Virus-induced Glucocorticoid Insensitivity in the Airways

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ABSTRACT

Chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) pose a major public health burden. Hospitalization, mortality and increased health care costs owing to these diseases are amongst the consequences of exacerbations. Exacerbations are usually elicited by respiratory infections, allergens, environmental pollutants or occupational sensitizers. These factors may work in either an additive or synergistic manner. Regular management using inhaled glucocorticoids (GCs) has an unquestioned benefit in alleviating exacerbations unless the respiratory viruses were the trigger. Due to the few alternative anti-inflammatory therapies available, restoring glucocorticoid (GC) efficacy is indispensable. Among the respiratory viruses implicated in asthma/COPD exacerbations is respiratory syncytial virus (RSV); the prime respiratory virus associated with bronchiolitis and pneumonia in infants, young children, immunocompromised adults and the elderly worldwide. Despite the fact that RSV-induced pathogenesis is in part inflammatory, the beneficial effect of GCs in alleviating the symptoms or altering the course of illness is still debatable. Several reports suggested that RSV infection has a detrimental effect on GC signalling through mechanisms that are not yet defined.

Being the prime locus for viral infection and replication and the crucial target for glucocorticoid therapy as well, airway epithelium has been suggested as a key cell type in which virus-induced glucocorticoid resistance may occur. The airway epithelium releases a surplus of cytokines and chemokines leading to airway inflammation and airway structural changes seen in asthma. Among these inflammatory mediators is the transforming growth factor β1 (TGF-β1). Our laboratory has identified TGF-β1 as the key mediator of glucocorticoid insensitivity in bronchial epithelial cells where it ablates glucocorticoid activity at pathophysiologically relevant picomolar concentrations. We, therefore, investigated the possible contribution of this key cytokine to RSV-induced GC resistance.

Data presented within this thesis has provided the first evidence that TGF-β1 drives RSV-induced GC-insensitivity in bronchial epithelium transformed with Ad12-SV40 2B (BEAS-2B) cell line. However, blanket inhibition of TGF-β1 using TGF-β receptor (activin-like kinase 5 (ALK5/ TGF-βR1 kinase) selective inhibitor, SB431542 compromises its beneficial pleiotropic effects leading to serious adverse effects. Our group has recently identified casein kinase 1delta /epsilon (CK1δ/ε) as a downstream effector of TGFβ-induced remodelling and steroid resistance. Our data also implicated CK1δ/ε in RSV triggered

impairment of GC responsiveness where pharmacological inhibition of CK1δ/ε by PF670462 has inhibited the expression and activity of TGF-β1 and subsequently prevented the GC impairment provoked by RSV infection in our *in vitro* model. Moreover, PF670462 has significantly subdued RSV-induced expression of pro-inflammatory gene suggesting the potential for additional beneficial anti-inflammatory activities mediated by PF670462. This potential was further confirmed *in vivo*, where we have provided evidence of an interaction between PF670462 and dexamethasone to achieve greater control of the pulmonary inflammation following RSV infection.

Moreover, we evaluated the contribution of different epithelial pattern recognition receptors (PRRs) involved in RSV sensing in the apparent resistance using a wide array of PRRs ligands. Among the ligands tested was polyinosinic:polycytidylic acid (poly I:C); a synthetic analogue of double-stranded RNA (dsRNA) that is frequently used as a viral mimic. Our results showed that that viral pathogen-associated molecular patterns (PAMPs), most importantly double-stranded RNA (dsRNA), activates toll-like receptor 3 (TLR3), activates toll-like receptor 3 (TLR3) that in turn signals through the extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent pathway to promote TGF-β activity hence mediating GC resistance in airway epithelial cells. Genetic approaches established that targeting TLR3 may restore the responsiveness to glucocorticoids.

In conclusion, we demonstrated that RSV impairs glucocorticoid sensitivity in airways epithelium partly through TLR3 activation with subsequent release of TGF-β1, a potent mediator of glucocorticoid resistance. We also implicated CK1δ/ε in the apparent resistance. This thesis provided novel insights into mechanisms of glucocorticoid insensitivity in the airway epithelium. These findings offered two novel anchor points; CK1δ/ε and TLR3, for a potential treatment for the prevention/treatment of GC insensitivity occurring during RSV infection-induced bronchiolitis or asthma/ COPD exacerbations.

DECLARATION

This is to certify that

i. the thesis comprises only my original work towards the Doctor of Philosophy except where indicated in the Preface;

ii. due acknowledgement has been made in the text to all other material used; and

iii. the thesis is fewer than 100,000 words in length, exclusive of tables, figures and bibliographies, and complies with the stipulations set out for the degree of Doctor of Philosophy by the University of Melbourne.

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PREFACE

In order to present a cohesive set of experiments, the following people contributed to this thesis.

Preparation of rat-tail collagen was performed by Ms Trudi Harris. Preparation of DH5α competent cells was performed by Dr Nik Veldhuis. Transformation of competent cells and preparation of glycerol stocks was performed by A/Prof Josephine Mok. Experiments examining the phosphorylation of ERK following RSV infection presented in Figure 3.19 were performed by Dr Yuxiu C. Xia. The *in vivo* study involving RSV infection in BALB/c mice was performed by Ms Shenna Y. Langenbach.

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CONFERENCE PRESENTATIONS

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ABBREVIATIONS

·OH	hydroxyl free radical
3′ UTR	3' untranslated region
AD	transactivator domain
AECOPD	acute exacerbations of chronic obstructive pulmonary disease
AECs	airway epithelial cells
AHR	hyperresponsiveness of airways
AHS	Australian Health Survey
Akt	Ak strain transforming
AJC	apical junctional complex
ALI	air-liquid interface
ALK5	activin-like kinase 5
AMs	alveolar macrophages
AP-1	activator protein-1
APCs	antigen-presenting cells
APS	ammonium persulfate
ARDS	acute respiratory distress syndrome
ASL	airway surface liquid
ATCC®	American Type Culture Collection
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BAFF	B-cell activating factor
BCi-NS1.1	basal cell immortalized-nonsmoker 1.1
BEAS-2B	Bronchial Epithelium transformed with Ad12-SV40 2B
BIR	a baculoviral inhibitor of apoptosis repeat
BPL	beta propiolactone
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
$C_{18}H_{13}ClFN$	thiophenecarboxamidopro
O_3S	pionic acid
CARDs	caspase-recruitment domains
CBP	cAMP Response Element-Binding Protein (CREB)-binding protein
CCL5	C-C motif ligand 5
CD14	cluster of differentiation 14
CDKN1C	cyclin-dependent kinase inhibitor 1C
cDNA	complementary DNA
CK1 δ/ε	casein kinase 1 δ/ε
CMV	cytomegalovirus
COPD	chronic obstructive pulmonary disease
CoA	co-activator
CoR	co-repressor
CPE	cytopathic effect
CREB	cAMP Response Element-Binding Protein ()-binding protein (CBP)
CRISP	Clustered Regularly Interspaced Short Palindromic Repeats
CS	Corticosteroids
CVB	coxsackieviruses B
CXCR	Chemokine receptor
CXCL	C-X-C motif chemokine ligand
DAMPs	danger-associated molecular patterns
DC	dendritic cells
DEPC	diethylpyrocarbonate

dH_20	Distilled water
DMEM	Dulbecco's modified Eagle's media
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DEPC	diethylpyrocarbonate
dsRNA	double-stranded RNA
DTT	1,4-Dithiothreitol
EBV	Epstein-Barr virus
ECP	Eosinophil Cationic Protein
EDN	Eosinophil-derived neurotoxin
EDTA	ethylene-diamine-tetra-acetic acid
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
ENaCα	epithelial sodium channel α subunit
ER	endoplasmic reticulum
ERK 1/2	Extracellular signal-regulated kinase 1/2
EV71	enterovirus 71
FADD	Fas-associated death domain-containing protein
FCS	foetal calf serum
FEV1	forced expiratory volume in 1s
FGF	fibroblast growth factor
FKB52	FK506-binding protein 52
FKBP5	FK506 binding protein 5
GAGs	Glycosaminoglycan Chains
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA3	GATA-binding protein 3
GC	Glucocorticoid
GCs	Glucocorticoids
G-CSF	Granulocyte-colony stimulating factor
GILZ	glucocorticoid-inducible leucine zipper
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR-α	Glucocorticoid receptor-alpha
GRE	glucocorticoid response element
GRIP1	Glucocorticoid receptor-interacting protein-1
H_2O_2	Hydrogen peroxide
hAEC	human airway epithelial cells
HAV	hepatitis A virus
HBE	human bronchial epithelial cells
HBECs	human bronchial epithelial cells
hCECs	human corneal epithelial cells
HCV	hepatitis C virus
HDAC2	Histone deacetylase 2
HDM	house dust mite
HEp-2	Human epithelial type 2
HIFCS	Heat-inactivated foetal calf serum
HMGB1	High mobility group proteins
HRP	horseradish peroxidase

HSE	herpes simplex encephalitis
HSPs	heat shock proteins
HSV-1	herpes simplex virus type 1
HSV-TK	herpes simplex virus thymidine kinase
IAV	Influenza A virus
IC	Inflammatory cytokines
ICAM-1	Intercellular Adhesion Molecule 1
IE	Intestinal epithelium
IFN	interferon
IFN-β	interferon-β
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
iHBEC	immortalized human bronchial epithelial cells
IKK	IκB kinases
IKK-i	inducible IκB kinase
IKKε	IκB kinase ε
IL	interleukin
IL6R	IL6 receptor
ILC2s	type 2 innate lymphoid cells
IP-10	Interferon gamma-induced protein 10
IPF	idiopathic pulmonary fibrosis
IPS-1	IFN-b promoter stimulator-1
IRAK2	IL-1 receptor-associated kinase-like 2
IRF-3	IFN- regulatory factor-3
ISGs	interferon-stimulated genes
ISREs	IFN-sensitive response elements
IκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
JAK	Janus kinase
JAM-A	Junctional Adhesion Molecule A Protein
JNK	c-Jun amino-terminal kinase
K_2HPO_4	Dipotassium hydrogen phosphate
kb	Kilobase
KCNB1	potassium channel shab-related subfamily B member 1
KH_2PO_4	Potassium dihydrogen phosphate
KO	knockout
KSHV	Kaposi's sarcoma-associated herpesvirus
LA	Luria Agar
LABA	long-acting β2-adrenoceptor agonists
LAP	latency-associated peptide
LB	Luria-Bertani medium
LCMV	lymphocytic choriomeningitis virus
LGP2	laboratory of genetics and physiology-2
LMW	low molecular weight
LPS	lipopolysaccharide
LRR	leucine-rich repeats
LTA	Lipoteichoic acid
LTBP	latent TGF-β binding protein

Mal	MyD88-adaptor like
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signalling protein
MBP	Major Basic Protein
MC	Mast cells
MCMV	murine cytomegalovirus
MDA5	melanoma differentiation-associated gene 5
MDP	muramyl dipeptide
MDR1	multidrug resistance 1
MEK1/2	mitogen-activated protein kinase kinase-1/2
$MgCl_2$	magnesium chloride
MKP-1	mitogen-activated protein kinase phosphatase-1
MOI	multiplicity of infection
MPO	Myeloperoxidase
mRNA	Messenger RNA
mTOR	mammalian target of rapamycin
MyD88	Myeloid Differentiation factor 88
МΦ	macrophages
NAC	N-acetyl-L-cysteine
NACHT	NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein)
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAP1	NF-jB-activating kinase-associated protein-1
NBD	nucleotide-binding domain
NF-κB	nuclear factor-κB
nGRE	negative glucocorticoid response element
NHMRC	National Health and Medical Research Council
NK	natural killer
NLRs	Nucleotide-binding oligomerization domain (NOD)-like receptors
NO	nitric oxide
NOD	Nucleotide-binding oligomerization domain
Nrf2	Nuclear erythroid-2-Related Factor 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
ODN	oligodeoxynucleotides
PAI-1	plasminogen activator inhibitor-1
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pDCs	plasmacytoid dendritic cells
PDE4	Phosphodiesterase 4
PDK4	pyruvate dehydrogenase kinase isozyme 4
PGE_2	Prostaglandin E_2
PI3K	phosphoinositide-3-kinase
PIV	Parainfluenza viruses
PLZF	Promyelocytic leukaemia zinc finger

Poly I:C	Polyinosinic: Polycytidylic Acid
PRRs	pattern recognition receptors
PTV	Punta Toro virus
PV	poliovirus
PVM	pneumonia virus of mice
PYDs	pyrin domains
Ras	Rat sarcoma
RD	repressor domain
RhoA	Ras Homolog Gene Family Member A
RICK	receptor-interacting serine/threonine kinase
RIG-I	Retinoic acid-inducible gene I
RIP1	receptor-interacting protein 1
RLRs	helicase receptors
RNA	Ribonucleic acid
ROCK	Rho-associated coiled-coil kinase
ROS	reactive oxygen species
rRNA	Ribosomal RNA
RSV	respiratory syncytial virus
RTA	replication and transcription activator
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
RV	Rhinovirus
SEM	standard error of the means
SDS	sodium dodecyl sulphate
SEAP	secreted embryonic alkaline phosphatase
SERPINA3	serpin family A member 3
siRNA	Small interfering RNA
SLPI	secretory leukoprotease inhibitor
Smad	Mothers against decapentaplegic
SOD	superoxide dismutase
SPGA	Sucrose-Phosphate-Glutamate-Albumin
ssRNA	single-stranded RNA
STAT3	Signal transducer and activator of transcription 3
TAK1	TGF-β-activated kinase 1
TB	transcription blocker
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing 0.1% tween
$TCID_{50}$	50% tissue culture infectious dose
TEMED	tetramethylethylenediamine
TβRII	TGFβ receptor type-2
TGF-β1	transforming growth factor-β
Th2	type 2 helper T
TIR	Toll/IL-1 receptor
TIRAP	TIR-containing adaptor protein
TLR1	Toll-like receptor 1
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TLR6	Toll-like receptor 6
TLR7	Toll-like receptor 7
TLR8	Toll-like receptor 8
TLR9	Toll-like receptor 9
TLR10	Toll-like receptor 10
TLRs	Toll-like receptors
TMEV	Theiler's murine encephalomyelitis virus
TNF	tumour necrosis factor

TNFRI	tumour necrosis factor receptor I
TRADD	TNFRI associated death domain
TRAF3	TNF-receptor associated factor 3
TRAF-6	TNF receptor-associated factor 6
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
TRAM	translocating chain-associating membrane
TRIF	TIR domain-containing adaptor protein interferon-β
Tregs	TGF-β dependent regulatory T cells
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
TRIM38	tripartite motif 38
tRNA	Transfer RNA
TSLP	thymic stromal lymphopoietin
UV	Ultraviolet radiation
VACV	vaccinia virus
VEGF	Vascular endothelial growth factor
VSV	vesicular stomatitis virus
WB	Western blotting
WNV	West Nile virus
ZBTB16	Zinc finger and BTB domain-containing protein 16
ZO-1	Zonula occludens-1

ETHICS STATEMENT

All experiments using cells and tissues from animal or human origin were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the National Statement on Ethical Conduct in Human Research.

CHAPTER 1

General Introduction

1 General introduction

1.1 Overview

1.1.1 Thesis statement

Glucocorticoids (GC) are poorly effective in alleviating respiratory syncytial virus (RSV)-induced bronchiolitis and wheezing. In this thesis, we demonstrated that RSV impairs glucocorticoid sensitivity in airways epithelium partly through Toll-like receptor 3 (TLR3) activation with subsequent release of transforming growth factor-β1 (TGF-β1), a potent mediator of glucocorticoid resistance. We also implicated casein kinase 1δ/ε (CK1δ/ε) in the apparent resistance. In this study, we have offered anchor points for novel therapeutic strategies to restore GC sensitivity during RSV infection.

1.1.2 Overview of general introduction

Firstly, this chapter focuses on reviewing the pathophysiology of asthma exacerbations and the contribution of viral infections in disease pathogenesis. Next, we will review the current knowledge of the molecular mechanisms of GC insensitivity. The respiratory synthetical virus, the prime cause of asthma exacerbation in infants will be then discussed. Current understanding of the immune response of airway epithelial cells to RSV infection will then be critically reviewed. The final sections of this chapter will be devoted to the rationale of examining the molecular mechanism of RSV-induced glucocorticoid resistance in the epithelium and the rationale of examining the different pattern recognition receptors (PRRs) involved in RSV sensing for a role in the reported GC insensitivity.

1.2 Chronic respiratory diseases; an overview

Chronic respiratory diseases are a diverse group of illnesses affecting the airways and other lung structures. The most significant of these disorders are asthma, chronic obstructive pulmonary diseases (COPD), pulmonary hypertension and idiopathic pulmonary fibrosis (IPF). Among these disorders, asthma is the most prevalent in Australia with one in nine (equivalent to 2.8 million people) has asthma based on data reported from the 2017–18 Australian Health Survey (AHS) (Health and Welfare, 2020). Despite being incurable, asthma is largely manageable except for exacerbations that respond poorly to steroids. This fact sparks our interest to explore treatment improvement for steroid-resistant asthma exacerbations. Hence, in this section, we will review the epidemiology, pathogenesis and treatment options of asthma exacerbations with a focus on exacerbations of viral aetiology.

1.2.1 Asthma exacerbations: definition

A major burden of asthma is its exacerbations that vary from being mild, moderate, severe, or life-threatening. In certain cases, oral glucocorticoid intervention may be required (Edwards et al., 2012). Asthma exacerbation is the transient worsening of asthma symptoms that may develop following exposure to "triggers". According to the Global Initiative for Asthma (GINA) guidelines, asthma exacerbations are defined as "episodes characterized by a progressive increase in symptoms of shortness of breath, cough, wheezing or chest tightness and progressive decrease in lung function" (GINA, 2020). A joint task force of the American Thoracic Society and European Respiratory Society has defined asthma exacerbations as events characterised by a change from the individual's previous status and categorized exacerbations into:

"1. Severe asthma exacerbations are defined as events that require urgent action on the part of the patient and physician to prevent a serious outcome, such as hospitalization or death from asthma.

2. Moderate asthma exacerbations are defined as events that are troublesome to the patient, and that prompt a need for a change in treatment, but that is not severe. These events are clinically identified by being outside the patient's usual range of day-to-day asthma variation" (Reddel et al., 2009).

1.2.2 The epidemiology of asthma exacerbations

Around 20% of asthma patients experience an exacerbation that often requires medical consultation or hospitalization that account for 80% of the asthma total costs (Rodrigo et al., 2004). Exacerbation occurs similarly across different races when taking the socioeconomic factors into account. The incidence of asthma exacerbations is higher in the obese population and women taking hormone replacement therapy (Dougherty and Fahy, 2009).

Exacerbations are usually self-reinforcing and increase the risk of future recurrence (Miller et al., 2007). Particular patients are found to be more 'exacerbation prone'. Usually, they are distinguished by irreversible limitation of airflow, chronic sinusitis, psychological disorders, and resistance to non-steroidal anti-inflammatory drugs (Koga et al., 2006; Kostakou et al., 2019). Exacerbations of asthma may result in long term effects on the lung. Patients prone to exacerbation are more liable to accelerated loss of lung function as a consequence of the greater inflammatory burden and the activation of auxiliary

inflammatory pathways during exacerbation of asthma (Bai et al., 2007; Holgate et al., 2008; Suau and DeBlieux, 2016).

1.2.3 Aetiology of asthma exacerbations

Numerous triggers have been identified for asthma exacerbations including occupational sensitizers (cleaning products, grains, flours), high doses of allergens (dust mites, pollen, animal dander), medications [β-blockers, Nonsteroidal anti-inflammatory drugs (NSAIDs)], environmental pollutants (diesel particulates), exercise and stress. Nevertheless, respiratory viruses are the most prevalent cause of asthma exacerbation especially among infants and children (Johnston et al., 1995; Freymuth et al., 1999; Rakes et al., 1999; Kotaniemi-Syrjänen et al., 2003; Thumerelle et al., 2003; Heymann et al., 2004; Jartti et al., 2004; Johnston et al., 2005; Lemanske et al., 2005; Kusel et al., 2006; Allander et al., 2007; Chung et al., 2007; Khetsuriani et al., 2007; Kusel et al., 2007; Bosis et al., 2008; Jackson et al., 2008; Papadopoulos et al., 2011; Turunen et al., 2014; Christensen et al., 2019). Other pathogens reported to mediate exacerbations are the gram-negative *Mycoplasma pneumoniae* and *Chlamydophila pneumonia*, however, they play a minor role **(**Figure 1.2**)** (Douwes et al., 2002). Moreover, the 'two-hit hypothesis' may act in some situations where some exposures may modify the effect of other exposures and there may be synergistic interactions that may lead to a more severe asthma exacerbation (Papadopoulos et al., 2011). As an example, viral-induced inflammation has been proved to prime the airways for a potentiated allergic response to exposure to allergens leading to severe asthma exacerbation (Green et al., 2002; Dougherty and Fahy, 2009). The interaction of some aeroallergens with other environmental components, e.g., high levels of air pollutants has also led to more severe asthma exacerbations in people of different age groups and gender (Murray et al., 2006; Cakmak et al., 2012).

Some individual characteristics have been proposed as potential modifiers of asthma exacerbations severity. Individuals with a sensitivity to certain allergens will probably experience more severe allergic reactions hence increase their risk to develop exacerbations (Medrek et al., 2017). Gender and age have been found to be potential modifiers possibly due to biological differences in levels of sex hormones (Osman, 2003), lung and airway size, volume and growth rates (Vink et al., 2010), or to socially-linked differences in exposures to physical activity, cigarette smoking (Clougherty, 2010), cosmetic products, or obesity (Cakmak et al., 2005).

1.2.4 Role of respiratory viruses' infection in asthma exacerbations

Asthma exacerbation is often associated with concomitant respiratory infections with the respiratory viruses being the main trigger in severe cases. Clinical studies have reported that the percentage of asthma exacerbations caused by viral infection is around 95% in infants and preschool-aged children, 60-75% in school-aged children and 80 to 85% in adults (Caramori et al., 2006). Viral infections commonly contributing to exacerbation include rhinovirus (RV), RSV, influenza, parainfluenza, coronaviruses, adenoviruses, human metapneumovirus, human bocavirus and enterovirus. A systematic review of 456 studies published between 2001-2011 revealed that RV are the most frequently detected virus type at all ages, with the exception of infants hospitalized with bronchiolitis, where RSV prevails . Most other agents have small to moderate involvement (Papadopoulos et al., 2011). The relative contribution of other viruses to exacerbations of acute asthma across different age groups is depicted in Figure 1.1. RV is the most common virus; being detected in 65% of cases (Johnston et al., 1995; Guibas and Papadopoulos, 2017). Parainfluenza viruses (PIV) are the second major cause of hospitalization in children with respiratory tract infection (Pecchini et al., 2015). Among the respiratory viruses involved in asthma exacerbations, RSV is especially crucial in premature infants and young children and will be the focus of the current thesis (Figure 1.1) (Stowell et al., 2009).

1.2.5 Mechanisms of virus-induced asthma exacerbations

Several mechanisms have been involved in the progression from a viral respiratory infection to an acute asthma exacerbation. In response to respiratory viral infections, an integrated response including the respiratory, the immune and the nervous systems occurs to quickly and efficiently clear the invading pathogen (Velissariou and Papadopoulos, 2006). In asthmatics, several elements of this integrated response are altered leading to airway hyperresponsiveness (AHR) that contributes to the exacerbations developed following viral infection (Figure 1.1) (Togias, 2004). AHR may develop secondary to airway wall oedema, metaplasia of goblet cell with alteration in mucus composition due to cell debris and plasma protein excess, increased epithelial and endothelial permeability and impaired small airway patency (Folkerts et al., 1998; Dakhama et al., 2005). The epithelium damage induced by viral infection results in shedding of dead epithelial cells into the lumen and impaired mucociliary clearance. Moreover, damage of the airway epithelium decreases the production of endogenous bronchodilators [Prostaglandin E_2 (PGE_2), endopeptidase, nitric oxide (NO)] and impairs the catabolism of bronchoconstrictor-like neurokinin A and substance P (Hegele et al., 1995). The absence of airway epithelium may

also increase the exposure of sensory nerves to allergens. The released kinins can trigger smooth muscle contraction. Lastly, viral infection triggers the release of a surplus of cytokines and chemokines which induce airway remodelling and promote an influx of inflammatory cells namely eosinophils and neutrophils to the site of infection. Degranulation of eosinophils release mediators that deposit on airway smooth muscle and interfere with the activity of the muscarinic acetylcholine receptor M2 leading to increased cholinergic responsiveness and continued bronchoconstriction (Papadopoulos et al., 2007).

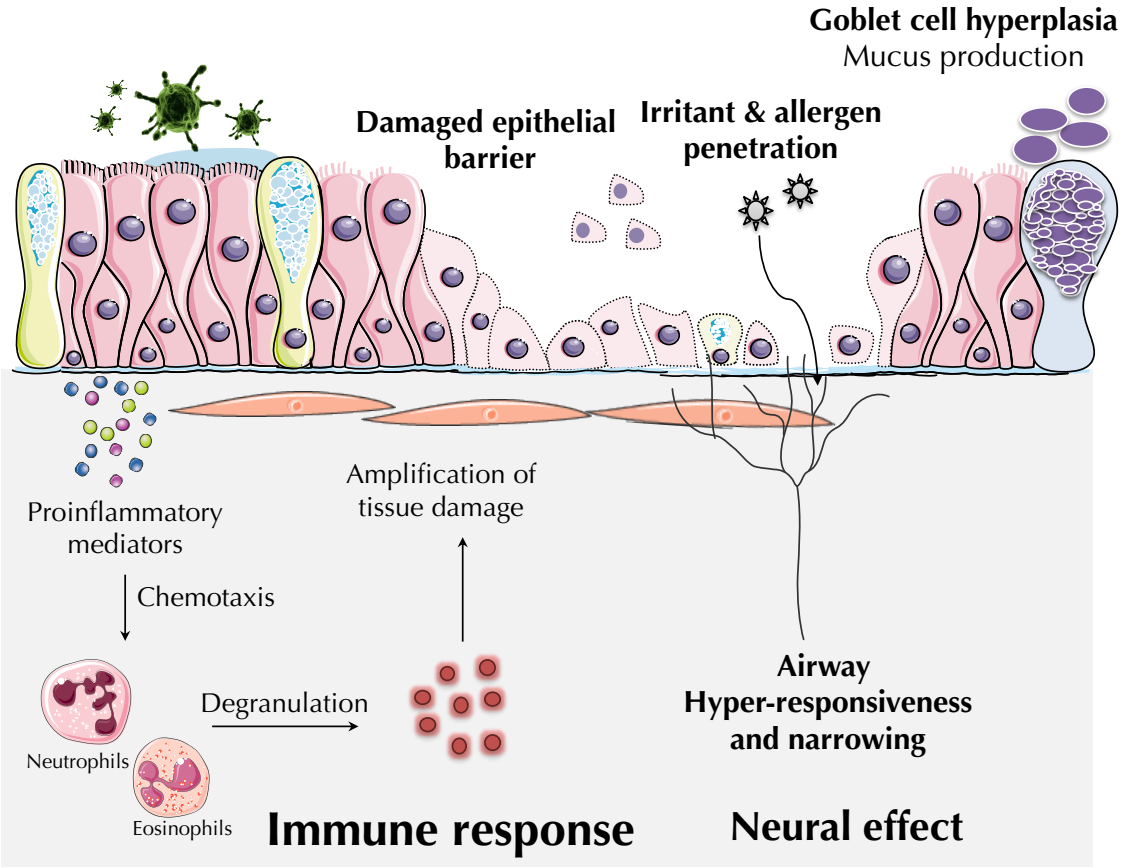


Figure 1.1| A conceptual model for asthma exacerbations upon viral infection.
Modified from (Papadopoulos et al., 2007)

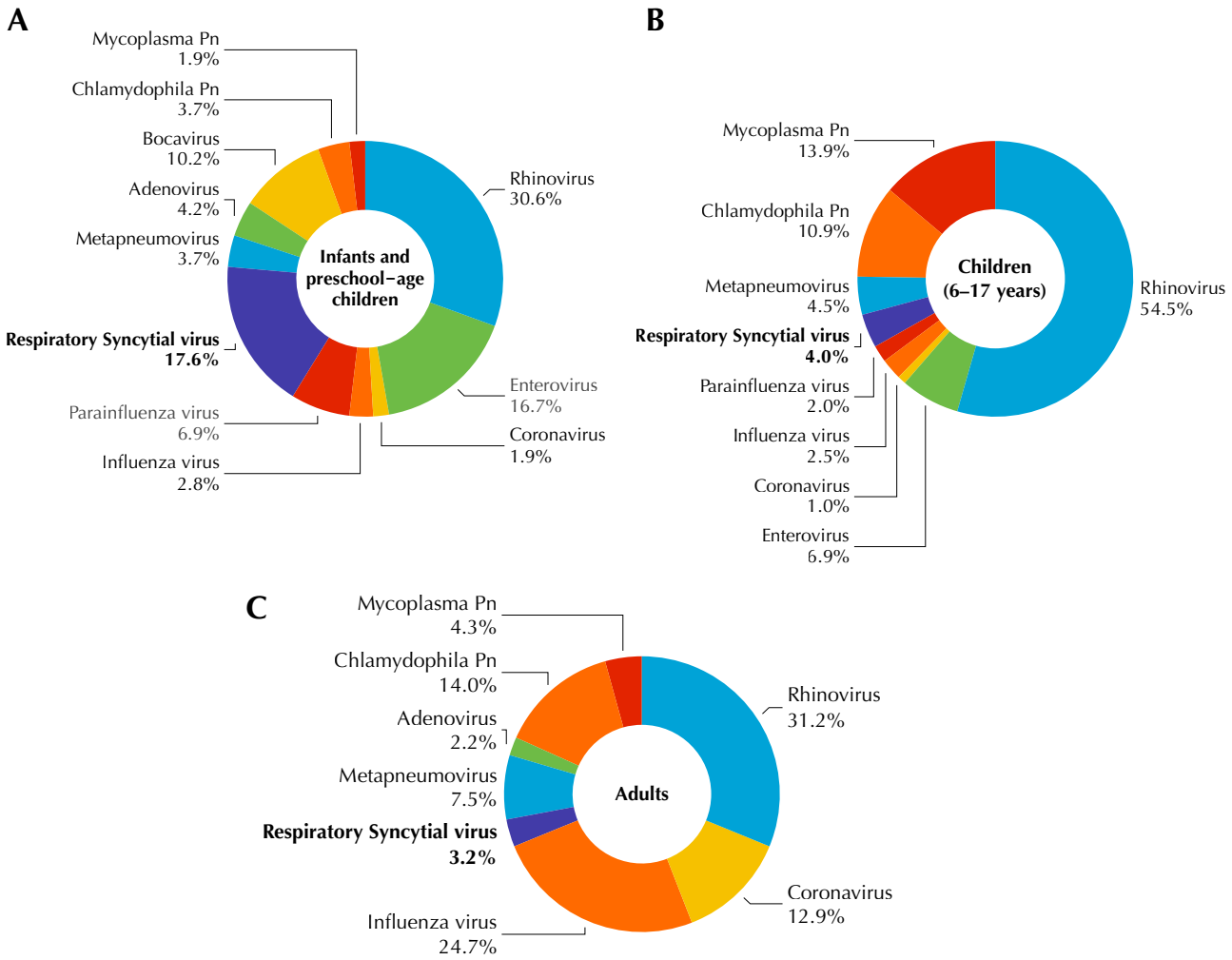


Figure 1.2| Prevalence of viruses and bacteria in asthma exacerbation across different age groups.
Modified from Edwards *et al.* (2012)

1.2.6 The therapeutic regimens for virus-induced exacerbations

Corticosteroids are so far the most protective against the development of asthma exacerbations leading to a 50% reduction in occurrence compared to β2 agonist or placebo (Sin et al., 2004; Tamm et al., 2012). Additionally, they are reported to improve the AHR leading to better control of exacerbations (Sont et al., 1999). Nonetheless, the effective treatment of asthma exacerbations remains unmet. The poor compliance of asthmatic patients to the inhaled drug treatment represents one problem (Lindsay and Heaney, 2013; Gaude et al., 2014; Engelkes et al., 2015). Accordingly, glucocorticoid treatment begins only when asthma exacerbations symptoms have already emerged resulting in a poor therapeutic outcome. The efficacy of corticosteroids in managing exacerbations remains controversial, particularly if they are virus-induced, despite the reported efficacy of inhaled corticosteroid in treating the respiratory viral infection (McKean and Ducharme, 2000; Alangari, 2014; Hasegawa et al., 2021). In animal models, virus-induced exacerbations have failed to respond to the inhaled corticosteroids (Singam et al., 2006). Partial resistance to even systemic corticosteroids has been reported in Lipopolysaccharide (LPS)-induced asthma exacerbations (Komlósi et al., 2006; Ueda et al., 2016; Hadjigol et al., 2020).

Alternative strategies are emerging for managing viral exacerbations. The first approach is to boost the innate immunity via the use of inhaled interferon-β as a complementary strategy to corticosteroids for managing virus-induced asthma exacerbations in difficult-to-treat patients (Boxall et al., 2013; Djukanovic et al., 2014). This strategy was implemented following a study that reported the *in vitro* anti-inflammatory and anti-viral properties of interferon-β on primary bronchial epithelial cells obtained from asthmatic patients (Cakebread et al., 2011). Djukanovic and colleagues had reported potential deleterious effects of inhaled IFN-β in subgroup of patients with mild asthma, where asthma symptoms have increased, although non-significantly compared to placebo group (Djukanovic et al., 2014). Epithelial cell derived from patients with mild asthma have normal IFN production (Sykes et al., 2014). It is possible that the addition of exogenous IFN-β in this subgroup could lead to greater inflammation and subsequent worsening of symptoms (Djukanović et al., 2014; Jackson, 2014). Watson et al. recently demonstrated that intermittent prophylactic doses of exogenous IFN-β could modulate viral infection in an *in vitro* model. Moreover, they reported that chronic dosing with IFN-β was more effective than dosing post viral infection while it was not associated with inflammatory mediators production (Watson et al., 2019).

The second approach aims at decreasing the sensitivity to inhaled allergens via the use of omalizumab to attenuate immunoglobulin E (IgE)-mediated allergic response. This anti-IgE drug has successfully reduced steroid use for managing viral exacerbations (Busse et al., 2001; Soler et al., 2001).

The third approach to reduce the risk of exacerbations is to alleviate pulmonary inflammation via different anti-inflammatory strategies. These include inhibition of type-2 cytokines such as the anti-Interleukin (IL)-5 (IL-5) drugs (reslizumab or mepolizumab) to reduce exacerbations and control refractory eosinophilic asthma (Haldar et al., 2009; Pavord et al., 2012; Castro et al., 2015), the anti-IL13 drugs (tralokinumab, lebrikizumab) (Corren et al., 2011) and the anti-IL4 /-13 receptor (dupilumab) (Wenzel et al., 2013). Another anti-inflammatory strategy is the inhibition of pro-inflammatory cytokines in COPD via the use of Anti-IL6 (siltuximab) and anti-IL-6 receptor (IL6R) (tocilizumab) that have been previously studied in other neutrophilic conditions such as Rheumatoid arthritis (Yao et al., 2014). Inflammation reduction is also achieved via disrupting chemokine signalling by antagonists of C-X-C motif chemokine ligand 10 (CXCL10)/ Interferon gamma-induced protein 10 (IP-10) and its receptor chemokine receptor type 3 (CXCR3) (Laragione et al., 2011; Yellin et al., 2012) or antagonists of CXCL8/IL-8 and its receptors CXCR1/2 that showed mixed results in COPD (Magnussen H, 2010; Rennard SL, 2013). Other anti-inflammatory strategies include blocking of the pattern recognition receptors (PRRs), i.e. toll-like receptor 4 (TLR4) antagonist (eritoran) that prevented mortality in influenza-infected mice (Shirey et al., 2013), inhibition of different components of inflammatory pathway, such as phosphoinositide 3-kinase (PI3K) inhibitors, p38 mitogen-activated protein kinase (MAPK) inhibitors or Phosphodiesterase 4 (PDE4) inhibitors. PI3K inhibitors have reversed steroid irresponsiveness *in vitro* in COPD (Marwick et al., 2010) and reduced the inflammatory response in mice infected with RV (Ganesan et al., 2012). Inhibition of p38 MAPK showed mixed outcomes regarding lung function and sputum neutrophilia (Chung, 2011; Lomas et al., 2012). Roflumilast; a PDE4 inhibitor, reduce the exacerbation risk and improved the forced expiratory volume in severe COPD (Calverley et al., 2009). There is increasing evidence that antioxidants, such as Nuclear erythroid-2- Related Factor 2 (Nrf2) activator, decrease COPD exacerbations (Poole and Black, 2006; Tse et al., 2013; Zheng et al., 2014) and reversed steroid insensitivity in COPD patients macrophages *in vitro* (Malhotra et al., 2011). Macrolides have also been shown to decrease COPD exacerbations frequency (Herath and Poole, 2013).

The last approach to reduce the risk of virus-induced asthma exacerbations is to target the virus itself via vaccination, use of antiviral drugs or preventing the entry of the virus. Influenza vaccine has decreased the frequency of COPD exacerbations (Poole et al., 2006) and immunizing mice with rhinovirus capsid protein has improved viral clearance following challenge with the virus (Glanville et al., 2013). Blocking Intercellular Adhesion Molecule 1 (ICAM-1), i.e. tremacamra, prevent viral entry and hence alleviating symptoms and reduce the viral titers in nasal lavage, yet expensive (Turner et al., 1999). *In vivo,* anti-ICAM-1 prevented RV infection in mice (Traub et al., 2013). Antivirals that target rhinovirus capsid (pleconaril and vapendavir) and rhinovirus protease 3C inhibitors (rupintrivir) had been developed (Thibaut et al., 2012; Casanova et al., 2018). Although capsid binders are attractive and potent early-stage inhibitors of RV replication *in vitro*, *in vivo* results were disappointing due to problems with pharmacodynamics, *in vivo* efficacy, and resistance development (Ortega et al., 2020). Similarly, 3C protease inhibitors are effective *in vitro*, while ineffective in clinical trials (Glanville et al., 2013). The benefits of influenza neuraminidase inhibitors (oseltamivir, zanamivir) have been proved (Dobson et al., 2015). Palivizumab; anti-RSV F protein, is licensed to be used in high-risk infants (Olszewska and Openshaw, 2009).

1.3 Glucocorticoids and asthma management

Glucocorticoids represent the mainstay for asthma therapy and other inflammatory lung diseases. The therapeutic efficacy of GCs can be attributed to their impressive anti-inflammatory and immunosuppressive effects. However, around 5-10% of asthmatic patients are unresponsive to inhaled corticosteroids and poorly respond to oral corticosteroids, a condition referred to as “steroid insensitivity” (Henderson et al., 2020). Glucocorticoid insensitivity or resistance is precisely described as not only the decrease in the maximum response to GCs but also to a rightward shift in the dose-response curve (Keenan et al., 2012). GC-insensitive asthma is clinically defined as the persistent airflow obstruction along with a less than 15% increase in the forced expiratory volume in 1s (FEV1) following a 14-day course of high-dose oral GCs (Chung et al., 2014). This asthma cohort may experience poorly controlled asthma due to the few alternative anti-inflammatory therapies available. This in turn leads to disproportionate health care costs due to hospitalisations and emergency services use (Godard et al., 2002). In this section, we will discuss mechanisms of GC actions and different molecular underpinnings influencing GC sensitivity. Understanding of the complexity in the GC signalling

mechanisms and the multiple pathways involved in GC insensitivity may help the identifications of appropriate targets to improve GC sensitivity in steroid-resistant diseases.

1.3.1 Glucocorticoid mechanism of action

GC exert their profound anti-inflammatory effects via either *transactivation* of anti-inflammatory genes or *transrepression* of proinflammatory genes (Figure 1.3). Glucocorticoids penetrate the cell membrane where they can engage with their cytoplasmic glucocorticoid receptor-α (GRα). The GRα dissociates from its molecular chaperones i.e., heat shock proteins (HSPs); HSP70, HSP90, p23 and FK506-binding protein 52 (FKB52)], to allow GC binding thereby activating it. The GC-GRα complex then migrates to the nucleus.

Through transactivation, the activated GC-GR complex homodimerize and binds to Glucocorticoid Response Elements (GRE); a specific DNA sequences (5′-GGTACAnnnTGTTCT-3′) found in the 3' untranslated region (3′ UTR) of steroid-sensitive genes. This leads to the recruitment of several coactivators with intrinsic histone acetyltransferase activities, such as cAMP Response Element-Binding Protein (CREB)-binding protein (CBP) that elicit chromatin remodelling to facilitate binding of RNA polymerase II and subsequent gene transcription. Additionally, GR in a monomeric form interacts with other transcription factors, namely Signal transducer and activator of transcription 3 (STAT3) or STAT5 to trigger transcription through "tethering" or "composite" mechanisms (Stoecklin et al., 1999; Langlais et al., 2012; Ratman et al., 2013). Among the steroid-sensitive genes upregulated through transactivation are genes encoding anti-inflammatory mediators including IL-10, annexin-1 (lipocortin-1), the inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), secretory leukoprotease inhibitor (SLPI), mitogen-activated protein kinase phosphatase-1 (MKP-1) and glucocorticoid-induced leucine zipper (*GILZ*) (Ito et al., 2000).

The GR-GC complex can also mediate transrepression via protein/protein interaction or tethering mechanism. GR can directly interact with other transcription factors to inhibit its activity without contacting the deoxyribonucleic acid (DNA) itself. These immune-regulating transcription factors include NF-κB (nuclear factor-κB), IRF3 (interferon regulatory factor 3), AP-1 (activator protein 1) and GATA3 (GATA binding protein 3) (Reily et al., 2006; Liberman et al., 2009). Alternatively, the activated GR (the GC-GR complex) can compete with the transcription factors (NFκB and IRF3) for the nuclear coactivators; CBP and GR-interacting protein-1 (GRIP1) hence prevent the uncoiling of chromatin by

histone acetylation, therefore rendering the gene incapable of transcription (Barnes, 2006; Uhlenhaut et al., 2013). This effect can be enhanced by histone deacetylase 2 (HDAC2), reversing the histone acetylation, leading to DNA coiling and hence inhibiting transcription (Ito et al., 2000). Transrepression can also occur through binding of dimerised GC-GR complex to the negative GRE (nGREs) as in the case of repressing the expression of thymic stromal lymphopoietin (Surjit et al., 2011).

