication compared to RV alone. However, there is no significant difference of RV replication in RV+H2O2 compared to RV alone.

*Figure 3.3.21 RV1B replication in pBECs following exposure to the oxidative stressors, CSE and H2O2; measured at 24 hours*
3.4 Discussion

In this study, we have investigated the role of oxidative stress, in the form of CSE and H$_2$O$_2$, on mitochondrial function and innate immune responses following RV infection in healthy pBECs. Using pBECs from healthy subjects, we have demonstrated that CSE/H$_2$O$_2$ lead to mitochondrial dysfunction, reflected by impaired mitochondrial membrane integrity, increased release of cytochrome c, ATP and increased expression of mitochondrial transcription factors. This corresponded with an abnormal innate immune response to RV1B infection. However, there was no difference of MAVS cleavage or MDA5 expression in all conditions.

Two sensors for viral RNA have been detected, MAVS and MDA5. MAVS and MDA5 are two cytoplasmic proteins which have significant roles in defence against viruses and initiation of antiviral responses. MDA5 which is also known as interferon-induced helicase C domain1 (IFIH1) is a cytoplasmic receptor which belongs to RNA helicases in pathogen recognition receptor (PRRs) family and plays a significant role in detection of viral nucleic acids through its helicase domain as well as initiation of signalling pathways through its caspase recruitment domain (CARD) [140]. The helicase domain possesses ATPase activity which binds to the 5’of dsRNA and then transmits signals through the CARD domain, which subsequently activates interferon response factor 3 (IRF-3) [149]. MAVS which is located on the outer membrane of mitochondria is also important in defence against virus infection. It consists of an N-terminal CARD-like domain, a C-terminal transmembrane domain and a proline-rich region [154]. The CARD-like domain leads to interferon induction. It activates the kinases, IKKε and TBK1 which are required for the phosphorylation of IRF3. pIRF3 then dimerizes and translocates to the nucleus leading to the activation of type I IFNs [284]. Similarly the C-terminal transmembrane domain attaches to the mitochondrial outer membrane [154]. The interaction of the proline rich region with several signalling molecules
like TNF receptor associated factor (TRAF)-6, TRAF-2, TRAF-3, receptor interacting protein 1 (RIP1) and Fas-associated protein with death domain (FADD), indicates the role of MAVS in TLR3-mediated pathways, in addition to RIG-I/MDA5-mediated signalling [155]. Once the attachment of viral dsRNA to MDA5 is complete, they translocate to the mitochondrial surface and interact with the MAVS protein. MAVS has a crucial role in defence against virus infection, through the recognition and inhibition of viral replication in the cytosol of different cells and its function is dependent on its mitochondrial localisation [276]. Scott, et al. [157] have already demonstrated that several viruses such as hepatitis A (HAV) and hepatitis C (HCV) cleave MAVS from the outer mitochondrial membrane. Drahos et al. [284] has also shown that RV1A can cleave MAVS leading to an impaired type I IFN response [284]. However, in this study we have demonstrated that exposure of healthy pBECs to RV1B, CSE and H2O2 had no effect on the further cleavage of MAVS. In our study, CSE alone resulted in mitochondrial damage; with increased release of ATP, while, H2O2 alone didn’t induce mitochondrial damage.

We observed that RV+CSE impaired mitochondrial membrane integrity and the co-localisation between MAVS, MDA5 and mitochondria. Also, RV+CSE/H2O2 further increased the release of ATP and the expression of mTFs which were greater in RV+CSE as well as the release of cytochrome-c which was greater in RV+H2O2. These results suggest that there is a greater effect between exposure to ROS in the form of CSE/H2O2 and viral infection that induces further mitochondrial dysfunction and may lead to impaired immune responses. However, RV, CSE and H2O2 had no effect on the signalling pathway through MDA5 and the downstream signalling pathways through the kinases TBK1, IKKe, pIRF3 and p65 expression. Exposure of ROS to healthy pBECs demonstrated impaired IFN responses with, reduced CXCL-10 and IFN-λ and an increase in CXCL8 and IL6 release. This led to an increase in
RV replication particularly with CSE in response to deficient antiviral responses. Thus, it appears that CSE is more specific and therefore a more effective oxidative stress in healthy pBECs. The comparison of Beas-2B cell lines and healthy pBECs showed that pBECs are more susceptible to the effects of oxidative stress compared to Beas-2B cells in terms of increased expression of mitochondrial transcription factors, the release of cytochrome c and ATP. However, the western blotting results showed that the cleavage of MAVS or the protein expression of MDA5 was not different in pBECs and Beas-2B cells. Inflammatory responses, antiviral responses and RV replication were also compared in pBECs and Beas-2B cells. The results showed that pBECs have more reduced antiviral responses which led to increased RV replication compared to Beas-2B cell. However, inflammatory responses were not different in pBECs and Beas-2B cells.

Studies have suggested an association between exposure to tobacco smoke and respiratory symptoms during childhood [285]. Studies have shown that there might be a relation between parental smoking and lower respiratory infections in children, while, viral upper respiratory tract infections (URTIs) are implicated in acute exacerbations of asthma with a rate of 80-85% in school age children [286]. Studies have shown that there is an association of parental smoking, particularly maternal smoking, with asthma severity. Tobacco smoke increases the prevalence of wheezing and cough in early life children because of the immaturity of their immune system and low levels of lung function during childhood, however, there is a greater risk of hospitalisation in children when exposed to smoking households due to severe symptoms [285, 287-290]. There are some possible mechanisms in which tobacco smoke can cause asthma exacerbations, such as increased levels of neutrophils, decreased levels of eosinophils, increased transcription of pro-inflammatory genes such as NF-kB and increased resistance to corticosteroids by changes in the glucocorticoid receptor α and β ratio [286, 291, 292].
Taken together, these results suggest that mitochondrial dysfunction which is caused by oxidative stress is likely to be an important factor impairing immune responses to RV infection [184]. However, there are still some complications about ROS due to their capability to act in many pathways and in many ways simultaneously, thus additional studies are needed to further investigate the exact mechanisms which lead to mitochondrial dysfunction in healthy pBECs. This will help to establish new therapeutic strategies which can be targeted to the mitochondria, to prevent mitochondrial oxidative damage.
Chapter 4

Comparing the effects of oxidative stress on mitochondrial function and antiviral responses in primary bronchial epithelial cells of asthmatics and non-asthmatics
4.1. Introduction:

Asthma is a chronic inflammatory airway disease. It has been shown that numerous cell types and inflammatory mediators are involved in the pathogenesis of asthma, with endogenous or exogenous ROS/RNS playing an important role [293]. RVs are among the most frequent triggers of acute asthma exacerbations. They are known to increase intracellular oxidative stress [104, 105] which can exert severe lower-airway dysfunction in inflammatory respiratory disorders such as asthma [271]. Asthmatics are more likely to have significant clinical symptoms of lower respiratory tract infection. Studies have revealed that pBECs of asthmatics are more susceptible to RV infections as they are not able to release effective type I (interferon-β) and type III (interferon λ1/3) responses to RV infection [294]. The mechanism of this impaired immune response and increased susceptibility to RV infection is unknown, but appears to involve a host innate immune defect in the airway epithelium which can cause acute exacerbations of asthma [294].

Human rhinovirus (HRV), a member of Picornaviridae family, is a non-enveloped virus and it has a positive sense single strand RNA virus with a genome length of 7.2 to 8.5 kb [110]. The point of HRV entry and initial site of infection is the upper respiratory tract [116]. After binding to the relevant receptor the viral RNA enters the cytoplasm of airway epithelial cells (AECs) and initiates innate immune responses [295]. Viral infection in AECs leads to IFN-β production which promotes apoptosis of infected cells and inhibits virus replication. In contrast, impaired IFN-β production and cell apoptosis leads to greater viral replication, which can cause cell necrosis and release of inflammatory mediators [294]. Furthermore, IFN-λs (IL-λ1-3, IL-28A/B and IL-29) are a novel group of cytokines which are secreted by plasmacytoid, dendritic and epithelial cells. These IFNs have type I IFN-like antiviral activities and can inhibit virus replication by signalling through a receptor complex comprising of IL-10Rβ and the IL-28Rα chains[296].
Pathogen recognition receptors (PRR), like toll-like receptors (TLRs) and the RNA helicases MDA5 and RIG-I, detect viral nucleic acids. TLR3 which is located within intracellular endosomes of BECs, dendritic cells (DCs), macrophages and lymphocytes can detect dsRNA and initiate downstream signalling pathways by activation of specific kinases [144, 297]. TBK1 and IKK-i (also known as IKKe) are responsible for the phosphorylation of IRF-3, leading to phosphorylation of the IRF3 at some specific serine sites in its C-terminal regulatory domain which forms a dimer and then translocates to the nucleus which is necessary for expression of type I IFNs, CXCL10 and inflammatory cytokines [147].