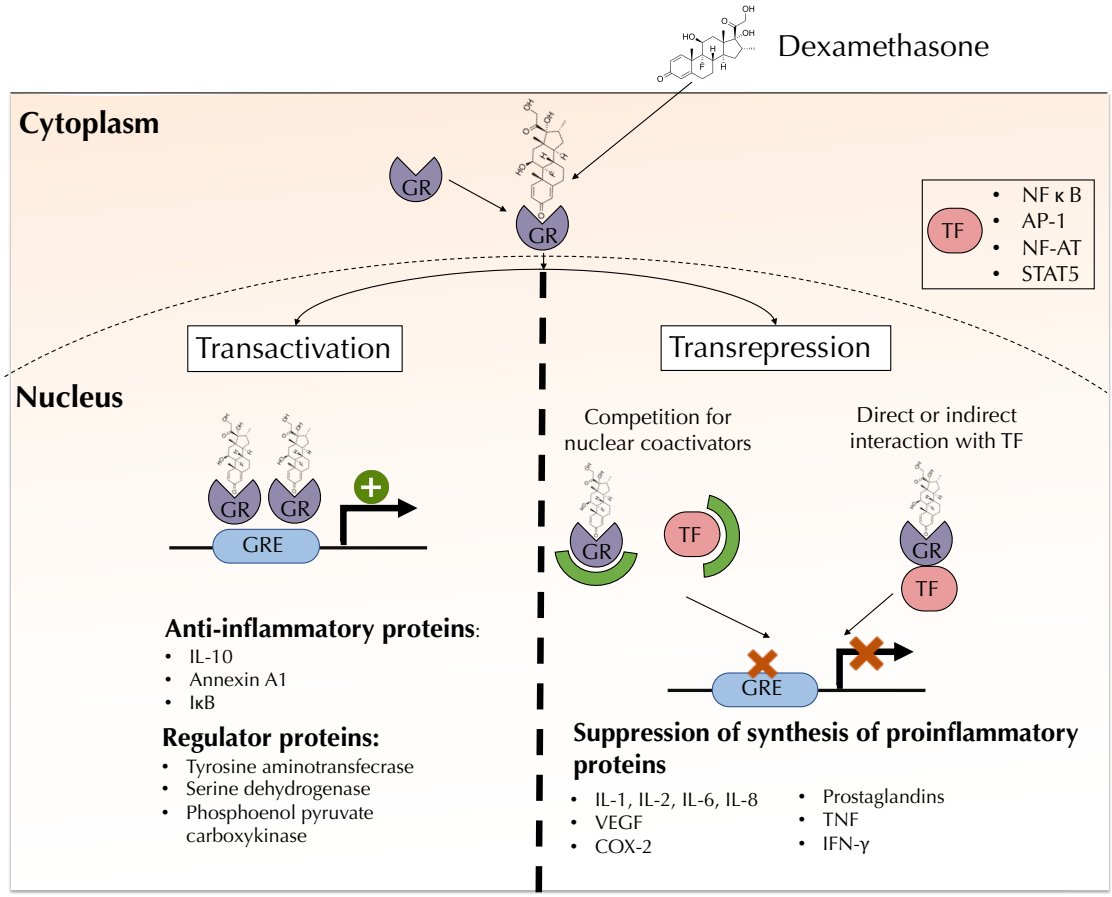


Figure 1.3| Mechanism of action of glucocorticoids.
Modified from (Patel et al., 2012)

1.3.2 Molecular mechanisms of GC insensitivity

Heterogeneous mechanisms have been involved in a diminished response to corticosteroids in asthma [reviewed in (Barnes, 2010; Keenan et al., 2012; Boardman et al., 2014)]. The importance of these mechanisms probably varies between patients and none of them are related to abnormal cortisol levels or Addison's disease. In this part, we will summarize the molecular mechanisms of GC resistance described to date.

1.3.2.1 Decreased GR binding

Glucocorticoids perform their action via binding to their cytoplasmic receptor GR. Therefore, it is possible that changes in GC-GR binding can result in GC insensitivity. For instance, the multidrug resistance 1 (MDR1) gene encodes the P-glycoprotein 170 pump that is responsible for the efflux of glucocorticoids from the cell. Upregulation of P-glycoprotein has been correlated to steroid resistance in inflammatory bowel disease, rheumatoid arthritis and nasal polyps due to the increased efflux of intracellular GCs (Farrell et al., 2000; Tsujimura et al., 2008; Kocharyan et al., 2014).

Another possible mechanism of the GC insensitivity is alternations in the expression of GRα/β. GRα is known as an active isoform while GRβ functions as a negative inhibitor by either competing with GRα for GRE binding (Leung et al., 1997) or forming a heterodimer with GRα thereby preventing it from binding to GRE sites (Oakley et al., 1999). Our laboratory has detected impaired GRα expression following the stimulation with TGF-β1 in cells of carcinomic human alveolar basal epithelial cell line A549 (Salem et al., 2012). GRα expression was also suppressed in mice treated with tumer necrosis factor-alpha (TNF-α) (Van Bogaert et al., 2011). Several studies, on the other hand, have detected an elevated level of GRβ in airway diseases that has been linked with modest response to GC treatment. Patients with GC-resistant asthma have shown an increased number of GRβ-positive bronchoalveolar lavage cells (BAL cells) and peripheral blood mononuclear cells (PBMCs) compared to healthy controls and GC-sensitive asthmatics (Christodoulopoulos et al., 2000; Sousa et al., 2000). Additionally, GRβ expression was increased in the epithelium and the submucosal inflammatory cells of severe asthmatics in comparison to patients with moderate asthma (Bergeron et al., 2006).

1.3.2.2 Impaired GRα nuclear translocation

GC function depends on efficient translocation of GR to the nucleus. PBMCs isolated from steroid-resistant asthmatics have shown defective GRα nuclear translocation (Matthews et al., 2004). GR nuclear translocation was 40% lower in airway smooth muscle cells (ASMs) from severe asthma than that measured in either healthy or non-severe asthma subjects (Chang et al., 2015). Given that GRα phosphorylation is well-documented to affect the nuclear trafficking of GR, it has been proposed as a mechanism of dysfunction. For example, the cytokine IL-2 was found to impair nuclear trafficking of GRα in $p38^{MAPK}$-dependent manner in PBMCs (Irusen et al., 2002). Similarly, phosphorylation of the GRα Ser226 residue in response to TNF-α activation of c-Jun amino-terminal kinase (JNK)

enhances nuclear export of GRα in inflammatory cell types (Barnes and Adcock, 2009). Additionally, our group has demonstrated that treatment of A549 cells with TGF-β has impaired GRα nuclear localisation (Salem et al., 2012).

The nuclear transport receptor Importin 13 controls the nuclear entry to GR. Decreased level of Importin 13 or degradation of Importin 7 caused by oxidative stress strongly potentiated glucocorticoid insensitivity via impairment of GR nuclear translocation (Tao et al., 2006; Hakim et al., 2013). Besides, Importin 13 genetic variation in children has resulted in better airway responsiveness in asthma that is equivalent to the improvement by long-time inhaled GCs (Raby et al., 2009).

1.3.2.3 Increased pro-inflammatory transcription factors

Peripheral blood mononuclear cells (PBMCs) and bronchial biopsies from glucocorticoid-resistant asthmatics have shown excessive activation of the transcription factor relative to steroid-sensitive asthmatic patients (Loke et al., 2006). AP-1 is thought to preferentially bind GRα thereby hindering GRα from interacting with other transcription factors or DNA (Adcock et al., 1995). Other transcription factors have been involved in GC resistance, such as NFκB, STAT5 and IRF1. The expression level of NFκB correlates inversely with glucocorticoid responsiveness (Hakonarson et al., 2005). STAT5 has been shown to bind GRα hence interfere with its translocation to the nucleus leading to defective DNA binding (Goleva et al., 2002). The TNFα and interferons (IFNs)-induced activation of the transcription factor IRF1 leads to sequestering the co-repressor GRIP-1, thereby inducing glucocorticoid irresponsiveness in airway smooth muscle cell (Bhandare et al., 2010).

1.3.2.4 Defective histone acetylation

GC transrepression depends mainly on recruitment of HDACs, especially HDAC2, to silence inflammatory genes. However, PBMCs isolated from patients with severe asthma or COPD have shown reduced HDAC2 activity that in turn results in poor GC responsiveness (Kobayashi et al., 2014; Tan et al., 2016). The impairment of HDAC2 function is highly correlated to PI3K signalling activation (Zijlstra et al., 2012). Additionally, the oxidative/nitrative stress can lead to S-nitrosylation of HDAC2 thereby impairing its activity impairs the effect of HDAC2 in facilitating GR transrepression in airway epithelial cells, which might contribute to poor GC response in these cells (Osoata et al., 2009).

1.3.2.5 Collagen signalling

Glucocorticoids have antimitogenic and anti-migratory effects on airway smooth muscle cells. Presence of denatured type 1 collagen impairs the GC responsiveness (Bonacci et al., 2003). Activation of integrin signalling by collagen has been reported to interfere with GC regulation of cyclin D1, a cell cycle and migration regulatory protein (Bonacci et al., 2006).

1.4 Human Respiratory Syncytial Virus

Respiratory syncytial virus is the prime respiratory virus associated with severe lower respiratory tract infections in infants, young children, immunocompromised adults and the elderly worldwide (Thompson et al., 2003; Falsey and Walsh, 2005). Additionally, transplant patients and patients with cystic fibrosis are at risk of severe RSV-induced airway disease (Garcia et al., 2007). RSV is estimated to cause 64 million cases of respiratory disease yearly (Nair et al., 2010). Moreover, RSV infections are linked to the development and exacerbations of asthma and COPD (Russell et al., 2017; Perk and Ozdil, 2018). In this section, we will review the virus structure and its replication, in addition to airway epithelial immune response to RSV infection.

1.4.1 Structure of RSV

Structurally, human RSV is an enveloped, pleomorphic virus of the family Paramyxoviridae, order Mononegavirales (Webster Marketon et al., 2014). The virion has single-stranded negative-sense RNA genome of a 15.2 kb containing 10 genes. The RNA core encodes 11 structural and non-structural proteins. The virus particles acquire their lipid bilayer envelop from the host cell plasma membrane. The virion has three transmembrane glycoproteins; the attachment G protein, fusion F protein and small hydrophobic integral SH protein. Besides, the matrix M protein resides in the inner layer of the viral envelope. The other two proteins, NS1 and NS2, are non-structural, not packaged into the virion and expressed only during cell infection. The nucleocapsid core has four proteins responsible for transcription and replication of the viral genome. These are the large polymerase subunit L, the antitermination factor M2-1, nucleocapsid protein N, and the phosphoprotein P (Figure 1.4) (Collins et al., 2001; Ghildyal et al., 2006).

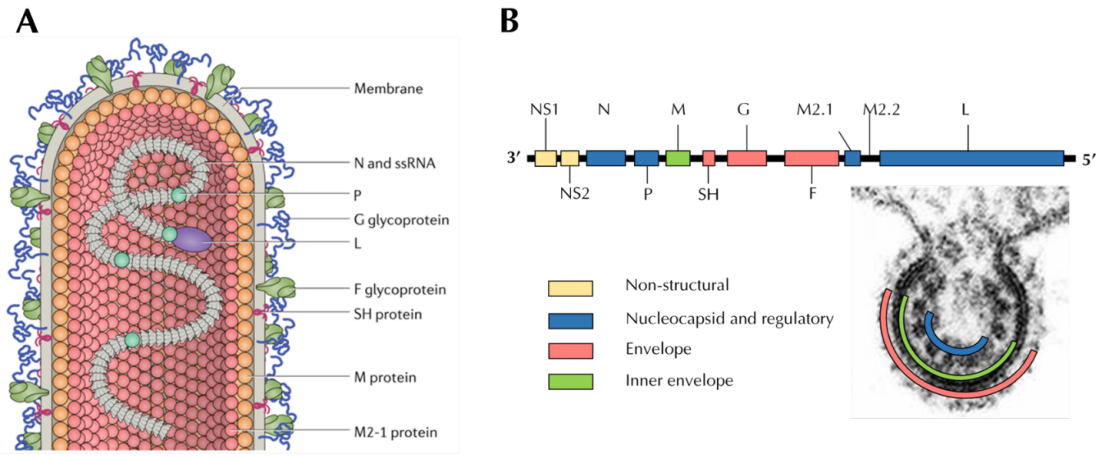


Figure 1.4| Schematic diagram of the respiratory syncytial virus (A) (Lay et al., 2013) and its genome structure together with the products of its replication and transcription (B). Adapted from (Lay et al., 2013) & (Battles and McLellan, 2019).

1.4.2 RSV replication cycle

RSV replication starts in the nasopharynx then extends to the epithelium of the small bronchioles (Dawson-Caswell and Muncie, 2011). The virion attaches to the host airway epithelial cells (AECs) via G protein that binds to Glycosaminoglycan Chains (GAGs); extracellular unbranched polysaccharides. Afterwards, the viral F protein binds to cell-surface RhoA nucleolin. Nucleolin is an element of a surface-signalling complex and is implicated in LPS internalization and signalling (Lay et al., 2013).

The mechanism of RSV entry is still an open question. Certain studies suggest that RSV fusion is related to the actin polymerization (San-Juan-Vergara et al., 2012), transient activation of macropinocytosis or clathrin-independent endocytosis (Krzyzaniak et al., 2013). After fusion into the plasma membrane, the virus nucleocapsid and the genome are released into AECs cytoplasm. The viral polymerase transcribes the genes into mRNAs by the sequential start-stop-restart synthesis. This generates a transcription gradient where transcription of starting genes is greater than that of downstream genes. Viral genome replication creates an anti-genome that serves as a template for genome synthesis. M, F and G proteins complex with the host cell membrane, whilst N, P, L and M2-1 proteins accumulates as cytoplasmic inclusions. M protein manages the assembly of the F and G envelope proteins with the nucleocapsid proteins N, P, and M2-1 before the budding of the mature virion. The virus spreads from cell to cell through fusion of plasma membranes of adjacent cells by F proteins, forming large syncytia (Figure 1.5) (Ghildyal et al., 2006).

Three to eight days after exposure to the virus, patients feel mild cold-like signs including rhinitis, dry cough, sore throat, low-grade fever and mild headache. Besides, RSV infections are usually accompanied by wheezing, dyspnea, hypoxemia due to the induced inflammation and small airways obstruction. The airways are usually clogged owing to smooth muscle contraction, mucus hypersecretion, the formation of oedema and lymphocyte infiltration around bronchi. The pulmonary clearance is often compromised because of the sloughing of ciliated epithelial cells raising the probability of secondary infections (Aherne et al., 1970). Premature infants and immunocompromised patients typically present with a serious lower respiratory tract illness, such as bronchiolitis and pneumonia. RSV infections have been involved in asthma exacerbations where asthmatics usually experience exaggerate the inflammatory response during concurrent viral infection (Hashimoto et al., 2004).

1.5 The immune response of airway epithelium to RSV infection

1.5.1 Structure of airway epithelium

The prime target of RSV infection is AECs, however, RSV can also infect other innate immune cells, such as dendritic cells (DC) and alveolar macrophages (AMs) (Marr et al., 2013). Depending on their locus, airway epithelium is categorized into bronchial epithelium (ciliated, secretory, Clara and basal cells) and alveolar epithelium (Type I, Type II cells). These cells work cooperatively to maintain the homeostasis in the airway and lung. The key cell type in the airway is ciliated epithelial cells, accounting for 50% of all epithelial cells. These cells harbour a maximum of 300 cilia per cell and have numerous mitochondria to produce the required energy for the effective mucus clearance via coordinated, directional cilia beating (Boers et al., 1998). Goblet cells are among the secretory cells of the respiratory tract that are characterized by electron-lucent acidic-mucin granules bounded to the apical membrane. These cells secrete mucus in order to trap foreign objects in the airway lumen. The normal human trachea has around 6800 goblet cells/mm^2 of the epithelial surface (Tam et al., 2011). Mucus cell hyperplasia commonly occurs in Asthma and COPD and is accompanied by continuous cough in these diseases (Knight and Holgate, 2003). Clara cells are located in bronchial and bronchiolar airways and secrete antiproteases and surfactant. Besides, they serve as a progenitor for both mucus-secreting and ciliated cells (Hong et al., 2001). Basal cells are frequent in the conducting epithelium that comprises about 50% in large airways and 81% in small airways (Tam et al., 2011). Similar to Clara cells, basal epithelial cells can differentiate into ciliated and mucous epithelial cells (Hong et al., 2004). The alveolar epithelium is

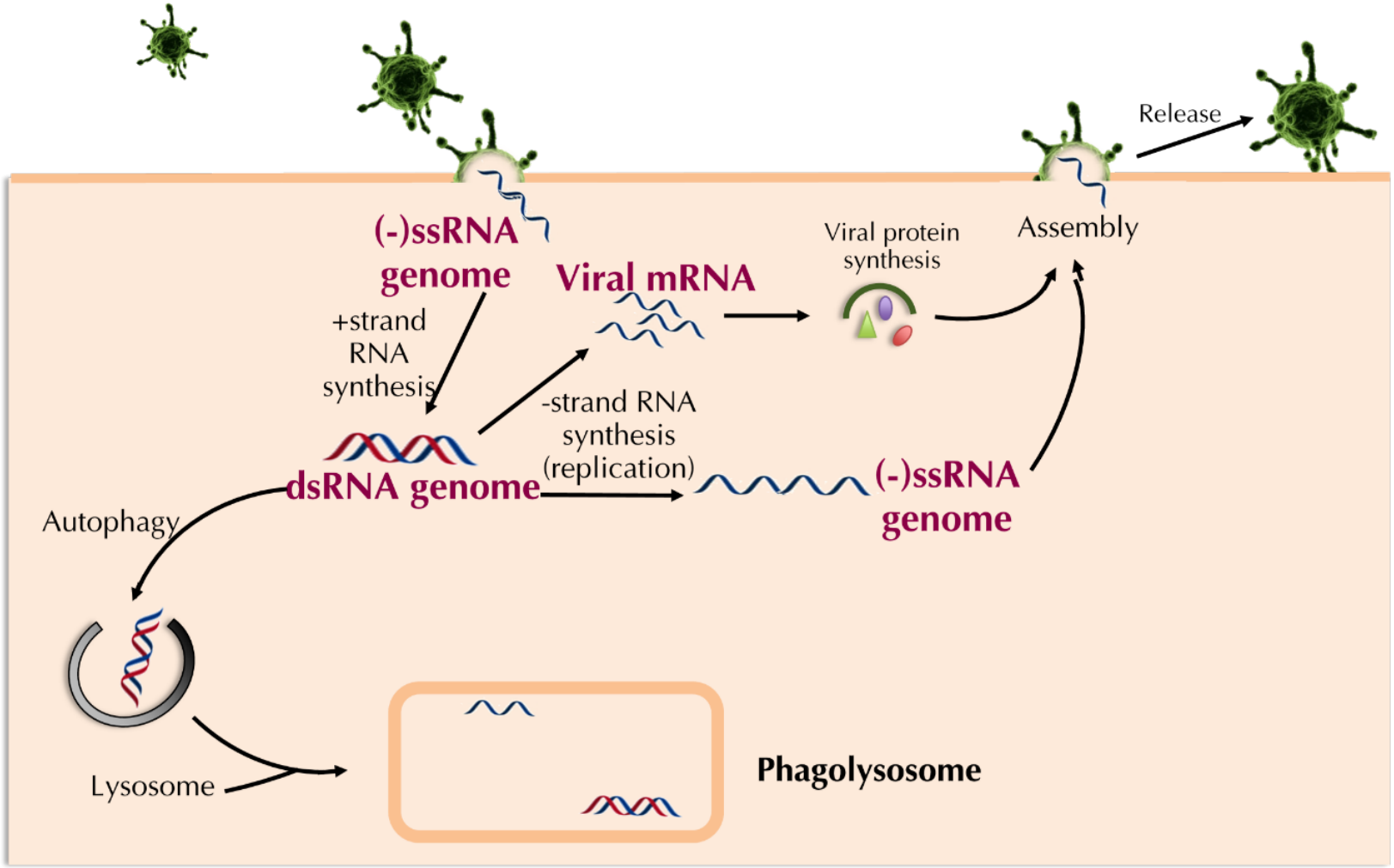


Figure 1.5| Replication of Respiratory Syncytial Virus in the lung epithelium.

comprised of two cell types, alveolar epithelial type I cells that form a very thin lining covering 95% of the human alveolus surface, and type II secretory cells that can also transdifferentiate into type I cells (Weibel, 2015).

1.5.2 The barrier function of the epithelium during viral infection

A healthy lung environment depends on the integrity and barrier functions of airway epithelium. This integrity is maintained by the apical junctional complex (AJC) that are formed of adherens and tight junctions. AJC governs the transfer of airborne environmental, microbial and viral antigens in the airways lumen to the underneath tissue and immune cells (Vareille et al., 2011). The clearance of inhaled foreign material and pathogens depends on the combined action of airway surface liquid (ASL), cilia and mucus (Bustamante-Marin and Ostrowski, 2017; Miura, 2019). Severe respiratory disease following viral infection has been reported in genetic polymorphism that negatively impacts the epithelial integrity (Zhang et al., 2016; Loxham and Davies, 2017). Early reports demonstrated that airway ciliated epithelial cells were the major locus for RSV replication (Henderson et al., 1978; Wright et al., 1997). RSV replication in human airway epithelial cells occurs at the apical cell surface of airway ciliated epithelial (Zhang et al., 2002; Wright et al., 2005). Furthermore, recent observations indicated that cilia might be a primary site of RSV morphogenesis (Smith et al., 2014; Jumat et al., 2015). In addition to preferential infection of ciliated cells via the apical membrane, RSV was shed exclusively from the apical surface and spread by the motion of the cilial beat to neighbouring epithelial cells (Zhang et al., 2002).

RSV infection has been shown to impair the mucociliary clearance of pathogens due to the accelerated ciliated cells loss, an effect that requires around 14 weeks to return to a normal state (Wong et al., 2005; Smith et al., 2014). Intriguingly, the integrity of epithelium has not greatly impacted by RSV infection. Indeed, RSV infection has upregulated the junctional proteins like Claudin-4 and Occludin while has no effect on others like E-cadherin, Junctional Adhesion Molecule A Protein (JAM-A) and Zonula occludens-1 (ZO-1) (Masaki et al., 2011; Guo-Parke et al., 2013). These results indicate that the epithelium barrier function is not heavily disrupted in response to RSV infection.

1.5.3 Viral detection by pattern-recognition receptors (PRRs) of the epithelium

RSV infection elicits weak adaptive immunity and, hence, the innate immunity has a vital role in the host defences against the virus (Hall et al., 1991b; Martin et al., 2006). The principal component of the innate immunity system is the pattern recognition

receptors. The PRRs detect invading pathogens and subsequently activate the innate immune response as the first defence line against those pathogens. The PRRs recognize the highly conserved pathogen-associated molecular pattern (PAMPs) in microbial nucleic acids, proteins, carbohydrates or lipids. In addition, they can recognize danger-associated molecular patterns (DAMPs), which are danger signals released from dying or necrotic cells. Binding of PRRs to PAMP-containing molecules trigger signalling cascades that ultimately result in the production of proinflammatory and antiviral cytokines. Secretion of these cytokines recruits natural killer (NK) cells and neutrophils at the infection site to eliminate pathogenic microbes and infected cells (Chow et al., 2015).

PRRs are primarily expressed by phagocytic, antigen-presenting-cells like macrophages and dendritic cells in addition to airway epithelium. Three main PRRs families are implicated in the recognition of viral infections; the membrane-associated TLRs and the cytosolic retinoic acid-inducible gene I-like helicase receptors (RLRs) and Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). The TLR system and RNA helicases confer antiviral responses where TLRs patrol the extracellular and endosomal compartments while melanoma differentiation-associated gene 5 (*MDA5*) and RNA helicases retinoic acid-inducible gene I (*RIG-I*) and survey the cytoplasm for the presence of viral RNAs (Kato et al., 2005). To date, 10 TLRs, 3 RLRs and 23 NLRs have been characterized in humans. Figure 1.6 summarizes the different PRRs implicated in RSV sensing. This section will only focus on the PRRs implicated in the innate immune response against RSV virions.

1.5.3.1 Toll-like Receptors (TLRs)

TLRs are a profound family of PRRs that detect various microbes and subsequently trigger the transcription of proinflammatory cytokines and antiviral type-I IFNs. Structurally, TLRs have a large horseshoe-shaped extracellular domain containing leucine-rich repeats (LRRs) that detect specific PAMPs, a transmembrane-spanning domain and a cytoplasmic signal-transduction tail with a conserved region named the Toll/IL-1 receptor (TIR) domain (Carty and Bowie, 2010). TLRs are predominant in tissues with immune function as blood leukocytes and spleen in addition to tissues with direct contact with the external environment as the gastrointestinal tract and lung. Different TLRs have been identified, ten in human (TLR1 to TLR10) and twelve in mice (TLR1 to TLR9, TLR11, TLR12 and TLR13). All TLRs are anchored to the plasma membrane except for TLR3, TLR7 and TLR9 that are localized in the endosome (Kawai and Akira, 2011). Table 1.1 lists different TLRs and their legends.

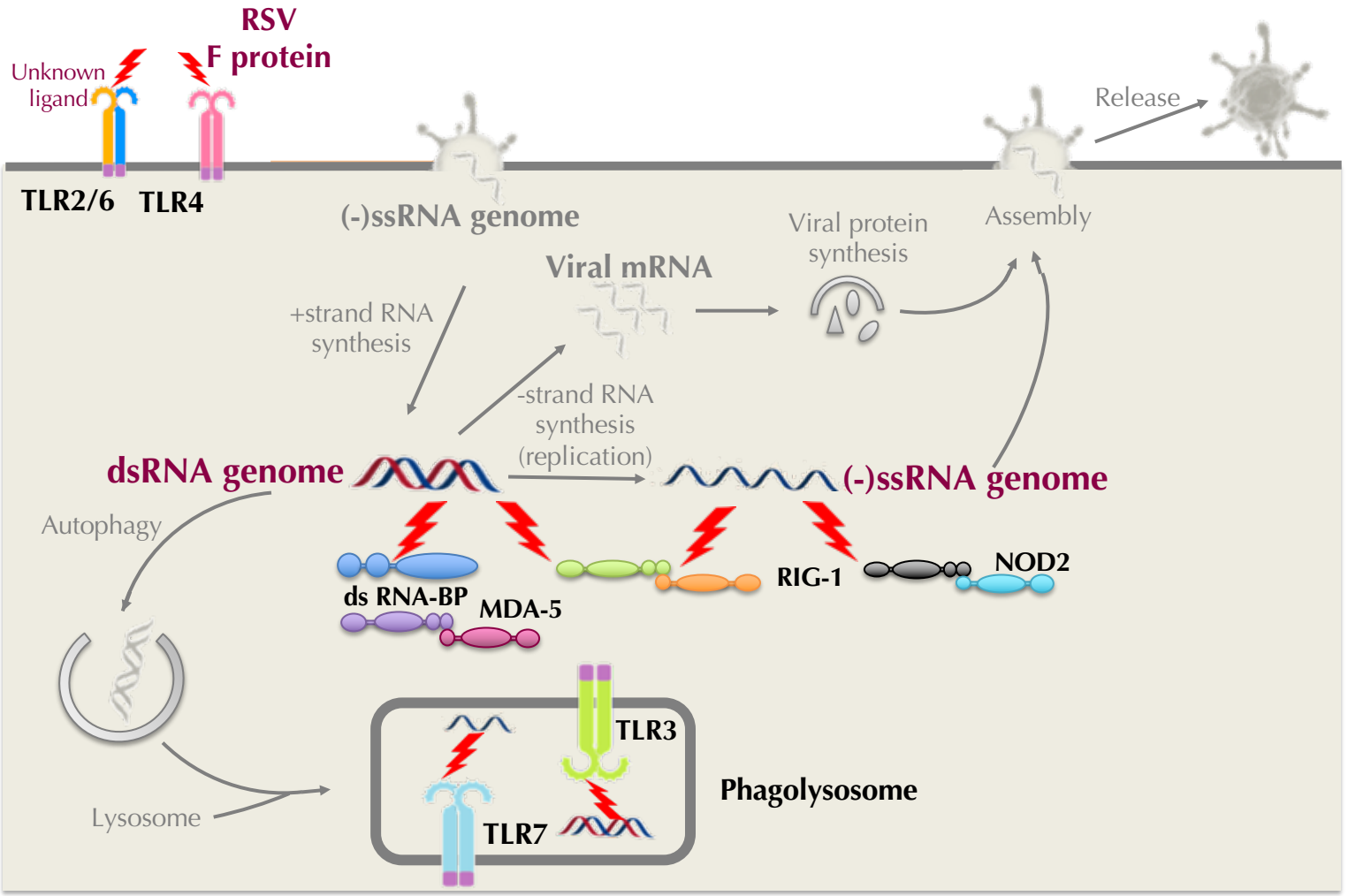


Figure 1.6| Pattern recognition receptors involved in Respiratory Syncytial Virus sensing

1.5.3.1.1 TLRs expression profile in the airway epithelium

TLR1 to TLR6 and TLR9 are expressed in AECs where their activation triggers the expression of diverse chemokines, cytokines and antimicrobial peptides. Loannidis and coworkers have studied the spatial distribution of individual TLR in the airway epithelium (Ioannidis et al., 2013). They reported that each TLR had its unique expression pattern and distribution on tracheal epithelial cells which is likely to contribute to the regulation of AECs innate response to commensal and pathogenic microorganisms in the pulmonary tract. As shown in Figure 1.7 B, TLR1 and TLR3 were detected on the surfaces and in the cytoplasm of epithelial cells throughout the whole width of the pseudostratified epithelium. TLR1 expression was more conspicuous on the apical surface of the tracheal epithelium. TLR3 was mainly expressed on the apical and basal mucosal surface. TLR2 and TLR6 showed a predominant basolateral distribution that may limit their interactions with PAMPs unless the epithelial barrier integrity is compromised. Although TLR2 was weakly expressed, TLR6 showed an intense basolateral staining. TLR4, TLR5, TLR7, TLR9, and TLR10 were weakly expressed in the apical surface of the tracheal mucosa; terminal plates and cilia. While TLR3, TLR7, and TLR9 are commonly expressed in the endosomal compartments of dendritic cells (Caramori et al., 2006; Blasius and Beutler, 2010), they were expressed both intracellularly and on the cell surfaces of AECs. These findings imply a tissue-specific distribution of TLRs. As demonstrated in Figure 1.7 C, in hAEC cultures, TLR1 and TLR3 demonstrated both apical and basolateral expression on the cell surface and in the cytoplasm. TLR2 and TLR6 were predominantly localized in the basolateral surface with only some apical expression. Expression of TLR4, TLR5, TLR7, TLR9, and TLR10 was mainly observed on the surface of the apical cells, especially in the terminal bars or in the cilia. No TLR8 expression was detected. Loannidis and coworkers suggested that the expression pattern of all TLRs in hAEC cultures corroborated findings from the trachea (Ioannidis et al., 2013). The morphology and differentiation of airway epithelium are crucial determinants to infection susceptibility and the elicited immune response. When AECs are cultured under air-liquid interface (ALI), they are differentiated into ciliated cells and become more resilient to viral invasions and elicit less exacerbated inflammatory responses (Lopez-Souza et al., 2004). Second, the apical surfaces of differentiated AECs are covered with mucin that has been shown to repress TLR signalling (Ueno et al., 2008; Ballester et al., 2021). Third, the differentiated epithelium allows for polarized positions of diverse receptors, e.g. IFN-α/β receptors giving a more real mimic to the microenvironment of airway epithelium *in vivo* (Ciencewicki et al., 2009).

Table 1.1 | Different types of TLRs and their PAMPs and DAMPs legends.

Adapted from (Narayanan and Park, 2015)

TLR	Localization/ Cell Expression	PAMPs	DAMPs	Signal Adaptor	Response
TLR1 & TLR2	Cell surface/ Monocytes, Macrophages, Dendritic cells, B cells	Lipopolysaccharides (LPS) Peptidoglycans Triacylated lipoproteins	• Proteoglycans (Versican, Hyaluronic Acid fragments) • High mobility group proteins (HMGB1) • Heat Shock Proteins (Gp96, HSP 60, 70)	TIRAP, MyD88, Mal	Inflammatory cytokines
TLR2 & TLR6	Cell surface/ Monocytes, Macrophages, Mast cells, B cells	Diacylated lipoproteins (FSL-1)			
TLR3	Endosomes/ B cells, T cells, Natural Killer cells, Dendritic cells	dsRNA (poly (I:C) tRNA, siRNA	mRNA tRNA	TRIF	Inflammatory cytokines, type1 IFN
TLR4	Cell surface/ endosomes Monocytes, Macrophages, Dendritic cells, Mast cells, Intestinal epithelium	Lipopolysaccharides (LPS) Paclitaxel	Heat Shock Proteins (HSP22, 60, 70,72, Gp96) High mobility group proteins (HMGB1) Proteoglycans (Versican, Heparin sulfate, Hyaluronic Acid fragments) Fibronectin, Tenascin-C	TRAM, TRIF TIRAP, MyD88 Mal	Inflammatory cytokines, type1 IFN
TLR5	Cell surface/ Monocytes, Macrophages, Dendritic cells, Intestinal epithelium	Flagellin		MyD88	Inflammatory cytokines

TLR (continued)	Localization/ Cell Expression	PAMPs	DAMPs	Signal Adaptor	Response
TLR7	Endosomes/ Monocytes, Macrophages, Dendritic cells, B cells, AEC	ssRNA Imidazoquinolines (R848) Guanosine analogues (Loxoribine)	ssRNA	MyD88	Inflammatory cytokines, type1 IFN
TLR8	Endosomes/ Monocytes, Macrophages, Dendritic cells, Mast cells	ssRNA, Imidazoquinolines (R848)	ssRNA	MyD88	Inflammatory cytokines, type1 IFN
TLR9	Endosomes/ Monocytes, Macrophages, Dendritic cells, B cells, T cells	CpG DNA CpG ODNs	Chromatin IgG complex	MyD88	Inflammatory cytokines, type1 IFN
TLR10	Endosomes/ Monocytes, Macrophages, Dendritic cells	profilin-like proteins		MyD88	Inflammatory cytokines

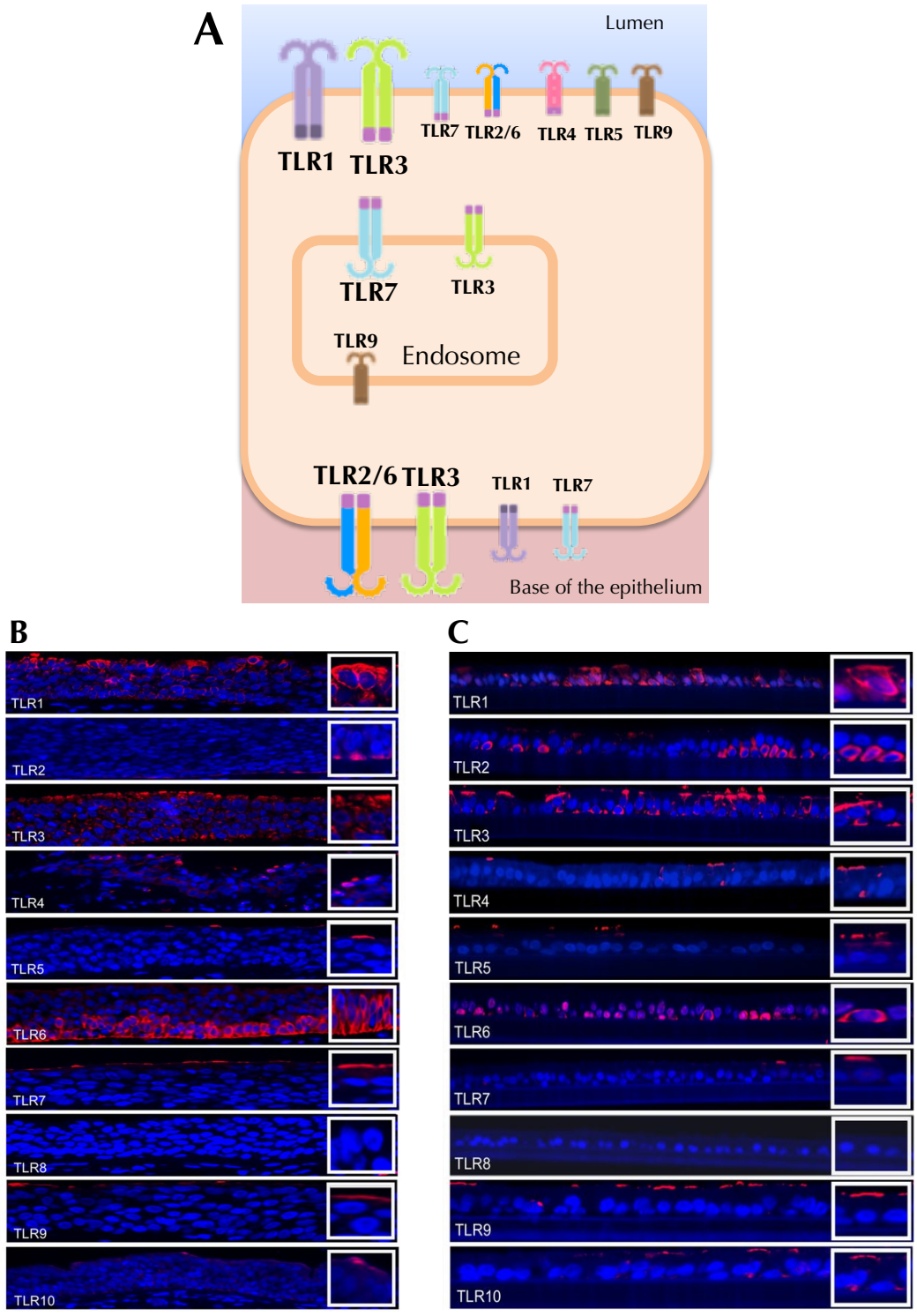


Figure 1.7| Pattern of TLRs expression and localization in the human airway epithelium. A, A schematic diagram illustrating the different TLRs in the airway epithelium, the size of TLRs is proportional to their abundance in the epithelial cells. TLR distribution in the airway epithelium of the human trachea (B) and human primary airway epithelial cells (AEC) cultures (C). Different TLRs are visualized by immunofluorescence utilizing anti-human TLR-specific antibodies. The apical surface is on the top (Ioannidis et al., 2013).

1.5.3.1.2 TLR signalling pathways

TLR signalling pathways are classified into two different pathways according to which cytosolic adaptor protein used; Myeloid Differentiation factor 88 (MyD88) or TIR-domain-containing adaptor protein inducing interferon-β (TRIF). All TLRs utilize MyD88 to transduce signal when binding to its ligand; except for TLR3 that harnesses TRIF for signalling (Figure 1.8) (Takeuchi and Akira, 2010).

The **MyD88-dependent pathway** starts with MyD88 recruitment to the TIR domain that comprises the cytosolic tail of TLRs in response to TLRs ligands binding. MyD88 then binds to the N-terminal death domain of IL1R-associated kinase (IRAK)-4 leading to its activation. The activated IRAK-4, in turn, stimulate the activation of other family members of IRAK including IRAK-1 and IRAK-2. After detaching from MyD88, IRAKs interact with TNF receptor-associated factor (TRAF-6) which interacts with TGF-β-activated kinase 1 (TAK1) afterwards. The activated TAK1 interact with the inhibitory κB (IκB) kinase (IKK) complex with subsequent phosphorylation of the NF-κB binding protein (IκBα/β). The proteasome then degrades the IKBα/β complex to liberate the transcription factor NF-κB. Following translocation of the free NF-κB to the nucleus, it stimulates transcription of proinflammatory cytokine genes including *CXCL8*. Beside its interaction with IKK complex, TAK1 also interact with MAPK and activate the downstream cascades that lead to activation of AP-1. Activated AP-1 then move to the nucleus to elicit the transcription of the proinflammatory cytokine genes (Loiarro et al., 2010).

The TRIF-dependent pathway begins when TLR3 binds to its ligand; dsRNA. The cytosolic tail of TL3 recruits the adaptor protein TRIF (Figure 1.8). TRIF then bind to TNF receptor associated factor 3 (TRAF3), which then activates the TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IκB kinase ε (IKKε). The two IKK-related kinases, in turn, phosphorylates IRF3 and induce its homodimerization. IRF3 homodimer, in turn, enters the nucleus, binds to IFN-sensitive response elements (ISREs) and triggers transcription of type I IFNs like IFN-β. Additionally, TRIF can also bind to TRAF6 and receptor-interacting protein 1 (RIP1) with the subsequent activation of the transcription factors NF-κB and AP-1. After translocation to the nucleus, they trigger the transcription of pro-inflammatory cytokines genes (Kawai and Akira, 2011).