The RNA helicases, such as MDA5 and RIG-I, detect viral dsRNA and single stranded RNA [140, 274]. After the viral RNA enters the cytoplasm of AEC, it binds to MDA5. Once the attachment of viral RNA to MDA5 is complete, they translocate to the mitochondrial surface and interact with the MAVS protein. MAVS has a crucial role in defence against virus infection, through the recognition and inhibition of viral replication in the cytosol of different cells [276]. Association of MAVS and MDA5 is required for the downstream activation of TBK1, IKKe, IRF3 and antiviral type I and III IFNs [153, 274, 275]. The function of MAVS is dependent on its mitochondrial localization. Thus, cleavage or suppression of MAVS expression inhibits interferon production and increases viral replication [277].

In this regard, initiation of an effective immune response to RV infection could be of great concern especially in asthmatics who have deficient innate immune responses to RV infection which increases lower respiratory tract symptoms and asthma exacerbations [107, 271]. Our aim was to compare the effects of oxidative stress on mitochondrial function and antiviral responses to RV infection in pBECs of asthmatics versus healthy subjects.
4.2. Materials and Methods:

Study design

- Confluent pBECs of 4 healthy and 4 asthmatic subjects
- 1 hour incubation with RV1B
- 4 hour incubation with 1% CSE and 0.2 mM H₂O₂
- 24 hour incubation with 1% CSE and 0.2 mM H₂O₂
- Analyse viability
- Analyse CXCL10 and IFN-λ
- Analyse CXCL8 and IL6
- RV replication
- Analyse MAVS, MDA5, pIRF3, TBK1, IKKe and p65 protein expression
- Analyse co-localisation of MAVS, MDA5 and mitochondria
- Analyse ATP level
- Analyse Cytochrome c
- Analyse mTFs
- Comparing the results of healthy subjects with asthmatics

pBECs from 4 asthmatics and 4 healthy controls were collected during bronchoscopy (as described in chapter 2). Cells were cultured into a 24 well tissue culture plate at a seeding concentration of 1x10⁵ cells/well, then they were incubated overnight at 37°C/5% CO₂ until they became 80% confluent, then they were infected with RV1B for 1 hr at room temperature and then were treated with 1% CSE and 0.2 mM H₂O₂. The cells were incubated at...
33°C/5%CO₂. Supernatants and cell lysates were harvested after 4hr and 24hr (as described in chapter 2). The viability of pBECs was measured by Annexin V-PE (methods described in chapter 2). Using ELISA kits the concentration of CXCL10, CXCL8, IL6, IFN-λ and cytochrome c were measured (methods described in chapter 2). ATP levels were measured by bioluminescent assay (methods described in chapter 2). Cleavage of MAVS, expression of MDA5, pIRF3, TBK1, IKKε and p65 were measured by western blotting (methods described in chapter 2). Co-localisation of MAVS, MDA5 and mitochondrial membrane integrity were measured by confocal microscopy (methods described in chapter 2). RV1B replication was measured by TCID50 (methods described in chapter 2). Gene expression of mitochondrial transcription factors was measured by Real-time PCR (methods described in chapter 2). All the experiments were performed at 4hr time-point, with the exception of the cells viability, RV replication, CXCL-8, IL-6, CXCL-10 and IFN-λ which were performed at 24hr.
4.3 Results

Determining the viability of pBECs of healthy and asthmatics by annexin V-PE:

pBECs were infected with RV1B at an MOI of 1 and were then treated with H$_2$O$_2$ (0.2mM) and CSE (1%) and then the viability was measured by Annexin V-PE after 24hr. Figure 4.3.1 shows that in cells from both a) asthmatics and b) healthy controls, there were no significant changes in pBECs viability in all conditions compared to media.

*Figure 4.3.1 pBECs viability following exposure to the oxidative stressors; a) asthmatic and b) healthy; RV, CSE and H$_2$O$_2$; measured at 24 hours*
The effect of oxidative stress on mitochondrial transcription factors in pBECs

The mRNA expression level of TFA, TB1 and TB2 were compared between asthmatics and healthy subjects. Figure 4.3.2 shows that RV infection prior to CSE/H$_2$O$_2$ exposure increased mTFA, mTB1 and mTB2 expression compared to media, whereas CSE or RV infection alone did not. Furthermore, the increase in mTFA, mTB1 and mTB2 expression following RV, then CSE/ H$_2$O$_2$ exposure was greater in asthmatics compared to healthy subjects.
Figure 4.3.2 The effect of the oxidative stressors, RV, CSE and $H_2O_2$ on the mRNA expression of mitochondrial transcription factors, a and d) TFA, b and e) TB1 and c and f) TB2, in healthy and asthmatic pBECs; measured at 4 hours.
The effect of oxidative stress on the release of cytochrome c

Four hour cell lysates were used to measure released cytochrome c in the asthmatic versus healthy control subjects. Figure 4.3.3 shows that RV, CSE and \( \text{H}_2\text{O}_2 \), in asthmatics, resulted in the release of cytochrome c into the cytosol compared to both media and healthy subjects. Thus, the release of cytochrome c increased in RV and ROS, however the combination was much greater in asthma.

Figure 4.3.3 The effect of the oxidative stressors, a) RV, CSE and RV+CSE and b) RV, \( \text{H}_2\text{O}_2 \) and RV+\( \text{H}_2\text{O}_2 \) on the release of cytochrome c from mitochondria in healthy and asthmatic pBECs; measured at 4 hours

a)
ATP release from pBECs following exposure to oxidative stress

Four hour supernatants were used to measure ATP levels in asthmatic versus healthy subjects. Figure 4.3.4 shows that RV, CSE/H$_2$O$_2$ alone as well as RV+CSE and RV+H$_2$O$_2$ induced the release of ATP into the cytosol in both the asthmatic and healthy groups, but as Figure 4.3.4.b shows, in asthmatics RV+H$_2$O$_2$ resulted in a further release of ATP from mitochondria compared to HC subjects. Thus, the release of ATP increased in RV and ROS, however the combination of RV and H$_2$O$_2$ led to a greater release of ATP in asthma.
Figure 4.3.4 The release of ATP from mitochondria in healthy and asthmatic pBECs following exposure to the oxidative stressors, a) RV, CSE and RV+CSE and b) RV, H$_2$O$_2$ and RV+H$_2$O$_2$; measured at 4 hours

4hr protein lysates were used to determine the presence of cleaved MAVS and MDA5 expression by western blot. Densitometry was measured and compared with β-actin control.
and expressed as a fold change compared to media. Figure 4.3.5 shows that CSE/H$_2$O$_2$ had no effect on MDA-5 expression or cleavage of MAVS in both groups.

**Figure 4.3.5** a) MAVS cleavage and b) MDA5 expression in healthy and asthmatic pBECs following exposure to the oxidative stressors RV, CSE, H$_2$O$_2$, RV+CSE and RV+H$_2$O$_2$; measured at 4 hours.
Co-localisation of MDA5, MAVS and mitochondria in asthmatic and healthy pBECs by confocal microscopy

Using confocal microscopy, we were able to visualise the association of MAVS, MDA5 and mitochondria in asthmatic subjects compared to HC subjects. Figure 4.3.6 b) shows that there is less MAVS in the CSE in asthmatics compared to healthy cells. Also, there is less MAVS
in the RV+H₂O₂ in HC cells. Furthermore Figure c shows that there is less MDA5 in the CSE and H₂O₂ in asthmatics compared to healthy cells. Also, figure d) shows that, RV+CSE impaired mitochondrial membrane integrity in healthy cells, while in asthmatics in figure e), CSE, H₂O₂, RV, RV+CSE and RV+H₂O₂ all led to mitochondrial membrane damage. Also, figure f) shows that in asthmatics, the co-localisation between MAVS, MDA5 and mitochondria decreased in the CSE/H₂O₂ alone as well as in the combination of RV+CSE and RV+H₂O₂ compared to media.

Figure 4.3.6 a) Co-localisation of MAVS, MDA5 and mitochondria in pBECs, following exposure to RV, CSE and H₂O₂, b) MAVS, c) MDA5, d) Mitochondrial membrane integrity in healthy cells, e) Mitochondrial membrane integrity in asthmatics, f) co-localisation of MAVS, MDA5 and mitochondria; measured at 4 hour. White arrows show the co-localisation of MAVS/MDA5 and mitochondria.
a) Media CSE RV RV+CSE

Healthy

MAVS MDA5 Mitochondria Merged

Media CSE RV RV+CSE

Asthatics

Media CSE RV RV+CSE
b) In the graph, the integrated density of MAVS is shown for different conditions. The results indicate significant differences between the healthy and asthmatic groups, with p-values of 0.02, 0.0009, and 0.004.

c) Similarly, the graph for MDA5 shows significant differences with p-values of 0.0002 and 0.0001, comparing healthy and asthmatic groups.
d) healthy

![Mitochondrial membrane integrity graph for healthy condition]

- Integrated density
- Media
- 1% CSE
- 0.2m M H$_2$O$_2$
- RV1B
- RV1B + 1% CSE
- RV1B + 0.2m M H$_2$O$_2$
- $p = 0.01$


e) asthmatic

![Mitochondrial membrane integrity graph for asthmatic condition]

- Integrated density
- Media
- 1% CSE
- 0.2m M H$_2$O$_2$
- RV1B
- RV1B + 1% CSE
- RV1B + 0.2m M H$_2$O$_2$
- $p = 0.001$
- $p = 0.0008$
- $p = 0.007$
- $p = 0.005$
- $p = 0.001$


**Colocalisation of MAVS, MDA5 and mitochondria**

![Graph showing integrated density](image)

$p = 0.002$

$p = 0.005$

$p = 0.001$

$p = 0.002$

**pIRF3, TBK1, IKKe and p65 expression in asthmatic and healthy pBECs following exposure to oxidative stress**

4hr protein lysates were used to determine pIRF3, TBK1, IKKe and p65 expression from 3 different asthmatics and healthy subjects by western blot. Densitometry was measured and compared with β-actin control and expressed as a fold change compared to media. Figures 4.3.7 shows that CSE/H$_2$O$_2$ had no effect on pIRF3, TBK1, IKKe and p65 expression.
Figure 4.3.7 a) pIRF3, b) TBK1, c) IKKε, d) p65 expression following exposure to RV, CSE and \(H_2O_2\) in healthy and asthmatic pBECs; measured at 4 hours.

a)
b)

**Healthy**

<table>
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<tr>
<th>Media</th>
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<th>H2O2</th>
<th>RV</th>
<th>RV+CSE</th>
<th>RV+H2O2</th>
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**Asthmatic**

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<td>B-actin</td>
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**TBK1**

![Graph showing fold change from media for healthy and asthmatic samples.](image)
c)

Healthy

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<th>H₂O₂</th>
<th>RV</th>
<th>RV+CSE</th>
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Asthmatic

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<th>RV</th>
<th>RV+CSE</th>
<th>UV</th>
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</table>

B-actin

Healthy

Asthmatic

IKKβ

Fold change from media

[Graph showing fold change for healthy and asthmatic conditions]
d)

**Healthy**
- Media
- CSE
- H$_2$O$_2$
- RV
- RV+CSE
- RV+H$_2$O$_2$

**B-actin**

**Asthmatic**
- Media
- CSE
- H$_2$O$_2$
- RV
- RV+CSE
- RV+H$_2$O$_2$

**B-actin**

**P65**

<table>
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</tr>
<tr>
<td>1% CSE</td>
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<tr>
<td>0.2 mM H$_2$O$_2$</td>
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<td>RV+CSE</td>
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</tr>
<tr>
<td>RV+H$_2$O$_2$</td>
<td>1.20</td>
</tr>
<tr>
<td>UV</td>
<td>1.00</td>
</tr>
</tbody>
</table>

- **healthy**
- **asthmatic**
CXCL8 release from pBECs following exposure to oxidative stress

24hr supernatants were used to measure CXCL8 in asthmatics versus healthy controls. Figure 4.3.8 shows that CSE and H$_2$O$_2$ alone did not increase the level of CXCL8 compared to media in both groups. However, in asthmatics, RV+CSE and RV+H$_2$O$_2$ resulted in a marked (two fold) increase in CXCL8 compared to media, compared to either CSE or H$_2$O$_2$ alone or compared to healthy controls.

Figure 4.3.8 CXCL8 release in pBECs following exposure to a) RV, CSE and RV+CSE and b) RV, H$_2$O$_2$ and RV+H$_2$O$_2$; measured at 24 hours.

a)}
IL6 release from pBECs following exposure to oxidative stress

24hr supernatants were used to measure IL6 in asthmatics versus healthy controls. Figure 4.3.9 shows that RV, CSE and H\textsubscript{2}O\textsubscript{2} alone did not increase the level of IL6 compared to media in both groups. However, RV+CSE and RV+H\textsubscript{2}O\textsubscript{2} increased IL6 compared to media or compared to either CSE or H\textsubscript{2}O\textsubscript{2} alone in both groups. However, in asthmatics, there is a marked increase in IL6 compared to healthy controls.
Figure 4.3.9 IL6 release in pBECs following exposure to a) RV, CSE and RV+CSE and b) RV, H₂O₂ and RV+H₂O₂; measured at 24 hours.

a)

b)
CXCL10 release in pBECs following exposure to oxidative stress

24hr supernatants were used to measure CXCL10. Figure 4.3.10 shows that RV infection increased CXCL10 release, which was greater in healthy controls than asthmatics, demonstrating impaired antiviral responses in asthmatics. When cells were infected to RV prior to CSE or H\textsubscript{2}O\textsubscript{2} exposure, there was a marked reduction in CXCL-10, compared to RV alone, in both groups.

*Figure 4.3.10 CXCL10 release from pBECs exposed to the oxidative stressors, a) RV, CSE and RV+CSE and b) RV, H\textsubscript{2}O\textsubscript{2} and RV+H\textsubscript{2}O\textsubscript{2}; measured at 24 hours*
IFN-λ response in pBECs following exposure to oxidative stress

24hr supernatants were used to measure IFN-λ. Figure 4.3.11 shows that RV infection increased IFN-λ release, which was greater in healthy controls than asthmatics, demonstrating impaired antiviral responses in asthmatics. When cells were infected to RV prior to CSE or H₂O₂ exposure, there was a marked reduction in IFN-λ response, compared to RV alone, in both groups.
Figure 4.3.11 IFN-λ release from pBECs exposed to the oxidative stressors, a) RV, CSE and RV+CSE and b) RV, H$_2$O$_2$ and RV+H$_2$O$_2$; measured at 24 hours

a) IFN-λ

![IFN-λ graph](image)

b) IFN-λ

![IFN-λ graph](image)
RV replication was measured using TCID50. Figure 4.3.12 shows that RV replication increased in asthmatic cells compared with HC cells. In asthmatics, RV+CSE and RV+H₂O₂ resulted in a further increase of RV replication compared to RV alone. In HC, RV+CSE only led to elevated RV replication compared to RV alone.

Figure 4.3.12 RV1B replication in healthy and asthmatic pBECs following exposure CSE and H₂O₂, measured at 24 hours
4.4. Discussion:

Oxidative stress is involved in the development of asthma and COPD [41]. A combination of several factors, such as viral infection, exposure to CSE or allergens may contribute to asthma exacerbations in the absence of effective type I and III IFN responses [42]. RV infections are associated with the majority of acute asthma exacerbations in children and adults [298, 299]. Epidemiological evidence demonstrates that asthmatic subjects develop more severe lower respiratory symptoms to RV infection thus are more susceptible to getting colds [104, 294]. The recruitment of inflammatory cells lead to the release of ROS which can further damage airway epithelial cells and exacerbate asthma symptoms in response to RV infection [300]. Mitochondria are known as one of the most important sources of endogenous ROS through the electron transport chain (ETC) and ATP production. Furthermore, they play a significant role in antiviral defence, via induction of innate immune responses through MDA5 and MAVS that result in a downstream activation of type I and type III IFNs in epithelial cells. Our group has previously shown that pBECs of asthmatics are more susceptible to RV infections as they are not able to release effective type I (interferon-β) and type III (interferon λ1/3) responses to RV infection. However, the exact mechanism of this impaired immune response is unknown. It is also known that RV infection may lead to an increased production of ROS which may worsen lower-airway inflammation and cell dysfunction [104, 105, 294]. To explore the mechanisms behind this increased susceptibility of epithelial cells to RV1B infection and impaired antiviral immune responses, we studied the influence of ROS in the form of CSE/H$_2$O$_2$ on mitochondrial function in pBECs from both subject groups. In this study, we have demonstrated that exposure of pBECs to CSE/H$_2$O$_2$ resulted in mitochondrial damage; with increased release of cytochrome-c and ATP which cytochrome c
release was considerably greater in asthmatic pBECs. RV+CSE/H₂O₂ further increased mTFs and ATP release which again was exaggerated in asthmatics compared to HC. In asthmatics, pBECs demonstrated a marked impairment in IFN responses with, reduced CXCL-10 and IFN-λ compared to HC. In contrast they led to an exaggerated release of pro-inflammatory mediators, with increased CXCL8 and IL-6 release.

These impaired immune responses were more pronounced in asthmatics which led to heightened virus replication compared to normal subjects. However, CSE and H₂O₂ had no effect on the cleavage of MAVS or the protein expression of MDA5, pIRF3, TBK1, IKKε and p65.