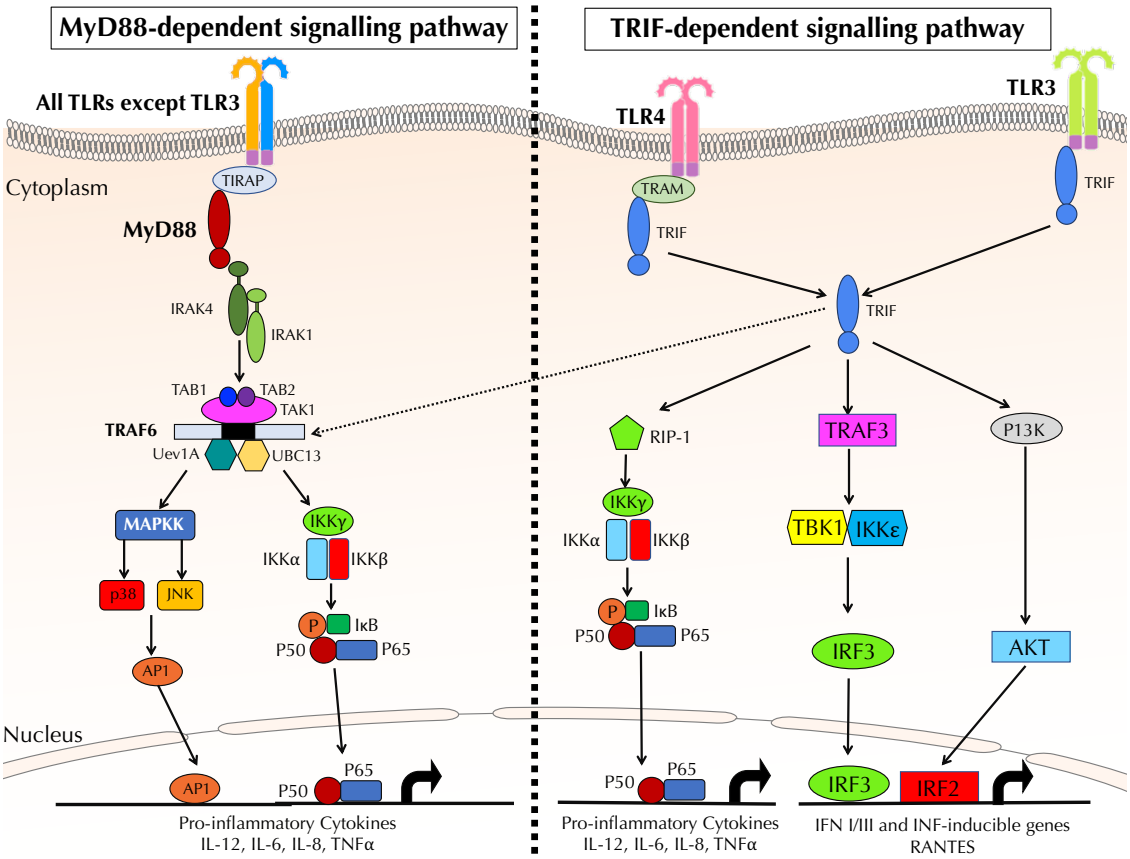


Figure 1.8 | Toll-like receptor signalling pathway.
Modified from (Patel et al., 2012)

1.5.3.1.3 Recognition of viral protein capsid by TLR2

TLR2 is a cell-surface TLR that requires heterodimerization with either TLR1 or TLR6 to function. TLR2 can recognize viral proteins, e.g. as human cytomegalovirus (CMV) glycoproteins B and H, measles virus hemagglutinin protein, Hepatitis C virus (HCV) core and NS3 proteins and Epstein-Barr virus (EBV) UTPase with subsequent formation of proinflammatory cytokines and chemokines via the MyD88 mediated pathway, as previously mentioned in section 1.5.3.1.2 (Shimojo et al., 2006; Murawski et al., 2009; Kawai and Akira, 2011). Several reports have demonstrated the upregulated TLR2 expression in airway epithelial cells after RSV infection (Arnold and König, 2006; Liu et al., 2007). Moreover, TLR2-blocking antibodies have diminished the induction of proinflammatory cytokines in airway epithelium following exposure to heat-inactivated RSV (Inoue et al., 2006). Following RSV infection, peritoneal macrophages isolated from TLR2- or TLR6 knockout (KO) mice have shown a diminished secretion of proinflammatory cytokines and chemokines compared to their wild-type counterparts (Murawski et al., 2009). Indeed, RSV-infected TLR2-deficient mice have shown uncontrolled viral replication and impaired dendritic cell activation and neutrophil migration in the lung.

Previous studies have highlighted a crucial function for TLR2 in RSV-antiviral immunity, however how TLR2 recognizes RSV virions remains unclear (Kim and Lee, 2014).

1.5.3.1.4 Recognition of viral double-stranded RNA by TLR3

TLR3 is an endosomal PRRs that can recognize double-stranded RNA (dsRNA) that is found in the dsRNA viruses genome. As discussed in section 1.5.3.1.2, TLR3 signalling is the TRIF-dependent and leads ultimately to the expression of type I IFNs and IFN-inducible genes by phosphorylation. Bermejo-Martin and colleagues (2007) have suggested the involvement of toll-IL-1 receptor domain-containing adaptor inducing TRIF that activate NF-κB and IRF-3 pathways, and successively potentiate the expression of IFN-β, C-C motif ligand 5 (CCL5), CXCL10 and CCL12.

Although RSV has a single-stranded RNA (ssRNA) genome, it forms a double-stranded intermediate during its replication cycle in the cytosol. When engulfed by the endosome, the dsRNA replication intermediate is recognized by TLR3 (Aeffner et al., 2011). TLR3 expression is upregulated in RSV-infected airway epithelium, hence, sensitize the epithelial cells to later exposure to viral dsRNA via promoting IL-8 production and NF-κB-related inflammation (Groskreutz et al., 2006). Dou *et al*. reported that TLR3 activation and production of TNF-α held responsible for severe clinical RSV syndrome in mouse lung (Huang et al., 2009; Dou et al., 2013). Downregulation of TLR3 dampens the amounts of released chemokines including CCL-5 and CXCL10 released in response to RSV infection compared to mock cells with no impact on RSV infection and replication (Rudd et al., 2005). Taken together, TLR3 likely has an adjunct role in RSV sensing and initiation of the innate immunity but is not required for viral clearance.

1.5.3.1.5 Recognition of viral Fusion protein by TLR4

TLR4 is among the TLRs residing on the cell surface. It detects LPS of gram-negative bacteria. TLR4-mediate signals through MyD88- or TRIF-dependent pathways. The downstream expression of NF-κB triggers a cascade of inflammatory and innate immune responses, as illustrated in section 1.1.1.1.1 (Zeng et al., 2012).

TLR4 is the first PRR shown to be involved in RSV recognition. An *in vitro* study has identified the interaction the purified RSV fusion protein F with TLR4 utilizing cluster of differentiation 14 (CD14) as a co-receptor (Hinck et al., 1996; Kurt-Jones et al., 2000b). RSV infection upregulates the expression and membrane localization of TLR4 in airway epithelium (Monick et al., 2003). RSV-TLR4/CD14 interaction elicits production of proinflammatory cytokines, such as TNF-α, IFNs, p35, CCL8 and interleukins 8, 10, 12 &

18, by airways epithelium (Numata et al., 2010). In infants with RSV bronchiolitis, blood monocytes have shown upregulated TLR4 expression that correlated to the disease severity (Gagro et al., 2004). TLR4-deficient mice (C57BL10/ ScCr) has shown impaired $CD14^+$ and NK cells recruitment, weakened NK function, diminished inflammatory cytokine expression and prolonged viral clearance in the lung following RSV infection (Haynes et al., 2001). Another report has claimed that the early NF-κB response induced by RSV infection was primed on TLR4 expression in the lung of TLR4-deficient mice (C3H/HeJ) (Haeberle et al., 2002). Nevertheless, TLR4-deficient mice (C57BL10/ScN and BALB/c LPS) have shown contradictory results. The diminished TLR4 expression has no impact on RSV-induced NK cell and other pulmonary inflammatory cells recruitment or activity and has not affected RSV viral clearance (Ehl et al., 2004). Another study has argued against the role of TLR4 in innate immunity against RSV. This study has reported that direct interaction of the human TLR4 with the intact RSV virions, not the purified RSV F protein, has no impact on RSV-induced NF-κB activation, viral entry, or replication (Marr and Turvey, 2012). Hence, the contribution of TLR4 in RSV sensing and immunity initiation is still inconclusive despite the great effort made to date.

1.5.3.1.6 Recognition of viral single-stranded RNA by TLR7

TLR7 and TLR8 are structurally-related TLRs sharing the same endosomal localization. TLR7 is expressed mainly in lung, spleen and placenta whilst TLR8 is mainly expressed in lung and peripheral blood leukocytes, particularly monocytes (Xagorari and Chlichlia, 2008). The ssRNA of viruses are natural ligands of TLR7 and 8 (Heil et al., 2004; Lund et al., 2004). The sequence specificity of targeted ssRNA remains poorly defined. ssRNA with poly(U)- or GU-rich sequences or synthetic small interfering RNA (siRNA) with a 5'-UGUGU-3' internal motif have been shown to activate TLR7 and 8. Since GU-rich sequences may be found in host RNAs, localization of viral RNAs to the endosome seems to be a molecular signature (Hornung et al., 2008). The nucleoside modification level is another factor to discriminate the host RNAs and the viral ones. Unlike the mammalian RNAs that are abundant in modified nucleosides, the viral RNAs are rarely modified (Karikó et al., 2005).

RSV virions possess ssRNA genome, hence it can be recognized by TLR7. Following engulfment of RSV virion particles by endocytosis, the viral envelope and capsid proteins are digested by host cell enzymes. Inside the endosomal vacuole, the released RNA from internalized virions is detected by TLR7 (Reis e Sousa, 2007). Following nucleic acid recognition, TLR7 recruits the adapter MyD88 that in turn complexes with IRAK1/4 and

TRAF6. These interactions result ultimately in NF-κB activation and the secretion of proinflammatory cytokines and chemokines, as discussed in section 1.1.1.1.1 (Lindell et al., 2009).

The interaction between RSV with TLR7 signalling pathway has first been detected in human plasmacytoid dendritic cells (pDCs) (Schlender et al., 2005). In mice infected with a pneumonia virus of mice (PVM), a mimic to RSV, the induction of IFN I and proinflammatory cytokines in pDCs, as well as CD8+ T cell response, are mediated by TLR7-MyD88 pathway (Davidson et al., 2011). Another study has reported upregulated TLR7 expression in eosinophil recruited to the lung after RSV infection. Recognition of viral ssRNA by TLR7 has resulted in the activation and degranulation of eosinophil to promote viral clearance (Phipps et al., 2007; Kvarnhammar and Cardell, 2012; Flores-Torres et al., 2019). Furthermore, upregulated TLR7 expression has been observed in lung cells from RSV-infected BALB/c mice with consequent NF-κB activation (Huang et al., 2009). The crucial role of TLR7 in RSV sensing and initiation of innate immunity was further supported by silencing experiments. TLR7$^{-/-}$ mice have developed a more pathogenic response in the lung during RSV infection including mucus overproduction and pulmonary inflammation. This pathology was attributed to the altered T cell responses resulting in increased secretion of mucogenic cytokines namely IL-4, IL-13 and IL-17. Neutralization of IL-17 in the same mouse model has alleviated the mucogenic response in the lungs upon RSV infection (Lukacs et al., 2010). Therefore, in absence of TLR7-mediated immune response, RSV infection may lead to significant remodelling of airway epithelium and goblet cell hyper- or metaplasia.

1.5.3.2 Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs)

TLRs have a limited ability to detect viruses since they are only expressed in limited cell types and survey only the extracellular space. On the contrary, the RLRs are expressed in most cell types (Chow et al., 2015). To date, three RLR members have been defined; RIG-I, MDA5 and laboratory of genetics and physiology-2 (LGP2) (Kawai and Akira, 2009). Both MDA5 and RIG-I have two N-terminal caspase-recruitment domains (CARDs), a DExD / H-box RNA helicase domain, and a C-terminal repressor domain (RD). The helicase domain and RD recognize viral RNAs, while the CARDs trigger intracellular signalling cascades (Yoneyama and Fujita, 2010). Both MDA5 and RIG-I detect the cytoplasmic dsRNA, however, they can distinguish their specific ligands by size; MDA5 recognizes long dsRNA, whereas RIG-I discriminates 5′-triphosphate ssRNA and short dsRNA (Kato et al., 2008). The third member of RLR family; LPG2, shares the same structure of MDA5 and

RIG-I but lacks any CARDs. Since LGP2 lacks a CARD, it was first postulated to acts as a negative regulator of RIG-I / MDA5 signalling. One report using LGP2 deficient mice have shown elevated induction of INF I upon exposure of Poly I:C while LGP2 overexpression has inhibited the Newcastle disease virus and Sendai virus signalling (Rothenfusser et al., 2005). However, another report has reached a contradictory conclusion that LGP2 enhances viral RNA recognition by MDA5 and RIG-I via its ATPase domain (Satoh et al., 2010). Therefore, the role of TPG2 as a positive or negative regulator in RLR signalling still needs more clarification.

RIG-I and MDA-5 share the same signalling pathway although recognizing different ligands (Figure 1.9). Following viral RNAs detection, MDA5 and RIG-I employ an adapter protein known as mitochondrial antiviral signalling (MAVS) or IFN-β promoter stimulator-1 (IPS-1). MAVS possess a N-terminal CARD that is homologous to the first CARDs of RIG-I and MDA5 utilized in CARD–CARD interaction. This protein is localized to the outer membrane of mitochondria, adding an immune function to the mitochondria besides their original metabolic roles. The activated MAVS harness tumour necrosis factor receptor I (TNFRI) associated death domain (TRADD) protein. TRADD in turn complexes with a death domain kinase, RIP1, Fas-associated death domain-containing protein (FADD) and TRAF3 (Sun and López, 2017).

Downstream of TRAF3, IκB kinases (IKK); IKKαβ, TBK1 and inducible IκB kinase (IKK-i), are activated. IKKαβ phosphorylates the inhibitory IκBα protein resulting in the dissociation of IκBα (inhibitor of kappa B) from NF-κB. The released NF-κB migrates to the nucleus and induces the expression of cytokine. On the other hand, TBK1 and IKKε activate IRF-3 and IRF-7 and induce their homo- and heterodimerization. IRF-3⁄-7 homo- and heterodimers migrate to the nucleus where they bind to IFN-sensitive response elements (ISREs) and trigger the expression of type I IFNs and IFN-inducible genes(Sun and López, 2017). Ling et al. (2009) have stated that RSV NS1 and NS2 can hinder the RIG-1 interaction with MAVS through binding to the CARD leading to repression of RIG-I-induced IFN pathway. These findings imply the crucial role of RIG-I-MAVS pathway in the innate immunity to virion particles.

Literature provides several pieces of evidence for the vital role the cytosolic receptor RIG-I plays in the replication-dependent detection of RSV. RIG-I colocalized with RSV genomic RNA throughout the early phase of infection in A549 cells (Kuenzel et al., 2010; Yoboua et al., 2010a; Lifland et al., 2012). A combined ultraviolet (UV)-crosslinking

and immunoprecipitation assay has also confirmed the physical interaction with the RSV N transcript when RIG-I ectopically expressed in A549 cells (Kuenzel et al., 2010). The role of RIG-I in RSV sensing was further proven by silencing experiments. In RIG-I-silenced cells, RSV failed to trigger the NF-κB and IRF-3 transcription factors during the early stage of infection and hence block the downstream formation of IFN-β, chemokine CCL5, interferon-stimulated genes (ISGs) and thymic stromal lymphopoietin (TSLP) (Liu et al., 2008; Kuenzel et al., 2010; Yoboua et al., 2010a; Lee et al., 2012a). Similarly, knockdown of MDA5 in A549 cells has diminished RSV-induced NF-κB activation. Intriguingly, MDA5 activates NF-κB by a yet uncharacterized pathway alternative to the classical one triggered by RIG-I (Yoboua et al., 2010a; b). Unlike RIG-I, the physical interaction between RSV nucleic acids and MDA5 has not yet been confirmed. In line with the role of RLRs, knockdown of the adaptor MAVS, the adaptor that transmits the signal immediately downstream of RLRs, also revokes NF-κB activation (Yoboua et al., 2010a; b). These findings endorse the conserved role of RLRs in RSV sensing.

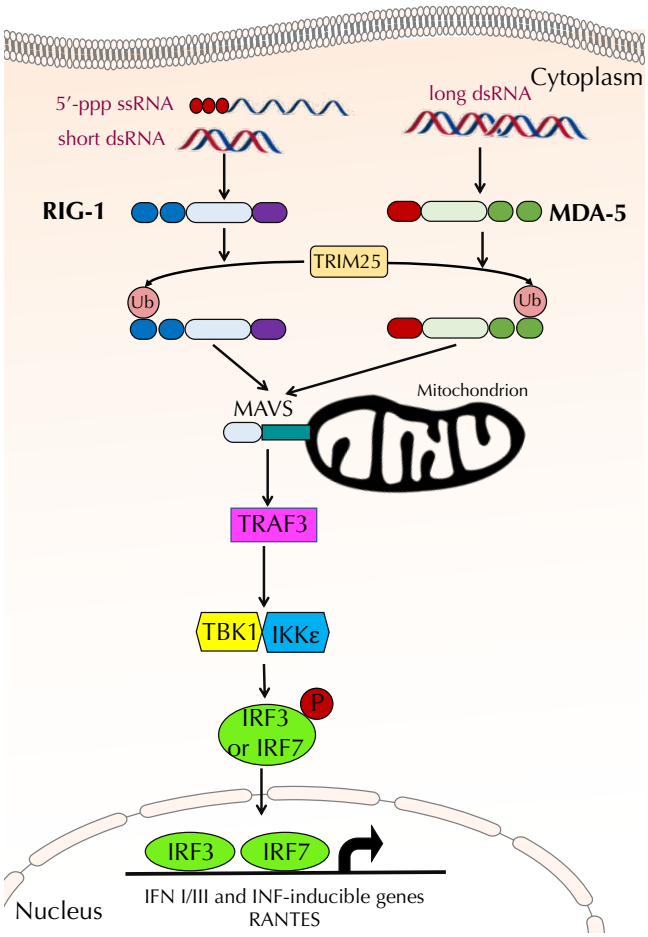


Figure 1.9| RIG-1-Like receptor signalling pathway.

Modified from (Shrivastav and Niewold, 2013)

1.5.3.3 Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)

NLRs are a major type of cytosolic PRRs that sense viral ssRNA and trigger innate immune activation. Structurally, the NLRs have three domains; the N-terminal effector, a central nucleotide-binding (NBD) domains and C-terminal LRR. The LRR domain is the sensor region of the NLRs. A NACHT [NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein)] protein is located centrally and mediates activation and oligomerization of NLRs. The N-terminal effector-binding domain signals downstream following activation and oligomerization of the NLRs (Fritz et al., 2006; Kuenzel et al., 2010; Jensen and Thomsen, 2012). Four effector domains have been characterized in NLRs; CARDs, pyrin domains (PYDs), a baculoviral inhibitor of apoptosis repeat (BIR) domains, or the acidic transactivator domain. Twenty-two human NLRs have been discovered to date. According to the effector domain, NLR can be divided into four subfamilies: NLRA (having acidic transactivating domain), NLRB (having BIR domains), NLRC (having CARD), and NLRP (having PYD).

NOD2 is a profound member of the NLRC subfamily with two CARD domains on its N-terminal. NOD2 acts as a general sensor for a bacterial invasion via recognizing the muramyl dipeptide structures found in all bacterial species. NOD2 activation by their legends induces conformational changes that allow for the interactions between the CARD domains of NOD2 and receptor-interacting serine/threonine kinase (RICK). Once activated, RICK induces ubiquitinylation of IKKγ that in turn phosphorylate IKKβ and its downstream IκB leading to its proteasomal degradation. These cascades result in the release of NF-κB form IκB and NF-κB translocation to the nucleus and then promote the transcription of interleukins IL-1β, -8, -10 and α-defensins (Zeng et al., 2012).

Numerous reports have pointed towards the important role of NODs in the immune response against RSV, influenza A virus, and parainfluenza virus 3 making NOD2 the only NLRs to respond to viral invasions. Sabbah and colleagues have given the first evidence that NOD2 in the human bronchial epithelium can sense viral ssRNA and elicit an antiviral response (Sabbah et al., 2009). NOD2 expression has been shown to increase 2hr post RSV infection with subsequent IFN production. NOD2-KO mice have experienced enhanced RSV pathogenesis and greater lung pathology, compared to their wild-type counterparts. Primary RSV infection of human peripheral blood monocytes has upregulated NOD2 expression and induced higher proinflammatory cytokines when stimulated with the

bacterial muramyl dipeptide (MDP) (Vissers et al., 2012). Non-replicating RSV has failed to induce mucosal immunity. However, beta propiolactone (BPL)-inactivated RSV has induced stronger antigen-presenting cells (APCs) activation *in vivo* and induction of IgA in the respiratory tract *in vitro* when supplemented with a TLR9 agonist [CpG oligodeoxynucleotides (ODN)] and NOD2 ligands (L18-MDP). These results suggest that NOD2/TLR9 ligands can induce RSV-specific immunity (Chen et al., 2009).

1.5.4 Epithelium-derived inflammatory mediators during RSV Infection

1.5.4.1 Secretion of inflammatory mediators

The airway epithelium release over 20 pro-inflammatory cytokines, chemokines, and growth factors upon the interaction of RSV PAMPS with their corresponding epithelial cell PRRs (Oshansky et al., 2010; Sun and López, 2017). Additionally, the cytokines and chemokines secreted during early RSV infection amplify and diversify of inflammatory mediators during the rest course of infection in an autocrine (Patel et al., 1998). IL-6, CCL5, CXCL8 and CXCL10 have been consistently detected in nasopharyngeal secretions of children with RSV induced bronchiolitis (Hornsleth et al., 2001; Bermejo-Martin et al., 2007; Murai et al., 2007; Kerrin et al., 2017). Genetic polymorphisms in genes coding these cytokines are linked to severe asthma phenotype in infants (Hattori et al., 2011). In addition to the aforementioned cytokines, the inflammatory mediators as TSLP, B-cell activating factor (BAFF) and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) are implicated in modulating the inflammatory responses during RSV infection. TRAIL sensitize epithelium to apoptosis hence it contributes to epithelial cells shedding due to RSV infection (Kotelkin et al., 2003). TSLP is responsible for T_h2-dominated immune responses the development during RSV infection (Lee et al., 2012a), whilst BAFF has been shown to modulate B-cell responses (McNamara et al., 2013a; b). Additionally, the airway epithelium releases diverse growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and TGF-β, however, their secretion patterns and functions are still underexplored especially in relation to RSV infection (Lee et al., 2000; Dosanjh et al., 2003).

1.5.4.2 Autocrine signalling amplifies inflammatory mediator release

Cytokines and chemokines released in response to RSV infection are categorized into primary and secondary cytokines. The secretion of primary cytokines is induced by the RSV

infection whilst secondary cytokines are prompted by other inflammatory mediators. In the context of RSV infection, CXCL8 and IL-6 expression are amplified via autocrine actions of IL-1α, IL-1β and TNF-α (Jiang et al., 1998; Patel et al., 1998). Neutralizing antibodies against IL-1α, IL-1β and TNF-α diminish the secretion of IL-6 and CXCL8 following RSV infection (Jiang et al., 1998; Patel et al., 1998). TNF-α release from RSV infected airway epithelial cells, on the other hand, results in increasing CCL5 secretion (Das et al., 2005). Among the primary cytokines released in RSV infection, IL-1α is the most potent in inducing the expression of CXCL8 and IL-6 (Jiang et al., 1998; Patel et al., 1998). Intriguingly, the secretion of CXCL8 during RSV infection is biphasic where TNF-α inhibits the CXCL8 secretion during the 24 h of RSV infection whilst amplify its secretion 48 h post-infection (Fiedler et al., 1995; Patel et al., 1998; Mellow et al., 2004). The autocrine effects of IL-1α and TNF-α on the release of inflammatory mediators from the airway epithelium are depicted in Figure 1.10 (Glaser et al., 2019).

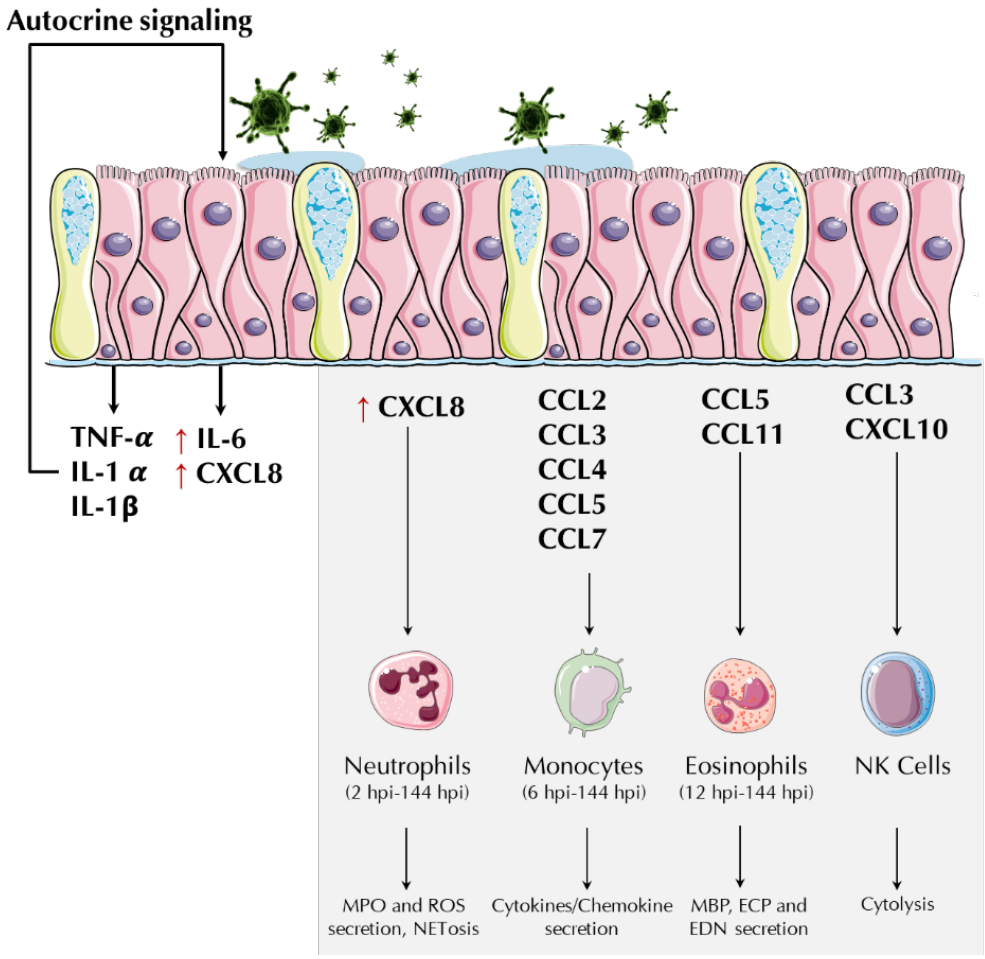


Figure 1.10| Cytokine and chemokine release from epithelial cells in response to RSV infection.

Eosinophil Cationic Protein (ECP), Major Basic Protein (MBP), Myeloperoxidase (MPO), Eosinophil-derived neurotoxin (EDN), reactive oxygen species (ROS).

1.5.4.3 IFNs potentiate the release of cytokine and chemokine release from the epithelium

Type III interferons namely IFNλ1 and IL-29 are the chief interferons released by the airway epithelium in response RSV infection (Carreño et al., 2007; Villenave et al., 2012; Guo-Parke et al., 2013; Villenave et al., 2015). Nonetheless, the exact underpinning of IL-29 induction and secretion kinetics during RSV infection downstream effects remain elusive. However, the downstream effects of IL-29 signalling involve Mx protein responses (Villenave et al., 2015). Type I interferons (IFNα/β) trigger the expression of inflammatory mediators. Yet, *in vitro* models of RSV-infected epithelial cells have shown limited expression of IFNα and a better expression of IFN-β. Mouse models have reported major secretion of type I INF from the alveolar macrophages (AM) following RSV infection (Mackay et al., 2015b). Yet, Schijf *et al*. reported a significant secretion of IFN-β from RSV-infected A549 cell lines *in vitro* (Badovinac et al., 2004). INF -β secretion has been cell line-specific and depend on a high multiplicity infection (Klonowski et al., 2004; Carreño et al., 2007). Recent reports noted that type I IFN responses following RSV infection was quite limited in differentiated primary airway epithelial cell cultures (Villenave et al., 2012; Guo-Parke et al., 2013; Villenave et al., 2015). Nevertheless, it was recently shown that in IFNARI knockout mice, the number of inflammatory mediators is substantially reduced during RSV infection in the lungs of infected animals, suggesting that IFNα/β affects airway epithelial cells in a paracrine fashion (Mackay et al., 2015a). Moreover, during RSV infection, airway epithelial cells interact with other resident immune cells including interstitial macrophages and dendritic cells, contribute to the inflammatory environment (Schenkel et al., 2016).

1.5.5 Induction of oxidative stress during RSV Infection

Reactive oxygen species (ROS) are ubiquitous, highly diffusible, and reactive molecules, including free radical superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydroxyl radicals ($OH^{\bullet}$), perhydroxyl radicals ($HO_2^{\bullet}$) and hydrogen peroxide (H_2O_2) (Dröge, 2002). These species are generated upon one electron reduction of molecular oxygen ($2O_2 + 2e^- \rightarrow 2O_2^{\bullet-}$) and formation of the parent ROS $O_2^{\bullet-}$ (Halliwell and Gutteridge, 1999). Due to its negative charge, the radical superoxide ($O_2^{\bullet-}$) cannot diffuse across cellular membranes and acts only at the site of production (Vlahos and Selemidis, 2014). One electron reduction of $O_2^{\bullet-}$ produces hydrogen peroxide ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$). H_2O_2 is uncharged so it can penetrate cell membrane (Vlahos and Selemidis, 2014).

Inside the cell, ROS are generated in the mitochondria, peroxisomes and microsomes as by-products of the normal O_2 metabolism of or from dysfunctional enzymes (Turrens, 2003; Bedard and Krause, 2007; Vlahos and Selemidis, 2014). The members of NADPH oxidase (NOX) family function solely to generate ROS (Bedard and Krause, 2007; Vlahos and Selemidis, 2014). The enzyme NOX2, a major source of $O_2^{\bullet-}$, is highly abundant in all cell types of the airways and in the residing alveolar macrophages or in the phagosomes of neutrophils and macrophages recruited to the lungs upon inflammation (Bedard and Krause, 2007; Vlahos and Selemidis, 2014; Singel and Segal, 2016). ROS at low levels are crucial for regulation of redox-sensitive genes involved in proinflammatory, immune responses and apoptosis (Dröge, 2002). At excessive levels, ROS cause oxidative stress, inflammation and tissue damage (Bedard and Krause, 2007; Vlahos and Selemidis, 2014). Hence, cells are well equipped with mechanisms to keep ROS levels under control. The first mechanism aims at limiting the generation of the parent ROS $O_2^{\bullet-}$ and its precursors, however it is crucial to balance the antiviral effects of ROS versus the detrimental effects of high ROS levels (Dröge, 2002; Bedard and Krause, 2007; Vlahos and Selemidis, 2014). An alternative approach is via simple stoichiometric scavenging by antioxidant molecules, such as vitamins A, C and E or the more complex antioxidant enzyme systems including superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase, thioredoxins, and peroxiredoxins (PRDXs) (Dröge, 2002; Bedard and Krause, 2007; Vlahos and Selemidis, 2014).

Oxidative stress plays a major role in the pathogenesis of RSV-associated lung inflammatory diseases, as evident by the surge of markers of oxidative damage that correlate to the disease severity in RSV-infected children (El Saleeby et al., 2011; Garofalo et al., 2013). Both transcriptomic and proteomic studies analysing host responses to RSV infection were able to detect virus-induced alterations in cell stress responses including transcriptional, ER and oxidative stress at mRNA transcript (Janssen et al., 2007; Martínez et al., 2007; Mayer et al., 2007; Mejias et al., 2013; Brand et al., 2015; Do et al., 2017; Mariani et al., 2017) and protein levels (Brasier et al., 2004; Jamaluddin et al., 2010; Munday et al., 2010a; Munday et al., 2010b; van Diepen et al., 2010; Ternette et al., 2011; Hastie et al., 2012; Wu et al., 2012; Diepen et al., 2015; Munday et al., 2015; Zhou et al., 2018). In addition, RSV infection of airway epithelial cells induced rapid production of intracellular ROS with a concomitant modification of expression and/or activities of antioxidant enzymes (Hosakote et al., 2009).

A study examining how RSV infection drives the rapid production of ROS suggested that macrophages are responsible for the oxidative damage to lung during RSV infection, most likely via $O_2^{\bullet-}$ production through phagocyte NOX2 (Nguyen et al., 2016). Inhibition of NOX2 activity or knocking down its expression significantly decreased ROS levels in AECs following RSV infection confirming the role of $O_2^{\bullet-}$-producing NOX2 in RSV-induced oxidative stress (Fink et al., 2008). In addition, NOX2 depletion in RSV-infected AECs also blocks IRF3 phosphorylation and its downstream antiviral responses and inhibits NF-kB activation leading to blockage of the downstream expression of proinflammatory cytokines and chemokines (Indukuri et al., 2006; Fink et al., 2008; Soucy-Faulkner et al., 2010). Hence, RSV infection causes oxidative stress due to the increased ROS production via $O_2^{\bullet-}$-producing NOX2, that activates the NF-kB and IRF3 signalling pathways producing proinflammatory and antiviral responses.

Another mechanism through which RSV infection induces oxidative stress is inhibition of antioxidant enzymes activity. This is evident from the reported reduction in the expression and activity of the antioxidant enzymes SOD1/3, PRDXS6, GPX1and catalase in RSV-challenged mice and children with RSV-induced bronchiolitis (Hosakote et al., 2011). How RSV infection interferes with the expression/activity of antioxidant enzymes is believed to involve the nuclear factor erythroid 2 (NFE2)-related factor 2 (NRF2) (Cho et al., 2009). NRF2 is a transcription activator that binds to the response element in promoter region of the antioxidant enzymes (Jaiswal, 2004). Under basal conditions, NRF2 is cytoplasmic and bounded kelch-like enoyl-CoA hydratase (ECH)-associated protein 1 (KEAP1) (Jaiswal, 2004). KEAP1 acts as an adaptor between NRF2 and cullin-3/RING box protein 1 (CUL3/RBX1), a ubiquitination ligase that ubiquitinates NRF2 and promotes its proteasomal degradation (Jaiswal, 2004). Oxidative stress promotes KEAP1 degradation and hence release of NRF2 from the KEAP2/ CUL3/RBX1 complex (Jaiswal, 2004; Kaspar et al., 2009). NRF2 then translocates to the nucleus and activates the transcription of antioxidant enzymes to protect the cells from oxidative stress (Jaiswal, 2004; Kaspar et al., 2009; Taguchi et al., 2011; Ma and He, 2012). RSV this mechanism via significantly increasing the expression and activity of histone deacetylase (HDAC) 2, which then deacetylases the nuclear NRF2 and targets it for proteasomal degradation. Dissociation of NRF2 from the response element sites of the antioxidant enzymes genes supress their transcription (Jamaluddin et al., 2005; Choudhary et al., 2009; Komaravelli et al., 2015; Feng et al., 2016). Indeed, treatment of RSV-infected airway epithelium with HDAC2 inhibitors restored NRF2 nuclear localisation and mRNA levels of antioxidant enzymes

(Komaravelli et al., 2015). In summary, RSV infection results in oxidative stress via increased ROS production with the concomitant inhibition of antioxidant enzymes transcription.

1.5.6 Role of mucus secretion in RSV pathogenicity

The inner lining of the respiratory tract is covered by a layer of a viscoelastic, gel-like substance called mucus (Rose, 1992). The viscosity and acidity of mucus protect the epithelial surface from energy and inhibits microbial attachment to the underlying cells. The viscoelastic properties for mucus are attributed to mucin (MUC). Mucins are a family of high molecular mass, heavily glycosylated macromolecules that are the main components of mucus (Evans and Koo, 2009). Twenty-two MUC proteins are characterized in human and classified according to their subcellular localization into two groups. The membrane-bound mucins possess a transmembrane domain to attach them to the cell membranes. This group includes MUC1, MUC3A/B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC18, MUC20, MUC21, and MUC22. The second group is secreted mucins and includes MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, and MUC19. Apart from MUC6, MUC7, and MUC17, all mucins are expressed in the airways (Hauber et al., 2006; Linden et al., 2008). Mucus overproduction is often observed in inflammatory lung diseases like bronchial asthma, chronic obstructive pulmonary disease, bronchiectasis, and cystic fibrosis (Hauber et al., 2006) in addition to several malignancies like breast, gastric, colorectal, pancreatic, lung, small bowel, and ovarian cancers (Rakha et al., 2005; Chauhan et al., 2009).

RSV is the leading cause of bronchiolitis and later stages of infection are characterized by the airways blockage by mucus plugs containing mucus, fibrin, cellular debris and lymphocytes (Pickles and DeVincenzo, 2015). In mice, RSV-induced lung damage has been correlated to raised neutrophil infiltration and elevated levels of TNF-α, a neutrophil recruitment factor that trigger the formation of MUC1 and MUC5AC (Hotard et al., 2015). The RSV G protein has been linked to mucus secretion in RSV-infected mice (Boyoglu-Barnum et al., 2013). This is believed to be triggered by the expression of type 2 cytokine, mainly IL-4, IL-5 and IL-13, and the chemokine MCP-1 (Zuhdi Alimam et al., 2000). In RSV-infected mice, signalling through the chemokine receptor CXCR2 has increased MUC5AC expression and in turn the mucus production (Miller et al., 2003). Other studies have highlighted the role of IL-17, a pro-inflammatory cytokine secreted by activated T cells, in mucus production in lung (Chen et al., 2003; Hashimoto et al., 2004).

Lately, it has been reported that infecting A549 cells with the structurally-similar RNA viruses; RSV and human metapneumovirus (hMPV), triggered differential expression of MUC2, MUC5AC and MUC5B as well as membrane bound mucins (Baños-Lara Mdel et al., 2015). To summarize, RSV infection can change the composition of infected airway epithelium via increasing MUC5AC-secreting cells suggesting a crucial role of mucus secretion in pathogenicity (Persson et al., 2014).

1.6 RSV-induced cytokine production and the pathophysiology of asthma

As discussed in section1.5.4, RSV infection triggers the production of a surplus of pro-inflammatory cytokines and chemokines by airway epithelium, fibroblasts, myofibroblasts, and leukocytes at infection sites. These cytokines are thought to be the key mediators of complicated pathophysiological events in asthma including airway remodelling (Kuo et al., 2011). Moreover, some of these cytokines have been reported to render the epithelium insensitive to anti-inflammatory action of glucocorticoids: TNFα suppress glucocorticoid transactivation in BEAS-2B cells and A549 cells (Rider et al., 2011); IFN-λ causes GC insensitivity through activation of the Janus kinase (JAK)-STAT signalling pathways leading to GC resistance in ALI-differentiated primary human bronchial epithelial cells (HBECs) and A549 cells (O'Connell et al., 2015); and IL-17 activates the PI3K pathways that reduce HDAC2 activity leading to GC insensitivity in 16HBE14o- human bronchial epithelial cells (Zijlstra et al., 2012). Of the cytokines investigated to date, TGF-β1 demands most attention due to its extraordinary capacity at picomolar concentrations to induce glucocorticoid insensitivity in airway epithelial cells (Salem et al., 2012; Keenan et al., 2014). Of relevance to this thesis, only TGF-β1 are summarised below.

1.6.1 Transforming growth factor-beta (TGF-β)

TGF-β proteins are among the TGF-β superfamily with 3 human isoforms characterized to date: TGF-β1, TGF-β2 and TGF-β3. TGF-β proteins are potent pleiotropic cytokines that regulate various arrays of processes including cell proliferation, inflammation, angiogenesis, autophagy, epithelial-to-mesenchymal transition and apoptosis. TGF-βs are produced by most cell types including epithelium, smooth muscle, fibroblasts and other inflammatory cell types and can bind to decorin, collagen, elastin, fibronectin and biglycan leading to TGF-βs sequestration into the extracellular matrix (Miyazono et al., 1993). The three TGF-β isoforms share 70-80% amino acid composition and bind to the same receptors whilst induce non-identical signalling pathways. Structurally, they have the same two cysteine-knotted monomers linked by a single

disulphide bond (Hinck et al., 1996). TGF-β molecules are secreted inactive as a part of a latent complex with latency-associated peptide (LAP) fused to the N-terminal of form mature TGF-β monomer. The small TGF-β-LAP complex is covalently bonded to a high molecular weight latent TGF-β binding protein (LTBP). The liberation of TGF-β from this large latent complex is achieved by either oxidation, acidification, proteolytic cleavage or integrins-mediated tractions depending on the microenvironment (Tatler and Jenkins, 2012).

TGF-βs signals via binding to a heterotetrameric receptor complex comprising of two types I and two types II transmembrane serine/threonine kinases. Binding of TGF-β to type II receptor (TGF-β RII) results in phosphorylation of type I receptor (TGF-β RI) which then recruits and phosphorylates Mothers against decapentaplegic homolog 2 (Smad2)/ Smad3 or Smad1/Smad5 depending on cell type. Phosphorylated Smads then binds to Smad4 before translocation to the nucleus. Smad proteins recruit chromatin remodelling factors, DNA-binding transcription factors, co-repressor (CoR) and co-activators (CoA) leading to the expression of several target proteins. In addition to Smad-dependent signalling pathway, TGF-β signals through PI3K/Ak strain transforming (Akt)/mammalian target of rapamycin (mTOR), Rat sarcoma (Ras)/MAPK, JNK, Ras Homolog Gene Family Member A (RhoA)/ Rho-associated coiled-coil kinase (ROCK) and p38 MAPKs in a context-dependent and cell type-specific manner (Massague, 1990). These pathways collectively contribute to the diverse cellular responses to TGF-βs.

1.6.1.1 Role of TGF-βs in asthma

A large cohort of studies has reported the increased levels of TGF-β1 and the activation of TGF-β/Smad pathways in asthmatic patients (Rosendahl et al., 2001; Bossé and Rola-Pleszczynski, 2007). TGF-β1 contributions to asthma pathogenesis have been well-documented and questioned (Reviewed in (Al-Alawi et al., 2014; Aschner and Downey, 2016). Elevated TGF-β1 levels in asthmatics result in recruitment of inflammatory cells to the epithelium implying a possible role in the granulocyte activation and trafficking (Gagliardo et al., 2013). Moreover, TGF-β1 elicits the release of pro-inflammatory cytokines, including IL-6 and IL-8 from lung epithelium, airway smooth muscle cells and fibroblast in asthmatics (Goulet et al., 2007; Ge et al., 2010). The physiological changes seen in severe asthma have been in part contributed to TGF-β1 activity in the airways and the coupled increase in oxidative burden (Michaeloudes et al., 2011). Moreover, TGF-β1is a prime driver of airway remodelling since it induces epithelial cell apoptosis promotes

epithelial-mesenchymal transition and induces the prefiltration of smooth muscle cells of the airways (Undevia et al., 2004; James et al., 2012).