We know from other studies, that asthmatic pBECs can up-regulate signalling pathways through MDA5 in response to RV infection as well as pBECs from healthy controls, but they are still not able to initiate effective downstream release of type I IFNs [301]. Thus, these observations suggest that impaired antiviral responses are not due to signalling pathways through MDA5. On the other hand, the integrated density of our confocal images showed that there is more MDA5/MAVS in the CSE/H₂O₂ in healthy cells compared to asthmatics. Also in asthmatics, CSE, H₂O₂, RV, RV+CSE and RV+H₂O₂ impaired mitochondrial membrane integrity, while, in HC cells, RV+CSE only led to mitochondrial membrane damage, also in asthmatics, CSE, H₂O₂, RV+CSE and RV+H₂O₂ decreased the co-localisation of MAVS, MDA5 and mitochondria, while in HC cells, RV+CSE only led to decreased level of co-localisation between MAVS, MDA5 and mitochondria suggesting that there is more mitochondrial oxidative damage in asthmatics compared to healthy cells.

Taken together, ROS generation through endogenous or exogenous sources can cause mitochondrial dysfunction leading to inflammation and impaired CXCL10 and type III IFN (interferon λ1/3).
Studies have shown that ROS such as CSE play an important role in the severity of respiratory disorders such as asthma. It has been shown that asthma mortality is increased in people who smoke compared with never-smokers [292]. Several studies have shown that active smokers have more severe symptoms with a greater need for hospitalisation and medication as well as more resistant to corticosteroids compared with asthmatic non-smokers [302, 303]. Cytokines and mediators such as CXCL8, IL-4 and TNF-α are increased in asthmatic smokers [304-307], they have more severe airway remodelling [308] and less eosinophils [309], which indicate altered and enhanced inflammatory responses that may be intrinsically resistant to therapy with corticosteroids in asthmatic smokers compared with asthmatic non-smokers. Our findings also showed that asthmatic cells are more susceptible to oxidative damage in response to RV infection. In addition, these results indicate that there is a synergism between exposure to ROS in the form of CSE/H₂O₂ and viral infection that induces further mitochondrial dysfunction and potentially enhances epithelial inflammatory responses, all of which would compound airway inflammation in the context of an acute asthma exacerbation. However, the exact mechanisms of this synergistic mechanism remain to be established. Further studies are needed to understand the role of ROS on mitochondrial function and the exact mechanisms which cause more susceptibility to RV infection in asthmatics. This may help us to establish a role for antioxidants as therapeutic interventions which target mitochondria to prevent from oxidative damage and reduce the severity of asthma exacerbations.
Chapter 5

The effects of lycopene and L-carnitine supplementation on mitochondria and pBECs of healthy and asthmatic subjects
5.1 Introduction

Mitochondrial oxidative damage can cause a wide range of degenerative diseases and is also important in the progression of inflammatory diseases such as asthma and COPD [184, 310]. Mitochondrial ROS can damage proteins, lipids and DNA. This can cause mitochondrial damage and impaired mitochondrial function leading to the further release of ROS into the cytosol [38, 39, 310, 311]. All organisms have cellular defences against ROS which are known as antioxidants. However, an imbalance between ROS and antioxidants lead to oxidative stress [40, 61].

These antioxidants include endogenous metabolites such as glutathione, L-carnitine, ubiquinone, exogenous antioxidants such as vitamin C, E, carotenoids and flavonoids, enzymatic antioxidants such as SOD, catalase and GPx and synthetic antioxidants such as butylated hydroxynasole (BHA) [199].

Carotenoids are natural fat-soluble antioxidants which are found abundantly in fruits and vegetables [200, 201]. Lycopene, found primarily in tomatoes and tomato-based products, is the most potent antioxidant amongst the carotenoid family and has potential therapeutic roles in a wide range of diseases such as atherosclerosis, cancers, diabetes and inflammatory disorders [312].

There is a growing body of evidence which indicates the consumption of fruits and vegetables may have protective effects against wheezing, chronic lung disease onset and asthma prevalence [195, 313-315]. One of the possible mechanisms by which fruit and vegetables may protect against disease is the presence of carotenoids, such as lycopene. Lycopene may reduce the level of oxidative stress as well as inflammation [316-319]. Indeed, lycopene has been shown to reduce neutrophilic airway inflammation in asthma in vivo [320] and has been shown to reduce RV-induced inflammation in vitro [321].
There are a variety of known anti-inflammatory mechanisms of lycopene. ROS are important in NF-kB activation and lycopene can scavenge free radicals, leading to the suppression of NF-kB which, in turn, can suppress the production of cytokines such as IL-6 and CXCL-8, which are known to induce airway neutrophilia [312]. Similarly, it has been shown that lycopene can decrease the inflammatory responses caused by environmental triggers such as cigarette smoke, by quenching singlet oxygen and free radicals which are generated by CSE and inhibiting the binding activity of NF-kB [202, 322, 323]. Our group has previously shown that lycopene can reduce airway inflammation following RV infection [200], by reducing IL-6 and CXCL-8 level and decreasing viral replication [200, 312]. Thus, lycopene-rich tomatoes or tomato products may be useful as anti-inflammatory treatments or protective agents against the airway inflammation that is associated with asthma [312, 324].

L-carnitine (L-3-hydroxy-4-N-N-N-trimethylaminobutyrate) is another antioxidant which is an essential nutrient, mainly found in red meat. L-carnitine acts as carrier of fatty acids across the inner mitochondrial membrane, which is important for β-oxidation and ATP production [261, 325]. Studies have shown that L-carnitine is an effective scavenger of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), superoxide and hydrogen peroxide radicals [248]. Therefore, it is an important antioxidant for protecting cellular components, particularly the mitochondria, against oxidative stress. Furthermore, it protects endogenous enzymes such as SOD and catalase from utilisation [325].

It has been demonstrated that levels of nonenzymatic antioxidants like vitamin C, vitamin E, lycopene, lutein, α-carotene and β-carotene are lower in asthmatics compared with controls[195]. Furthermore, the activities of enzymatic antioxidants, including SOD, GPx and catalase are decreased in asthma compared to controls [196, 199]. Wood et al, also demonstrated that the withdrawal of lycopene from daily diet can cause neutrophilic airway inflammation and exacerbate asthma symptoms [312, 326]. Thus, supplementation with
antioxidants which can prevent oxidative damage to cellular components, including the mitochondria, may have therapeutic potential in patients with asthma [184]. In this study, we evaluated the possible antioxidant effects of lycopene and L-carnitine on pBECs of healthy and asthmatic subjects to see whether the oxidative damage to mitochondria, that we have observed in Chapter 3 and 4, can be reversed using these antioxidants.
5.2. Materials and Methods:

Study design

pBECs from 5 different HC and 5 different asthmatic subjects were incubated for 4 hours with 2.5µg/ml lycopene dissolved in THF and 0.5mM L-carnitine dissolved in BEBM minimal media. These subjects are different from the subjects in chapter 4. The concentration of lycopene had already optimised in our group by Ahmad Saedi’s PhD research [327].
We also pre-treated the Beas-2B cells with 0.1mM, 0.5mM and 1mM of L-carnitine and then we measured CXCL10 and CXCL8 to determine the most effective concentration of L-carnitine. We found that 0.5mM L-carnitine was trending to increase CXCL10 (n=1), while it was trending to decrease CXCL8 (n=1). Thus, we chose 0.5mM of L-carnitine for our pBEC experiments. Cells were then infected with RV1B at an MOI 1 for 1hour which, as described in chapter 2, MOI 1 was shown to induce appropriate immune responses without causing significant cytopathic effects. Cells were then exposed to 1%CSE and 0.2mM H2O2 which, as described in chapter 2, these concentrations were also chosen based on their ability to induce immune responses without causing significant cytopathic effects. Cells and supernatants were then harvested after 4 hours, 2hours and 24 hours. pBEC viability was measured by Annexin V-PE after 24hr (Methods described in Chapter 2). Using ELISA kits the concentration of CXCL10, IL6, CXCL8 and IFN-λ were measured after 24hr (Methods described in Chapter 2). ATP levels were measured by bioluminescent assay after 4hr (Methods described in Chapter 2). Co-localisation of MAVS, MDA5 and mitochondria were assessed by confocal microscopy after 4hr (Methods described in Chapter 2). RV1B entry was measured after 2hr by confocal microscopy (Methods described in Chapter 2). RV1B replication was measured by TCID50 after 24hr (Methods described in Chapter 2).
5.3 Results

*Determining the viability of healthy pBECs by annexin V-PE:*

pBECs were pre-treated with 2.5µg/ml lycopene in THF and 0.5mM carnitine in BEBM minimal media for 4hr, then infected with RV1B at an MOI of 1 for 1hr. They were then treated with H₂O₂ (0.2mM) or CSE (1%) and then the viability was measured by annexin V-PE after 24hr. Figure 5.3.1(a) shows that there was no difference in the pBECs viability in all controls, with and without THF, compared to media. Also, as Figure 5.3.1b) and 5.3.1 c) show, there is no difference in the pBEC viability in all conditions with lycopene or carnitine compared to media.

Figure 5.3.1 a) pBEC viability following exposure to the oxidative stressors, with and without THF, b) pBEC viability following exposure to the oxidative stressors, with and without lycopene, and c) pBEC viability following exposure to the oxidative stressors, with and without L-carnitine.; measured at 24 hours.
RV1B entry into healthy pBECs pre-treated with lycopene and carnitine, then RV infection followed by exposure to CSE/H$_2$O$_2$.

Healthy pBECs were pre-treated with 2.5µg/ml lycopene and 0.5mM carnitine for 4hr, then they were infected with tagged RV1B for 1hr and then exposed to 1% CSE and 0.2mM H$_2$O$_2$ for 2hr. The entry of RV1B into the healthy pBECs was then visualised by confocal microscopy. Figure 5.3.2 shows that the entry of RV1B into healthy pBECs was not different in all conditions. DAPI stains the nuclei in blue and RV1B is stained in red.
Figure 5.3.2 RV1B entry into healthy pBECs pre-treated with lycopene and carnitine, then RV infection followed by exposure to CSE/H$_2$O$_2$.
ATP release from healthy pBECs pre-treated with lycopene and carnitine, then RV infection followed by exposure to CSE/H$_2$O$_2$

Four hour supernatants were used to measure ATP levels in 5 asthmatics and 5 HC controls. Figure 5.3.3 a) shows that there was no difference in all controls with and without THF.

Figure 5.3.3 (b and c) show that CSE+lycopene, H$_2$O$_2$+lycopene, RV+lycopene, RV+CSE+lycopene and RV+H$_2$O$_2$+lycopene reduced the release of ATP into the cytosol in healthy controls. In asthmatics, RV+lycopene, RV+CSE+lycopene and RV+H$_2$O$_2$+lycopene reduced the release of ATP into the cytosol. Figure 5.3.3 (d and e) show that CSE+carnitine, H$_2$O$_2$+carnitine, RV+carnitine, RV+CSE+carnitine and RV+H$_2$O$_2$+carnitine reduced the release of ATP from the mitochondria into the cytosol in both groups.

Figure 5.3.3 The release of ATP from mitochondria in healthy and asthmatic pBECs following pre-treatment with lycopene and carnitine, then RV infection followed by exposure to CSE/H$_2$O$_2$, a) controls with and without THF, b) healthy controls+ healthy samples in 2.5µg/ml lycopene, c) asthmatic controls+ asthmatic samples in 2.5µg/ml lycopene, d) healthy controls +healthy samples in 0.5mM carnitine, e) asthmatic controls +asthmatic samples in 0.5mM carnitine; measured at 4 hours.

a)
b) Healthy relative ATP levels

![Healthy relative ATP levels graph]

- **p < 0.0001**
- **p < 0.0001**
- **p = 0.002**
- **p = 0.01**
- **p < 0.0001**

![Healthy relative ATP levels graph]

![Healthy relative ATP levels graph]

p < 0.0001
p < 0.0001
p < 0.0001


c) Asthmatic relative ATP levels

![Asthmatic relative ATP levels graph]

- **p < 0.0001**
- **p < 0.0001**
- **p < 0.0001**
d) Healthy relative ATP levels

\[ P = 0.0002 \]
\[ P = 0.03 \]
\[ P < 0.0001 \]
\[ P < 0.0001 \]
\[ P < 0.0001 \]

![Bar graph showing healthy relative ATP levels with statistical significance](image)

---

e) Asthmatic relative ATP levels

\[ P = 0.0003 \]
\[ P = 0.0001 \]
\[ P < 0.0001 \]
\[ P < 0.0001 \]
\[ P < 0.0001 \]

![Bar graph showing asthmatic relative ATP levels with statistical significance](image)
Co-localisation of MDA5, MAVS and mitochondria in asthmatic and healthy pBECs pre-treated with lycopene, then RV infection followed by exposure to CSE/H$_2$O$_2$

Healthy and asthmatic pBECs were pre-treated with lycopene, then they were infected with RV1B for 1hr, then they were exposed to 1% CSE and 0.2mM H$_2$O$_2$ for 4hr. After 4hr, using confocal microscopy, we were able to visualise the association of MAVS, MDA5 and mitochondria as described in Chapter 4. Figure 5.3.4 (a-n) show healthy and asthmatic pBECs which were pre-treated with lycopene. These figures show that RV+H$_2$O$_2$+lycopene increased the amount of MAVS in both healthy and asthmatic cells. Also, RV+lycopene increased MDA5 in HC cells. Furthermore, RV+lycopene, RV+CSE+lycopene, and RV+H$_2$O$_2$ lycopene increased mitochondrial membrane integrity in HC cells, while, in asthmatics, all conditions which were pre-treated with lycopene increased mitochondrial membrane integrity. Also, the co-localisation of MAVS, MDA5 and mitochondria increased in the H$_2$O$_2$+lycopene and RV+H$_2$O$_2$+lycopene in asthmatics and in the RV+CSE+lycopene in both groups.

Figure 5.3.4 Co-localisation of MAVS, MDA5 and mitochondria in pBECs, following pre-treatment with lycopene, then RV infection followed by exposure to CSE/H$_2$O$_2$. (a-b) HC pBECs and (c-d) asthmatic pBECs; a) CSE and lycopene, b) H$_2$O$_2$ and lycopene, c) CSE and lycopene, d) H$_2$O$_2$ and lycopene, e) MAVS, CSE, f) MAVS, H$_2$O$_2$, g) MDA5, CSE, h) MDA5, H$_2$O$_2$, i) mitochondrial membrane integrity, CSE in HC cells, j) mitochondrial membrane integrity with the H$_2$O$_2$ in HC, k) mitochondrial membrane integrity with the CSE in asthmatics, l) mitochondrial membrane integrity with the H$_2$O$_2$ in asthmatics, m) co-localisation of MAVS, MDA5 and mitochondria with the CSE, n) co-localisation of MAVS, MDA5 and mitochondria with the H$_2$O$_2$; measured at 4 hours. White arrows show the co-localisation of MAVS/MDA5 and mitochondria.
### b) 

<table>
<thead>
<tr>
<th>MAVS</th>
<th>MDA5</th>
<th>Mitochondria</th>
<th>Merged</th>
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<td><img src="image" alt="Media MDA5" /></td>
<td><img src="image" alt="Media Mitochondria" /></td>
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<td><img src="image" alt="H₂O₂ MDA5" /></td>
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<td><img src="image" alt="RV MDA5" /></td>
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<td><img src="image" alt="RV+H₂O₂ lycopene MDA5" /></td>
<td><img src="image" alt="RV+H₂O₂ lycopene Mitochondria" /></td>
<td><img src="image" alt="RV+H₂O₂ lycopene Merged" /></td>
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</tbody>
</table>

**Healthy**
Asthmatics

MAVS
MDAS
Mitochondria
Merged

Media
CSE
RV
RV+CSE
CSE lycopene
RV lycopene
RV+CSE lycopene
g) In the graph labeled **MDA5**, the integrated density bars for different treatments are compared between healthy and asthmatic conditions. The p-values indicate statistical significance:

- **CSE** group: $P = 0.0004$
- **CSE lycopene** group: $P = 0.0006$

h) Another graph labeled **MDA5** shows the integrated density for different treatments under healthy and asthmatic conditions. The p-values are:

- **H₂O²⁻** group: $P = 0.006$
- **RV + H₂O²⁻ lycopene** group: $P = 0.01$
i) Healthy

Mitochondrial membrane integrity

![](chart_i)

j) Healthy

Mitochondrial membrane integrity

![](chart_j)
k) Asthmatic

Mitochondrial membrane integrity

![Graph showing mitochondrial membrane integrity with integrated density values for different conditions: Media, CSE, RV1B, RV1B+CSE, CSE lycopene, RV lycopene, RV+CSE lycopene. The graph includes statistical significance levels such as p < 0.0001 and p = 0.005.]

l) Asthmatic

Mitochondrial membrane integrity

![Graph showing mitochondrial membrane integrity with integrated density values for different conditions: Media, H₂O₂, RV, RV + H₂O₂, H₂O₂ lycopene, RV lycopene, RV + H₂O₂ lycopene. The graph includes statistical significance levels such as p = 0.04 and p = 0.005.]