Other studies have focused on the contributions of other TGF-β isoforms to asthma pathology. Elevated levels of TGF-β2 has been detected in bronchial mucosa and bronchoalveolar lavage fluid from asthmatic patients (Balzar et al., 2005; Torrego et al., 2007). Using neutralizing antibodies specific for each TGF-β isoform, TGF-β1 alone has specifically triggered the macrophages recruitment and increased the sub-epithelial deposition of decorin in a validated ovalbumin mouse model. On the contrary, TGF-β1 and TGF-β2 together have recruited lymphocytes and eosinophils coupled with the increase in sub-epithelial deposition of collagen (Bottoms et al., 2010). The third TGF-β isoform; TGF-β3 has shown no expression difference in asthmatic patients compared to controls despite its expression in macrophages and bronchial epithelium (Torrego et al., 2007).

A study investigating the intrinsic differences between the paediatric healthy and asthmatic bronchial epithelium has detected the markedly diminished production of TGF-β in Asthmatic epithelial cells compared to healthy nonatopic samples. This difference was consistent over several passages indicating the independency on an *in vivo* environment (Kicic et al., 2006). Given the crucial function of TGF-β1 in pAEC wound repair *in vitro*, the blunted production of TGF-β1 by paediatric asthmatic epithelium subsequently contributes to the dysregulated airway epithelial repair leading ultimately to a defective airway epithelium; one of the fundamental factors of the development and persistence of asthma (Ling et al., 2016).

1.6.1.2 TGF-β1 as an inducer of epithelial glucocorticoid resistance

Glucocorticoid treatment fails to decrease TGF-β1 levels in the airways of asthmatics (Chakir et al., 2003; Yamaguchi et al., 2008). Moreover, the epithelial-to-mesenchymal transition (EMT) triggered by TGF-β1 has not been inhibited in BEAS-2B, A549 cell line or primary bronchial epithelium following glucocorticoid treatment (Doerner and Zuraw, 2009; Salem et al., 2012). Our laboratory was the first to identify TGF-β1 as a potent inducer of glucocorticoid insensitivity in airway epithelium. In 2012, we reported findings made many years earlier indicating that TGF-β dampens GRE-dependent transactivation and glucocorticoid regulation of IL-6 and IL-8 release in A549 cells, partly through decreased nuclear translocation of GRα. This observation was held true in other cell types as TGF-β1 has impaired glucocorticoid regulation of thrombin and

IL-1 stimulated GM-CSF release from BEAS-2B bronchial epithelial cells (Salem et al., 2012) and air-liquid-interface differentiated human bronchial epithelial cells (Keenan et al., 2014). Although inhibiting TGF-β1 activity in the airways could serve as a therapeutic strategy to treat glucocorticoid-resistant asthma, its global inhibition carries known risks including autoimmune diseases, mitral valve defects and increased cancer risks (Anderton et al., 2011; Akhurst and Hata, 2012). Due to the beneficial pleiotropic effects of TGF-β1, such as immunosuppression and proliferation modulation, TGF-β1 inhibition results in acceptable benefits for only high-mortality cancer and idiopathic pulmonary fibrosis (IPF) that outweighs the severe adverse effects, such as bleeding and skin disorders (reviewed in Lachapelle et al. (2018). However, the burden of complications associated with TGF-β1 inhibition is too high especially with the long-term administration anticipated in case of chronic respiratory diseases (Anderton et al., 2011; Akhurst and Hata, 2012). Therefore, the identification of signals downstream of the TGF-β1 receptor that specifically suppress the glucocorticoid activity may lead to safer therapeutic targets without disrupting multiple vital physiologic functions of TGF-β1.

1.6.1.3 The interplay between RSV infection and TGF-β1 in airway epithelial cells

The first evidence for the interaction between RSV and TGF-β was provided by a study that reported an approximately four-fold increase in RSV replication upon human epithelial cells exposed to TGF-β (McCann and Imani, 2007). The beneficial TGF-β effect on RSV replication was attributed to cell cycle arrest (Gibbs et al., 2009); a well-known function of TGF-β. The cytokine TGF-β activates a downstream signalling pathway that arrest cell cycle at G0/G1 and G2/M phases (Laiho et al., 1990; Saltis, 1996; Desai et al., 2002; Matsumoto et al., 2002; Davy and Doorbar, 2007). Gibbs et al. (2009) have observed that pharmacological inhibitors of the cell cycle have augmented RSV replication. The authors have also detected the secretion of TGF-β in its active form following RSV infection and the reduced RSV replication upon treatment of epithelial cells with TGF-β1 receptor blocker. These studies have generated the hypothesis that RSV harnesses TGF-β1 in the lung epithelium to promote its replication leading to the exaggerated inflammatory response during RSV infections seen in patients with asthma. The interplay between the TGF-β1-induced cell cycle arrest and viral load may lead to a more severe and prolonged infection. Besides, the cell cycle arrest of lung cells hinders lung repair leading to severe bronchiolitis.

It is acknowledged that asthma exacerbation is often caused by viral infections and respond poorly to glucocorticoid therapy. Despite the clear correlation between TGF-β1 and glucocorticoid insensitivity, the contribution of this key cytokine in the RSV-induced GC resistance is yet to be established.

1.7 Hypothesis and aims

Respiratory syncytial virus is the main cause of paediatric bronchiolitis as well as a leading trigger of exacerbations of chronic airway inflammatory diseases; including asthma and COPD (Padem and Glick Robison, 2019). Although RSV-induced pathogenesis is partly inflammatory, the potential benefits of glucocorticoids in modulating the disease course remains doubtful (Ermers et al., 2009; Fernandes et al., 2013). Recent reports have attributed glucocorticoids compromised therapeutic potential to RSV interference with GC signalling precluding steroid responsiveness through mechanisms that are still far from clear (Hinzey et al., 2011; Webster Marketon and Corry, 2013a; b; Webster Marketon et al., 2014). Better insight into the mechanisms underlying GC insensitivity during RSV infection may offer anchor points for new therapeutic strategies.

Since airway epithelium is the major site for viral infection and replication and the crucial target for glucocorticoid therapy as well, we hypothesized that the emergence of resistance may occur through epithelium activation by viral PAMPs with subsequent release of cytokines, including TGF-β, which has a profound inhibitory effect on the cellular responses to glucocorticoids.

This thesis aimed at contributing to bridging the above-mentioned knowledge gap by:

- defining the contribution of TGF-β in the RSV-induced GC resistance. The results of this work are presented in Chapter 3.
- systematically evaluating the impact of activation of different PRRs involved in RSV sensing by their respective ligands, on GC responsiveness in human bronchial epithelial cells. The results of this work are presented in Chapter 3.
- characterizing the novel role of the casein kinase 1 δ/ε (CK1δ/ε) inhibitor; PF670462, in counteracting RSV-mediated attenuation of steroids response in the human airway epithelial cells. The results of this work are presented in Chapter 3.
- confirming the intracellular signalling cascade involved in RSV-mediated ablation of GC responsiveness in human airway epithelial cells. This was done by siRNA-mediated gene silencing. The results of this work are presented in Chapter 4.
- ascertaining the impact of the CK1δ/ε inhibitor; PF670462, on airway inflammation in murine models of RSV infection and poly I:C acute lung injury. The results of this work are presented in Chapter 5.

CHAPTER 2

General Methods

2 General Methods

2.1 Cell Culture

2.1.1 Heat inactivation of foetal calf serum (FCS)

Foetal calf serum (Sigma-Aldrich, Castle Hill, NSW, Australia) was defrosted in a 37°C water-bath and then mixed thoroughly prior to heating at 56°C for 30 min. Heat-inactivated foetal calf serum (HIFCS) was aliquoted under aseptic conditions and kept refrigerated until use.

2.1.2 Regeneration of cell cultures from cryopreserved stock

Cryovials were removed from the liquid nitrogen storage tank and defrosted in a 37°C water-bath. The cryovial contents were transferred to a 15mL centrifuge tube containing 5mL of HIFCS and spun down at 1500 rpm for 5 min to pellet the cells and remove them from cryoprotectant cocktail. The supernatant was discarded, and the cells were gently resuspended in 5 mL of culture medium. The cell suspension was transferred to a sterile T25 flask and incubated at 37°C in humidified 5% CO_2. Cells were allowed to adhere overnight. The culture medium was then substituted with fresh pre-warmed culture medium to get rid of residual Dimethylsulphoxide (DMSO) and dead cells.

2.1.3 Culture of BEAS-2B human bronchial epithelial cells

The BEAS-2B cell line was purchased from ATCC®; American Type Culture Collection (CRL9609™, VA, USA). BEAS-2B cells, an adenovirus-12 SV40 hybrid virus-transformed nontumorigenic human bronchial epithelial cells, were cultured as previously described (Salem et al., 2012). Briefly, BEAS-2B were maintained in LHC-9 medium (Gibco by Life Technologies) supplemented with 2% (v/v) HIFCS (Sigma-Aldrich, Castle Hill, NSW, Australia), 2 mM L-glutamine (Sigma-Aldrich, Castle Hill, NSW, Australia), penicillin-streptomycin 50 IU/mL and 50µg/ml respectively (Sigma-Aldrich, Castle Hill, NSW, Australia), and maintained at 37°C in humidified 5% CO_2. Cells were passaged twice a week by washing confluent cell monolayers twice with pre-warmed Dulbecco's phosphate-buffered saline (DPBS) (Gibco by Life Technologies) then incubating with trypsin (0.12% w/v) (Sigma-Aldrich, Castle Hill, NSW, Australia) for 5 min or until cells detached. Trypsin was neutralized with complete LHC-9 medium and the cell count was determined using a haemocytometer. Cells were then re-seeded at density of 8,000 cells/cm^2 in 75cm^2 flasks. Cells were kept up in culture till passage 40 before bringing up fresh cells.

2.1.4 Culture of HEp-2 human epithelial type 2 cells

Human epithelial type 2 (HEp-2) cells (ATCC® CCL-23™) are the standard cell line used for growth and quantitation of RSV. HEp-2 cells are originated from epidermoid cancerous tissue from the larynx of a 56-year-old human male (Mahy, 2001).

HEp-2 cells were maintained in DMEM/Ham's F-12 medium (Life Technologies, 10565-018) supplemented with 4% (v/v) HIFCS (Sigma-Aldrich, Castle Hill, NSW, Australia), 10 mM HEPES (Sigma-Aldrich, Castle Hill, NSW, Australia), penicillin-streptomycin 50 IU/mL and 50µg/ml respectively (Sigma-Aldrich, Castle Hill, NSW, Australia), and maintained at 37°C in humidified 5% CO_2. Cells were passaged twice a week by washing confluent cell monolayers twice with pre-warmed DPBS (Gibco by Life Technologies) then incubating with trypsin (0.12% w/v) (Sigma-Aldrich, Castle Hill, NSW, Australia) for 5 min or until cells detached. Trypsin was neutralized with complete growth medium. Cells suspension was spun down at 1500 rpm for 5 min to pellet the cells and remove residual trypsin. Cells were resuspended in complete growth medium and sub-cultured at a ratio of 1:20 in 75cm^2 flasks.

2.1.5 Freezing cell cultures for long term storage

Adherent cells from early passages were detached, pelleted at 1500rpm for 5min and gently resuspend in ice-cold sterile-filtered cryopreservation media composed of either 90% HIFCS and 10% DMSO for BEAS-2B cells, or DMEM/Ham's F-12 medium supplemented 8% (v/v) HIFCS and 5% (v/v) DMSO for HEp-2 cells. The cell suspension was aliquoted into sterile cryogenic vials at a density of 400,000 cells per mL and allowed to freeze overnight at a rate of -1°C per min in a -80 °C freezer using a cryogenic freezing container (Frosty Boy). Cryogenic vials were then transferred to liquid nitrogen tanks for longer-term storage.

2.1.6 Mycoplasma Testing

Cell cultures were routinely monitored for Mycoplasma contamination using MycoAlert™ Mycoplasma detection kit (Lonza, Mt Waverley, VIC, Australia) as per manufacturers' instructions. Culture medium supernatants used in the assay had been in contact with the cells for at least 24 h under normal culture conditions.

2.2 RSV Inocula preparation

Human RSV, A2 strain (ATCC® VR-1540™), was used in the current study. RSV strain was isolated from the lower respiratory tract of a hospitalized infant with bronchiolitis and bronchopneumonia (Lewis FA, 1961).

To prepare viral stocks, HEp-2 cells were detached from the tissue culture flask using trypsin (0.12% w/v) and supplemented with maintenance medium as previously mentioned in Section 2.1.4. Cell suspension was spread in appropriate volumes to cell culture flasks and was incubated at 37°C overnight. Once the cells became ~90% confluent, the maintenance medium was aspirated and then washed once in warm PBS. RSV inoculum stock was thawed at 37°C, diluted in infection medium; DMEM/Ham's F-12 medium supplemented with 2% (v/v) HIFCS, 10 mM HEPES, penicillin-streptomycin 50 IU/mL and 50 µg/ml respectively, and then added to each flask (2ml per flask). Flasks were gently rocked every 10-15 min for 1 h, after which, a further 12.5 ml of infection media was added. The cells were incubated at 37°C, 5 % CO_2 and observed daily for development of syncytia; the characteristic morphological cytopathic effects caused by the virus (CPE).

As most RSV infectivity is cell-associated (Collins et al., 2001), the virus is usually harvested as a clarified cell lysate. Once a moderate level of CPE was observed, the medium was aspirated off. Inoculated HEp-2 cells were gently rinsed once in chilled VP-SFM (Gibco by Life Technologies) supplemented with 4 mM L-glutamine and 25 μg/ml gentamycin. The virus was then harvested by scraping the cells into 2 ml chilled VP-SFM using a 15 mm cell scraper (Corning Inc.). An equal volume of chilled Sucrose-Phosphate-Glutamate-Albumin (SPGA) stabilizer (218 mM sucrose, 7.1 mM K_2HPO_4, 3.76 mM KH_2PO_4, 4.9 mM sodium glutamate and 1% (w/v) bovine albumin) was then added to avoid undue losses in infectivity (Tannock et al., 1987). The cell debris disrupted as much as possible by pipetting up and down 10 times at least. The collected suspension was clarified by centrifugation at 1,000g for 15 min at 4°C. The resulting RSV inoculum was aliquoted into cryovials, snap-frozen and then stored at -80°C.

The mock inoculum was identically prepared by collecting cell culture supernatant from lysed, uninfected HEp-2 cells to serve as a control for possible effects of cellular components in the inoculum.

2.3 50% tissue culture infectious dose ($TCID_{50}$) assay

Viral titres were determined *in vitro* by estimating the virus infectious dose. For virus strains that are tissue culture-adaptable, the viral titre is ascertained in cell culture through an endpoint dilution assay. The dilution of the virus that will produce detectable cytopathic effects in 50% of the inoculated cell cultures is termed 50% the tissue culture infectious dose, or $TCID_{50}$. $TCID_{50}$ is the minimum virus concentration required to detect a viral cytopathic effect (CPE) and it gives a rough estimation of the overall concentration of virus present in the virus stock suspension.

To set up for the assay, HEp-2 cells were suspended in maintenance medium, as described in 2.1.4, and seeded into flat bottomed 96-well plates at a density of 25,000 cells/100µl/well. Viral stocks, prepared as described in Section 2.2, were serially diluted in log increments using infection media (DMEM/Ham's F-12 medium supplemented with 2% (v/v) HIFCS, 10mM HEPES, penicillin-streptomycin 100 IU/mL and 100 µg/ml respectively). Using a multistep pipettor, 30 µl aliquots of the diluted virus were added to 8 wells for each dilution starting with the most diluted virus samples. Mock was diluted at the same concentration as the stock virus and applied to control cell wells as a virus-free control. Plates were gently rocked so that the virus settles into the cells. Plates were then incubated at 37˚C/ 5%CO_2 and examined for the development of characteristic CPE on a daily basis. Too short exposure period may lead to suboptimal CPE, while too long exposure may adversely affect the viability of cells. Both scenarios adversely affect the dynamic range of the Assay. Hence, depending upon the kinetics of CPE, viral titrations were incubated for 3-5 days.

The $TCID_{50}$ assay merely requires recording of whether or not there is CPE, not the extent of CPE. At least one good-sized syncytium per well was scored as positive. The viral titre was calculated by the method of Reed-Muench and expressed as $TCID_{50}$/ml as mentioned in Figure 2.1 (Reed and Muench, 1938).

$$TCID_{50} = 10^{\text{log total dilution above 50\% - (}\mathbf{I}\text{ x log }\mathbf{h}\text{)}}$$

Where, **I** = Interpolated value of the 50% endpoint and calculated as follows,

$$\mathbf{I} = \frac{\text{(\% of wells infected at dilution above 50\% - 50\%)}}{\text{(\% of wells infected at dilution above 50\% - \% of wells infected at dilution below)}}$$

Figure 2.1 | Formulae for $TCID_{50}$ calculation according to Reed & Muench Method.

2.4 Transient transfection of reporter constructs

2.4.1 Reporter construct plasmid vectors

pGRE-SEAP plasmid vector (Figure 2.2 A) (Clontech, Mountain View, CA, USA) was designed to monitor GRE induction and the glucocorticoid-mediated signal transduction pathway. This vector contains multiple tandem copies of GRE consensus sequences fused to a TATA-like promoter region (P_{TAL}) from the herpes simplex virus thymidine kinase (HSV-TK) promoter located upstream of the secreted embryonic alkaline phosphatase (SEAP) reporter gene. Being followed by the SV40 late polyadenylation signal, the SEAP coding sequence can be properly and efficiently processed within the transfected cells. The transcription can be induced, and reporter genes are transcribed and translated, following transcription factors binding to GRE. In order to reduce background transcription, a synthetic transcription blocker (TB), composed of adjoining polyadenylation and transcription pausing sites, is located upstream of the GRE. Additionally, the vector backbone includes f1 ori; an f1 origin for ss-DNA generation, pUC replication origin, as well as Amp(R)-gene conferring ampicillin-resistance in *E. coli*.

The Amaxa pMAX-GFP vector (Figure 2.2 B) was purchased from Lonza and contains CMV promoter upstream of the max GFP gene encoding the new green fluorescent protein from copepod Pontellina sp. The pGL3 reporter vector (Figure 2.2 C) (Promega #E1741, San Luis Obispo, CA, USA) contains SV40 promoter and enhancer sequences controlling the expression of modified firefly (*Photinus pyralis*) luciferase. Both plasmids also contain polyadenylation signal/transcription pause site, an origin of plasmid replication in *E. coli* (ori), and kanamycin/Neomycin (pMAX-GFP) or ampicillin (pGL3) resistance genes for bacterial selection. pmax GFP and pGL3 vectors were co-transfected in BEAS-2B cells as internal controls for qualitative and quantitative assessment of transfection efficiency respectively.

2.4.2 Transformation of Competent Cells

DNA constructs were transformed into MAX efficiency® DH5α™ (Invitrogen #18258-012) or JM109 (Promega # L2001) competent cells. The transformation was performed by adding 1 ng of DNA construct to 35 µL of competent cells in pre-chilled Eppendorf tubes. Cells were immediately incubated for 30 min on ice, heat-shocked in a 42°C water bath for 45 seconds, and then returned to ice for a further 5 min. 450 µL of antibiotic-free S.O.C medium (Invitrogen #15544-034) pre-warmed to room temperature

was then added to each transformation reaction and incubated for 60 min at 37°C in an orbital shaking incubator adjusted at 225rpm. Transformed cells were spread on Luria Agar (LA) plates (1.5% (w/v) agar, 0.17M NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract) containing appropriate antibiotics (150 µg/mL ampicillin or 50 µg/mL kanamycin) and incubated inverted for 16 h at 37°C.

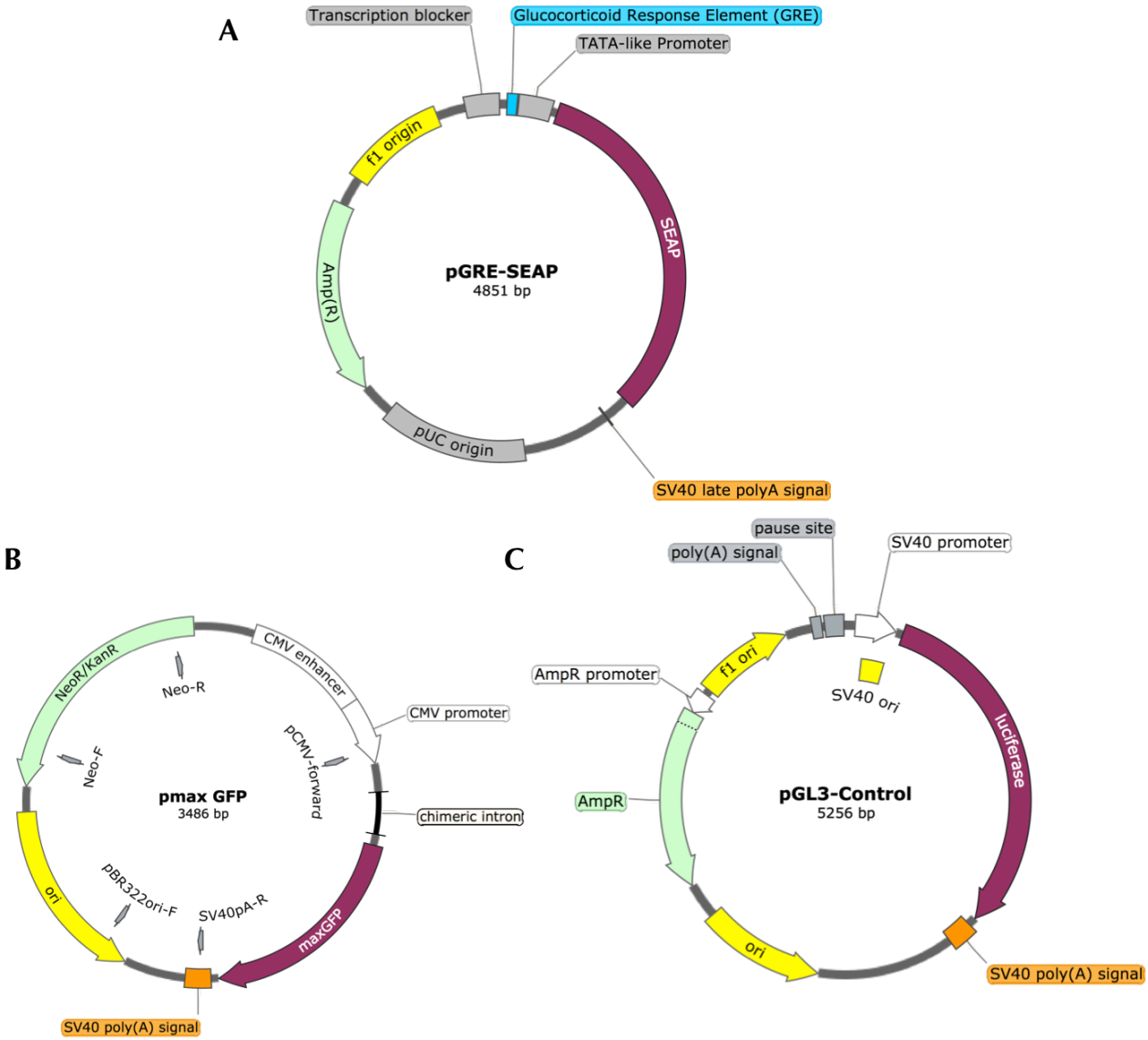


Figure 2.2| Maps of pGRE-SEAP (A), pmax GFP (B) and pGL3-control (C) vectors. Arrows indicate the transcription direction; the arrow in f1 ori indicates the single-stranded DNA (ssDNA) strand synthesis direction. Maps were created by SnapGene software based on the sequences published by the manufacturers.

2.4.3 Growth of transformed cells and purification of cloned DNA vectors

A single discrete bacterial colony produced from the transformation was inoculated into 3mL of Luria-Bertani (LB) medium (0.17M NaCl, 1%(w/v) tryptone, 0.5% (w/v) yeast extract) containing appropriate antibiotics (150µg/mL ampicillin or 50µg/mL kanamycin). Culture media were incubated at 37°C in an orbital shaking incubator adjusted at 225rpm for 16 h. 200 µL of the culture was then inoculated into 200mL of LB containing antibiotics and incubated at 37°C in an orbital shaking incubator adjusted at 225rpm for 16 h. After spinning down the overnight transformed cell cultures, the supernatant was decanted. DNA constructs were purified from the pelleted transformed cells using QIAprep® Spin Miniprep and Maxiprep kits (Qiagen, Doncaster, VIC, Australia) according to the manufacturer's instructions. DNA yield was quantitated using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia) and stored at -80°C.

Glycerol stocks of transformed cells was generated by mixing the remaining un-purified transformed cell culture with an equal volume of 50% (v/v) sterile glycerol and stored at -80°C.

2.4.4 Preparation of charcoal-stripped FCS

Dextran-coated charcoal was prepared by adding 1 g of activated charcoal (Sigma) to 50 mL pre-cooled sterile saline containing 0.4% (w/v) dextran (Clinical Grade C average MW 70kDa, Sigma, MO, USA) in the biohazard cabinet to maintain sterility. The mixture was stirred for 30 min at room temperature to allow dextran to fully coat the activated charcoal prior to centrifugation at 2,000g for 10 min. The supernatant was decanted, and the pelleted dextran-coated charcoal was resuspended in 50ml heat-inactivated FCS, incubated for 20 min, and then centrifuged at 2,000g for 10 min. The charcoal-stripped FCS was then decanted, passed through a 0.2µm filter twice, then stored at 4°C. FCS stripping by dextran-coated charcoal results in a more defined serum since lipophilic materials (certain growth factors, hormones and cytokines) that may impact the results are not in the culture system.

2.4.5 Transient transfection of reporter constructs

BEAS-2B cells were seeded in a 24-well cell culture plates at a density of 75,000 cells/well in penicillin and streptomycin free LHC-9 medium supplemented by 2% (v/v) charcoal-stripped FCS and 2 mM L-glutamine. BEAS-2B cells were washed once by sterile pre-warmed phosphate-buffered saline (PBS). The cells were equilibrated in 0.5 mL phenol red-free DMEM containing 0.2% (w/v) sodium bicarbonate and 15mM HEPES for 1 h before transfection. Cells were co-transfected for 4 h with GRE-secreted embryonic alkaline phosphatase (SEAP) reporter construct (pGRE-SEAP, Clontech Laboratories Inc., Mountain View, CA) and pmax-GFP (Amaxa, Lonza, Waverley, Aus) or pGL3-Luc (Promega, Alexandria, NSW, Aus) as internal transfection controls. The transfection mixture was prepared by incubating the reporter constructs (150ng/well each) with Lipofectamine® 2000 (1 µL/well) (Invitrogen, Carlsbad, CA) in 100 µL/well of Opti-MEM media for 30 min at room temperature and then added dropwise to each well. Four hours post-transfection, the medium was replaced with 1 mL/well of DMEM supplemented with 15 mM HEPES, 0.2% (v/v) sodium bicarbonate, 2 mM L-glutamine, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, 0.25% (w/v) bovine serum albumin (BSA) and penicillin-streptomycin 50 IU/mL and 50 µg/mL respectively. In experiments involving RSV inoculation, the transfection medium was replaced with Dulbecco's modified Eagle's media (DMEM) supplemented with 1% (v/v) heat-inactivated FCS, 15 mM HEPES, 0.2% (v/v) sodium bicarbonate, 2 mM L-glutamine, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, 5 IU/mL penicillin and 50 mg/mL streptomycin.

At the end of each experiment, samples of the conditioned cell culture media were collected to quantify SEAP by SEAP reporter gene assay. Transfection efficiency was assessed qualitatively by GFP expression in cells co-transfected with the pMAX-GFP control vector using the Leica DMI6000B fluorescence microscope (Figure 2.3), or quantitatively by determining luciferase expression in cell lysates from cells co-transfected with the pGL3 control vector by luciferase reporter gene assay.

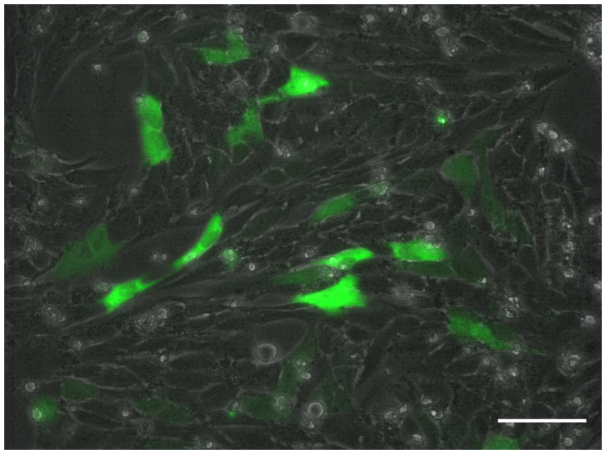


Figure 2.3| BEAS-2B cells co-transfected with GRE-secreted embryonic alkaline phosphatase (SEAP) reporter construct (pGRE-SEAP) and pmax-GFP.
Scale bar is 100µm

2.4.6 SEAP reporter gene assay

The SEAP Reporter Gene Assay is a chemiluminescent assay that provides a highly sensitive and convenient method to quantify transcriptional activity in SEAP-transfected cells (Figure 2.4) (Cullen and Malim, 1992; Bronstein et al., 1994). SEAP was quantitatively assessed in conditioned cell culture medium collected from pGRE-SEAP transfected cells 24 h after treatment with dexamethasone (30 nM) using a chemiluminescence kit (Roche Applied Science, NSW, Australia) according to manufacturer's protocol. Briefly, 15 µL of supernatants were diluted with 45 µL dilution buffer provided in the kit and then incubated in a water bath for 30 min at 65°C. Ten µL of heat-inactivated samples were transferred to a black and white isoplate (Perkin Elmer, Glen Waverley, VIC, Australia) and 10 µL of the inactivation buffer were added. Both the heat inactivation and the use of specific inhibitors in the inactivation buffer confirmed almost complete elimination of contaminating alkaline phosphatase activity, which is generally present in the culture medium, without significantly affecting SEAP activity.

Alkaline phosphatase substrate; CSPD; [3-(4-methoxyspiro[1,2-dioxetane-3,2'(5'-chloro)-tricyclo(3.3.1.13,7) decane]-4yl)phenyl phosphate], was diluted 1 in 20 with substrate buffer. After incubation for 5 min at room temperature, substrate reagent (10 µL) was added to each well and allowed to incubate for a further 10 min at room temperature. The plate was gently tapped to ensure mixing of the reagents. The chemiluminescence was measured using Top count (Perkin Elmer, Glen Waverley, VIC, Australia). The SEAP level was used as an estimate for GRE activity and was represented as a percentage of the SEAP induced by dexamethasone (30 nM).

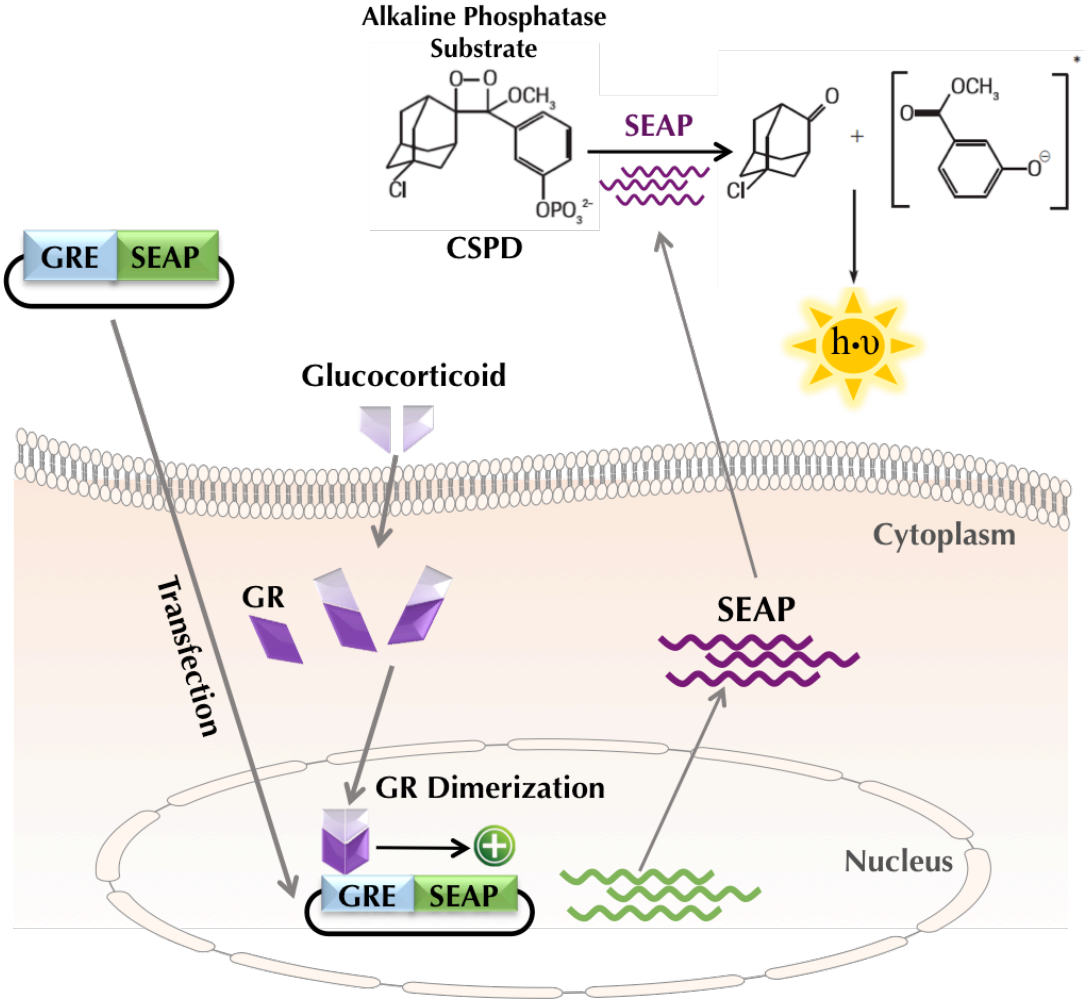


Figure 2.4| Schematic diagram of SEAP reporter gene assay principle.

Alkaline phosphatase (AP) dephosphorylate CSPD leading to the formation of unstable dioxetane anion that decomposes and emits light that can be measured at a wavelength of 477 nm.

2.4.7 Luciferase reporter gene assay

At the conclusion of each experiment, pGL3-transfected cells were first washed once with ice-cold PBS, and then incubated with lysis buffer (25 mM tris-phosphate (pH 7.8), 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mg/mL (w/v) BSA, 2 mM EDTA, 2 mM DTT) on ice for 5 min. Cells were scraped and 50 µl of the cell lysates were transferred to a black and white 96-well isoplate (Perkin Elmer, Glen Waverley, VIC, Australia). Beetle luciferin (Promega #1601) (0.47 mM) was added to the reagent buffer (20 mM Tris-base (PH 7.8), 33.3 mM DTT, 8 mM $MgCl_2$, 0.13 mM EDTA) and protected from light. After adding 25 µl of the luciferin reagent to each well, luciferase activity was quantified by measuring the luminescence using the Topcount™ instrument (Perkin Elmer, Glen Waverley, VIC, Australia).

2.5 Small interfering RNA (siRNA)-mediated gene silencing

Predesigned Silencer® select siRNA oligonucleotides targeting TLR3 (s236, sense: 5′-CCGAUGAUCUACCCACAAAtt-3′, anti-sense: 5′-UUUGUGGGUAGAUCAUCGGgt-3′), and silencer® select negative control no. 1 (4390843) were purchased from Ambion (Austin, TX, USA). As off-target and non-specific effects are more likely to occur with higher siRNA concentration, preliminary experiments were performed to determine the optimum siRNA concentration, as well as the appropriate negative control (scrambled) sequence was used to optimize the transfection protocol. BEAS-2B cells were subcultured in a 24-well cell culture plate at a density of 75,000 cells/well and incubated overnight in penicillin and streptomycin free LHC-9 medium supplemented by 2% (v/v) charcoal-stripped FCS and 2 mM L-glutamine. One hour before siRNA transfection, cells were washed once with pre-warmed PBS incubated in 500 µL/well of phenol red-free DMEM containing 15 mM HEPES, 0.2% (w/v) sodium bicarbonate.

Cells were transfected with 10 nM siRNA for 4 h using 1 µL/well Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. Briefly, 10 nM siRNA was pre-complexed with 1 µL/well Lipofectamine™ RNAiMAX (Invitrogen) in 100 µL/well Opti-MEM reduced serum media for 20 min before drop-wise addition to each well. Transfection medium was then replaced with phenol red-free DMEM supplemented with 15 mM HEPES, 0.2% (v/v) sodium bicarbonate, 2 mM L-glutamine, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, 0.25% (w/v) BSA and penicillin-streptomycin 50 IU/mL and 50 µg/mL respectively. The efficiency of siRNA silencing was determined at 24h, 48h and 72h post-transfection by real-time qPCR. siRNA (10 nm) was chosen for knockdown experiments after performing an initial concentration-response study.

2.6 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

2.6.1 Total RNA extraction

Total RNA extraction was done using TRIzol® reagent (Invitrogen, Doncaster, VIC, Australia) according to the manufacturer's protocol. Briefly, confluent monolayers of cells in 24 well plates were washed with once with PBS and lysed in 500 µL TRIzol® reagent. Cell lysates were transferred to RNase-free tubes and incubated at room temperature for 5 min to ensure complete dissociation of the nucleoprotein complex and 100 µL chloroform was added to generate phase separation. Samples were then vortexed and centrifuged at 13,000 rpm for 15 min at 4°C. The upper aqueous phase was then transferred to a fresh

tube and 10µg RNase-free glycogen (Invitrogen #AM9510) was added before RNA precipitation using 500 µL isopropanol. Samples were then incubated for 10 min and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was then aspirated, and the RNA pellet was washed with 1mL of 75% (v/v) ethanol in diethylpyrocarbonate (DEPC)-treated water and centrifuged at 7,500g for 5 min. RNA pellets were then air-dried, resuspended in 30µL DEPC-treated water. Total RNA was purified from animal tissue using QIAGEN RNeasy Mini-kits (Qiagen Inc., Valencia, CA) according to manufacturer's instructions. RNA was eluted in 30 µL RNase-free water. Thereafter, RNA concentration was quantified by the NanoDrop 1000. Samples were stored at -80°C until further analysis.

2.6.2 Reverse Transcription of RNA

RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen #4387406) in a 5 µl reaction containing 2 µL total RNA (containing 100 ng RNA), 2.5 µL 2x RT Buffer, 0.5 µL 20x RT Enzyme Mix. Reverse transcription was performed on an Eppendorf Mastercycler® Pro according to the following thermal protocol: 0–5 min, 25°C; 5–60 min, 50°C; 60–75 min, 75°C. For subsequent RT-qPCR, the resulting cDNA was diluted with 145 µL DEPC-treated H_2O and stored at -20°C.

2.6.3 RT-qPCR

Messenger RNA (mRNA) transcript levels of genes of interest were quantified by Real-time PCR using either ABI Prism 7900HT sequence detection system (Applied Biosystems) or QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed in triplicates in 384-well plate using 2 µl diluted cDNA in a 6 µl reaction containing 2.5 µL Platinum SYBR® Green qPCR Supermix-UDG (Invitrogen, Mulgrave, VIC, Australia) and 0.75 µl of each of the relevant forward and reverse primer (1 nM). Primers used were either obtained from the literature or pre-validated KiCqStart® SYBR® Green Primers from Sigma-Aldrich (Table 2.1 and Table 2.2).

The thermal PCR amplification protocol was as follows: 50°C for 2 min then 95°C for 10 min followed by 40 cycles of 95°C for 0.15 min, and 60°C for 1 min. Threshold (Ct) values for each reaction were estimated using SDS 2.0 software (Applied Biosystems) or QuantStudio™ 6 and 7 Flex software (Applied Biosystems). Generation of specific PCR products was confirmed through dissociation curve analysis. All the Ct values of the target genes were normalized to the Ct value of 18S rRNA, which was used as a housekeeping gene. 18S rRNA was chosen as a housekeeping gene since, first, it has a low turnover rate,

while the large 18S rRNA pool is less prone to substantial changes from physiological perturbations. Second, its use has been validated by numerous studies that showed its invariant expression across various organisms, tissues, developmental stages, and treatments (Butte et al., 2001; Kuchipudi et al., 2012; Hounkpe et al., 2020).

2.7 Western Blotting

2.7.1 Preparation of total protein lysates

For preparation of lysates, cells monolayers were first rinsed twice in ice-cold PBS and incubated on ice for 15 min with a lysis buffer containing 100 mM sodium chloride NaCl (Sigma), 10 mM Tris-HCl (pH 7.5; Sigma), 2 mM EDTA (Sigma), 0.5% (w/v) deoxycholate (Sigma), 1% (v/v) Triton-X (Sigma), 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich #P1860), 1% (v/v) phosphatase inhibitor cocktail (Sigma-Aldrich #P5726, Castle Hill, Australia), and 1 mM magnesium chloride (Sigma). Cell lysates were collected into Eppendorf tubes and centrifuged at 10000 rpm for 10 min at 4°C to remove cellular debris. Clarified supernatants were aspirated and stored at -20°C. 3 µl of each sample were aliquoted to measure the total protein concentration using the Bradford protein assay protocol described in Section 2.7.2.

2.7.2 Bradford protein assay

The protein content in the cell lysates was determined by the Bradford dye-binding method (Bradford, 1976). Briefly, 7 µl of cell lysates were reconstituted in 93 µl of PBS. BSA standard curve, spanning 0-20 µg, was constructed from a stock solution of BSA (200 µg/mL) diluted in PBS. Both standards and samples (100 µl each) were prepared in duplicate and solubilized by incubation with NaOH (0.2 M; 100 µL/sample) for 15 min at room temperature. Distilled water (600 µL/sample) and Protein Assay Dye Reagent Concentrate (Bio-Rad #500-0006) (200 µL/sample) were added to the standards and samples. Tubes were then vortexed thoroughly and 200 µL of each standard and sample were transferred to a 96-well plate. The absorbance at 595 nm was measured on a Multiskan Ascent plate reader (Thermo Scientific). A standard curve was generated by fitting the absorbance of the BSA standards to a linear equation, and the protein content of each sample was estimated with reference to the standard curve.

Table 2.1| *Homo sapiens* primer sequences used in RT-qPCR.