Colocalisation of MAVS, MDA5 and mitochondria

- **Media:**
  - CSE
  - RV1B
  - RV1B+CSE
  - CSE lycopene
  - RV lycopene
  - RV+CSE lycopene

- **Healthy vs. Asthmatic:**
  - CSE: p = 0.03
  - RV1B: p = 0.01
  - RV1B+CSE: p = 0.01
  - CSE lycopene: p = 0.01
  - RV lycopene: p = 0.01
  - RV+CSE lycopene: p = 0.01

Colocalisation of MAVS, MDA5 and mitochondria

- **Media:**
  - H₂O₂
  - RV1B
  - RV1B+H₂O₂
  - H₂O₂ lycopene
  - RV lycopene
  - RV+H₂O₂ lycopene

- **Healthy vs. Asthmatic:**
  - H₂O₂: p = 0.01
  - RV1B: p = 0.07
  - RV1B+H₂O₂: p = 0.07
  - H₂O₂ lycopene: p = 0.07
  - RV lycopene: p = 0.07
  - RV+H₂O₂ lycopene: p = 0.07
Co-localisation of MDA5, MAVS and mitochondria in asthmatic and healthy pBECs pre-treated with carnitine, then RV infection followed by exposure to CSE/H$_2$O$_2$

Healthy and Asthmatic pBECs were pre-treated with carnitine, then they were infected with RV1B for 1hr, then they were exposed to 1% CSE and 0.2mM H$_2$O$_2$ for 4hr. After 4hr, using confocal microscopy, we were able to visualise the association of MAVS, MDA5 and mitochondria as described in Chapter 4. Figure 5.3.5 (a-n) show healthy and asthmatic pBECs which were pre-treated with carnitine.

These figures show that RV+carnitine and RV+H$_2$O$_2$+carnitine increased the amount of MAVS in both HC and asthmatic cells. Also, RV+carnitine, H$_2$O$_2$+carnitine and RV+H$_2$O$_2$+carnitine increased the amount of MDA5 in asthmatics, while there was no difference in HC cells. Furthermore, CSE+carnitine and RV+CSE+carnitine increased mitochondrial membrane integrity in HC cells, while, in asthmatics, all conditions which were pre-treated with carnitine increased mitochondrial membrane integrity. Also, H$_2$O$_2$+carnitine and RV+CSE+carnitine increased the co-localisation between MAVS, MDA5 and mitochondria in both groups. CSE+carnitine and RV+H$_2$O$_2$+carnitine led to increased level of co-localisation in asthmatics.

Figure 5.3.5 Co-localisation of MAVS, MDA5 and mitochondria in pBECs, following pre-treatment with carnitine, then RV infection followed by exposure to CSE/H$_2$O$_2$. (a-b) HC pBECs and (c-d) asthmatic pBECs; a) CSE and carnitine, b) H$_2$O$_2$ and carnitine, c) CSE and carnitine, d) H$_2$O$_2$ and carnitine, e) MAVS, CSE, f) MAVS, H$_2$O$_2$, g) MDA5, CSE, h) MDA5 H$_2$O$_2$, i) mitochondrial membrane integrity with the CSE in HC cells, j) mitochondrial membrane integrity with the H$_2$O$_2$ in HC cells, k) mitochondrial membrane integrity with the CSE in asthmatics, l) mitochondrial membrane integrity with the H$_2$O$_2$ in asthmatics, m) co-localisation of MAVS, MDA5 and mitochondria with the CSE, n) co-localisation of MAVS, MDA5 and mitochondria with the H$_2$O$_2$; measured at 4 hours. White arrows show the co-localisation of MAVS/MDA5 and mitochondria.
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<th>Treatment</th>
<th>Healthy</th>
<th>Media</th>
<th>H₂O₂</th>
<th>RV</th>
<th>RV+H₂O₂</th>
<th>H₂O₂ carnitine</th>
<th>RV carnitine</th>
<th>RV+H₂O₂ carnitine</th>
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d) MAVS  MDAS5  Mitochondria  Merged

Asthmatics

Media

$H_2O_2$

RV

$RV+H_2O_2$

$H_2O_2$ carnitine

RV carnitine

$RV+H_2O_2$ carnitine
g) MDA5

Integrated density

Media | CSE | RV+1B | RV+CSE | CSE carnitine | RV carnitine | RV+CSE carnitine

1.0 × 10^1
1.0 × 10^2
1.0 × 10^3
1.0 × 10^4
1.0 × 10^5
1.0 × 10^6

healthy
asthmatic

p = 0.0008

h) MDA5

Integrated density

Media | H2O | RV | RV+H2O | H2O+carnitine | RV carnitine | RV+H2O+carnitine

1.0 × 10^1
1.0 × 10^2
1.0 × 10^3
1.0 × 10^4
1.0 × 10^5
1.0 × 10^6

healthy
asthmatic

p < 0.0001

p = 0.01

p < 0.0001
i) Healthy

![Mitochondrial membrane integrity graph](image)

j) Healthy

![Mitochondrial membrane integrity graph](image)
k) asthmatic

Mitochondrial membrane integrity

l) asthmatic

Mitochondrial membrane integrity
Colocalisation of MAVS, MDAS and mitochondria

![Graph showing colocalisation of MAVS, MDAS and mitochondria with healthy and asthmatic samples.](image)

- p = 0.004
- p = 0.002
- p = 0.004
- p = 0.02
- p = 0.03
- p = 0.005

Healthy
Asthmatic
CXCL8 release from pBECs following pre-treatment with lycopene and carnitine, then RV infection followed by exposure to CSE/H\textsubscript{2}O\textsubscript{2}

24hr supernatants were used to measure CXCL8 in the pBECs of 5 asthmatics and 5 HC pre-treated with 2.5µg/ml lycopene and 0.5mM carnitine following RV infection and then exposure to 1% CSE and 0.2mM H\textsubscript{2}O\textsubscript{2}. Figure 5.3.6 a) shows that there was no difference in all controls with and without THF.

Figure 5.3.6 (b and c) show that the release of CXCL8 was reduced in RV+lycopene and RV+CSE+lycopene compared to RV and RV+CSE respectively in both asthmatics and healthy controls. Figure (c and d) show that the release of CXCL8 was reduced in RV+carnitine, RV+CSE+carnitine and RV+H\textsubscript{2}O\textsubscript{2}+carnitine compared to RV, RV+CSE and RV+H\textsubscript{2}O\textsubscript{2} respectively in both asthmatics and healthy controls.

Figure 5.3.6 CXCL8 release in healthy and asthmatic pBECs pre-treated with lycopene and carnitine, then RV infection followed by exposure to CSE/H\textsubscript{2}O\textsubscript{2}. a) controls with and without THF, b) healthy controls+ healthy samples in 2.5µg/ml lycopene, c) asthmatic controls + asthmatic samples in 2.5µg/ml lycopene, d) healthy controls+healthy samples in 0.5mM carnitine, e) asthmatic controls +asthmatic samples in 0.5mM carnitine; measured at 24 hours.
b) healthy

![CXCL8 concentration graph for healthy subjects.](image)

- **CXCL8 Concentration (pg/ml)**
- **Conditions**: media, CSE, H₂O₂, RV, RV+CSE, RV+H₂O₂, CSE lycopene, H₂O₂ lycopene, RV lycopene, RV+CSE lycopene, RV+H₂O₂ lycopene, UV
- **Significance Levels**: p = 0.03, p = 0.01

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c) asthmatic

![CXCL8 concentration graph for asthmatic subjects.](image)

- **CXCL8 Concentration (pg/ml)**
- **Conditions**: media, CSE, H₂O₂, RV, RV+CSE, RV+H₂O₂, CSE lycopene, H₂O₂ lycopene, RV lycopene, RV+CSE lycopene, RV+H₂O₂ lycopene, UV
- **Significance Levels**: p = 0.02, p = 0.01
d) healthy

![CXCL8 graph for healthy condition](image)

- $p = 0.02$
- $p = 0.02$
- $p = 0.003$

e) asthmatic

![CXCL8 graph for asthmatic condition](image)

- $p = 0.04$
- $p = 0.004$
- $p = 0.04$
IL6 release from pBECs following pre-treatment with lycopene and carnitine, then RV infection and exposure to CSE/H$_2$O$_2$

24hr supernatants were used to measure IL6 in the pBECs of 5 asthmatics and 5 HC pre-treated with 2.5µg/ml lycopene and 0.5mM carnitine followed by RV infection and then exposure to 1% CSE or 0.2mM H$_2$O$_2$. Figure 5.3.7 a) shows that there was no difference in all controls with and without THF.

Figure 5.3.7 (b and c) show that the release of IL6 was reduced in RV+CSE+lycopene and RV+H$_2$O$_2$+lycopene compared to RV+CSE and RV+ H$_2$O$_2$ respectively in both asthmatics and HC. Figure 5.3.7 (d and e) show that the release of IL6 was reduced in RV+CSE+carnitine and RV+H$_2$O$_2$+carnitine compared to RV+CSE and RV+H$_2$O$_2$ respectively in both asthmatics and HC.
Figure 5.3.7 IL6 release in healthy and asthmatic pBECs pre-treated with lycopene and carnitine, then RV infection followed by exposure to CSE/H\textsubscript{2}O\textsubscript{2}, a) controls with and without THF, b) healthy controls+ healthy samples in 2.5µg/ml lycopene, c) asthmatic controls+ asthmatic samples in 2.5µg/ml lycopene, d) healthy controls +healthy samples in 0.5mM carnitine, e) asthmatic controls +asthmatic samples in 0.5mM carnitine; measured at 24 hours.

a)

![Graph of IL6 concentration with legend indicating healthy and asthmatic categories.]

b) healthy

![Graph of IL6 concentration showing statistical significance with p-values p = 0.02 and p = 0.01.]

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c) asthmatic

IL 6

Concentration (pg/ml)

p = 0.03

p = 0.0008

d) healthy

IL 6

Concentration (pg/ml)

p = 0.03

p = 0.02
CXCL10 response in pBECs following pre-treatment with lycopene and carnitine, then RV infection and exposure to CSE/H$_2$O$_2$

24hr supernatants were used to measure CXCL10 in the pBECs of 5 asthmatics and 5 HC pre-treated with 2.5µg/ml lycopene and 0.5mM carnitine followed by RV infection and then exposure to 1% CSE or 0.2mM H$_2$O$_2$. Figure 5.3.8 a) shows that there was no difference in all controls with and without THF.

Figure 5.3.8 b) and c) show that the release of CXCL10 was unchanged in pBECs pre-treated with 2.5µg/ml lycopene or 0.5mM carnitine in both the asthmatics and HC.
Figure 5.3.8 CXCL10 release in healthy and asthmatic pBECS following pre-treatment with lycopene and carnitine, then RV infection followed by exposure to CSE/H$_2$O$_2$, a) controls with and without THF, b) controls+ all samples in 2.5µg/ml lycopene, c) controls +all samples in 0.5mM carnitine; measured at 24 hours

a)

![Graph showing CXCL10 concentration in healthy and asthmatic pBECS with various treatments](image1)

b)

![Graph showing CXCL10 concentration in healthy and asthmatic pBECS with various treatments](image2)
**IFN-λ response in pBECs following pre-treatment with lycopene and carnitine, then RV infection and exposure to CSE/H₂O₂**

24hr supernatants were used to measure IFN-λ in the pBECs of 5 asthmatics and 5 HC pre-treated with 2.5µg/ml lycopene and 0.5mM carnitine, followed by RV infection and then exposure to 1% CSE or 0.2mM H₂O₂. Figure 5.3.9 a) shows that there was no difference in all controls with and without THF.

Figure 5.3.9 (b and c) show that lycopene and carnitine had no effect on the release of IFN-λ in either the asthmatics or HC.
Figure 5.3.9 IFN-λ release in healthy and asthmatic pBECs pre-treated with 2.5µg/ml lycopene and 0.5mM carnitine, then RV infection followed by exposure to CSE/H₂O₂. a) controls with and without THF, b) controls+ all samples in 2.5µg/ml lycopene, c) controls +all samples in 0.5mM carnitine; measured at 24 hours.
RV1B replication in pBECs following pre-treatment with lycopene and carnitine, then RV infection and exposure to CSE/H$_2$O$_2$

24hr supernatants were used to measure RV1B replication in the pBECs of 5 asthmatics and 5 HC pre-treated with 2.5µg/ml lycopene and 0.5mM carnitine, followed by RV infection and then exposure to 1% CSE or 0.2mM H$_2$O$_2$. Figure 5.3.10 a) shows that there was no difference in all controls with and without THF. However, lycopene in Figure 5.3.10 (b and c) and carnitine in Figure 5.3.10 (d and e) both decreased RV replication in RV, RV+CSE and RV+H$_2$O$_2$ in both asthmatics and HC.
Figure 5.3.10 RV1B replication in healthy and asthmatic pBECs pre-treated with 2.5µg/ml lycopene and 0.5mM carnitine, then RV infection followed by exposure to CSE/H2O2. 
a) controls with and without THF, b) healthy controls+ healthy samples pre-treated with 2.5µg/ml lycopene, c) asthmatic controls+ asthmatic samples pre-treated with 2.5µg/ml lycopene, d) healthy controls+ healthy samples pre-treated with 0.5mM carnitine, e) asthmatic controls+ asthmatic samples pre-treated with 0.5mM carnitine; measured at 24 hours.
c) 

\[ \log_{10} \text{RV TCID}_{50} / \text{ml} \]

RV
RV+CSE
RV+H
2O
2
RV lycopene
RV+CSE lycopene
RV+H
2O
2

\[ 1 \times 10^1 \]
\[ 1 \times 10^2 \]
\[ 1 \times 10^3 \]
\[ 1 \times 10^4 \]
\[ 1 \times 10^5 \]
\[ 1 \times 10^6 \]
\[ 1 \times 10^7 \]
\[ 1 \times 10^8 \]
\[ 1 \times 10^9 \]

\[ p = 0.01 \]
\[ p < 0.0001 \]
\[ p = 0.0003 \]

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d) 

\[ \log_{10} \text{RV TCID}_{50} / \text{ml} \]

RV
RV+CSE
RV+H
2O
2
RV carnitine
RV+CSE carnitine
RV+H
2O
2

carnitine

\[ 1 \times 10^1 \]
\[ 1 \times 10^2 \]
\[ 1 \times 10^3 \]
\[ 1 \times 10^4 \]
\[ 1 \times 10^5 \]
\[ 1 \times 10^6 \]
\[ 1 \times 10^7 \]
\[ 1 \times 10^8 \]
\[ 1 \times 10^9 \]

\[ p = 0.04 \]
\[ p = 0.007 \]
\[ p = 0.002 \]
5.4. Discussion:

Lycopene and carnitine, which act as free radical scavengers, are potent antioxidants which can reduce oxidative damage. This study demonstrated that pre-treatment of pBECs from healthy and asthmatic cells with lycopene and carnitine followed by RV infection and CSE/H₂O₂ exposure, led to increased protein expression of MAVS and MDA5, compared to control conditions. In addition, both lycopene and carnitine improved mitochondrial membrane integrity, the amount of MAVS, MDA5 and the co-localisation between MAVS, MDA5 and mitochondria in HC and asthmatics. Furthermore, more ATP was retained in the mitochondria in both healthy and asthmatic cells pre-treated with lycopene/carnitine compared to control conditions. These data suggest that enrichment of pBECs with lycopene and carnitine can restore mitochondrial function by improving the membrane integrity and restoring the ATP contents, which is important for providing cellular energy and metabolism.
ROS generation through exogenous factors or endogenous mechanisms lead to inflammatory responses by activation of redox-sensitive transcription factors and pro-inflammatory signalling pathway [199]. It has been shown that RV infection of human respiratory epithelial cells resulted in increased ROS generation leading to increased CXCL8 and IL6 production[105]. In our study, lycopene and carnitine enrichment of airway epithelial cells infected by RV1B and exposed to CSE/H2O2 decreased inflammation by reducing pro-inflammatory cytokines, CXCL8 and IL6. This confirms the results of other studies in which lycopene and carnitine decreased inflammation [200, 316, 317]. Unexpectedly however we found that enrichment of pBECs with lycopene and carnitine didn’t restore antiviral IFN responses, including CXCL10 and IFN-λ. Nonetheless, our TCID50 results showed that lycopene and carnitine significantly decreased RV replication.

To further explore the mechanisms behind this decreased level of RV replication, we tagged RV1B to investigate the effects of lycopene and carnitine on RV1B entry into the airway epithelial cells. Our confocal images showed that RV1B entry into the pBECs was not affected by either lycopene or carnitine. Therefore, RV1B was still able to enter the cells and replicate. Hence, viral entry does not explain the low level of RV replication with antioxidant supplementation. These findings also suggest that although mitochondrial dysfunction is an important factor in airway epithelial injury and asthma pathogenesis and can be corrected using antioxidants, it appears dissociated from antiviral responses.

ROS can act on many pathways simultaneously, so it is likely that ROS also affect other organelles. One of these organelles is the endoplasmic reticulum (ER) which is important in ROS generation and may be involved in the progression of stress signalling pathways and inflammatory diseases [328-331]. The ER is an intracellular organelle which is important for protein folding by forming the disulphide bonds which provide an oxidizing folding-environment leading to the generation of ROS [331]. It is possible that CSE/H2O2 may affect
the ER, which may not be restored by the function of lycopene/carnitine. This may explain the reduced antiviral responses we showed following RV infection. However, this hypothesis requires further investigation.