Gene ID		Primers sequence (5′→3′)	
		Forward	**Reverse**
18s rRNA	18S ribosomal RNA	CGC CGC TAG AGG TGA AAT TC	TTG GCA AAT GCT TTC GCT C
CDKN1C	Cyclin-dependent kinase inhibitor 1C	TCT GAT CTC CGA TTT CTT CG	CTC TTT GGG CTC TAA ATT GG
ENaCα	Epithelial sodium channel subunit alpha	AGC ACA ACC GCA TGA AGA C	TGA GGT TGA TGT TGA GGC TG
FKBP5	FK506 binding protein 5	AGATTGAGCTCCTTGATTTC	TGAATATCCCTCTCCTTTCC
GILZ	Glucocorticoid-induced leucine zipper	TCC TGT CTG AGC CCT GAA GAG	AGC CAC TTA CAC CGC AGA AC
IFN-β	Interferon beta	CAG CAA TTT TCA GTG TCA GAA GC	TCA TCC TGT CCT TGA GGC AGT
IL-6	Interleukin 6	AGC TCT ATC TCG CCT CCA GGA	CGC TTG TGG AGA AGG AGT TCA
IL-8	Interleukin 8	CTG GCC GTG GCT CTC TTG	CCT TGG CAA AAC TGC ACC TT
KCNB1	Potassium voltage-gated channel subfamily B member 1	AAG ATC CTT GCC ATA ATT TCC	GAA CCT CAG CAG GTA CTC
PAI-1	Plasminogen activator inhibitor-1	TCA GGC TGA CTT CAC GAG TCT TT	CTG CGC GAC GTG GAG AG
PDK4	Pyruvate Dehydrogenase Kinase 4	CTT GGG AAA AGA AGA CCT TAC	GTG CAG TGG AGT ATG TAT AAC
SERPINA3	Serpin Family A Member 3	TCA AGA CAA GAT GGA GGA AG	TCA CCT ATC TCT CTG AAC TC
TGF-β1	Transforming growth factor-beta 1	CCC AGC ATC TGC AAA GCT C	GTC AAT GTA CAG CTG CCG CA
TLR1	Toll-like receptor 1	CCCTACAAAAGGAATCTGTATC	TGCTAGTCATTTTGGAACAC
TLR2	Toll-like receptor 2	CTTTCAACTGGTAGTTGTGG	GGAATGGAGTTTAAAGATCCTG
TLR3	Toll-like receptor 3	CCTGGTTTGTTAATTGGATTAACGA	TGAGGTGGAGTGTTGCAAAGG
TLR4	Toll-like receptor 4	GATTTATCCAGGTGTGAAATCC	TATTAAGGTAGAGAGGTGGC
TLR5	Toll-like receptor 5	ATCTTTCACATGGGTTTGTC	TTCCCCCAGAAGGTTATATG
TLR6	Toll-like receptor 6	AGAGATCTTGAATTTGGACTC	TGTCTTTGGTCATGATGTTG
TLR7	Toll-like receptor 7	AGATATAGGATCACTCCATGC	CTTCCAAAATGGAATGTAGAGG
TLR8	Toll-like receptor 8	TGGAAAACATGTTCCTTCAG	TGCTTTTTCTCATCACAAGG
TLR9	Toll-like receptor 9	AAATCCCTCATATCCCTGTC	TTGTAATAACAGTTGCCGTC
TLR10	Toll-like receptor 10	CATCTGTAAGGGTTTTGAGC	CTTTCTTAGAGACATGTTGGAG
ZBTB16	Zinc finger and BTB domain-containing protein 16	GTT CCT GGA TAG TTT GCG	CAT GTC AGT GCC AGT ATG

Table 2.2| *Mus musculus* primer sequences used in RT-qPCR.

Gene ID		Primers sequence (5′→3′)	
		Forward	**Reverse**
18s rRNA	18S ribosomal RNA	TCC GGC GAG GGA GCC TG	CCT GCT GCC TTC CTT GGA T
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	GTACCTCTGCAAGGTGGAACTC	CCAAAGGAGGAAGTCAGAATCCG
FOXP3	Forkhead box P3	CCTGGTTGTGAGAAGGTCTTCG	TGCTCCAGAGACTGCACCACTT
IL-6	Interleukin 6	CTG CAA GAG ACT TCC ATC CAG TT	TTG TCA CCA GCA TCA GTC CC
INF-α	Interferon alpha	CTTCCACAGGATCACTGTGTACCT	TTCTGCTCTGACCACCTCCC
INF-β	Interferon beta	CTGGAGCAGCTGAATGGAAAG	CTTCTCCGTCATCTCCATAGGG
INF-γ	Interferon-gamma	TGA GTA TTG CCA AGT TTG AGG TCA	GTG GAC CAC TCG GAT GAG CT
INF-λ2 (IL-28A)	Interferon lamda 2	AAG GAT GCC ATC GAG AAG	GTC ATG TTC TCC CAG ACC
INF-λ3 (IL-28B)	Interferon lamda 3	AAG TCT CTG TCC CCA AAA G	TTA TGT TCT CCC AGA CCT TC
KC	Keratinocyte chemoattractant	TGC ACC CAA ACC GAA GTC A	GCA AGC CTC GCG ACC AT
TBX21	T-Box Transcription Factor 21	CCACCTGTTGTGGTCCAAGTTC	CCACAAACATCCTGTAATGGCTTG
TNF-α	Tumour necrosis factor-alpha	CAG ACC CTC ACA CTC AGA	TGC TAC GAC GTG GGC TAC AC
ICAM-1	Intercellular Adhesion Molecule 1	TGA TCC CTG GGC CTG GT	GGA AGG CTT CTC TGG GAT GG

2.7.3 Preparation of 12% (w/v) polyacrylamide gels

Gels, 1.5mm thick, were prepared using Mini-PROTEAN® Tetra hand-cast system (Bio-Rad). Glass plates were first washed with dH_20 then with 70% ethanol and wiped dry with Kim wipe. Plates were assembled as described in the manual. The gel space was filled with dH_20, left for 5min to test for leaks. After removing water, the running gel consisting of 12% (w/v) acrylamide/bisacrylamide (Bio-Rad #161-0158), 375 mM Tris, 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.05% (w/v) ammonium persulfate (APS), 0.0005% (v/v) tetramethylethylenediamine (TEMED) was cast in glass plates with 100 µL isopropanol layered gently on top without disturbing the interface. After the gel had set, isopropanol was removed by tipping apparatus upside down and the gel was flushed twice with dH_20. Stacking gel consisting of 4% (w/v) acrylamide/bisacrylamide, 125 mM Tris, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.0015% (v/v) TEMED was poured on top and 10 or 15 well combs were placed, and the stacking gel was allowed to set for 15 min. When the stacking gel had set, combs were removed, and the gel was flushed twice with dH_2O. The gels were transferred to a running tank and topped up with running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3). In some instances, Any kD™ Mini-PROTEAN® TGX stain-free™ precast polyacrylamide gels were used with Biorad Mini-PROTEAN electrophoresis cells.

2.7.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting

Thirty microgram of protein samples were mixed with sample buffer (0.125 M Tris-HCL (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 0.1% (w/v) bromophenol blue, 1% (v/v) β-mercaptoethanol) and denatured by heating to 95°C for 3 min. Protein samples were loaded alongside 5 µL of Kaleidoscope™ precision plus protein™ prestained standards for molecular weight comparison of the sample protein bands and monitoring of electrophoresis and transfer efficiency.

Proteins were then resolved by SDS-PAGE on 12% polyacrylamide gel at 200V for 45 min until the dye front ran to the end of the gel. Resolved proteins were then transferred onto nitrocellulose membranes (Amersham/GE Health Care, Rydalmere, NSW, Australia) by wet transfer method using a Mini Trans-Blot® cell (Bio-Rad) in transfer buffer containing 25mM Tris, 0.2M Glycine, 0.1% (w/v) SDS, 30% (v/v) methanol at 100V for 1 h on ice. Successful protein transfer was confirmed by staining the nitrocellulose membranes with Ponceau stain. To eradicate non-specific binding, the membrane is blocked by 5% (w/v)

skimmed milk in Tris-buffered saline (TBS) containing 0.1% tween (TBS-T) for 1 h at room temperature. Membranes were washed three times in TBS-T before the addition of the primary antibody for the target protein which was diluted in 5% BSA in TBS-T solution and incubated overnight at 4°C. Following incubation with the primary antibody, membranes were washed three times in TBS-T prior to incubation with horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h. The primary and secondary antibodies used were listed in Table 2.3. Membranes were then rewashed with TBS-T and immunoreactive bands were detected by Thermo Scientific™ Pierce™ Enhanced Chemiluminescence (ECL) Western Blotting Substrate (GE Healthcare, NSW, Australia) using Bio-Rad ChemiDoc™ MP imaging system (# 731BR01006, NSW, Australia). To estimate the subsequent exposure time, blots were initially exposed from less than one minute to a maximum of five minutes. Membranes were stripped in stripping buffer (50 mM glycine, 0.1% (w/v) SDS, 1% Tween-20, pH 2.2) for 15 min twice, followed by two 10 min PBS washes and two 5 min TBS-T washes. Stripped membranes were then re-blocked with 5% skim milk and probed with primary antibodies.

Densitometry was carried out on captured images to measure the band intensities using Image J program (1.48v, National Institute of Health, Bethesda, MD, USA). The expression level of protein, β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was applied as housekeeping controls for normalization to account for variation in the amount of protein sample loaded in each lane, protein transfer efficiencies from the gel to the membrane among different samples/lanes, antibody incubation (for primary or secondary antibody), and signal detection across separate samples/lanes. The housekeeping control was chosen based on the size of the protein(s) of interest (i.e., they should have substantially different molecular weights) to ensure that the detection bands of loading controls do not interfere with those of the protein(s) of interest. Hence, β-actin (45 KDa) was chosen as loading control for Smad2/3 (60, 52 KDa), while GAPDH (37 KDa) was chosen as loading control for p44/42 MAPK (Erk1/2) (42, 44 KDa).

Table 2.3| Primary and secondary antibodies applied in Western blotting (WB).

Antibody	Species/ Clonality	Dilution	Target protein MW (KDa)	Supplier
Phospho-Smad2 (Ser465/467) Antibody	Rabbit monoclonal	1:1000	60	Cell signalling (138D4) #3108
Smad2/3 Antibody	Rabbit monoclonal	1:1000	60, 52	Cell signalling (D7G7) #8685
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody	Rabbit monoclonal	1:1000	42, 44	Cell signalling (20G11) #4376
p44/42 MAPK (Erk1/2)	Rabbit monoclonal	1:1000	42, 44	Cell signalling (137F5) #4695
Anti-GAPDH antibody	Rabbit polyclonal	1:1000	37	Abcam #ab37168
Anti-beta Actin antibody	Mouse monoclonal	1:2000	45	Abcam #ab8226
Anti-mouse IgG HRP-linked Antibody	Goat polyclonal	1:5000	-	Dako #P0447
Anti-rabbit IgG, HRP-linked Antibody	Goat polyclonal	1:5000	-	Cell Signalling #7074

2.8 Statistical Analyses

Data are presented as means ± standard error of the means (SEM) for n independent biological replicate experiments in *in vitro* experiments, or for n mice per group in *in vivo* experiments. Statistical analysis was performed using GraphPad Prism version 8.4.2 for macOS (GraphPad Software, San Diego, California USA, www.graphpad.com). Statistical differences between study groups were tested for significance by either one-way or two-way analyses of variance (ANOVA) followed by Bonferroni's or Dunnett's multiple comparisons test as a post hoc test. Where appropriate, two-tailed t-test or non-parametric t-tests were applied to analyze the data. A p-value below 0.05 was considered as a criterion for a statistically significant difference.

CHAPTER 3

Pattern Recognition Receptors and Glucocorticoid Insensitivity

3 Pattern Recognition Receptors and Glucocorticoid Sensitivity

3.1 Introduction

Respiratory syncytial virus stands among the respiratory viruses implicated in exacerbations of asthma and chronic obstructive pulmonary disease (Kurai et al., 2013; Matsumoto and Inoue, 2014). Glucocorticoids remain central to treatment regimens for acute exacerbations unless RSV is the trigger. Glucocorticoids have usually no beneficial effect on alleviating the symptoms or altering the course of illness in RSV-triggered paediatric bronchiolitis (Buckingham et al., 2002; Patel et al., 2004; Steiner, 2004; Ermers et al., 2009; Panickar et al., 2009; Somers et al., 2009; Fernandes et al., 2013). In cell-based studies, glucocorticoids have been shown to attenuate cytokine production elicited by RSV in some reports (Jaovisidha et al., 1999; Wang et al., 1999) but not in others (Bonville et al., 2001; Carpenter et al., 2002). Though contradictory, these findings indicate that RSV may have a detrimental effect on GC signalling.

Being the prime locus for viral infection and replication and the crucial target for glucocorticoid therapy as well, airway epithelium has been proposed as the main cell type where virus-induced glucocorticoid resistance may occur. RSV has been shown to potentially interfere with the anti-inflammatory activity of steroids in epithelial cells (Bonville et al., 2001; Carpenter et al., 2002), yet with undefined mechanism.

Three potential pathways could contribute to RSV interference with GC signalling. These include production of autocrine factors such as cytokines; activation of intracellular signalling pathways; or via a direct viral proteins or RNA effect. Airway epithelial cells produce an array of pro- and anti-inflammatory cytokines and chemokines including the type-I and -III interferons (IFN-α/β and IFN-λ), TNFα, IL-4, IL-8, IL-13, IL-17, CCL3 and RANTES (Zeng et al., 2011). Some of these autocrine factors such as IL-2/IL-4, IL-13, TNFα, IL-6, IL-8, IFNγ and IFNα have been proved to interfere with epithelial response to GC (Rider et al., 2011; Zijlstra et al., 2012; O'Connell et al., 2015). This fact has tempted researchers to postulate that RSV-induced cytokines could impair GR function. When tested, RSV-conditioned media failed to repress expression of GC-responsive genes (Webster Marketon et al., 2014) despite being able to induce IL-8 transcription (Patel et al., 1998). These findings suggest that RSV-induced GC irresponsiveness is not triggered by a soluble autocrine factor.

RSV infection also activates a number of host signalling pathways, such as NF-κB, phosphoinositide 3-kinase (PI3K), and MAP kinase pathways (Garofalo et al., 1996;

Mastronarde et al., 1996; Thomas et al., 2002; Lindemans et al., 2006; Singh et al., 2007). Several studies have reported the mutual transcriptional antagonism between GR and these pathways, so GC resistance could be a result of the activation of these pathways (Ray and Prefontaine, 1994; Irusen et al., 2002; Adcock and Lane, 2003; Marwick et al., 2009; Ayroldi et al., 2012). However, Hinzey et al. (2011) have excluded the possible involvement of NF-κB pathway in repressing dexamethasone actions in RSV-infected lung epithelial cells.

Interference with the initiation of these signalling cascades has been also tested. MyD88 is a downstream adaptor molecule of TLRs or RIG-1 signalling pathways and its knocking-down did not restore dexamethasone-induction of *GILZ* expression in RSV-infected A549 cells ruling out its contribution to the apparent RSV-induced GC resistance. Knockdown of MAVS, another downstream adaptor of RIG-1 signalling cascade, has significantly reduced dexamethasone induction of *GILZ* in mock-infected cells that further reduced in case of RSV infection, albeit non significantly (Webster Marketon et al., 2014). Hence, we cannot exclude MAVS biological role in the RSV-induced GC insensitivity. Webster Marketon et al. (2014) have continued to test the possibility of a direct viral proteins or RNA effect. It is known that NS1 and NS2 proteins of RSV inhibit the interaction between RIG-I and MAVS with subsequent suppression of type I interferon response (Ling et al., 2009; Boyapalle et al., 2012). Overexpression of NS1, but not NS2, repressed GC induction of *GILZ*. This observation was held true when tested using viruses with deletions in the NS1, but not NS2 genes, that prevented RSV suppression of glucocorticoid-induced *GILZ* (Webster Marketon et al., 2014) suggesting that non-structural proteins mediate the RSV suppression of GR function.

We have previously established TGF-β as a profound mediator of glucocorticoid resistance in A549 cells (Salem et al., 2012), human immortalized bronchial epithelial BEAS-2B cells, and in ALI-differentiated primary HBECs (Keenan et al., 2014). Our group has unravelled a potential link between RSV infection and TGF-β, where TGF-β functions in an autocrine manner to mediate glucocorticoid insensitivity in RSV-infected BEAS-2B and primary bronchial epithelial cells (Xia et al., 2017). Since TGF-β is crucial to diverse cellular activities (Boxall et al., 2006), broad inhibition of TGF-β is not clinically feasible due to the known risk of autoimmune colitis (Shull et al., 1992; Kulkarni et al., 1993), tumorigenesis (Hahm et al., 2001; Oyanagi et al., 2014) and mitral valve lesion (Anderton et al., 2011).

Our group comprehensively reviewed TGF-β1 signalling pathways to define the downstream pathways by which TGF-β impairs epithelial GC actions, hence identify novel targets to circumvent GC resistance (Salem et al., 2012; Keenan et al., 2014). We have found that TGF-β profoundly impairs GC responsiveness not through known non-canonical pathways, nor through Smad4-dependent signalling, suggesting that a novel non-canonical signalling mechanism.

Comprehensive literature review revealed a little-known connection between TGF-β1 signalling and casein kinase (CK) activity. Of particular interest, casein kinase has also been implicated in the TGF-β1 signalling cascade in compelling studies. The immune-precipitated TβRII is reported to possess an intrinsic casein kinase activity, suggesting that casein kinase may be involved in one of the signalling cascades subserving the activity of TGF-β1 (Hall et al., 1996). Furthermore, CK1ε is able to phosphorylate the receptor-activated Smads and the TβRII *in vitro*. More importantly, inhibition of endogenous CK1 catalytic activity with IC261 blocked TGF-β-stimulated transcriptional activity (Waddell et al., 2004). Intriguingly, our laboratory found that inhibition of CK1δ/ε by PF670462, prevented TGF-β1-induced EMT in A549 human alveolar epithelial cells and TGF-β1-activated fibrogenic gene expression in human lung fibroblasts (Keenan et al., 2018). CK1δ/ε may therefore be an important component in the TGF-β signalling cascade. Hence, in the current study, we sought to selectively modulate TGF-β1 activity by targeting CK1δ/ε using PF670462, a dual inhibitor of CK1δ/ε activity, seeking to restore GC sensitivity in RSV-infected epithelial cells.

The airway epithelium orchestrates the innate immunity of the lung. Pulmonary epithelium expresses a repertoire of PRRs that directly activate innate immunity after recognising conserved virion motifs, referred to as the PAMPs. RSV can be recognised by heterogeneous types of PRRs, such as the Toll-like receptors, NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) (Kato et al., 2005).

RSV possesses various PAMPs that can activate different TLRs (Figure 3.1). TLR4 was the first cell-surface TLR shown to be involved in RSV recognition. It interacts with the viral fusion protein F and uses CD14 as a co-receptor (Kurt-Jones et al., 2000b; Kurt-Jones et al., 2000a). TLR2, a cell-surface TLR proved to play a key role in RSV-antiviral immunity, although via a hitherto uncharacterized ligand (Murawski et al., 2009). Viral RNAs are recognized by independent PRRs including TLRs; TLR3 and TLR7/8, and RNA helicases; MDA5 and RIG-I. The TLRs sample the extracellular and endosomal compartments while RIG-I and MDA5 sample the cytoplasm for viral RNAs. TLR3 recognizes dsRNA; an

intermediate formed in the RSV replication cycle (Aeffner et al., 2011) while TLR7/8 detect ssRNA RSV genome (Lindell et al., 2009). RIG-I and MDA-5 although sharing the same signalling features(Yoneyama et al., 2005) and structural homology (Yoneyama et al., 2004), recognize different RNA ligands. RIG-I detects short dsRNA and ssRNA that harbour 5′-triphosphate caps (Hornung et al., 2006) or RNAs with complex secondary structures (Saito et al., 2007). On the contrary, signalling by MDA5 is uniquely triggered by long kilobase-scale dsRNA; genomic or replication intermediates, without end specificity (Kato et al., 2006). Both RIG-I and MDA5 contribute to RSV sensing. RIG-I mediates the early innate immune reaction against RSV infection in bronchial epithelial cells (Liu et al., 2007). MDA-5 plays a crucial role in NF-κB-driven promoter regulation following RSV infection (Yoboua et al., 2010a; b). Moreover, MDA5 recognizes poly(I:C); a synthetic analogue of dsRNA that is commonly used as a viral mimic (Gitlin et al., 2006).

In this chapter, we hypothesised that RSV-induced steroid insensitivity may occur through epithelium activation by viral PAMPs with consequent release of cytokines, such as TGF-β, a potent inducer of glucocorticoid insensitivity. Thus, we systemically assessed the PRRs including TLR 2, 3, 4, 7 and RIG/MDA5 that may contribute to RSV sensing for their potential contribution to GC insensitivity following RSV infection in order to delineate the driving mechanisms of the apparent resistance.

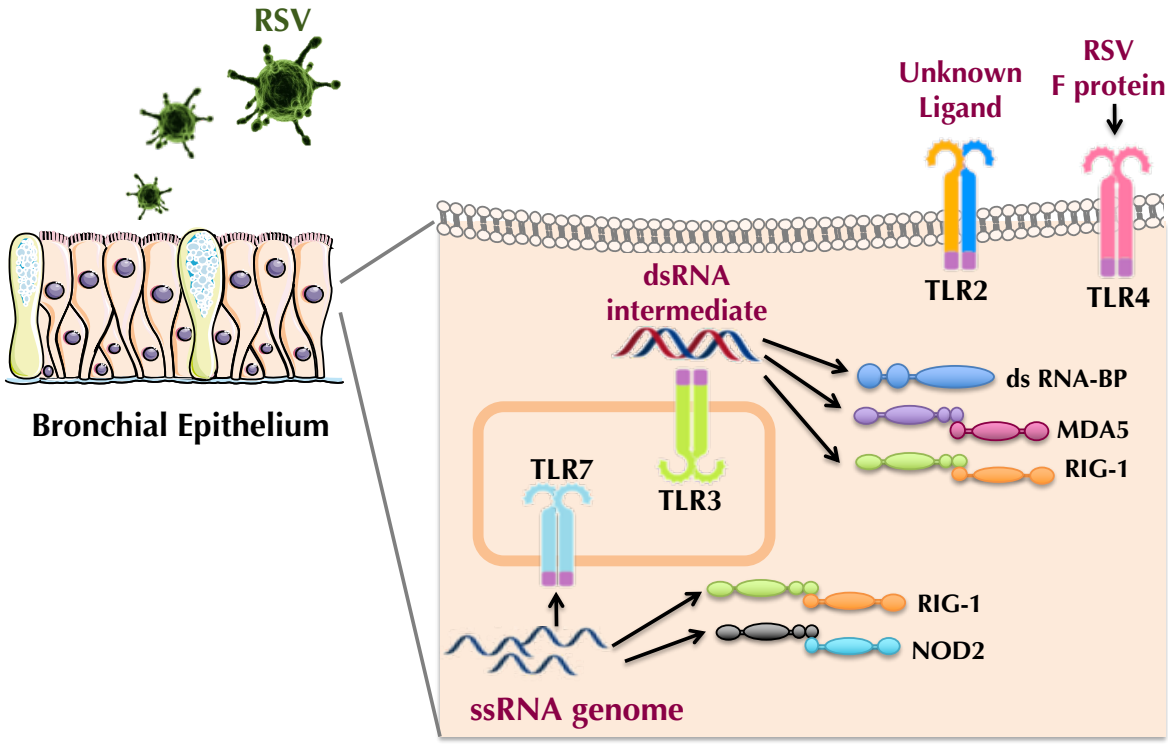


Figure 3.1| Pattern Recognition Receptors (PRRs) involved in the recognition of respiratory syncytial virus (RSV) in bronchial epithelium.

3.2 Outline and aims of the chapter

In the current chapter, we attempted to develop a better understanding of the role of different TLRs in RSV-induced glucocorticoid insensitivity in airway epithelium. This was addressed by examining the activation of various PRRs implicated in RSV sensing by their respective ligands, to assess their influence on GRE activity in human bronchial epithelial cells. To ascertain whether RSV-induced GC resistance is replication-dependent, the impact of heat-inactivated, and UV-irradiated RSV on GRE activity was explored in BEAS-2B. Furthermore, the modulation of transcriptional responses to glucocorticoids was also examined. Smad phosphorylation; a specific signalling response to TGF-β, has been explored in BEAS-2B to disentangle the contribution of endogenous TGF-β in the apparent resistance. It is anticipated that this knowledge can help in deciphering the underpinning mechanism of RSV-induced glucocorticoid insensitivity and ultimately defining novel targets to circumvent resistance.

3.3 Methods

3.3.1 Epithelial Cell culture

BEAS-2B (ATCC® CRL9609™, VA, USA), an SV40-transformed nontumorigenic human bronchial epithelial cell line was cultured as previously described in Section 2.1.3.

3.3.2 RSV Inocula Preparation and Inactivation

Human RSV, A2 strain (ATCC® VR-1540™), was used in the current study. RSV was propagated on HEp-2 cells (ATCC® CCL-23™) and stored, as previously described in Section 2.2. The mock inoculum was prepared following the same procedure as the RSV stock preparation, with the exception that the HEp-2 cells were not inoculated with replication-competent RSV as previously described in Section 2.2.

RSV replication was inhibited either by UV-irradiation or heat-inactivation. For UV irradiation, the RSV was aliquoted to an open 24-well cell culture plate and UV-irradiated for 1 hr. This protocol abolishes RSV infectivity without disrupting the conformation of viral proteins and mediators (Jaovisidha et al., 1999). RSV was heat-inactivated via incubation at 56°C for 30 min (Ausar et al., 2005). Inactivation of the virus infectivity of HI-RSV and UV-RSV was confirmed upon inoculation into HEp-2 cells. The efficiency of RSV inactivation was confirmed by $TCID_{50}$ assay as previously mentioned in Section 2.3. Previous study reported that when cell culture medium was irradiated with UV at relevant disinfection doses, the chemical composition of the media was affected with respect to

several carboxylic acids, and to a minimal extent, amino acids (Yen et al., 2014). Moreover, heat inactivation decomposes heat-liable medium components. Hence, the mock inoculum was subjected to the identical inactivation procedure either by heat or UV-irradiation to help serve as a negative control in these sets of experiments.

3.3.3 Infection of bronchial epithelial cells

The multiplicity of infection (MOI) refers to the ratio of the number of virus particles to the number of target cells. The number of virions that infect each cell at different MOI, is described by the Poisson distribution:

$$P(k) = e^{-m}m^k/k$$

where, P(k) is the fraction of cells infected by k virus particles, and m is the MOI.

Natural RSV infection occurs through inhalation of aerosolized droplets that contain viral particles, resulting in infection of small foci of apical respiratory epithelial cells (Johnson et al., 2007; González-Parra and Dobrovolny, 2018). Hence, we have chosen lower MOI (0.1) to simulate the natural infection and allow for multiple cycles of replication. Based on Poisson distribution ($P(k) = e^{-m}m^k/k$), high MOI (1) will infect around 65% of BEAS-2B cells, however lower MOI (0.1) will only infect only 9.5% of BEAS-2B cells which is more physiologically relevant.

In the current study, BEAS-2B were inoculated with RSV at a MOI of 0.1 as previously described (Xia et al., 2017). Briefly, BEAS-2B cells were seeded in the maintenance medium described in Section 2.1.3 and grown until ~90% confluent. To work out the MOI, cells in one well were washed once in warm PBS to remove detached cells and then harvested with Trypsin-EDTA. Maintenance medium was added to the cells suspension and cell count was performed using a haemocytometer. Proper viral stock dilution was calculated based on $TCID_{50}$ of RSV inoculum and cell count to achieve the required MOI.

RSV inoculum stock was thawed at 37°C, diluted in infection medium; DMEM, supplemented with 1% (v/v) heat-inactivated FCS, 0.2% (v/v) sodium bicarbonate, 15 mM HEPES, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 1% (v/v) sodium pyruvate, 50 mg/mL-1 streptomycin and 5 IU/mL-1 penicillin. For infection, the maintenance medium was aspirated and then washed once in warm PBS. Cells were inoculated with RSV at MOI 0.1 at a volume of 125 µL for 24 well plates or 200 µL for 6 well plates to ensure efficient viral binding. Culture plates were incubated at 37°C, 5 % CO_2 and gently rocked every 10-15 min for 1 h. One-hour post-infection, 1 mL/well of infection media was

added and cells were incubated at 37°C, 5 % CO_2 and observed daily for cytopathic effects induced by the virion until they were harvested for RNA or protein.

It is known that the clinical manifestation of RSV infection typically appears after four to six days after exposure to the virus (Piedimonte and Perez, 2014). Therefore, we chose 48 hours viral infection. Moreover, the RSV infection at MOI 0.1 longer than 48 hours induces apparent cytopathic effects, which impacts the cell viability, therefore, maximum of 48 hours viral infection had been conducted. Our group had conducted preliminary experiments to examine 24 hours RSV infection, in parallel to 48 hours RSV infection, with 4 hours dexamethasone treatment in BEAS-2B cells. The expression of GC-responsive genes was also decreased, however non-significantly, with 24 h RSV infection.

Moreover, our group also found that TGF-β mRNA at 24 h time point was higher than the expression at 48 h time point. Previous data suggests that TGF-β effect can be detected within 8 hours. Therefore, we could expect to see the secreted TGF-β-induced effect by 48 hours after viral infection. Expression of PAI-1 mRNA was used as a consistent marker for TGF-β activity, as expected; the expression level of PAI-1 was significantly increased with 48 hours viral infection.

3.3.4 PRRs Challenging

The following PRR ligands were used for challenging BEAS-2B cells: TLR2 agonists; Zymosan A from *Saccharomyces cerevisiae* (Z4250, Sigma) and Lipoteichoic acid from *Staphylococcus aureus* (L2515, Sigma), TLR3 agonist; double-stranded Poly (I).Poly (C) (4287, Tocris Bioscience), TLR4 agonist; LPS from Escherichia coli (L-2630, Sigma), TLR7/8 agonist; Imiquimod (RDS370050, Tocris Bioscience) and RLR agonist; poly I:C (LMW)/LyoVecTM (tlrl-picwlv, InvivoGen).

Table 3.1| Pattern recognition receptors (PRRs) ligands used in the current study.

Ligand	Target Receptor	Supplier	Vehicle
Zymosan A (from *Saccharomyces cerevisiae*)	TLR2	Sigma-Aldrich (Z4250)	PBS
Lipoteichoic acid (from *Staphylococcus aureus*)	TLR2	Sigma-Aldrich (L2515)	PBS
Polyinosinic: Polycytidylic Acid (Poly I:C)	TLR3	Tocris Bioscience (4287)	PBS
Lipopolysaccharide (LPS) (from *Escherichia coli*)	TLR4	Sigma (L-2630)	PBS
Imiquimod	TLR7	Tocris Bioscience (RDS370050)	DMSO
Poly I:C (LMW)/LyoVec ™	RIG/MDA5	InvivoGen (tlrl-picwlv)	Endotoxin-free water

3.3.5 Epithelial Cell Transfection and Reporter Gene Assay

BEAS-2B cells were seeded overnight in a 24-well cell culture plates at a density of 75,000 cells/well in penicillin and streptomycin free LHC-9 medium supplemented by 2% (v/v) charcoal-stripped FCS and 2 mM L-glutamine. BEAS-2B cells were co-transfected with pGRE-SEAP (Clontech Laboratories Inc., Mountain View, CA) and pmax-GFP (Amaxa, Lonza, Waverley, Australia) or pGL3-Luc (Promega, Alexandria, NSW, Australia) as internal transfection controls using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as mentioned previously in Section 2.4.5.

One-hour post-transfection, cells were either inoculated with RSV MOI 0.1 as described in Section 3.3.3 or challenged with TLR ligands for 24 h before treatment with dexamethasone (30 nM) (Sigma Aldrich, Castle Hill, NSW, Australia) or Vehicle (0.1% DMSO) for 24 h. Supernatants were then collected, and SEAP levels were determined using a chemiluminescence kit (Roche Applied Science, NSW, Australia) following manufacturer's protocol using Topcount (Perkin Elmer, Glen Waverley, VIC, Australia) as detailed in Section 2.4.4. The determined SEAP level was normalized to the level of luciferase expression as determined from cell lysates from cells transfected with pGL3 plasmid (described in Section 2.4.7). Cells transfected with pMAX-GFP were assessed qualitatively for equivalent transfection efficiency, as determined by GFP fluorescence using a Leica DMI6000B inverted fluorescence microscope. SEAP activity was used as a measure of GRE activity and was expressed as a percentage of the SEAP level induced by 30 nM dexamethasone.

3.3.6 Determination of endogenous gene expression in BEAS-2B cell line

BEAS-2B cells were seeded in 24 well culture plate at a density of 50,000 cells per well and allowed to adhere overnight in LHC-9 medium supplemented as detailed in Section 2.1.3. BEAS-2B cells were washed once by sterile pre-warmed PBS then rendered quiescent by starvation in serum-free DMEM medium containing 0.25% (w/v) BSA, 15 mM HEPES, 0.2% (v/v) sodium bicarbonate, 2 mM L-glutamine, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, 5 IU/mL penicillin and 50 mg/mL streptomycin for 24 h prior to treatment.

Serum-starved cells were either inoculated with RSV MOI 0.1 as described in Section 3.3.3 or challenged with different PRRs ligands. After 20 h of TLR challenging or 44 h of RSV infection, cells were treated with dexamethasone (30nM) or Vehicle (0.1% DMSO) for 4 h. The 4-hour time point for mRNA expression of the GC-inducible genes

was determined to be suitable by our group based on time-course evaluations (Salem et al., 2012; Keenan et al., 2014). In some set of experiments, SB431542 (1μM), PF670462 (10μM) or U0126 (1μM) was added 30 min before RSV infection or poly I:C (10 μg/mL). Total RNA was then extracted using TRIzol reagent (Invitrogen) (Section 2.6.1) and reverse-transcribed into cDNA (Section 2.6.2). Endogenous gene expression was then determined by RT-qPCR (Section 2.6.3) using specific primers listed in Table 2.1.

3.3.7 Determination of intracellular kinase phosphorylation

BEAS-2B cells were seeded in 6 well culture plates at a density of 100,000 cells/mL in LHC-9 medium supplemented as detailed in Section 2.1.3. Cells were allowed to adhere overnight and then serum-starved as described in Section 3.3.6 for 24 h before treatment. Cells were incubated with SB431542 (1 μM) for 30 min before RSV inoculation at MOI of 0.1 (Section 3.3.3) for 3 h, 24 h and 48 h or TLR3 challenging using poly I:C (10 μg/mL) for 2 h, 4 h, 6 h and 24 h. Smad and Extracellular signal-regulated kinase (ERK) phosphorylation were determined in cell lysates by Western blotting as detailed in Section 2.7 using specific antibodies listed in Table 2.3.

3.4 Results

3.4.1 Expression profiling and functional analysis of different TLRs in human bronchial epithelial cells.

To validate our *in vitro* model, we firstly assessed the expression profile of different TLRs in serum-starved unstimulated BEAS-2B cells by qRT-PCR. Our analysis revealed that BEAS-2B cells expressed all TLRs (1-10), albeit at varying levels. Based upon the basal mRNA expression ($2^{-\triangle CT}x10^{8}$), the most highly expressed TLRs were TLR2, TLR3 and TLR4, TLR 1, TLR5, TLR6 and TLR9 were also expressed but al lower levels, whereas TLR7, TLR8, and TLR10 mRNA appeared to be negligible as shown in Figure 3.2. Next, to confirm the functional expression of different TLRs, we stimulated BEAS-2B cells with highly purified specific ligands as listed in Table 3.1. Ligands for TLR2 (Zymosan A 10 μg/ml), –3 (poly I:C 10 μg/ml), –4 (LPS 10 μg/ml) were able to induce IL-6 and IL-8 expression with different efficacy. However, BEAS-2B cells showed a much weaker response to Imiquimod (10 μM), which is consistent with the weak expression of TLR7.

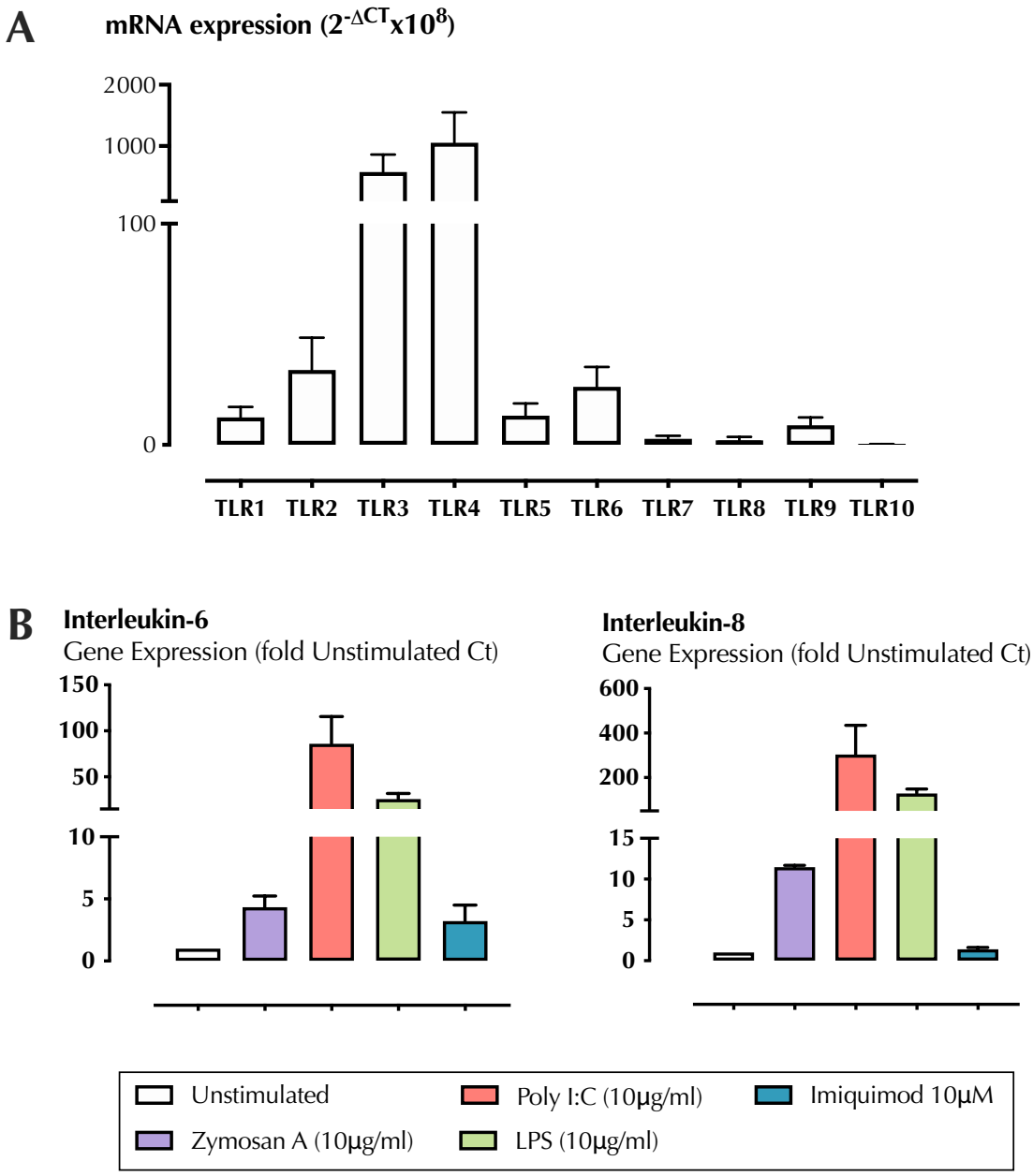


Figure 3.2| Expression profiling and functional analysis of Toll-like receptors in BEAS-2B cells.

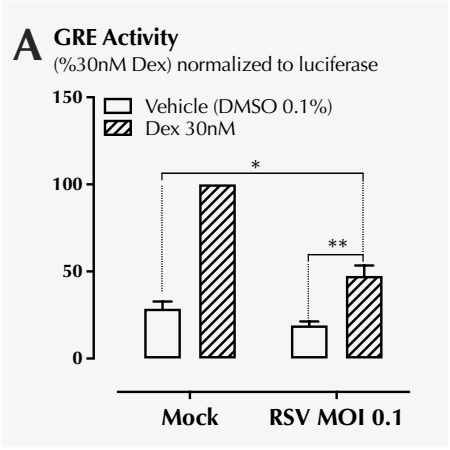
A, RNA was extracted from serum-starved unstimulated BEAS-2B cells and analysed by RT-qPCR. Results are expressed as $2\text{-}\Delta CTx10^8$ and presented as means ± SEM for n = 4-6 independent experiments. B, BEAS-2B cells with challenged with Zymosan A (10 µg/ml), poly I:C (10 µg/ml), LPS (10µg/ml) or Imiquimod (10 µM) for 24h. The expression level of pro-inflammatory genes; *IL-6* and *IL-8*, were determined by RT-qPCR. Data are expressed as fold change from the unstimulated control. Data are presented as means and SEM for n = 3-4 independent experiments.

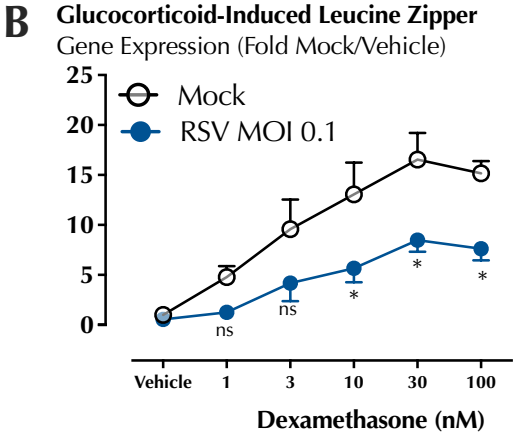
3.4.2 RSV Infection dampens glucocorticoid transactivation in bronchial epithelial cells

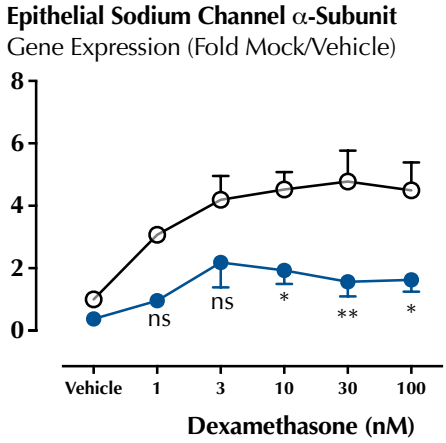
Dexamethasone (30 nM) was established previously as the concentration that optimally activates glucocorticoid response element (GRE) activity in BEAS-2B cells transfected with GRE-controlled SEAP expression vector (Salem et al., 2012; Keenan et al., 2014). Inoculation of the cells with RSV at MOI of 0.1 for 24 h prior to the addition of dexamethasone (30 nM) induced a profound suppression of GRE activity ($p<0.0001$). Noteworthy, GRE activity was not reduced to baseline levels in RSV-infected cells treated with dexamethasone (30 nM), and there was still some residual, however significant, induction of GRE activity compared to vehicle-treated RSV infected cells ($p=0.0047$) (Figure 3.3 A).