In summary, cells pre-supplemented with lycopene and carnitine and exposed to oxidative stress, had restored mitochondrial function and decreased inflammation with a marked reduction in CXCL8 and IL6. In addition, they showed reduced virus replication, however antiviral IFN responses were not restored. Thus, we conclude that lycopene and carnitine may have therapeutic utility in reducing inflammation and viral replication in virus-induced exacerbations of asthma. However, the exact mechanisms which lead to impaired antiviral responses should be further investigated. Furthermore, the role of other organelles in oxidative stress-induced impaired antiviral responses warrants further investigation and provides an interesting area for future research.
Chapter 6

General discussion and future directions
Asthma is associated with an increase in reactive oxidant species (ROS) in the airways which can lead to oxidative stress. A combination of several factors, such as viral infection, exposure to tobacco smoke or allergens may contribute to asthma exacerbations in the absence of effective type I and type III IFN responses [332]. The toxic components of tobacco smoke which were discussed in chapter 3 can produce ROS which are able to damage mitochondria and airway epithelial cell membranes [57, 278, 280]. Furthermore, tobacco smoke can increase the risk of wheezing and airway respiratory infections by affecting the host immune defence [283]. Rhinovirus infections (RVs) are associated with the majority of acute asthma exacerbations in children and adults [298, 299]. Our group has previously shown that pBECs of asthmatics are more susceptible to RV infections as they are not able to release effective type I (interferon-β) and type III (interferon λ1/3) responses to RV infection. However, the exact mechanism of this impaired immune response is unknown. It is also known that RV infection may lead to an increased production of ROS which may worsen lower-airway inflammation and cell dysfunction [104, 105, 294].

ROS are also known to affect mitochondrial function and this may impact on bronchial epithelial cell responses, including antiviral responses to RV infection [177, 270, 273]. It has been shown that mitochondria play a significant role in antiviral defence, via induction of innate immune responses through melanoma differentiation-associated gene 5 (MDA5) and mitochondrial antiviral signalling protein (MAVS), that result in a downstream activation of type I and type III IFNs in epithelial cells [153, 274, 276].

Pathogen recognition receptors (PRR), like toll-like receptors (TLRs) and the RNA helicases MDA5 (melanoma differentiation associated gene 5) and RIG-I (retinoic acid-inducible gene), detect viral nucleic acids [140]. After the virus injects its RNA into the cytoplasm of AEC, it binds to MDA5. Once the attachment of viral RNA to MDA5 is complete, they translocate to the mitochondrial surface and interact with the MAVS protein
which subsequently activates IRF-3, IRF-7 and NF-κB through the kinases IKKα/β and TBK1/IKKe (Figure 6.1) [150].

It has been shown that Cleavage or suppression of MAVS expression inhibits interferon production and increases viral replication [277]. In this regard, initiation of immune responses to RV infection could be of great concern especially in patients with impaired type I and III IFN immune responses, such as asthma.

*Figure 6.1. Initiation of downstream signalling pathway against viral RNA through RNA helicases and TLR3 pathway.*

Thus, the development of antioxidants which can prevent mitochondrial oxidative damage may have therapeutic potential in these patients.
Results presented in chapter 3 on the effects of oxidative stress on mitochondrial function and antiviral immune responses to RV infection in healthy pBECs confirmed that RV+CSE led to mitochondrial dysfunction by impairing mitochondrial membrane integrity and the co-localisation of MAVS, MDA5 and mitochondria. This effect was also associated with the increase in expression of mitochondrial transcription factors and the release of ATP and cytochrome c from mitochondria with RV+CSE/H$_2$O$_2$. However, our western blotting results showed that RV, CSE and H$_2$O$_2$ had no further effect on the cleavage of MAVS or the protein expression of MDA5, pIRF3, TBK1 and IKKe compared to media.

Exposure of healthy pBECs to oxidative stress led to inflammation by increasing the release of CXCL8 and IL6. They also reduced the release of CXCL10 and a type III IFN (IFN-λ1/3) which led to enhanced RV1B replication. The comparison of Beas-2B cell lines and healthy pBECs showed that pBECs are more susceptible to the effects of oxidative stress compared to Beas-2B cells in terms of increased expression of mitochondrial transcription factors, the release of cytochrome c and ATP. However, the western blotting results showed that the cleavage of MAVS or the protein expression of MDA5 was not different in pBECs and Beas-2B cells. Inflammatory responses, antiviral responses and RV replication were also compared in pBECs and Beas-2B cells. The results showed that pBECs have more reduced antiviral responses which led to increased RV replication compared to Beas-2B cell. However, inflammatory responses were not different in pBECs and Beas-2B cells.

In chapter 4, the effects of oxidative stress on mitochondrial function and antiviral immune responses to RV infection have been compared in healthy and asthmatic pBECs.

It was found that exposure of pBECs to CSE/H$_2$O$_2$ resulted in mitochondrial damage; with impaired mitochondrial membrane integrity and co-localisation of MAVS, MDA5 and mitochondria, increased expression of mTFs, release of cytochrome-c and ATP from mitochondria which was greater in asthmatic pBECs.
RV+CSE/H$_2$O$_2$ further increased mTFs, and ATP release which again was exaggerated in asthmatics compared to HC. In asthmatics pBECs demonstrated impaired IFN response with, the decrease in CXCL-10 and IFN-λ compared to HC, and rise in CXCL8, IL-6 release and RV replication. This was more pronounced in asthmatics compared to HC. CSE and H$_2$O$_2$ had no effect on MAVS cleavage or the protein expression of MDA5, pIRF3, TBK1 and IKKe in both groups. Thus, our experiments showed that asthmatic cells are more susceptible to oxidative damage in response to RV infection. In addition, we found that there is a greater effect when the cells are exposed to ROS in the form of CSE/H$_2$O$_2$ and viral infection that induces further mitochondrial dysfunction and potentially enhances epithelial inflammatory responses which can lead to an acute asthma exacerbation. However, further studies are needed to investigate the following questions:

1) The exact mechanisms of this synergistic mechanism.

2) The exact mechanisms causing greater susceptibility to RV infection in asthmatics.

3) The role of ROS on mitochondrial function.

In this thesis, we have also considered the role of antioxidants or therapeutic interventions which can prevent mitochondrial oxidative damage in asthma.

In this regard, chapter 5 describes the effects of lycopene and L-carnitine as potential antioxidants in pBECs of healthy and asthmatic subjects. Our aim, in this chapter, was to determine if the oxidative damage to mitochondria and antiviral responses to RV infection could be reversed using lycopene and carnitine.

Lycopene and carnitine, which are found abundantly in tomato products and in red meat respectively, have been shown to reduce oxidative stress in cells. They are considered to be potent singlet oxygen quenchers as well as scavengers of free radicals, such as the superoxide anion and hydrogen peroxide [229, 237-239, 248, 261]. It has been shown that carnitine specifically transports long chain fatty acids into the mitochondria which is important for the
oxidation of these fatty acids. Studies have shown that the accumulation of these long chain fatty acids in the cytosol, in the absence of carnitine, make the cell’s environment toxic leading to airway inflammation [262, 265].

The results of this chapter showed that RV1B entry into healthy pBECs which were pre-supplemented with lycopene and carnitine and exposed to CSE/H$_2$O$_2$ was not affected by oxidative stress, as the virus entered the cells and replicated quickly. On the other hand, we observed that lycopene and carnitine were able to exert their protective effects on mitochondrial function in healthy and asthmatic pBECs. These antioxidants restored mitochondrial membrane integrity leading to increased levels of ATP inside the mitochondria, which is essential for providing the cellular energy. Furthermore, the results of confocal microscopy showed that lycopene and carnitine were able to enhance the co-localisation between MAVS, MDA5 and mitochondria, which is important for the initiation of downstream signalling pathways. Thus, they may be beneficial in the prevention of mitochondrial oxidative damage. Also, we found that these antioxidants can decrease inflammation with a marked reduction in CXCL8 and IL-6. In addition, they led to reduced virus replication, however the release of type I IFN stimulated proteins (CXCL10) and a type III IFN (IFN-λ1/3) were not restored. Thus, we conclude that lycopene and carnitine may have therapeutic utility in improving mitochondrial function and decreasing inflammation. However, they could not restore antiviral responses. Although, there were some limitations in this study regarding the number of subjects in chapter 4 and 5, but further investigations are still required to determine the exact mechanisms which lead to impaired antiviral responses to RV infection, particularly in asthmatics which may help us to reduce exacerbations in these patients. In this regard, a direct assessment of mitochondrial function, IRF3 and NF-kβ binding to inflammatory or other gene promoters and the use of other mitochondria-directed anti-oxidants such as SS-31 would be helpful. Also, investigations of the effects of oxidative
stress on other organelles may provide us a clue for these impaired anti-viral immune responses to RV infection. Furthermore, the role of lycopene and carnitine in the treatment of inflammatory diseases such as asthma, rather than prevention, may provide a broader picture of the beneficial effects of these antioxidants which would be an interesting area for future research.

References


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Dutch CNSLD Study Group.}