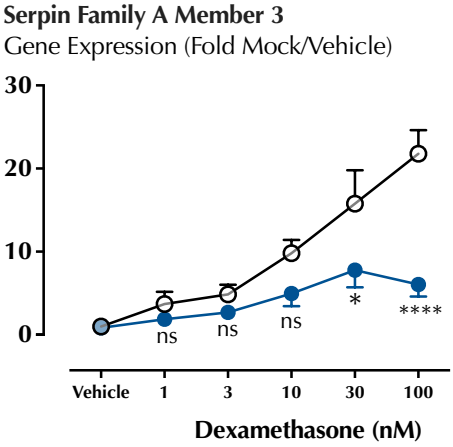
Since the artificial GRE promoter construct may respond differently from the promoters of endogenous GRE-regulated genes, the expression of GRE-regulated genes was measured to confirm the deleterious potential of RSV infection on dexamethasone-induced transactivation. Dexamethasone (1-100 nM) has induced the expression of the selected GC-inducible genes in a concentration-dependent manner. Inoculation of BEAS-2B with RSV MOI 0.1 for 48 h significantly impaired dexamethasone-induced transactivation of most of the GC-inducible genes assessed as shown in Figure 3.3 B.

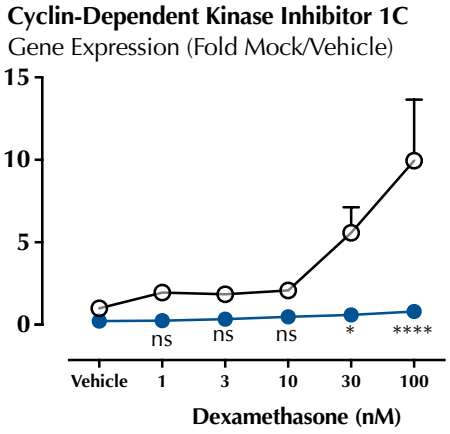
Genes dampened by RSV infection included genes encoding glucocorticoid-inducible leucine zipper (*GILZ*), which is thought to mediate a number of the anti-inflammatory actions of glucocorticoids by inhibiting the activations of the pro-inflammatory transcription factor NFκB; the epithelial sodium channel α subunit (*ENaCα*) which has a role to limit the accumulation of fluid in the airway surface through uptaking Na^+ ions; α-1 antichymotrypsin (serpin family A member 3; *SERPINA3*), which protects lower respiratory tract from damage caused by proteolytic enzymes through inhibiting proteases activity; cyclin-dependent kinase inhibitor 1C (*CDKN1C*), which encodes the cyclin-dependent kinase inhibitor, $p57^{kip2}$ which may mediate some of the growth inhibitory actions of glucocorticoids; pyruvate dehydrogenase kinase isozyme 4 (*PDK4*), that contribute to regulation of glucose metabolism; and the potassium channel shab-related subfamily B member 1 (*KCNB1*) that controls electrolyte transport in airway epithelial cells. Moreover, mRNA expression of promyelocytic leukaemia zinc finger protein (*PLZF*); a transcriptional repressor that mediates antiproliferative and apoptotic actions of GCs, showed a slight decrease, however non-significantly, in RSV-infected cells. Worth-mentioning, Mock was used as a control in the current study to ensure that the observed effects were due to the virus itself not to another RSV stock component since it is crudely prepared from infected HEp-2 cells.

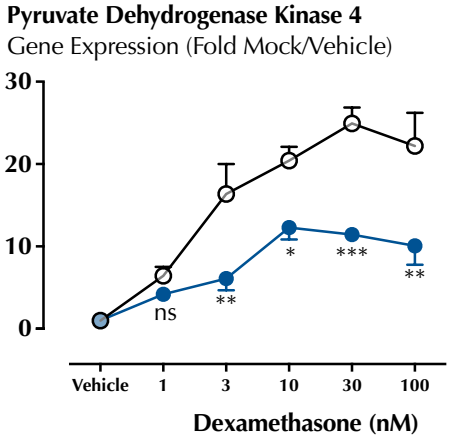

A
GRE Activity
(%30nM Dex) normalized to luciferase
Vehicle (DMSO 0.1%)
Dex 30nM
150
100
50
0
*
**
Mock
RSV MOI 0.1

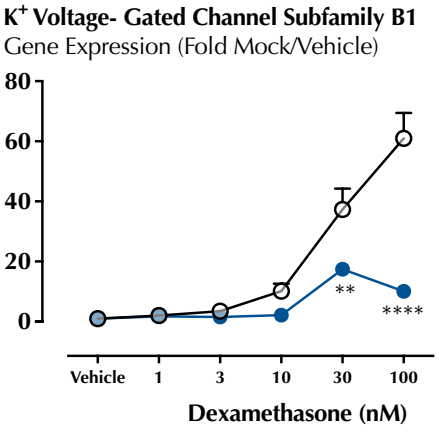

B
Glucocorticoid-Induced Leucine Zipper
Gene Expression (Fold Mock/Vehicle)
Mock
RSV MOI 0.1
25
20
15
10
5
0
ns
ns
*
*
*
Vehicle
1
3
10
30
100
Dexamethasone (nM)


Epithelial Sodium Channel α-Subunit
Gene Expression (Fold Mock/Vehicle)
8
6
4
2
0
ns
ns
*
**
*
Vehicle
1
3
10
30
100
Dexamethasone (nM)


Serpin Family A Member 3
Gene Expression (Fold Mock/Vehicle)
30
20
10
0
ns
ns
ns
*
****
Vehicle
1
3
10
30
100
Dexamethasone (nM)


Cyclin-Dependent Kinase Inhibitor 1C
Gene Expression (Fold Mock/Vehicle)
15
10
5
0
ns
ns
ns
*
****
Vehicle
1
3
10
30
100
Dexamethasone (nM)


Pyruvate Dehydrogenase Kinase 4
Gene Expression (Fold Mock/Vehicle)
30
20
10
0
ns
**
*
***
**
Vehicle
1
3
10
30
100
Dexamethasone (nM)


K+ Voltage- Gated Channel Subfamily B1
Gene Expression (Fold Mock/Vehicle)
80
60
40
20
0
**
****
Vehicle
1
3
10
30
100
Dexamethasone (nM)

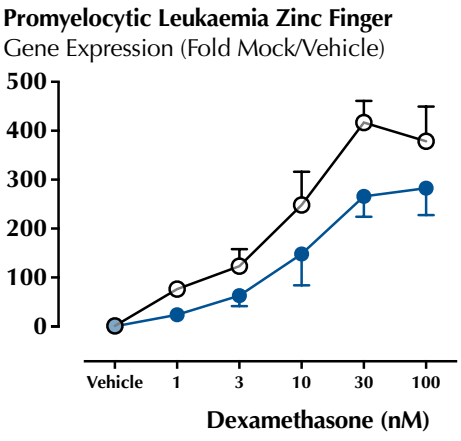

Promyelocytic Leukaemia Zinc Finger
Gene Expression (Fold Mock/Vehicle)
500
400
300
200
100
0
Vehicle
1
3
10
30
100
Dexamethasone (nM)

Figure 3.3| Respiratory syncytial virus (RSV) infection attenuates glucocorticoid transactivation in BEAS-2B cells.
A, BEAS-2B cells transiently transfected with GRE-controlled SEAP expression vector were inoculated with RSV at MOI (multiplicity of infection) of 0.1 or a mock-conditioned medium for 24 h then treated with dexamethasone for a further 24h. GRE activity was measured by the level of SEAP in the supernatants. Data are presented as a percentage the SEAP level induced in response to 30nM dexamethasone and shown as mean ± SEM for n = 3 independent experiments. B, BEAS-2B cells grown in cortisol deficient medium for 24 h were infected with RSV (MOI 0.1) for 44 h, before stimulation by Dexamethasone (1, 3, 10, 30 100 nM) for 4h. RNA was extracted and analyzed by RT-qPCR. Data are presented as fold change from Mock control and shown as mean ± SEM for n = 3 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance and p-values were coded as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.4.3 RSV-induced expression of inflammatory cytokine was largely replication-dependent

We thought to explore whether virus replication was needed to induce expression of inflammatory cytokine; IL-6 and IL-8 or the surface contact with viral components was sufficient. In order to do that, the replication competence of RSV was inhibited by either heat-inactivation or UV-irradiation as previously mentioned in Section 3.3.2. Heat inactivation of the virus denatures the viral proteins, hence, allows exploring the residual impact of ssRNA viral genome alone. On the contrary, UV inactivation damages the viral genome through dinucleotide crosslinking, while maintaining the viral proteins intact.

As seen in Figure 3.4 A, replication-competent RSV induces cell fusion and syncytium formation; the cytopathic effect characteristic of RSV-infected cells, in HEp-2 cells. Successful viral inactivation was proved by the absence of syncytium in HEp-2 cells inoculated with either HI-RSV or UV-RSV.

RSV infection induced a marked increase in the expression of pro-inflammatory cytokines, namely IL-6 and IL-8 mRNA in BEAS-2B cells, whilst modest up-regulation of IL-6 and IL-8 mRNA expression was observed in cells challenged with HI-RSV (MOI 0.1 and 1) and UV-irradiated RSV (MOI 0.1 and 1). UV and heat inactivation of the virus was associated with approximately 10-fold reduction in IL-6 and IL-8 gene expression, suggesting that RSV-induced expression of inflammatory cytokine was largely replication-dependent. Dexamethasone (30nM) remarkably dampened expression of pro-inflammatory cytokines; IL-6 and IL-8 in RSV-infected, HI-RSV- or UV-RSV-challenged BEAS-2B cells (Figure 3.4 B).

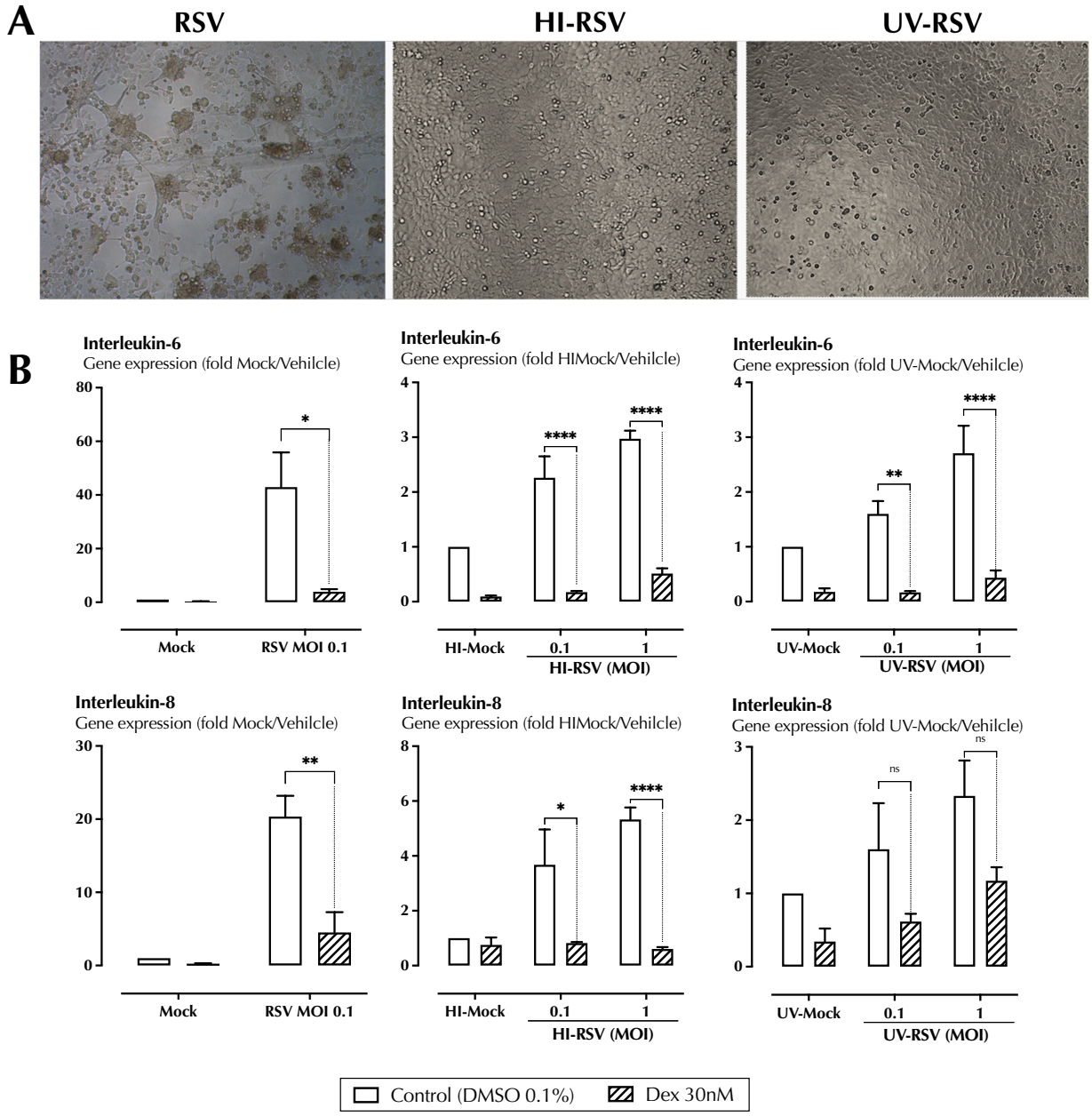


Figure 3.4| Impact of heat-inactivation or UV-irradiation on RSV replication competency and inflammatory response.

A, HEp-2 cells inoculated with replication-competent RSV, (left), heat-inactivated RSV (center) and UV-irradiated RSV (right). B, Serum-starved BEAS-2B cells were incubated with either RSV (MOI 0.1), HI-RSV (MOI 0.1 and 1), UV-irradiated RSV (MOI 0.1 and 1) or the corresponding Mock conditioned medium for 24 h before stimulation by dexamethasone (30 nM) for 4 h after which RNA was extracted and analyzed by qRT-PCR. Gene expression is calculated as fold change from control. Data are shown as mean and SEM for n = 3 - 6 independent experiments, Ordinary 2-way ANOVA, Bonferroni's multiple comparisons post-hoc test, *p<0.05, **p<0.01, ***p<0.001, ****, p<0.0001.

3.4.4 RSV repression of GR-mediated transactivation in BEAS-2B cells is replication-dependent

Next, we have investigated if the RSV-induced attenuation of GC-induced gene transactivation and GRE activity is linked to viral replication or it is merely the signalling caused by recognition of virus components.

To define the role of replication competence in the apparent resistance, GRE-SEAP transfected BEAS-2B cells were challenged with either HI-RSV (MOI 0.1 and 1) or UV-irradiated RSV (MOI 0.1 and 1) for 24h prior to treatment with dexamethasone (30 nM) for further 24h. HI-RSV (MOI 0.1 and 1) has ameliorated GRE activation mediated by dexamethasone (30 nM). On the contrary, UV irradiated RSV (MOI 0.1 and 1) has no detectable effect on GRE activity. In terms of endogenous gene expression, neither HI-RSV nor UV-RSV had altered transactivation of GRE-regulated genes *GILZ*, *CDKN1C* and Zinc finger and BTB domain-containing protein 16 (*ZBTB16*) (Figure 3.5). These data support the notion that RSV-induced impairment of GC sensitivity is at least partially replication-dependent. Although the fact that the sets of experiments using replication competent RSV, HI-RSV and UV-RSV were performed separately in different cell culture plates, which is a suboptimal experimental setup for comparison, during each experiment, the virus and mock used were originating from the same virus or mock batch. In addition, BEAS-2B cells were obtained from the same source, were of similar passage number, and were handled at the same facility. These might partly minimize the impact of confounders and make the comparison possible.

3.4.5 TLR3 and TLR4 challenging suppress glucocorticoid-mediated transactivation

It is known that RSV infection activates a large repertoire of innate immune responses specifically, TLR and RLR pathways (Hillyer et al., 2018). Therefore, we explored the role of these pathways in RSV-mediated suppression of glucocorticoid induction of GR-regulated genes. TLR3 and TLR4 are among the PRRs involved in RSV sensing, where TLR3 recognizes dsRNA intermediate, and TLR4 is RSV Fusion protein (F) sensor. In BEAS-2B cells transfected with GRE controlled SEAP expression vector, incubation with the TLR3 agonist; poly I:C for 24 h prior dexamethasone (30 nM) treatment markedly attenuated the GRE activity in a concentration-dependent manner, where complete inhibition was observed at 10 µg/mL (Figure 3.6). TLR4 challenging with LPS; a canonical TLR4 agonist, at a dose level of 0.1, 1 and 10 µg/mL had resulted in a significant reduction of GRE activation induced by Dexamethasone (30 nM). However, neither of the tested concentrations had led to complete inhibition of GRE activity.

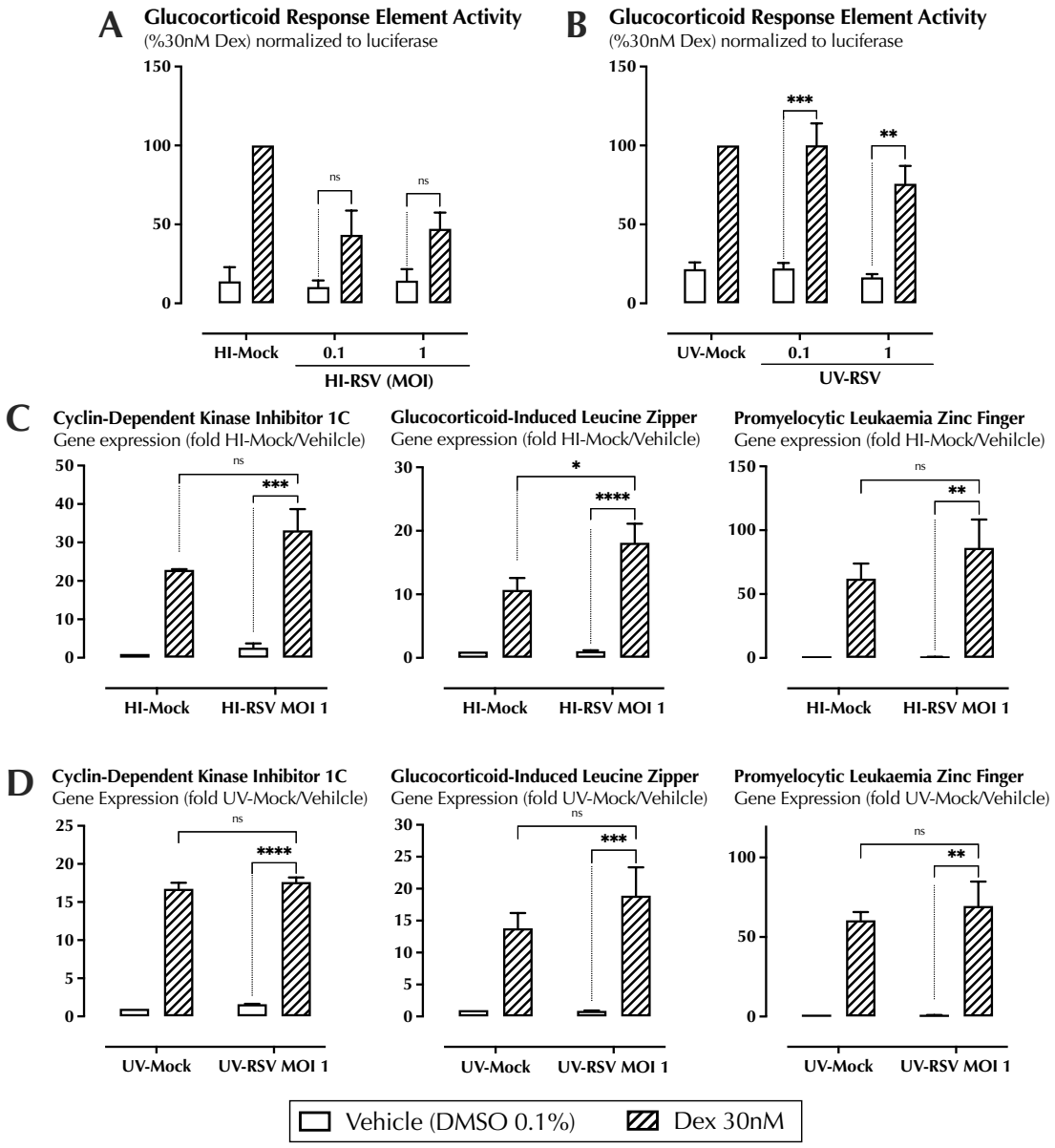


Figure 3.5| RSV infection impairs dexamethasone-induced gene transactivation in a replication-dependent manner.

BEAS-2B cells transiently transfected with a GRE-SEAP reporter construct were incubated with either (A) HI-RSV (MOI 0.1 and 1), (B) UV-irradiated RSV (MOI 0.1 and 1) or the corresponding Mock-conditioned medium for 24 h before stimulation by dexamethasone (30 nM) for a further 24 h. GRE activity was measured by the level of SEAP in the supernatants. Data were presented as a percentage the SEAP level induced in response to 30 nM dexamethasone and presented as mean ± SEM for n = 3 independent experiments. Serum-starved BEAS-2B cells were incubated with either (C) HI-RSV (MOI 1), (D) UV-irradiated RSV (MOI 1) or the corresponding Mock-conditioned medium for 24 h with Dexamethasone (Dex) (30 nM) added for the last 4 h. RNA was extracted and analyzed by RT-qPCR. Gene expression is expressed as fold change from control. Data are presented as mean and SEM for n = 3 - 6 independent experiments for C and n = 3-7 independent experiments for D. Ordinary 2-way ANOVA, Bonferroni's multiple comparisons post-hoc test, *p<0.01.

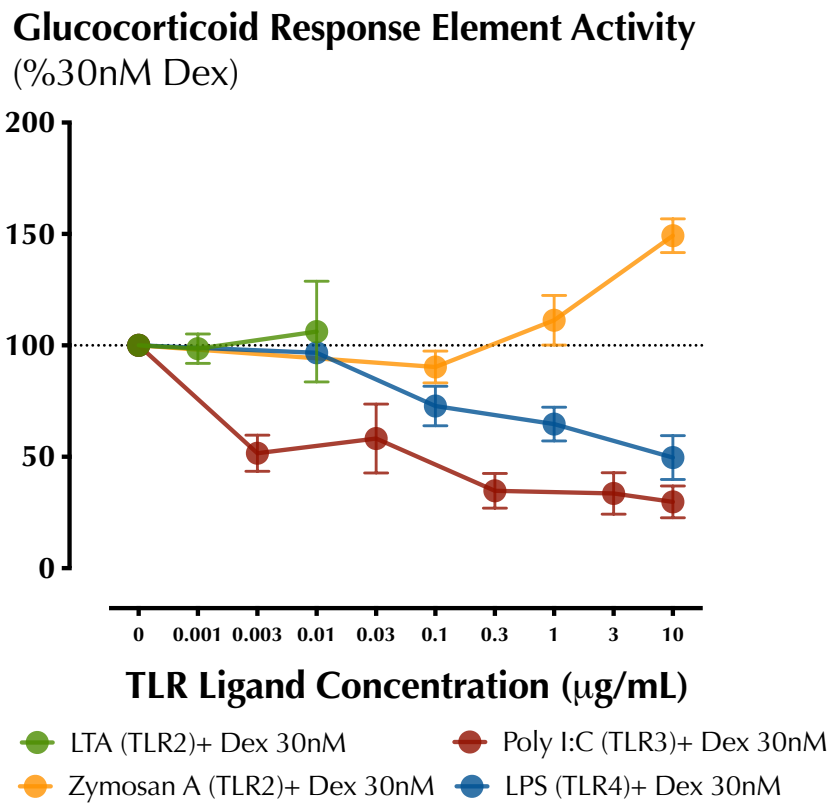


Figure 3.6| Differential impact of TLR ligands on GRE activity in BEAS-2B cells. BEAS-2B cells were transiently transfected with a GRE-SEAP reporter construct and incubated with LTA (1,10 nM), Zymosan A (0.1, 1, 10 μg/mL), Poly I:C (0.003-10 μg/mL) or LPS (0.01-10 μg/mL) for 24 h with Dexamethasone (Dex) (30 nM) added for further 24 h. GRE activity was measured by the level of SEAP in the supernatants. Data were presented as a percentage the SEAP level induced in response to 30 nM dexamethasone and shown as mean ± SEM for n = 3 - 4 independent experiments.

To further confirm these observations, expression of endogenous GRE regulated genes had been measured in BEAS-2B cells exposed to poly I:C (10 μg/mL) or LPS (1, 10 μg/mL). As shown in Figure 3.7, Dexamethasone (1-100 nM) induced the expression of the selected GC-inducible genes in a concentration-dependent manner. TLR3 activation by poly I:C (10 μg/mL) for 24 h significantly repressed dexamethasone-mediated transactivation of an array of the GC-inducible genes. Genes impaired by TLR3 challenging included *CDKN1C, ENaCα, GILZ, PDK4, KCNB1* and *FK506* binding protein 5 (*FKBP5*), which encodes FK506 binding protein that serves as a co-chaperone for the glucocorticoid receptor and reductions in its levels have been correlated with severe asthma. Additionally, poly I:C dampened dexamethasone-mediated mRNA expression of *ZBTB16*; also known as *PLZF*, that encodes pro-myeolocytic zinc finger protein; a transcriptional repressor that has been implicated in anti-inflammatory and growth-regulatory actions of glucocorticoids.

Challenging bronchial epithelial cells with LPS (1, 10 μg/mL) had a much-limited impact on GR-regulated genes transactivation, compared to that observed with Poly I:C, where only the expression of *CDKN1C* and *GILZ* were repressed, while *ENaCα* and *PLZF* did not (Figure 3.8).

3.4.6 Neither TLR2 nor TLR7 activation impairs GC transactivation

TLR2 and TLR7 have been shown to play a key role in RSV-antiviral immunity (Murawski et al., 2009). Hence, we have examined these receptors for possible involvement in RSV deleterious effects on GC signalling in bronchial epithelial cells. In order to address this question, we have used highly purified ligands for TLR2; Zymosan A and Lipoteichoic acid (LTA) and TLR7; Imiquimod.

Exposure of BEAS-2B cells transiently transfected with GRE-SEAP expression vector to TLR2 ligands; Zymosan A (0.1, 1 and 10 μg/mL), or Lipoteichoic acid (1 or 10 μg/mL), for 24hr before 24 h of dexamethasone induction (30 nM), contrary to TLR3 and TLR4 ligands, did not attenuate dexamethasone-induced GRE activity (Figure 3.6). Noteworthy, pre-incubation with TLR2 ligand; Zymosan A at 10 μg/mL had led to approximately 50% increase in GRE activity. Moreover, activation of both TLR2 by Zymosan A (10, 100 μg/mL) or TLR7 activation by Imiquimod (1 or 10μM) did not affect mRNA expression of GR-mediated transactivation of *CDKN1C*, *GILZ* or *ZBTB16* (Figure 3.9). These observations suggest that however contributing to RSV sensing, neither TLR2 nor TLR7 activation contributes to RSV-induced GC insensitivity.

3.4.7 RIG-1/MDA5 activation had no detectable effect on glucocorticoid induction of endogenous *GR-regulated genes*.

In addition to TLRs, both RIG-I and MDA5 contribute to RSV sensing, where RIG-I has been stated to mediates the early innate immune response to RSV infection in bronchial epithelial cells (Liu et al., 2007) and MDA-5 plays a crucial role in NF-κB-driven promoter regulation following RSV infection (Yoboua et al., 2010a). Poly(I:C)-LMW/LyoVec is a complex between poly(I:C)-LMW and the transfection reagent LyoVec™ 1:6 ratio (w/w). Poly(I:C)-LMW (low molecular weight) is poly(I:C) with an average size is 0.2-1 kb. This complex has been shown to induce the activation of the RIG-I/MDA-5 signalling pathway (Kato et al., 2005; Gitlin et al., 2006). RIG-1/MDA5 activation of BEAS-2B cells by poly I:C (LMW)/LyoVec for 24h had induced the expression of pro-inflammatory cytokines, namely IL-6 and IL-8 mRNA and antiviral cytokine; Interferon-beta (INF- β) (Figure 3.10 B). However, poly I:C (LMW)/LyoVec did not affect the GR-mediated transactivation of *ENaCα*, *GILZ* and *PLZF*, suggesting that RLRs might not contribute to RSV-induced GC insensitivity in epithelial cells. (Figure 3.10 A)

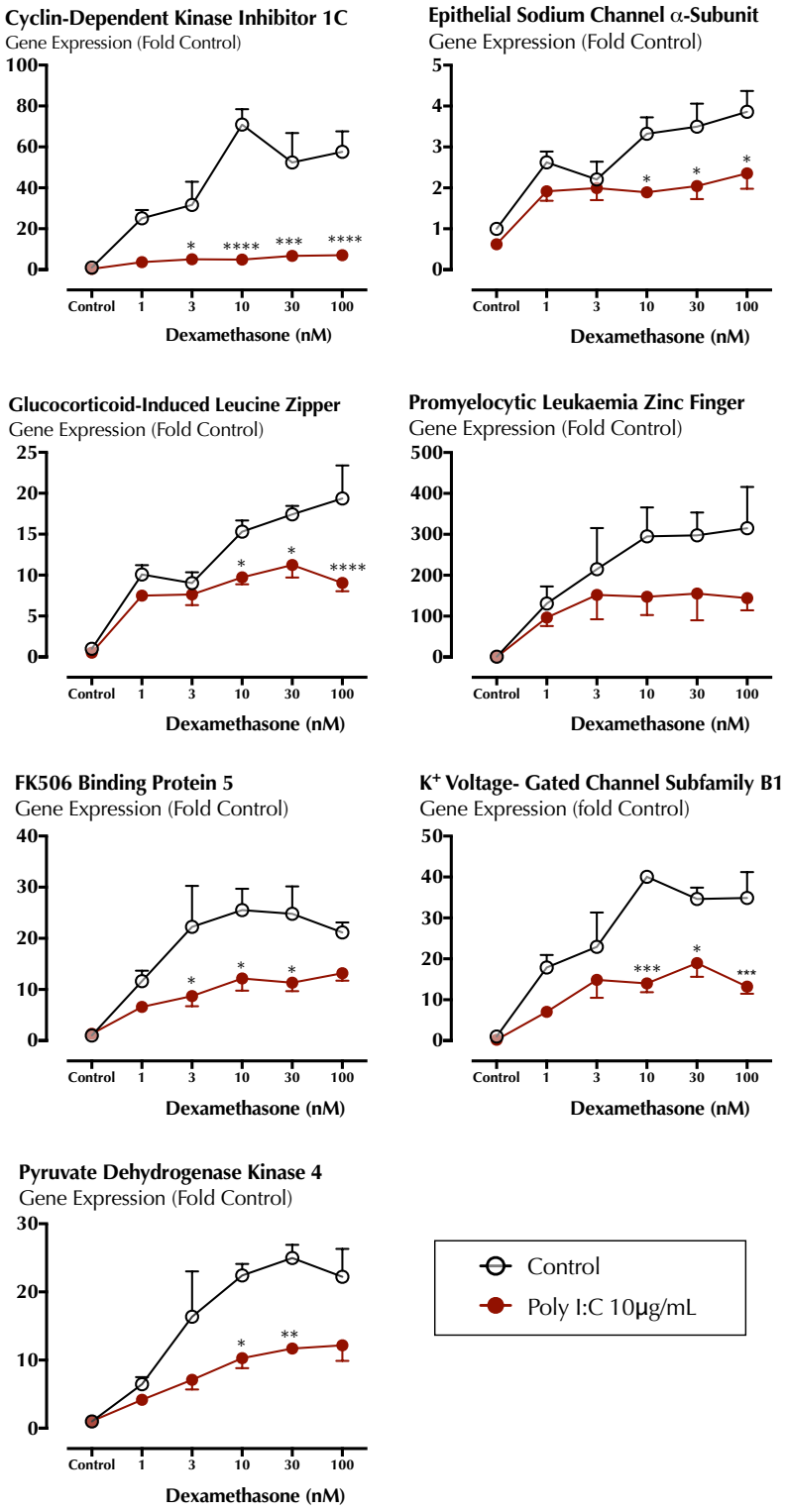


Figure 3.7| TLR3 activation by poly I:C impaired dexamethasone-induced gene transactivation in BEAS-2B cells.

Serum-starved BEAS-2B cells were challenged with poly I:C (10 µg/mL) for 20 h before stimulation by dexamethasone (1-100 nM) for 4 h. Total RNA was extracted and analyzed by RT-qPCR. Gene expression is presented as fold change from vehicle control and shown as mean ± SEM for n = 4 - 5 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance and p-values were coded as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

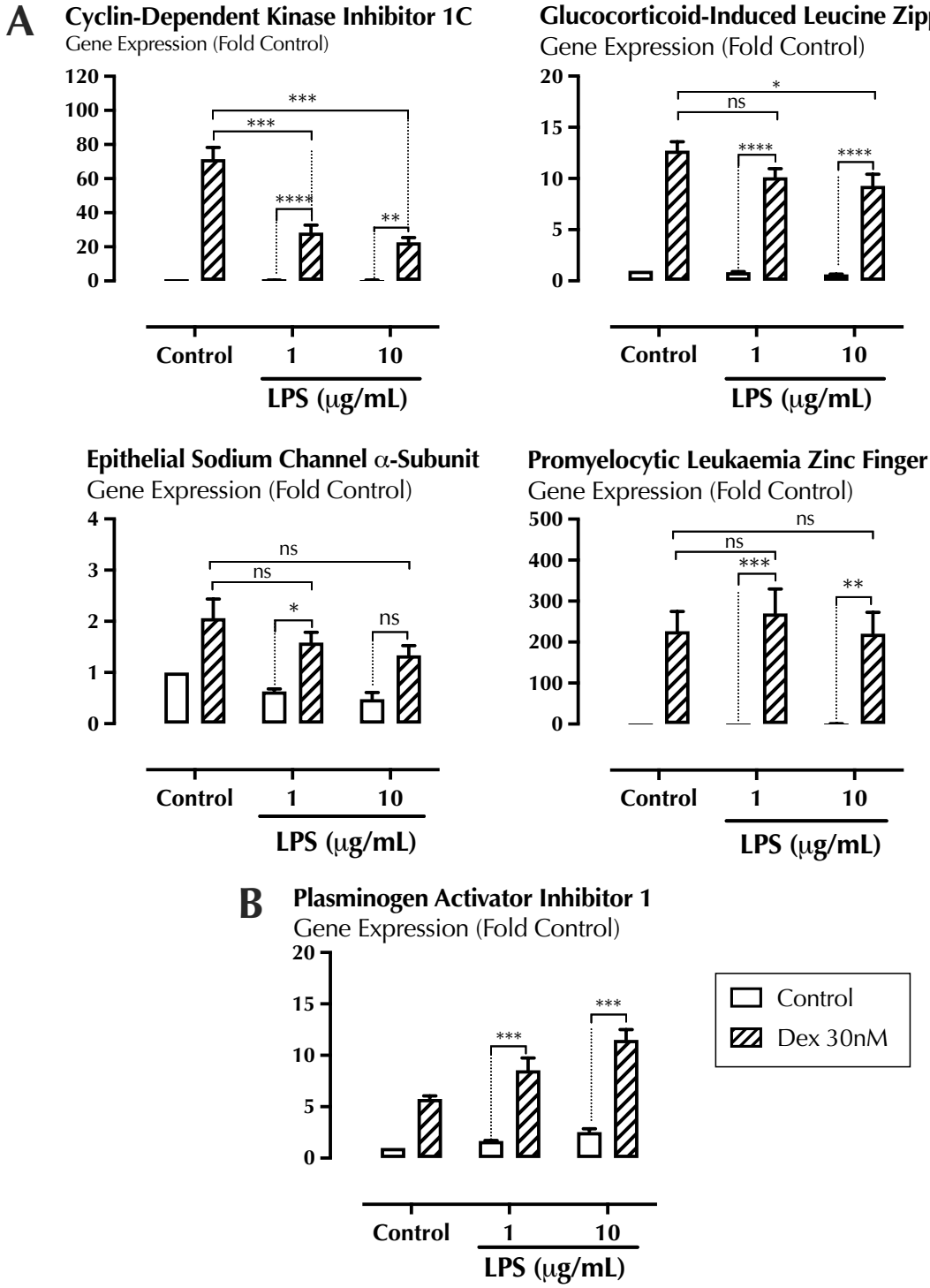


Figure 3.8| Effect of TLR4 activation on glucocorticoid-mediated transactivation in BEAS-2B cells.

Serum-starved BEAS-2B cells were challenged by LPS (1, 10 μg/mL) for 24 h with Dexamethasone (Dex) (30 nM) added for the last 4 h. Total RNA was extracted, transcribed and analyzed by RT-qPCR. Gene expression is presented as fold change from control and shown as mean ± SEM for at n = 4 -6 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance, and p-values were coded as follows: *, $p<0.05$; ***, $p<0.001$ of Control.

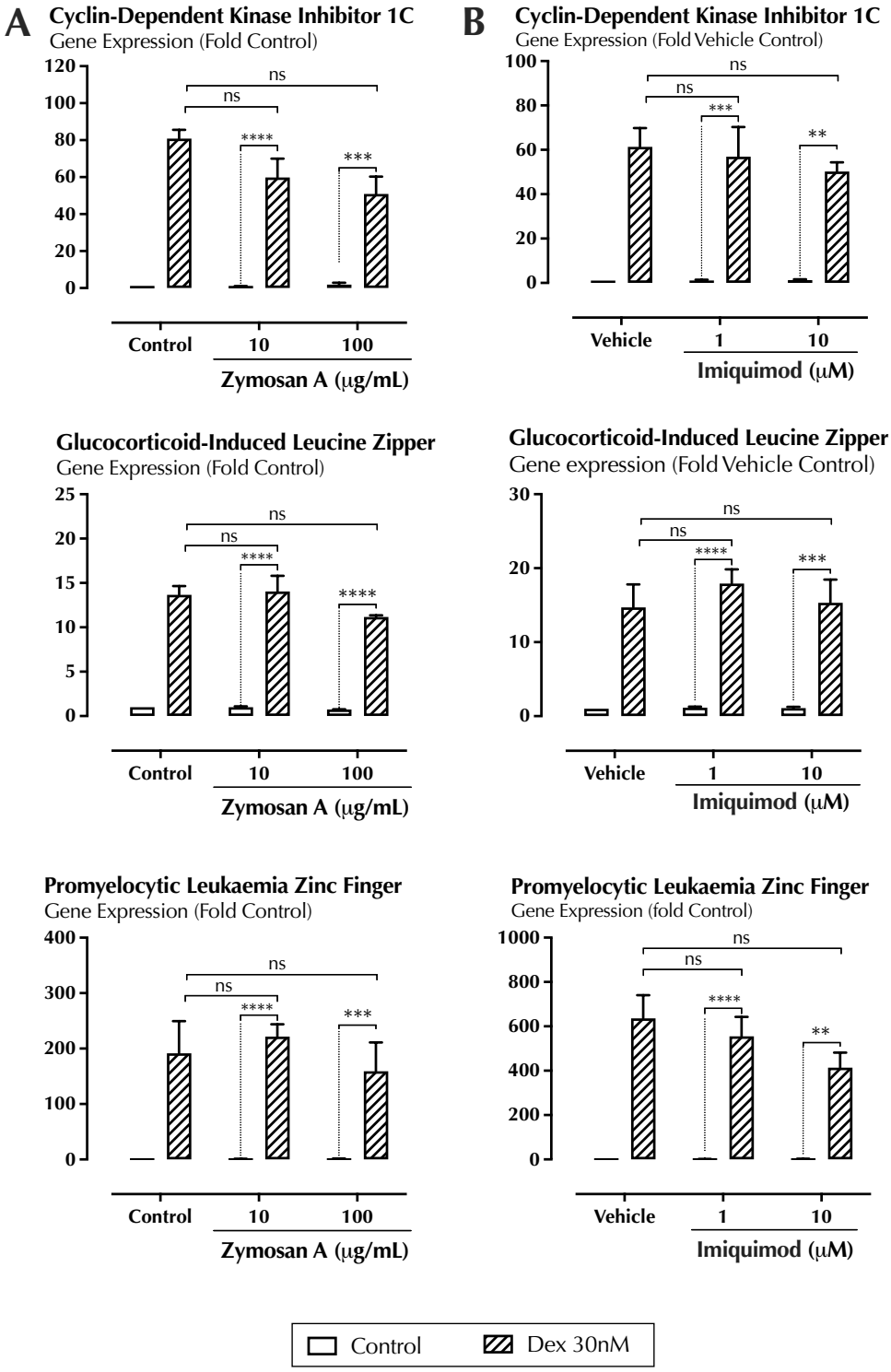


Figure 3.9| Impact of TLR2 and TLR7 on transactivation of the glucocorticoid receptor (GR)-mediated gene activation in bronchial epithelial cells.

Serum-starved BEAS-2B cells were challenged by (A) Zymosan A (10, 100 μg/mL) or (B) Imiquimod (1 or 10 μM) for 24 h with Dexamethasone (Dex) (30 nM) added for the last 4 h. Total RNA was extracted, transcribed and analyzed by RT-qPCR. Gene expression is presented as fold change from control and presented as mean ± SEM for n = 4 -5 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance.

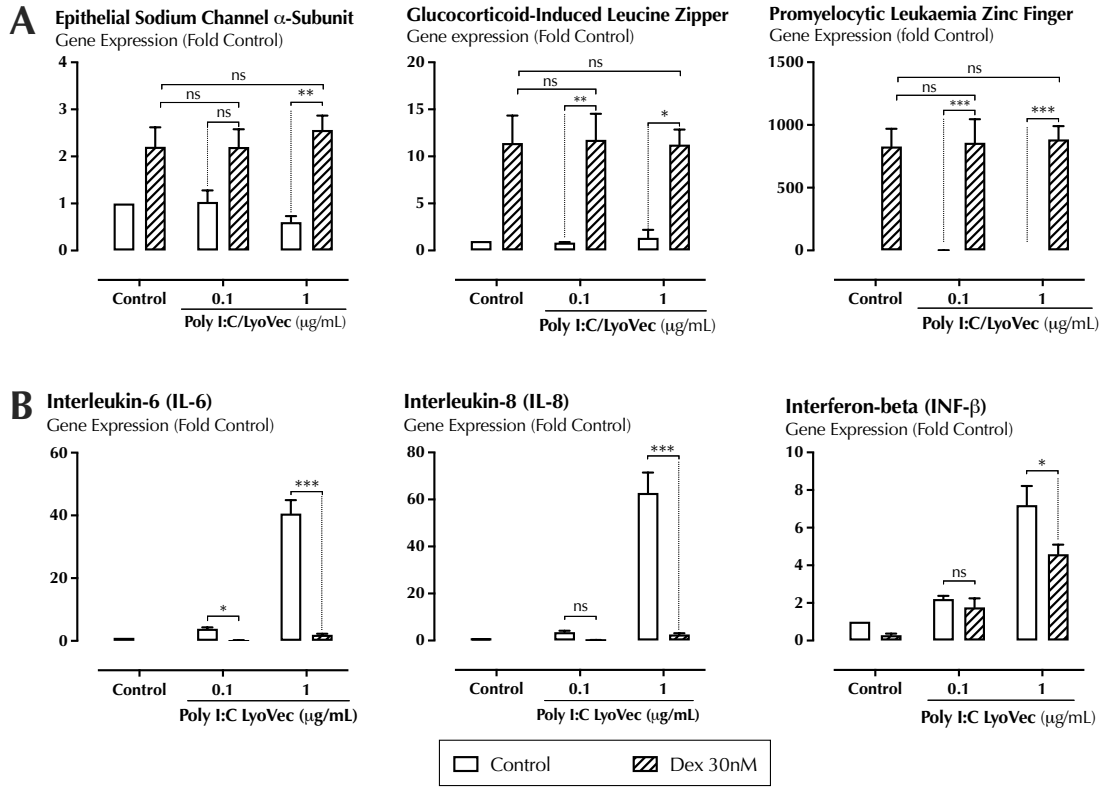


Figure 3.10| RIG-1/MDA5 activation by poly I:C (LMW)/LyoVec did not affect glucocorticoid transactivation (A), while it induced expression of the pro-inflammatory antiviral cytokines (B).

Serum-starved BEAS-2B cells were challenged with poly I:C (LMW)/LyoVec (0.1, 1 μg/mL) for 24 h with Dexamethasone (Dex) (30 nM) added for the last 4 h. RNA was extracted and analyzed by RT-qPCR. Gene expression is expressed as fold change from control and presented as mean ± SEM for n = 3 - 4 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance, and p-values were coded as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.4.8 RSV infection and poly I:C induced endogenous *TGF-β* expression and activity

We next sought to disentangle the contribution of endocrine *TGF-β* in the observed glucocorticoid insensitivity in RSV-infected or poly I:C-exposed cells. To that end, the expression of *TGF-β* and plasminogen activator inhibitor-1 (*PAI-1*), a consistent marker for TGF-β activity, was assessed. Our data revealed that RSV inoculation significantly induced mRNA expression of *TGF-β1* and *PAI-1* (Figure 3.11 A). This potential was further confirmed by incubating the cells with the TGF-β receptor (activin-like kinase 5 (ALK5/ TGF- β R1 kinase) selective inhibitor, SB431542 [1μM; concentration validated in previous studies done by our group (Salem et al., 2012; Keenan et al., 2014)] for 30 min prior to RSV inoculation, where ALK5 inhibition by SB431542 abolished RSV-induced *TGF-β1* and

PAI-1 expression. These findings suggested that RSV-induced TGF-β expression might be dependent on the activation of ALK5. Our group provided additional evidence of a role for ALK5 activation in RSV-induced TGF-β expression using a structurally distinct ALK5 inhibitor; GW788388 and ALK5 siRNA, where both methods had shown to ablate TGF-β expression in RSV-infected cells. Noteworthy, HI-RSV and UV-RSV did not affect the mRNA expression level of *TGF-β* or *PAI-1* in BEAS-2B cells (Figure 3.12), suggesting that RSV-induced *TGF-β* activity is replication dependent.

Similar findings were observed in BEAS-2B cells stimulated with poly I:C, where *TGF-β* and *PAI-1* expression shown significant induction abolished with ALK5 inhibition (Figure 3.11 B). Moreover, the phosphorylation of Smad2 was increased, albeit non significally, 24h post poly I:C exposure, in a manner markedly reduced by pre-incubation with SB431542 (Figure 3.11C).

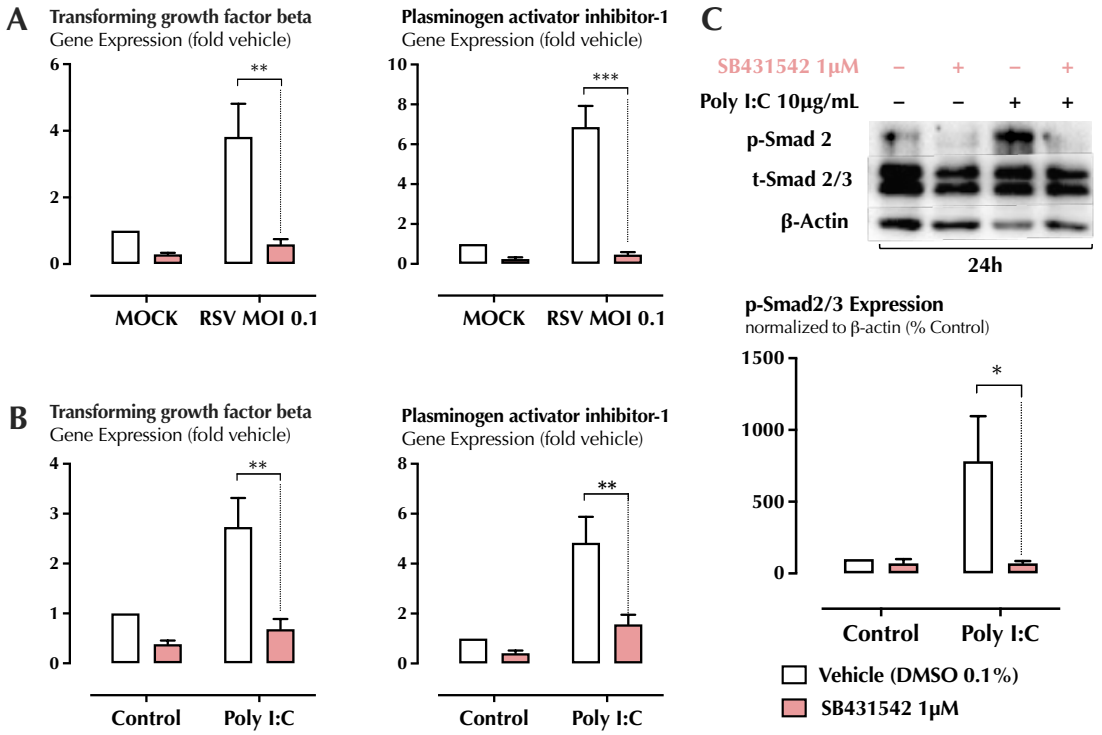


Figure 3.11| Induction of transforming growth factor-β (TGF-β)-responsive genes by RSV infection and Toll-like receptor 3 (TLR3) ligand in bronchial epithelial cells.
A, BEAS-2B cells were incubated with SB431542 before inoculation with RSV at MOI of 0.1 for 48 h. Total RNA was extracted and analyzed by RT-qPCR. B, Serum-starved BEAS-2B cells were incubated with SB431542 (1 μM) before challenging with poly I:C (10 μg/mL) for 24 h. Total RNA was extracted and analyzed by RT-qPCR. Gene expression data from A and B are expressed as fold change from vehicle control and presented as mean ± SEM for A, n = 3 – 5, B, n= 6 - 7 and C, n = 3 - 4 independent experiments. C, Serum-starved BEAS-2B cells were incubated with SB431542 (1 μM) before challenging with poly I:C (10 μg/mL) for 24 h. Smad phosphorylation was determined in cell lysates by Western blotting

using phospho-specific antibodies. Densitometry data were normalized to β-actin expression and then presented as mean and SEM of 4 experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance and p-values were coded as follows: *p<0.05, **p<0.01, ***p<0.001.

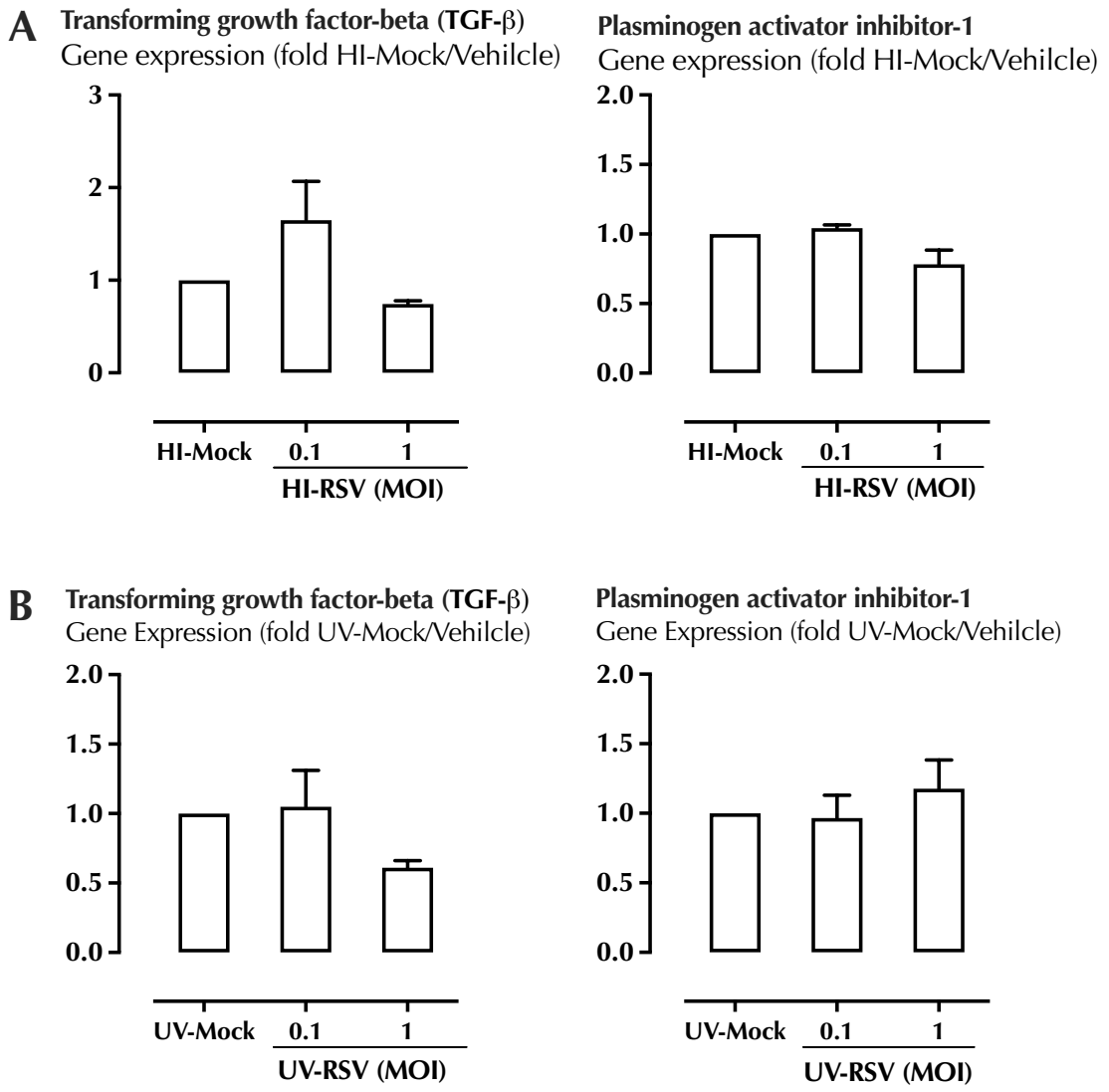


Figure 3.12| Induction of transforming growth factor-β (TGF-β)-responsive genes by RSV infection in bronchial epithelial cells is replication dependent.
Serum-starved BEAS-2B cells were incubated with either (A) HI-RSV (MOI 1), (B) UV-irradiated RSV (MOI 1) or the corresponding Mock-conditioned medium for 24 h with Dexamethasone (Dex) (30 nM) added for the last 4 h. RNA was extracted and analyzed by RT-qPCR. Gene expression was presented as fold change from control. Data are shown as mean and SEM for n = 3 – 4 independent experiments. Ordinary one-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance.

3.4.9 ALK5 Inhibition precludes RSV and poly I:C-induced impairment of glucocorticoid responsiveness in bronchial epithelial cells

Our laboratory has found that TGF-β attenuates glucocorticoid sensitivity in pulmonary epithelial A549 cells, bronchial epithelial BEAS-2B cells and primary ALI-HBECs. The observed attenuation of the transcriptional responses to glucocorticoid in these cell lines was inhibited with the SB431542 (Salem et al., 2012; Keenan et al., 2014).

Therefore, we examined the effects of ALK5 inhibition by SB431542 at a concentration (1 μM), to confirm its impact on glucocorticoid irresponsiveness induced by RSV infection and poly I:C exposure. We have found that inhibition of ALK5 by SB431542 (1 μM) prevent impairment of the GRE response to dexamethasone (30 nM) in RSV-infected and poly I:C-stimulated epithelial cells (Figure 3.13 A and Figure 3.14 A). Moreover, inhibition of ALK5 using SB431542 attenuated RSV infection and poly I:C-induced suppression of selected gene expression responses to dexamethasone (30 nM) exemplified by *ENaC-α* and *GILZ* expression (Figure 3.13B and Figure 3.14B). Collectively, these findings suggested that activation of ALK-5 contributes to the RSV infection and poly I:C-induced GC impairment. Our group provided additional evidence of a role for ALK5 activation in mediating RSV-induced GC resistance using a structurally distinct ALK5 inhibitor; GW788388 and ALK5 siRNA, where both methods had shown to ablate GC insensitivity in RSV-infected cells (Xia et al., 2017).

3.4.10 PF670462 abrogated RSV and poly I:C induced impairment of glucocorticoid actions

Our group have recently implicated casein kinase 1 δ/ε (CK1 δ/ε) in TGF-β 1 signals subserving glucocorticoid insensitivity (Li et al., 2019). Hence, we have harnessed PF670462 in the current study as a potent and selective dual CK1ε/δ inhibitor to explore the impact of the CK1 δ/ε inhibition on RSV-mediated attenuation of GC epithelial cell responses. To that end, BEAS-2B cells were treated with PF670462 (10 μM) (Abcam, Australia) for 30 min before RSV infection (MOI 0.1) or exposure to poly I:C (10 μg/mL). Data indicated that PF670462 fully prevented the deleterious effect of both RSV infection and poly I:C stimulation on dexamethasone-induced GRE activity (Figure 3.15 A and Figure 3.16 A). Moreover, it significantly precluded RSV- and poly I:C-mediated suppression of selected gene expression responses to Dex (30 nM), exemplified by *ENaC-α* and *GILZ* expression (Figure 3.15 B and Figure 3.16 B). Besides, PF670462 abrogated *TGF-β1* and *PAI-1* expression induced by RSV infection (Figure 3.17).

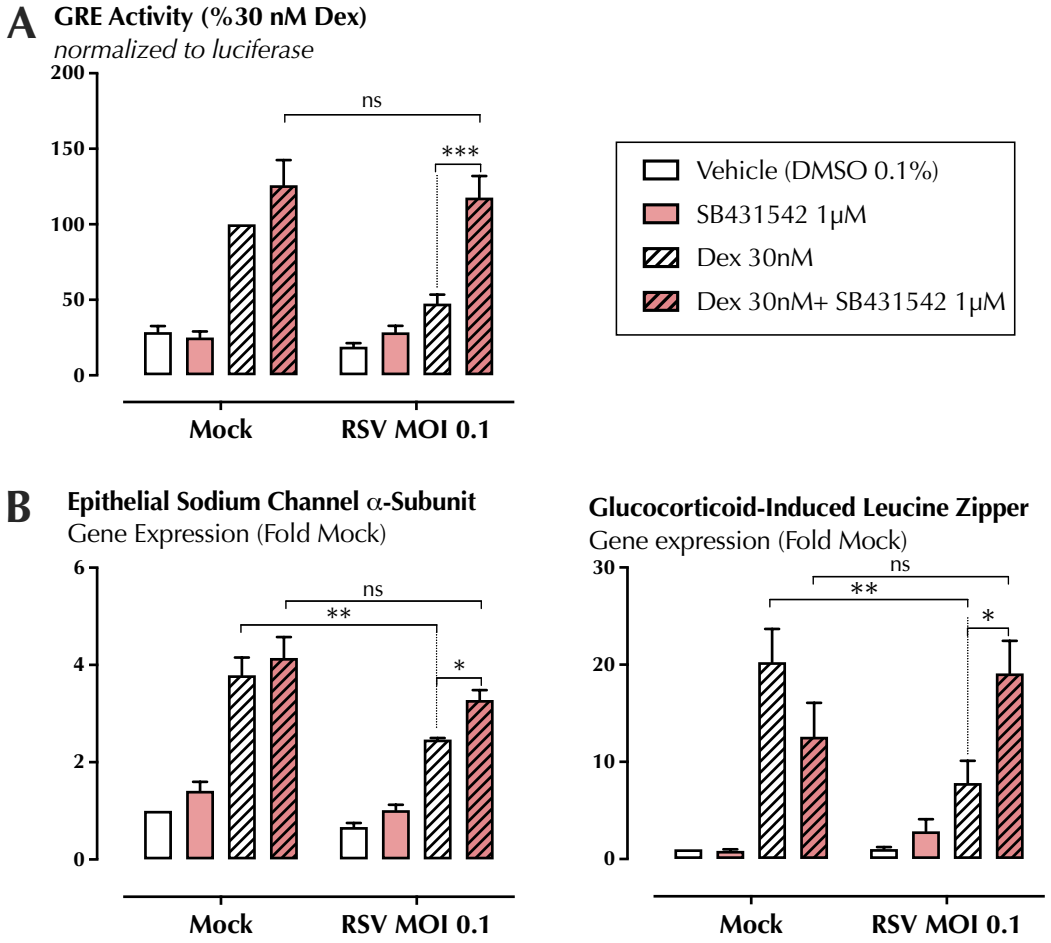


Figure 3.13| ALK5 inhibition prevents RSV-induced glucocorticoid irresponsiveness in bronchial epithelial cells.

A, BEAS-2B cells were transiently transfected with a GRE-SEAP reporter construct, incubated with SB431542 (1 µM) for 30 min before infection with RSV at MOI 0.1 for 24 h with Dexamethasone (Dex) (30 nM) added for further 24 h. GRE activity was measured by the level of SEAP in the supernatants. Data were presented as a percentage the SEAP level induced in response to 30 nM dexamethasone and shown as mean ± SEM for n = 3 independent experiments. B, BEAS-2B cells were incubated with SB431542 (1 µM) for 30 min before RSV infection with at MOI 0.1 for 48 h then were treated with Dexamethasone (30 nM) for the last 4 h. RNA was extracted and analyzed by RT-qPCR. Data are expressed as fold change from control and presented as mean ± SEM for n = 3 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance and p-values were coded as follows: *p<0.05, **p<0.01, ***p<0.001.

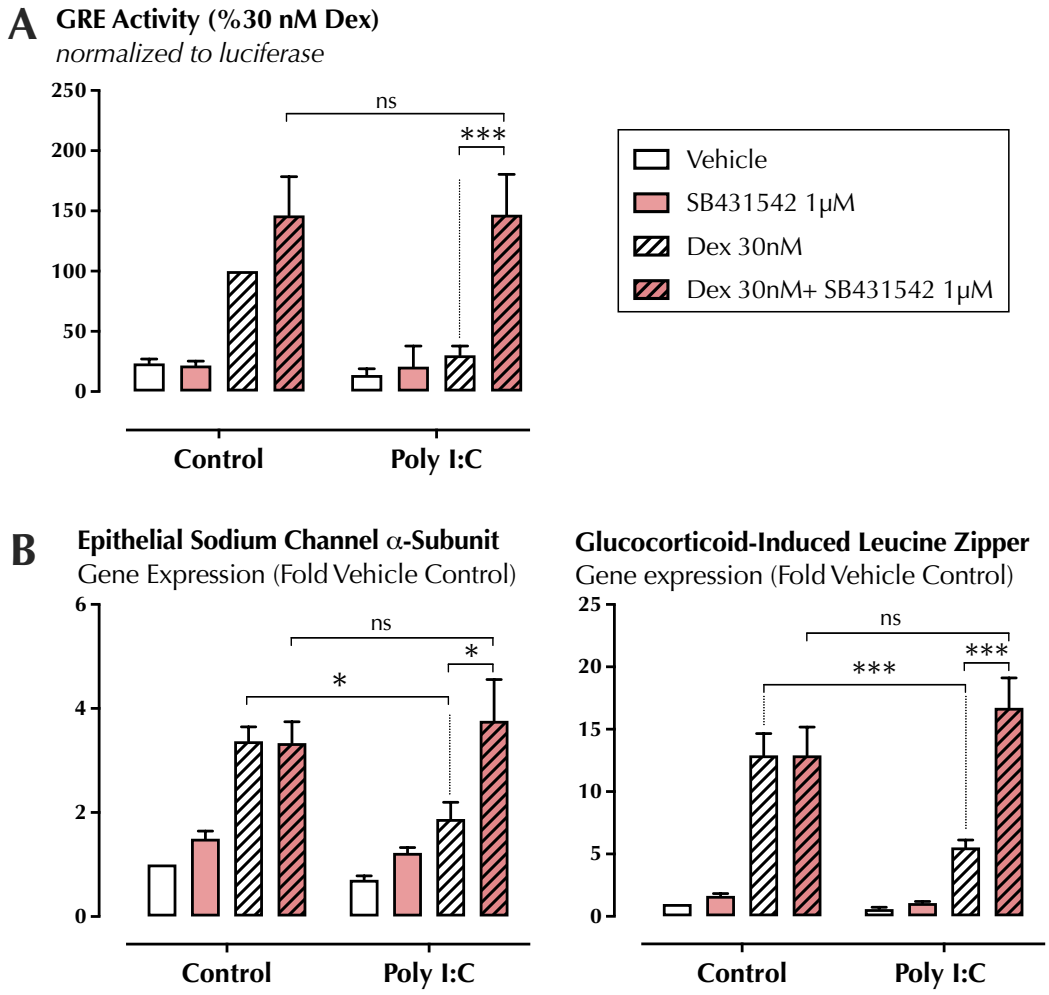


Figure 3.14| ALK5 inhibition attenuates glucocorticoid insensitivity induced by TLR3 activation in bronchial epithelial cells.

A, BEAS-2B cells were transiently transfected with a GRE-SEAP reporter construct, incubated with SB431542 (1 µM) for 30 min before challenging with poly I:C (10 µg/mL) for 24 h with Dexamethasone (Dex) (30 nM) added for further 24 h. GRE activity was measured by the level of SEAP in the supernatants. Data were expressed as a percentage the SEAP level in response to 30nM dexamethasone and presented as mean ± SEM for at least 3 independent experiments. B, BEAS-2B cells were incubated with SB431542 (1 µM) for 30 min before stimulation with poly I:C (10 µg/ml) for 24 h then were treated with Dexamethasone (30 nM) for the last 4 h. RNA was extracted and analyzed by RT-qPCR. Data are presented as fold change from control and shown as mean ± SEM for A, n = 3 - 6 independent experiments and B, n = 4 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance and p-values were coded as follows: *p<0.05, ***p<0.001.

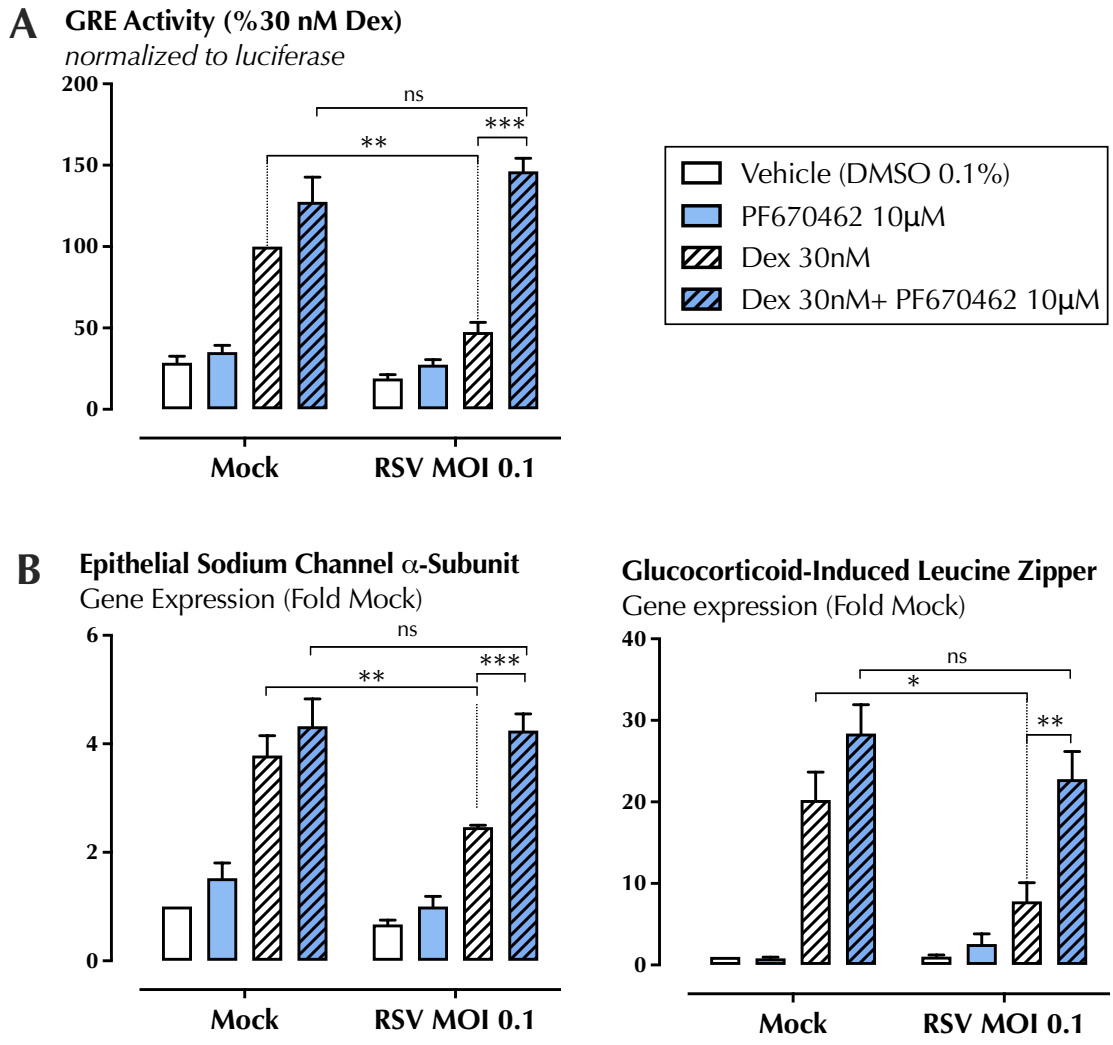


Figure 3.15| The dual CK1δ/ε inhibitor PF670462 attenuate RSV-mediated glucocorticoid insensitivity in bronchial epithelial cells.

A, BEAS-2B cells were transiently transfected with a GRE-SEAP reporter construct, incubated with PF670462 (10 µM) for 30 min before infection with RSV at MOI 0.1 for 24 h with Dexamethasone (Dex) (30 nM) added for further 24 h. GRE activity was measured by the level of SEAP in the supernatants. Data were presented as a percentage the SEAP level induced in response to 30nM dexamethasone and shown as mean ± SEM for n = 3 independent experiments. B, BEAS-2B cells were incubated with PF670462 (10 µM) for 30 min before RSV infection with at MOI 0.1 for 48 h then were treated with Dexamethasone (30 nM) for the last 4 h. RNA was extracted and analyzed by RT-qPCR. Data were presented as fold change from control and shown as mean ± SEM for n = 3 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance and p-values were coded as follows: *p<0.05, **p<0.01, ***p<0.001.

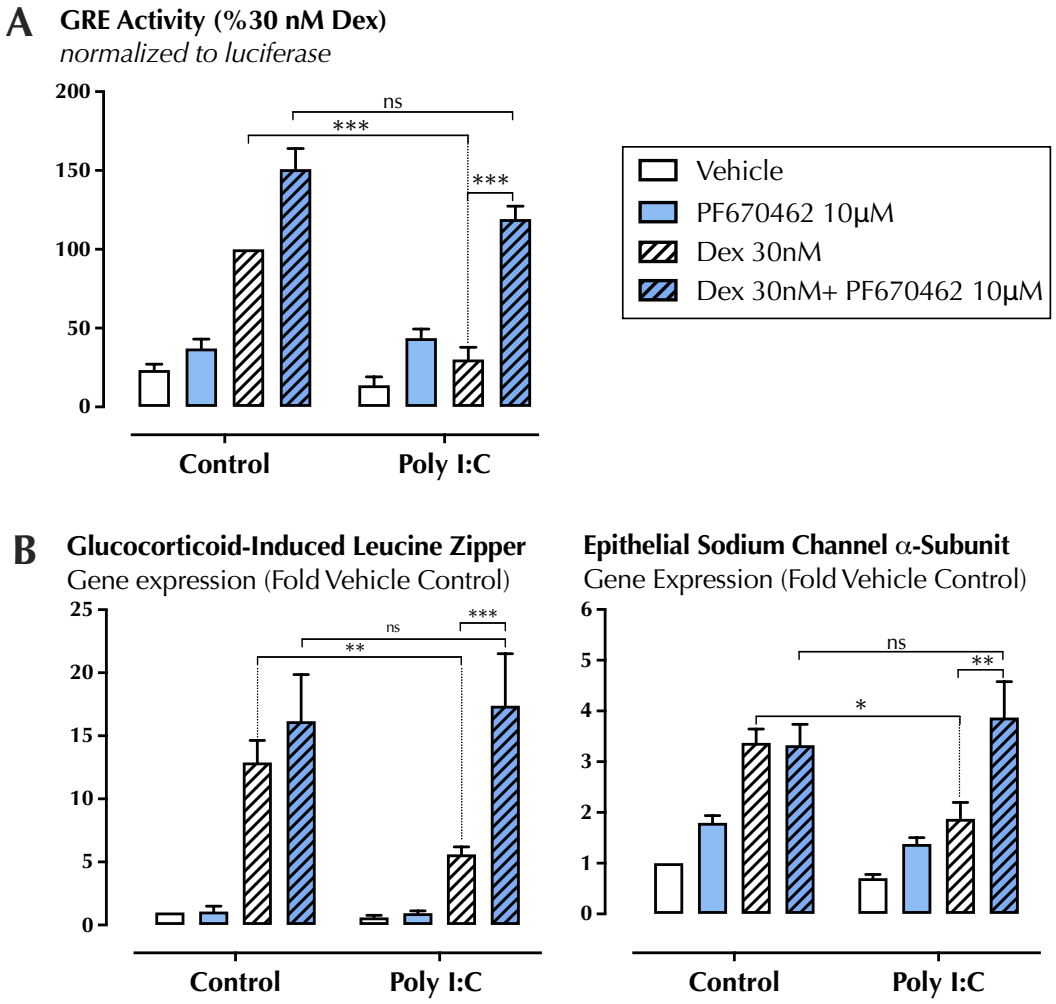


Figure 3.16| The dual CK1δ/ε inhibitor PF670462 attenuate glucocorticoid insensitivity induced by TLR3 activation in bronchial epithelial cells.

A, BEAS-2B cells were transiently transfected with a GRE-SEAP reporter construct, incubated with PF670462 (10 µM) for 30 min before challenging with poly I:C (10 µg/mL) for 24 h with Dexamethasone (Dex) (30 nM) added for further 24 h. GRE activity was measured by the level of SEAP in the supernatants. Data were presented as a percentage the SEAP level in response to 30nM dexamethasone and shown as mean ± SEM for n = 3 - 6 independent experiments. B, BEAS-2B cells were incubated with PF670462 (10 µM) for 30 min before stimulation with poly I:C (10 µg/ml) for 24 h with Dexamethasone (Dex) (30 nM) added for the last 4 h. RNA was extracted and analyzed by RT-qPCR. Data are expressed as fold change from control and presented as mean ± SEM for A, n = 3- 6 and B, n= 3-4 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance and p-values were coded as follows: *p<0.05, **p<0.01, ***p<0.001.

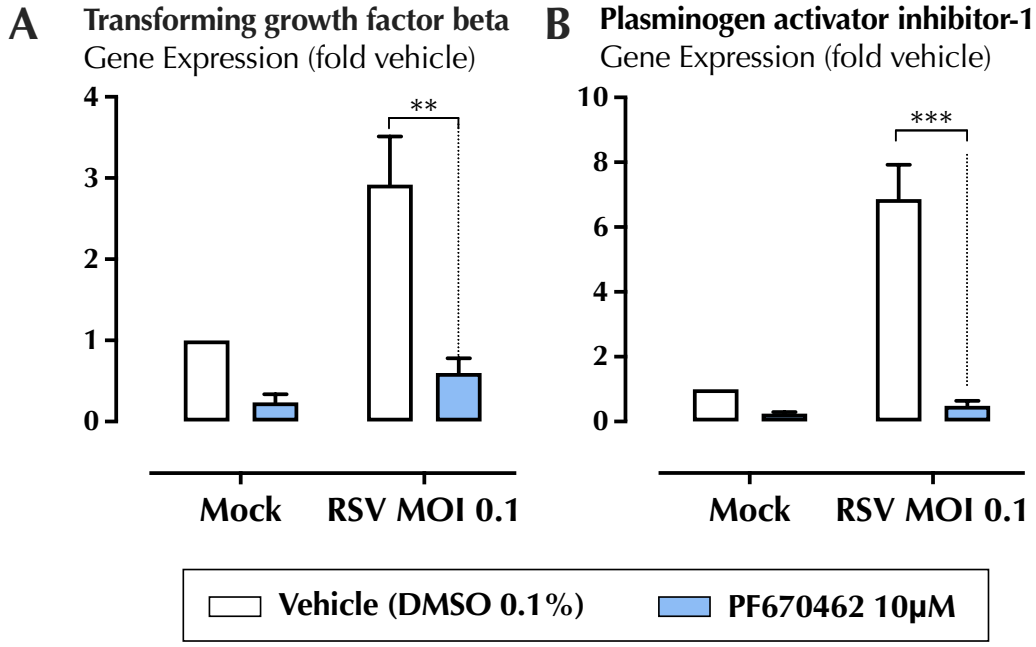
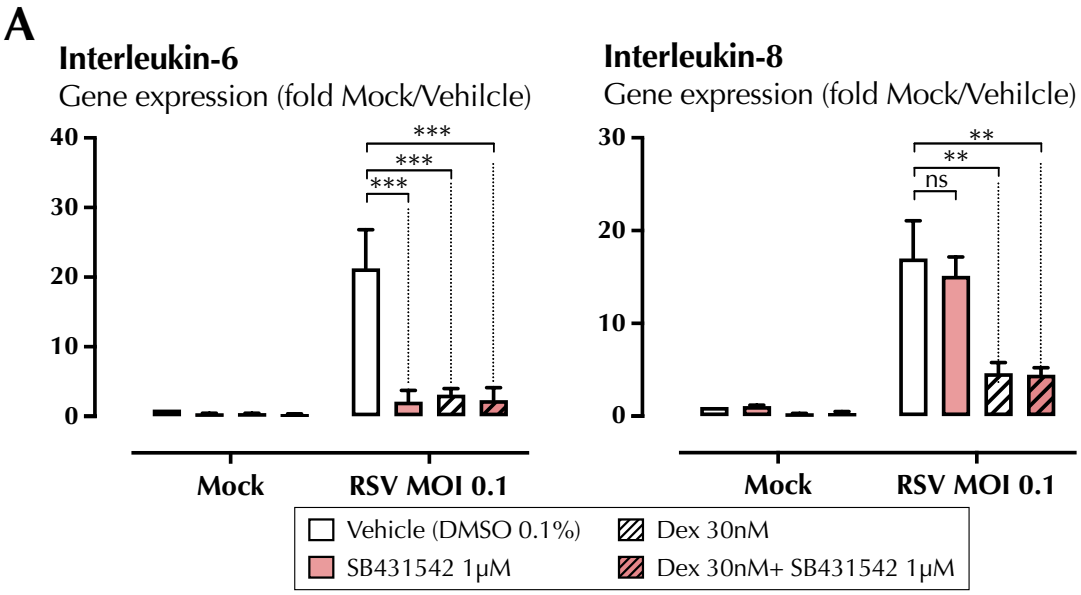


Figure 3.17| The dual CK1δ/ε inhibitor PF670462 prevents TGF-β activity provoked by RSV infection in bronchial epithelial cells.
BEAS-2B cells were incubated with PF670462 (10 µM) for 30 min before (A) RSV inoculation at MOI of 0.1 for 48 h with Dexamethasone (Dex) (30 nM) added for the last 4 h. RNA was extracted and analyzed by RT-qPCR. Data are presented as fold change from control and shown as mean ± SEM for A, n = 3 and B, n = 3 - 5 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance and p-values were coded as follows: *p<0.05, **p<0.01, ***p<0.001.

3.4.11 Inhibition of CK1δ/ε or ALK5 subdues pro-inflammatory cytokine expression in RSV-infected BEAS-2B cells

The expression of genes encoding IL-6 and IL-8 are known to be regulated by glucocorticoid transrepression mechanism (King et al., 2013). Therefore, we investigated whether RSV infection impairs the regulation of IL-6 and IL-8 expression in BEAS-2B cell. As expected, RSV infection prompted the expression of *IL-8* and *IL-6* mRNA in BEAS-2B cells. Dexamethasone (30 nM) subdued the RSV infection-induced *IL-6* and *IL-8* expression. Next, we explored whether the inflammatory response triggered by RSV infection can be inhibited by targeting ALK5 or CK1δ/ε. Interestingly, pre-treatment of cells with either SB431542 (1 µM) or PF670462 (10 µM) prior to RSV inoculation had no effect on *IL-8* mRNA expression, yet significantly repressed RSV-induced *IL-6* expression (Figure 3.18 A and Figure 3.18 B).



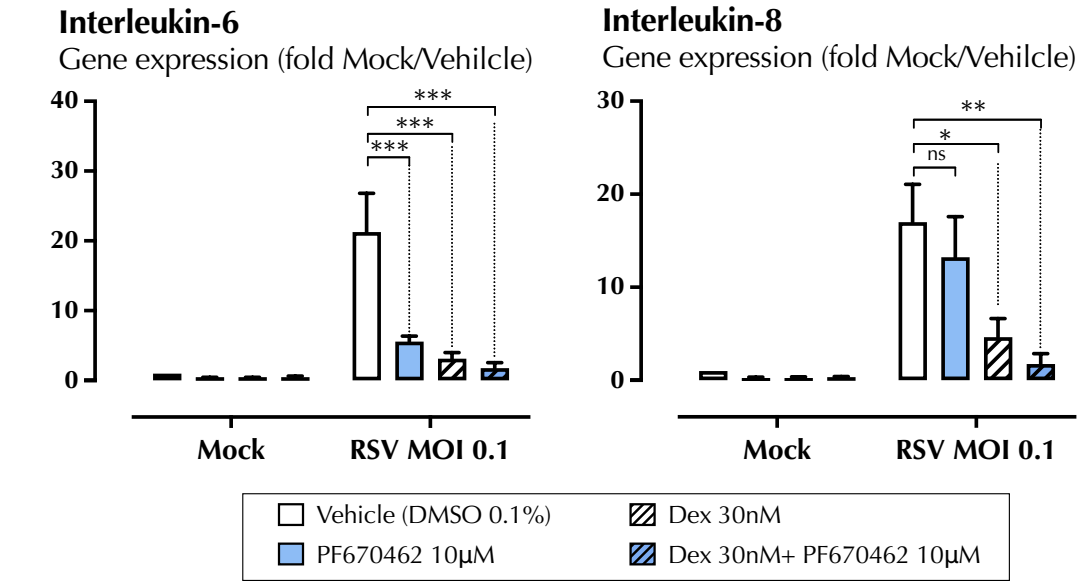


Figure 3.18| Effect of TGF-β modulators on dexamethasone regulation of RSV-induced pro-inflammatory cytokine expression in BEAS-2B cells.
BEAS-2B cells were incubated with (A) SB431542 (1 µM) or (B) PF670462 (10 µM) for 30 min before RSV inoculation at MOI of 0.1 for 48 h with Dexamethasone (Dex) (30 nM) added for the last 4 h. RNA was extracted and analyzed by RT-qPCR. Data were presented as fold change from Mock control and shown as mean ± SEM for n = 3 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance and p-values were coded as follows: *p<0.05, **p<0.01, ***p<0.001.

3.4.12 RSV infection elicits TGF-β expression and activity and mitigates glucocorticoid sensitivity via ERK1/2-dependent pathway

RSV infection of epithelial cells triggers a plethora of intracellular signalling cascades. A number of the underlying kinases are implicated in non-canonical TGF-β signalling pathways, including ERK1/2, $p38^{MAPK}$, Akt, JNK and NFκB. Therefore, the involvement of these pathways was investigated. Interestingly, while RSV infection had no significant effect on phosphorylation of $p38^{MAPK}$, Akt, JNK and NFκB (results not mentioned here), RSV infection triggered ERK1/2 phosphorylation in BEAS-2B cells, as demonstrated by Western blotting using corresponding antibodies and phospho-antibodies (Figure 3.19 A). The phosphorylation of the ERK1/2 was induced at 3h post RSV inoculation and become significant 24 h post-infection. At 48 h following exposure to RSV, ERK-1 and ERK-2 phosphorylation was not detected. Intriguingly, incubation of the BEAS-2B cells with SB431542 (1 μM) 30 min prior to RSV infection had no effect on the phosphorylation of ERK1/2 , albeit the data were too variable to provide a confident interpretation (1951.6 ± 910.4, 1348.7 ± 543.4, p=>0.9999). This suggests possible involvement in RSV-induced TGF-β expression and activity and subsequently GC insensitivity. We, therefore, explored this possibility using mitogen-activated protein kinase kinase-1/2 (MEK1/2) inhibitor (U0126, Tocris, Bristol, UK) at 1 μM, the concentration that was proved effective based on our previous experience (Kassel et al., 2008; Le Quément et al., 2008; Salem et al., 2012; Keenan et al., 2014). Pre-incubation of the cells with U0126 (1μM) 30 min prior to RSV exposure significantly reduced RSV-mediated expression of *TGF-β* and *PAI-1* mRNA (Figure 3.19 C) and abolished the RSV-induced impairment of budesonide-induced transactivation of *GILZ* (Figure 3.19 B).

Analogous observations were reported in BEAS-2B cells exposed to the TLR3 ligand; poly I:C stimulation (10 μg/ml). Poly I:C has also triggered EKR1/2 phosphorylation, however, at an earlier time point (6 h) and continued until 24 h post poly I:C stimulation (Figure 3.20 A). Pre-incubation with SB431542 (1 μM) 30 min prior to poly I:C challenging significantly attenuated the phosphorylation of ERK1/2. Furthermore, ERK1/2 inhibition with U0126 (1μM), added to the cells prior to poly I:C stimulation (10 μg/ml), had repressed the expression of *TGF-β* and *PAI-1* mRNA (Figure 3.20 C) and attenuated impairment of GR-mediated transactivation of *GILZ* mediated by TLR3 challenging (Figure 3.20 B).

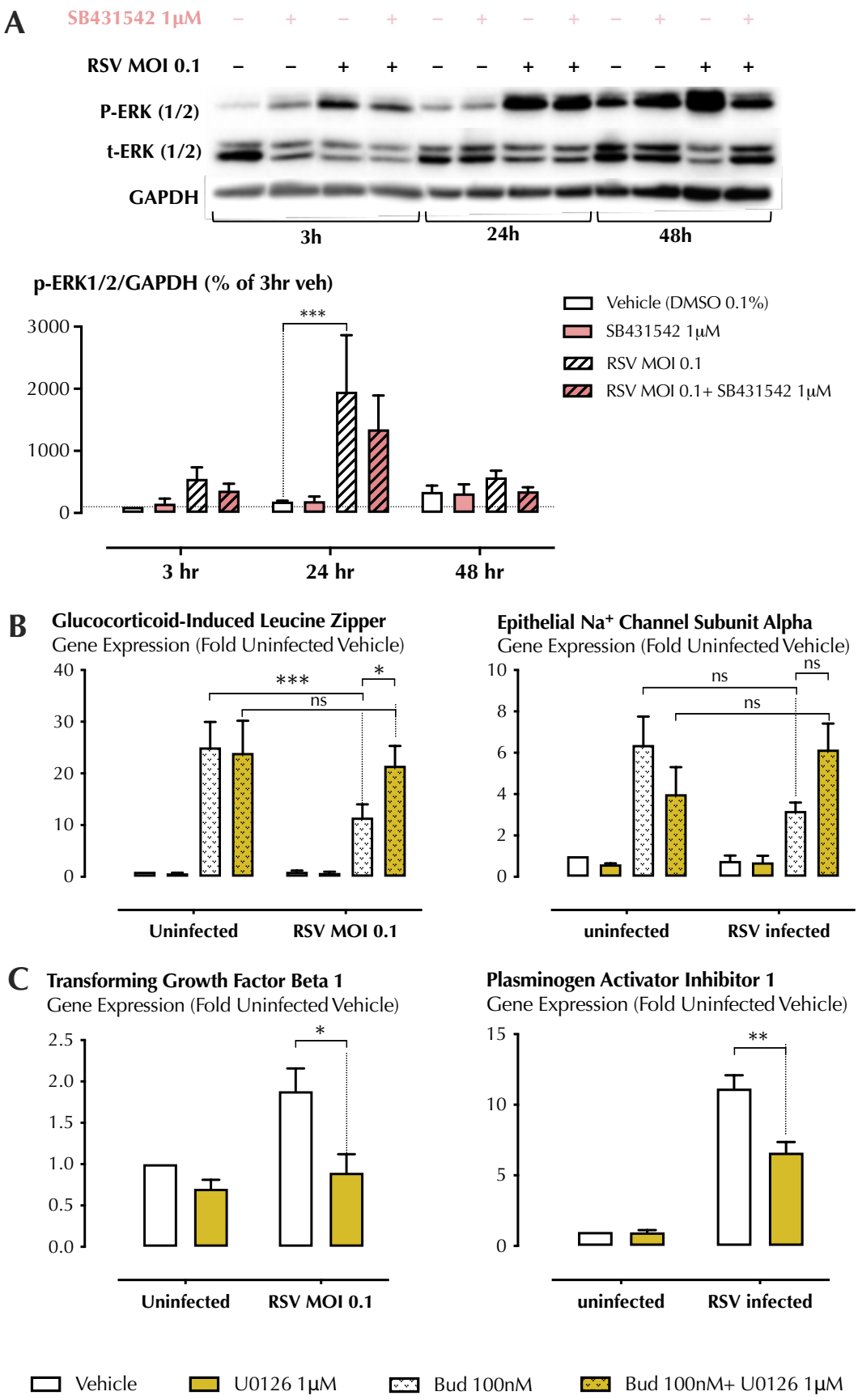

A
SB431542 1µM
RSV MOI 0.1
P-ERK (1/2)
t-ERK (1/2)
GAPDH
3h
24h
48h
p-ERK1/2/GAPDH (% of 3hr veh)
Vehicle (DMSO 0.1%)
SB431542 1µM
RSV MOI 0.1
RSV MOI 0.1+ SB431542 1µM
3 hr
24 hr
48 hr
B
Glucocorticoid-Induced Leucine Zipper
Gene Expression (Fold Uninfected Vehicle)
Uninfected
RSV MOI 0.1
Epithelial Na+ Channel Subunit Alpha
Gene Expression (Fold Uninfected Vehicle)
uninfected
RSV infected
C
Transforming Growth Factor Beta 1
Gene Expression (Fold Uninfected Vehicle)
Plasminogen Activator Inhibitor 1
Gene Expression (Fold Uninfected Vehicle)
Vehicle
U0126 1µM
Bud 100nM
Bud 100nM+ U0126 1µM

Figure 3.19| RSV infection induced TGF-β activity and subsequent impairment of glucocorticoid transactivation are associated with ERK1/2 phosphorylation.
A, BEAS-2B cells were incubated with SB431542 (1 μM) before RSV inoculation at MOI of 0.1 for 3 hr, 24 hr and 48 hr. ERK phosphorylation was determined in cell lysates by Western blotting using phospho-specific antibodies. Densitometry data were normalized to GAPDH expression and then presented as mean and SEM of 3 experiments. B and C, BEAS-2B cells were incubated with U0126 (1 μM) for 30 min before RSV inoculation at MOI of 0.1 for 48 h with Budesonide (Bud) (100 nM) added for the last 4 h. RNA was extracted and analyzed by RT-qPCR. Data are expressed as fold change from uninfected control and presented as mean ± SEM for n = 3 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance and p-values were coded as follows: *p<0.05, **p<0.01, ***p<0.001.

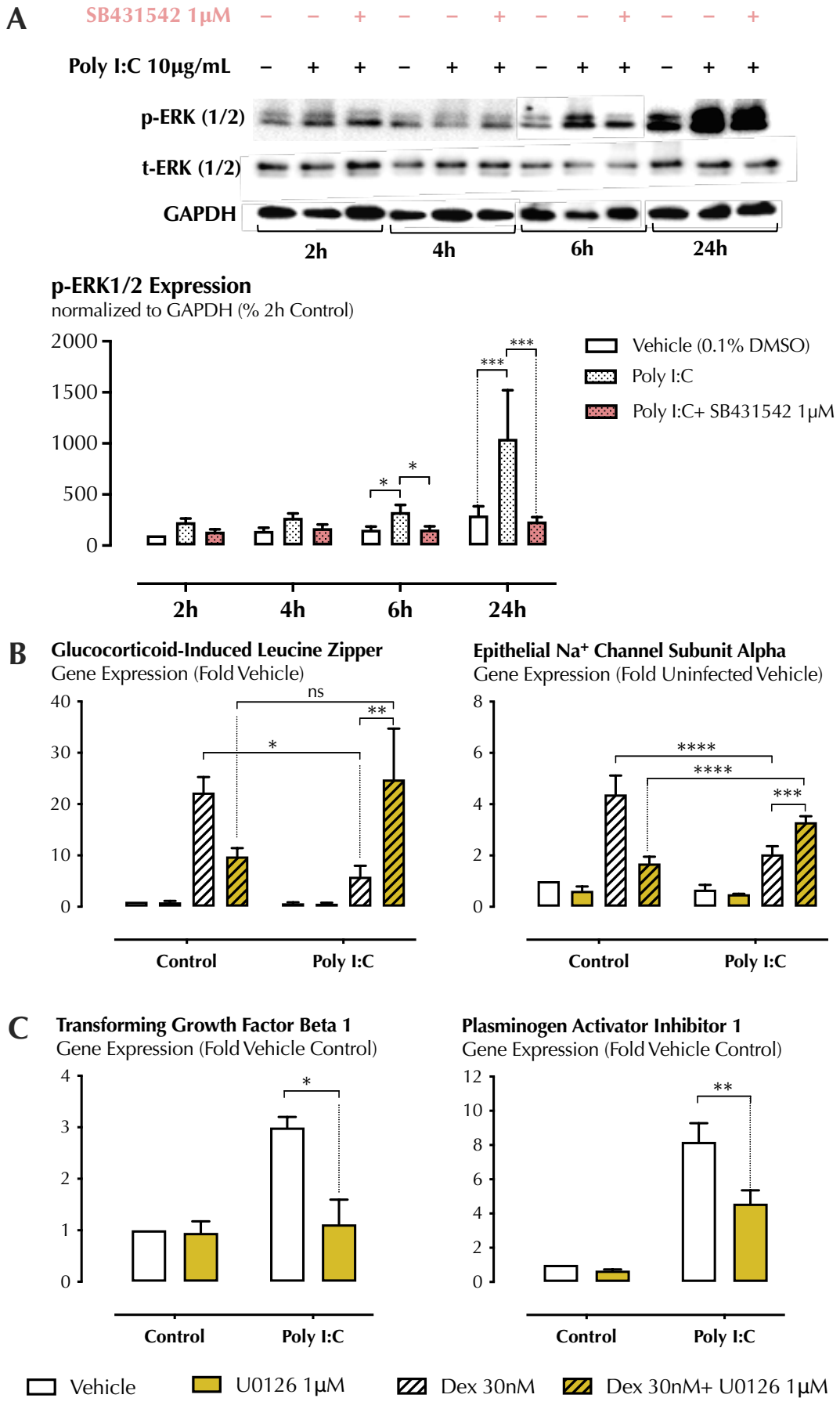

A
SB431542 1µM
Poly I:C 10µg/mL
p-ERK (1/2)
t-ERK (1/2)
GAPDH
2h
4h
6h
24h
p-ERK1/2 Expression
normalized to GAPDH (% 2h Control)
Vehicle (0.1% DMSO)
Poly I:C
Poly I:C+ SB431542 1µM
B
Glucocorticoid-Induced Leucine Zipper
Gene Expression (Fold Vehicle)
Epithelial Na+ Channel Subunit Alpha
Gene Expression (Fold Uninfected Vehicle)
Control
Poly I:C
C
Transforming Growth Factor Beta 1
Gene Expression (Fold Vehicle Control)
Plasminogen Activator Inhibitor 1
Gene Expression (Fold Vehicle Control)
Vehicle
U0126 1µM
Dex 30nM
Dex 30nM+ U0126 1µM

Figure 3.20| TLR3 activation induced TGF-β activity and subsequent impairment of glucocorticoid transactivation are associated with ERK1/2 phosphorylation.
A, BEAS-2B cells were incubated with SB431542 (1μM) before treatment with Poly I:C (10 μg/ml) for 2 h, 6 h and 24 hr. ERK phosphorylation was determined in cell lysates by Western blotting using phospho-specific antibodies. Densitometry data were normalized to GAPDH expression and then presented as mean and SEM of A, n = 8 – 11 independent experiments. B and C, BEAS-2B cells were incubated with U0126 (1 μM) for 30 min before treatment with Poly I:C (10 μg/ml) with Dexamethasone (Dex) (30 nM) added for the last 4 h. RNA was extracted and analyzed by RT-qPCR. Data are presented as fold change from vehicle control and shown as mean ± SEM for B, n = 3 - 5 independent experiments and C, n = 4 - 5 independent experiments. Ordinary two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was used to determine statistical significance and p-values were coded as follows: *p<0.05, **p<0.01, ***p<0.001.

3.5 Discussion

RSV infection has a deleterious impact on GC signalling via undefined mechanisms. The airway epithelium is the prime target for RSV infection and has been involved in the frontline defence against respiratory viruses (Vareille et al., 2011). Hence, these cells are likely to play a crucial role in RSV infection pathogenesis. In this chapter, we have attempted to decipher the mechanism of RSV-induced glucocorticoid insensitivity in airway epithelium. It is anticipated that this knowledge will ultimately define novel targets to circumvent steroid insensitivity.

The immortalised bronchial epithelial cell line BEAS-2B is the most commonly employed for the *in vitro* study of RSV infection. Hence, we have selected BEAS-2B cell line to conduct the current study as a surrogate model for hAECs. BEAS-2B cell line was generated from human bronchial epithelial cells by introducing viral H-ras (Reddel et al., 1988; Kaighn et al., 1989). BEAS-2B cells have been proved to share some characteristics of primary bronchial epithelial cells. For instance, BEAS-2B cells maintain the typical epithelial morphology and the same epithelial functional characteristics, however, they lack the individual variability observed in hAECs (Stewart et al., 2012). They also exhibit almost identical intracellular signalling pathways to that present in primary bronchial epithelial cells (Sauty et al., 1999; Schwarze et al., 2004) and show a similar responsiveness spectrum to numerous stimuli and pathogens (Thomas et al., 1998; Pichavant et al., 2003; Ieki et al., 2004; Schwarze et al., 2004). Moreover, BEAS-2B cells express all types of TLRs with the same spatial localization in the differentiated human AECs (Ioannidis et al., 2013), hence these cells present a satisfactory platform to test different TLRs ligands. BEAS-2B cells also mirror some hallmarks of RSV infection reported *in vivo*. For instance, in histopathological studies, the fatal cases airways have shown restriction of RSV infection

to small foci (Aherne et al., 1970; Johnson et al., 2007) and the same pattern is observed in RSV-infected BEAS-2B cells (Hillyer et al., 2018). Additionally, RSV infection of BEAS-2B cells triggers robust pro-inflammatory responses, characterized by cytokines and chemokines release and generates antiviral immune responses with the expression of interferon-stimulated genes (Aherne et al., 1970; Johnson et al., 2007; Hillyer et al., 2018). Most importantly, BEAS-2B cells produce diverse inflammatory mediators implicated in the asthma pathogenesis, e.g., TGF-α and -β, GM-CSF, IL-6, IL-8 and TNF-α. However, BEAS-2B cells are derived from healthy donor, so extrapolating findings to asthma is problematic due to intrinsic differences between non-asthmatic and asthmatic airway epithelial cells, i.e., the baseline expression levels of PRRs. A study has examined baseline expression of PRRs; TLR3, MDA5 and RIG-I in cultured pBEC from asthmatic and healthy subjects and found similar mRNA expression levels in the two subject groups. Similar pattern was observed in an *in vivo* setting by measuring the TLR3, RIG-I and MDA5 mRNA levels from endobronchial biopsies obtained from these subjects (Parsons et al., 2014). However, cultured pBECs from asthmatics released significantly reduced levels of types I and III IFNs when exposed to RV infection indicating the defective signalling through TLR3 and MDA5 in asthmatic pBEC, despite the normal expression and induction of these PRR (Wark et al., 2005; Contoli et al., 2006; Parsons et al., 2014). Another study has investigated the expression of TLR2, TLR3 & TLR4 in large and small airways of fatal asthmatics, mild asthmatic and deceased control subjects. The immunohistochemistry has shown higher TLR2 expression in the epithelial and outer layers of large and small airways of fatal asthmatic patients compared with deceased controls. Fatal asthmatic patients had greater TLR3 expression in the outer layer of large airways and no significant differences in the small airways. A greater TLR4 expression were detected in the outer layer of small airways of fatal asthmatics when compared to deceased controls. No bacterial DNA was detected in both groups. QRT-PCR had detected only a small difference in TLR3 expression between mild asthma patients and healthy controls (Ferreira et al., 2012a; Ferreira et al., 2012b). Upregulation of epithelial gene expression for TLR2 and TLR4, but not TLR5, was observed by examining the epithelial brushings obtained from the large airways at bronchoscopy from asthmatic and healthy subjects (Cottey et al., 2011). On the contrary, targeted gene expression arrays of asthmatic and non-asthmatic airway wall samples have examined the expression patterns of candidate genes hypothesized to be involved in the asthma pathophysiology. Of all TLRs tested, only TLR7 was down-regulated (Pascoe et al., 2017).

Given that the anti-inflammatory effects of glucocorticoids are mediated, at least in part, by the transactivation of anti-inflammatory genes, we examined the impact of human RSV and different PRRs ligands on both GRE-mediated promoter activity and GC-inducible gene expression. Noteworthy, we have chosen 48 h of RSV infection as a time point to conduct the current study since, first, the longer incubation periods induce apparent cytopathic effects and impact the cell viability, second, previous data suggested that TGF-β effect can be detected within 8 h post-infection (Gibbs et al., 2009; Mgbemena et al., 2011; Bakre et al., 2015). Therefore, we have expected to see the secreted TGF-β-induced effect by 48 h after viral infection.

RSV infection has been proved to have a detrimental effect on anti-inflammatory GR signalling (Hinzey et al., 2011; Marketon and Corry, 2013; Xia et al., 2017). In line with previous reports, RSV inoculation in BEAS-2B cell line had attenuated dexamethasone-induced GRE activity and impaired the expression of GC regulated genes, including *ENaCα*, *GILZ*, *SERPINA3*, *CDKN1C*, *PDK4*, *KCNB1* and *ZBTB16*, however, with varying degrees of inhibition. *GILZ* is a GC-responsive gene that mediates anti-inflammatory actions of glucocorticoids by inhibiting the activations of the pro-inflammatory transcription factor NFκB in macrophages, T cells and epithelial cells as well (Eddleston et al., 2007). Attenuation of *GILZ* expression by viral infection dampens the anti-inflammatory potential of glucocorticoids. In lung epithelial cells, ENaC channels regulate the level of airway surface fluids. Viral infection impairs *ENaCα* expression resulting in fluid accumulation on the airway surface and recurrent infections in the lung (Itani et al., 2002). The observed attenuation of GC action in the current study may explain clinical ineffectiveness of GC treatment in patients with RSV infection. The GC-inducible genes are not only regulated by GC, but also subject to complex regulation by other factors due to promoter complexity. It has been reported that *CDKN1C* can be down-regulated through a combination of epigenetic modifications such as promoter hypermethylation, histone deacetylation, repression of tumour suppressor genes by CCCTC-binding factor (CTCF) and noncoding RNA transcripts. We have previously shown that TGF-β-impaired GC-induced *CDKN1C* expression was not a result of epigenetic repression of gene transcription. However, there might be other factors released from the viral infected-cells that caused repression of the gene transcription through epigenetic modification. It is likely to explain why this gene was more profoundly suppressed by RSV infection.

Although RSV infection impaired the transactivation of most of the GC-regulated genes measured, we found that glucocorticoids might be beneficial in regulating RSV-induced cytokine expression, where dexamethasone markedly repressed the expression of RSV-induced mRNA encoding the pro-inflammatory cytokines; IL-6 and IL-8. The observed downregulation of pro-inflammatory cytokines (IL-6 and IL-8) would need to be validated at protein level.

To test whether the observed deleterious effect of RSV infection on GC responsiveness is replication-dependent, RSV was inactivated either by heat or UV irradiation. Challenging BEAS-2B cells with HI-RSV (MOI 0.1 and 1) and UV-irradiated RSV (MOI 0.1 and 1) have up-regulated *IL-6* and *IL-8* mRNA expression, however to a limited extent compared to the alive RSV. This shows that inactivated virion particles retain their ability to interact with surface receptors that elicit signalling cascades with the subsequent release of inflammatory mediators.

Indeed, literature provides several pieces of evidence for the interactions of RSV proteins with PRRs on the surface of airway epithelial cells. Several studies have reported the secretion of CCL5, CXCL8, CXCL10 and IL-6 in response to the UV-irradiated virus in addition to purified RSV F and G proteins (Arnold et al., 1994; Fiedler et al., 1995; Mellow et al., 2004; Oshansky et al., 2010). The IL-6 secretion induced by the recombinant RSV F-protein has been attributed to the interaction with TLR4 (Kurt-Jones et al., 2000b; Kurt-Jones et al., 2000a).

We have found that HI-RSV, but not UV-RSV, has mitigated Dexamethasone-induced GRE activity. However, neither HI-RSV nor UV-RSV had detectable effects on dexamethasone-induced transactivation of endogenous GR regulated genes; *GILZ*, *CDKN1C* or *ZBTB16*. The controversy between the suppression of GRE activity and the endogenous GR-regulated gene expression in case of HI-RSV is expected. Similar to any synthetic reporter constructs, GRE reporter constructs do behave differently to the endogenous promoters. The construct employed in the current study contains multiple tandem GRE sequences. Additionally, the endogenous GC inducible genes are subjected to the regulation complexity related to chromatin structure and epigenetic regulation, the expression regulation by cofactors and sensitivity to post-translational modifications of GR α whilst the GRE reporter constructs are not (Clark and Belvisi, 2012). In line with our observation, Hinzy and his colleagues had reported that UV irradiation prevented RSV mediated suppression of glucocorticoid responses in BEAS-2B cells (Hinzey et al., 2011). These findings suggest that RSV-induced GC insensitivity is replication dependent.

RSV infection induced a plethora of immune responses in airway epithelial cells, most importantly innate immune responses through recognition of viral PAMPs by epithelial cell PRRs, particularly TLR2, 3, 4 and 7. This has led to hypothesis that PAMPs can recapitulate RSV-induced compromised epithelial responses to glucocorticoids. To test this hypothesis, we have explored the impacts of different PRRs ligands on GC signalling.

TLR2 and TLR7 are among PRRs that play a key role in RSV-antiviral immunity (Murawski et al., 2009). These PRRs can recognize RSV capsid proteins and ssRNA respectively. Regrading RSV-induced GC resistance, we have found that neither activation of TLR2 with Zymosan A or LTA nor TLR7 with Imiquimod had attenuated GRE activity or GC-regulated genes transactivation suggesting that both receptors do not contribute to RSV-induced GC insensitivity. This comes in line with the aforementioned results that HI-RSV; a surrogate to ssRNA, and UV-irradiated RSV; a surrogate to viral proteins, have no role in the RSV-induced GC insensitivity in the airway epithelium.

TLR3 is an endosomal PRR that recognizes short intramolecular dsRNA segments that are formed during RSV replication (Martinez and Melero, 2002). Martinez and Melero (2002) showed that short intramolecular dsRNA segments were formed during RSV replication. dsRNA is then released into the extracellular space following RSV-mediated cell destruction and necrosis. Minute amounts of dsRNA, as little as one molecule per cell, can profoundly impact cellular biology (Jacobs and Langland, 1996). The microenvironment around the airway epithelial cells would have these very small quantities of dsRNA that can conjugate with the TLR3 to elicit antiviral and inflammatory responses. Poly I:C; a mimic to viral dsRNA, have been used in the current study as a TLR3 ligand. We have found that poly I:C markedly impaired dexamethasone GRE activity, with a complete inhibition achieved at 10 µg/mL. Moreover, transactivation of an array of endogenous GC regulated genes, including *GILZ*, *ENaCα*, *CDKN1C*, *PDK4*, *FKBP5*, *KCNB1* and *ZBTB16* were attenuated in BEAS-2B cells. Marketon and Corry had reported similar observations, where dexamethasone transactivation of *GILZ* and *FKBP5* in BEAS-2B and hAECs had been repressed following exposure to poly I:C (Webster Marketon and Corry, 2013a).

Worth mentioning, poly I:C had been added in our experiment without transfection. On the contrary to the exclusive endosomal localization of TLR3 in APCs, TLR3 was also detected on the cell surface of BEAS-2B cell line, as well as the apical and basolateral surfaces of differentiated AECs (Blasius and Beutler, 2010; Ioannidis et al., 2013). Other studies validated the localization of the TLR3 receptor on the epithelial cell

surface by flow cytometry. Hewson et al. (2005) demonstrated that TLR3 receptor was expressed on the cell surface of BEAS-2B cells exposed to RV. Ueta et al. (2005) showed that TLR3 receptor was expressed on the human corneal epithelial cells surface. Moreover, several studies have proved the effective internalization of poly I:C into endosomal TLR3 in the human corneal epithelial cells (hCECs) (Kumar et al., 2006). This also highlights the expanded ability of endosomal TLR3 to recognize viral dsRNA released from neighbouring infected dying cells in addition to the endogenously generated dsRNA (Kumar et al., 2006). These facts rationalize for the exogenous application of poly I:C without transfection used in the current study.

The host innate immunity harnesses the endosomal TLR3-signalling pathway in addition to cytoplasmic RNA helicases for poly I:C sensing (Le Goffic et al., 2007; Liu et al., 2007; Kalali et al., 2008; Wornle et al., 2009). Dauletbaev and colleagues (2015) have proven the ability of naked poly I:C; which is used in the current study, to leak from the endosome and become accessible to cytoplasmic RNA helicases; RIG-1 and MDA5, with consequent activation of MAVS pathway. Moreover, impaired induction of type I IFN has been reported in TRIF/IPS double knockout mice following intraperitoneal administration of poly I:C compared to mice deficient of either of them (Kumar et al., 2008). Slater and co-workers have proved that TLR3 can upregulate the expression of both *MDA5* and *RIG-1* genes in hBECs infected with RV (Slater et al., 2010). This raised the question as to whether the observed deleterious effects of poly I:C on GC actions were partly downstream of cytosolic RNA helicase mediated signalling. Therefore, we thought to explore the impact of poly I:C complexed with a transfection reagent on GC responsiveness. Transfected poly I:C predominantly reach the cytosol resulting in preferential activation of MDA-5 or RIG-1. Although challenging RLRs with poly I:C (LMW)/LyoVec promoted the expression of pro-inflammatory and antiviral genes, it did not affect the GC actions. These findings support the notion that poly I:C diminished GC actions downstream of TLR3.

Whilst TLR3 activation markedly diminished GRE-dependent transactivation, we have found that challenging bronchial epithelial cells with LPS; TLR4 ligand, partially impaired the GRE activity. In terms of endogenous genes expression, TLR4 activation had much-limited attenuation of GR-regulated genes transactivation, where only the expression of *CDKN1C* and *GILZ* among genes assessed were repressed. These data suggest that the contribution of TLR4 pathway to RSV-induced GC insensitivity may be limited.

Binding of TLR3 to its ligand activates the expression of multiple transcription factors such as MAPK, NF-κB and members of IRF family with subsequent production of inflammatory cytokines and type I/III IFNs. So, it is plausible that the RSV impairs GC signalling through the release of cytokine(s) that act in an autocrine manner. Among the cytokines induced by RSV infection, IL4, IL13, IL17, TNFα and IFNγ had been shown to interfere with GC action in epithelial cells (Rider et al., 2011; Zijlstra et al., 2012; O'Connell et al., 2015). However, the impact of TNFα, IL-4 and IL-13 on the GC actions was partially limited and cannot account for the profound GC impairment seen in RSV-infected epithelial cells.

Recently, extensive studies by our group have established TGF-β1 as a key mediator of glucocorticoid insensitivity in different epithelial cell types; A549 and BEAS-2B cells. The apparent resistance induced by TGF-β1 occur through ALK5 activation, whilst the signal transduction downstream of ALK5 could not be linked to any canonical or non-canonical pathways (Salem et al., 2012; Keenan et al., 2014). This led to the hypothesis that RSV may impair GC signalling through TGF-β1 release.

It has been proven that TGF-β1 expression and secretion is evident during RSV infection in epithelial cells (Gibbs et al., 2009). Coinciding with this, we have demonstrated that RSV infection triggered the expression of endogenous *TGF-β1* and *PAI-1*; a TGF-β1-responsive gene in BEAS-2B cells, while ALK5 inhibition by SB431542 abolished these effects. Similarly, poly I:C has been shown to provoke the expression of *TGF-β1* and *PAI-1* expression and significantly elevated levels of phosphorylated Smad2/3 indicative of TGF-β1 activity. Pre-incubation with SB431542 blocked poly I:C-induced TGF-β1 activity which in turn inhibited activation of Smad2/3 signalling. Intriguingly, SB431542 abrogated both RSV and poly I:C-evoked impairment of GRE activity, GR-induced transactivation of *GILZ* and *ENaC* expression, therefore enhancing GC therapeutic potential and airway fluid regulation.

We have shown that RSV-induced TGF-β expression was triggered by ERK1/2 phosphorylation, at least partially via TLR3 activation. Inhibition of ERK1/2 activation using U0126 repressed RSV- and poly I:C-induced TGF-β activity and prevented attenuation of GC responses by both stimuli. Our group has previously excluded ERK activation as a down-stream mediator of TGF-β1-induced GC insensitivity (Salem et al., 2012; Keenan et al., 2014). Therefore, we speculated that ERK1/2 activation occurs upstream of TGF-β expression, in signalling emerging from TLR3 activation by viral PAMP; dsRNA. This proposed mechanism is reinforced by our observation showing that poly I:C-induced

ERK1/2 activation became evident at 6 h; an earlier time point compared to Smad2/3 phosphorylation; 24 h. Of note, the kinetics of ERK phosphorylation are different between the poly I:C and RSV, and this is consistent with the time required for the virus replication process to occur and generate dsRNA replication intermediate.

In the light of the findings presented, there is compelling evidence towards the mobilization of endogenous TGF-β1 as a driving mechanism of both RSV infection and Poly I:C induced glucocorticoid insensitivity. Since TGF-β is crucial to several cell functions, global inhibition of TGF-β1 signalling through the inhibition of ALK5 receptor kinase activity using SB431542 is not clinically feasible due to the known risk of autoimmune disease, tumorigenesis (Hahm et al., 2001; Oyanagi et al., 2014) and mitral valve damage (Anderton et al., 2011). It is therefore imperative to define the downstream pathways incriminated in glucocorticoid insensitivity by TGF-β in the airway epithelium through hypothesis-free approaches, including proteomics and functional genomics (Keenan et al., 2015). Recently, our group had implicated casein kinase 1δ/ε (CK1δ/ε) in TGF-β1 signals subserving glucocorticoid insensitivity and epithelium-derived pro-inflammatory cytokine production (Li et al., 2019).

Intriguingly, we have shown for the first time that pre-incubation with PF670462; a potent and selective CK1δ/ε inhibitor, inhibited the expression and activity of TGF-β and subsequently prevented the GC impairment provoked by either RSV infection or poly I:C. Additionally, PF670462 significantly subdued RSV-induced expression of pro-inflammatory gene encoding IL-6 suggesting the potential for additional beneficial anti-inflammatory activity. These findings support the possible use of PF670462 for the prevention/management of GC resistance during RSV infection-induced bronchiolitis or exacerbations of asthma/COPD.

3.6 Limitations of the study

The morphology and differentiation of the airway epithelium impact susceptibility to viral infection and the ensuing immune response. Thus, while studies using BEAS-2B cell line may provide an appropriate and feasible approach to investigate RSV responses, it has some limitations. First, the initial infection of RSV is typically ciliated airway epithelium and the BEAS-2B cell line does not show normal epithelial differentiation with respect to ciliated, goblet and club cells and cultured as a submerged monolayer rather than at air-liquid interface. Second, BEAS-2B cells are derived from single donor, so they cannot capture all of the potential responses of the genetically heterogeneous human

population. Third, the dichotomy between adult and paediatric-derived primary cells highlights the difficulty in interpreting observations made with regard to childhood airway repair. Moreover, BEAS-2B cells are relatively normal and not diseased, hence extrapolating findings to asthma is problematic due to intrinsic differences between non-asthmatic and asthmatic airway epithelial cells, i.e. the baseline expression levels of genes/proteins of interest.

In the current chapter, we only examined the gene expression of inflammatory cytokines at mRNA level. However, changes in mRNA transcript levels do not always correspond to the changes in protein abundance due to post-transcriptional mechanisms controlling the rate of protein translation, different half-lives of mRNAs and the corresponding protein products, and differences in the intracellular locations of expressed proteins (Gygi et al., 1999). Hence, assessing the observed expressional changes at the protein level using ELISA or western blotting should be the focus of future experiments.

Lastly, we employed various small molecule kinase inhibitors including SB431542, PF670462, and U0126. While pharmacological inhibition can be immediate, it might have non-specific, "off-target" effects that can affect the cellular physiology, hence altering the experimental outcomes. Due to the potential non-specific, "off-target" effects, we recommend the combined use of two or more unrelated methodological approaches, such as siRNA gene silencing, expression of dominant-negative mutants, or gene knockouts.

3.7 Conclusions from chapter 3

In this chapter, we first demonstrated that RSV infection dampens glucocorticoid sensitivity in bronchial epithelial cells. This observation can partly explain the limited protection afforded by inhaled corticosteroids against RSV-induced asthma exacerbations. Next, we identified autocrine TGF-β as a key mediator of RSV detrimental effect on GC therapeutic potential through the activation of ALK5. We also implicated CK1δ/ε in the apparent resistance suggesting a novel target to restore GC sensitivity during RSV infection. Moreover, among various PRRs involved in RSV sensing, we have shown that activation of TLR3 extensively attenuates glucocorticoid responsiveness in bronchial epithelial cells. Based on the results presented in this chapter, we suppose that viral PAMPs, most importantly dsRNA, activates TLR3 which in turn signals through the ERK1/2-dependent pathway to promote TGF-β activity hence mediating GC resistance in airway epithelial cells. This suggests that dsRNA/TLR3 interactions play a central role in virus-induced glucocorticoid insensitivity.

CHAPTER 4

TLR3 Gene Silencing in Airway Epithelium

4 TLR3 gene silencing in Airway Epithelium

4.1 Introduction

In the preceding chapter, we have reported for the first time the evidence for the primacy of the cytokine TGF-β1 in RSV-induced steroid resistance in a surrogate model of the bronchial epithelium. Using a systematic approach, we examined the possible role of each of RSV-sensing PRRs in altering GC sensitivity during viral infection. Our data suggested that out of all PRRs tested, TLR3 and to a lesser extent TLR4 contribute, at least partially, to RSV-mediated GC resistance. TLR3 is the major sentinel against viral infections due to its ability to recognize dsRNA that present in some viruses with dsRNA genome or produced as an intermediate during DNA and ssRNA viruses replication (Thompson et al., 2011). TLR3 also sense the endogenous dsRNA released from cells upon necrosis (Karikó et al., 2004; Cavassani et al., 2008). TLR3 is highly conserved and broadly expressed in immune and non-immune cells (Mikami et al., 2012). We here review the TLR3 signalling, the regulatory networks that viruses encompass to modulate the TLR3-mediated antiviral responses and finally discuss TLR3 contribution to viral pathogenesis.

4.1.1 TLR3 signalling cascade

In unstimulated cells, TLR3 inhabits the endoplasmic reticulum (ER). When stimulated with dsRNA, TLR3 interact with UNC-93B; an ER-membrane protein, that facilitates its translocation to the endosome where it dimerizes via pH-independent mechanisms before binding to its ligand; the viral dsRNA (Kim et al., 2008). Structural studies have revealed that the minimum length of dsRNA essential for signalling is TLR3 at least 40 to 50 base pairs (Luo et al., 2012; Tatematsu et al., 2013; Tatematsu et al., 2018). The crystal structure of two mouse TLR3-dsRNA complex has shown that the dsRNA binds to TLR3 ectodomains at the opposite ends of the TLR3 horseshoe and the dimer is stabilized by an intermolecular contact between the two TLR3-ECDs C-termini. This juxtaposition allows the cytoplasmic TIR domains to dimerize and thereby starts the signal transduction (Figure 4-1) (Liu et al., 2008).

For recruitment of TRIF, the epidermal growth factor receptor (EGFR) and c-Src are crucial for phosphorylation of the Tyr858 and Tyr759 residues of TLR3. Other kinases are involved in TLR3 activation that includes phosphoinositide Bruton's tyrosine kinase (BTK) and 3-kinase (PI3K) (Lee et al., 2012b).

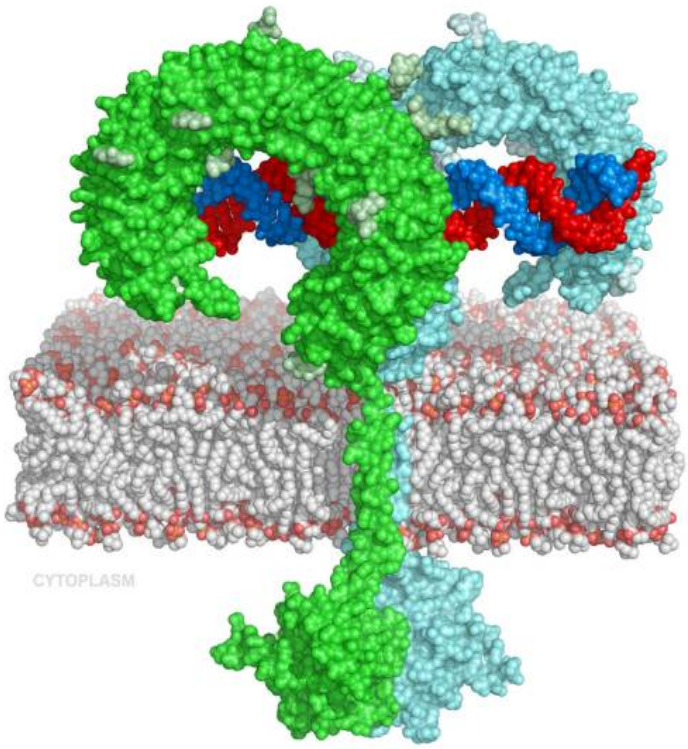


Figure 4.1| Model of TLR3:dsRNA signalling complex.
Adapted from (Liu et al., 2008)

TLR3-TRIF signalling cascades leads ultimately to AP-1, IRF3 and NF-κB activation via two branches. NF- κB-activating kinase-associated protein-1 (NAP1) triggers the association of TRIF with TRAF3 and TBK1/IKKε complex and thereby elicits phosphorylation and dimerization of IRF3. The activated IRF3 dimer then translocate to the nucleus and trigger an antiviral response through the production of type I IFNs (Sato et al., 2003). The second branch drives NF-κB and AP-1 activation and involves the association of RIP1 with TRAF6 that interacts with TRIF with the subsequent recruitment of TAB2, TAB3 and TAK1. The TRIF branch activates the MAPK pathway and its downstream AP-1. This branch also mediates IKK complex activation with further NF-κB activation. Activated NF-κB and AP-1 translocate to the nucleus where they trigger a pro-inflammatory response by inducing proinflammatory cytokines production (Alexopoulou et al., 2001).

Interestingly, the TLR3-TRIF axis also triggers apoptosis via a RIP1/FADD)/caspase-8-dependent and mitochondrion-independent pathway (Han et al., 2004). Collectively, the literature demonstrates that the stimulation of TLR3–TRIF axis has three major endings: (i) the generation of antiviral response triggered by IRF3 activation and subsequent IFN type I release; (ii) the production of a pro-inflammatory environment via AP-1 and NF-κB activation; (iii) a cytopathic effect via RIP1-induced caspase-8 activation.

4.1.2 Viral-driven regulators of TLR3 signalling cascade

Viruses have developed mechanisms to modulate the host immune responses for their own interest and diminish the antiviral responses at several levels. In this section, we illustrate the multiple strategies viruses evolved to control the TLR3 signalling cascade. One strategy involves downregulation of TRIF expression. Some viruses possess non-structural proteins that can target TRIF for proteolysis via their serine protease activity. Examples of these viral proteins include the protease-polymerase processing intermediate (3CD) of hepatitis A virus (HAV) (Qu et al., 2011), the HCV NS3/4A (Li et al., 2005), the 3C protease of enterovirus 71 (EV71) (Lei et al., 2011) and the coxsackievirus B3 (CVB3) 3C(pro) cysteine protease (Mukherjee et al., 2011a). In addition, Kaposi's sarcoma-associated herpesvirus (KSHV) owns the replication and transcription activator (RTA) that shortens the TRIF half-life through targeting it for degradation by the ubiquitin-proteasome system (Ahmad et al., 2011). The second viral strategy is to regulate the expression of the tripartite motif 38 (TRIM38). This E3 ligase triggers the polyubiquitination of NAP1 and TRIF and targets them for degradation by the proteasome. The vesicular stomatitis virus (VSV) induces the expression of TRIM38 that intern reduces TLR3-induced type I IFN response (Xue et al., 2012).

Other viruses modulate the antiviral response via sequestering the key proteins TRAF3 and TRAF6. As an example, the coronavirus M protein hinders the assembly of the TRAF3-TANK-TBK1/IKKε complex and thereby suppressing the downstream IRF3 and IRF7 activation (Siu et al., 2009). The protein A52R of vaccinia virus (VACV) sequesters TRAF6 and IL-1 receptor-associated kinase-like 2 (IRAK2) resulting in inhibition of TLR3-mediated NF-κB activation (Harte et al., 2003).

4.1.3 The role of TLR3 in host immunity to viruses: defence or offence?

Since TLR3 recognizes dsRNA produced by most viruses, TLR3 signalling holds the potential to master the immunity in most viral infections caused by herpes virus, flaviviruses, rotavirus, hepatitis B and C viruses, retroviruses, poxviruses and orthomyxoviruses among others (Perales-Linares and Navas-Martin, 2013). However, it is quite challenging to understand the protective versus pathogenic contribution of TLR3 in viral infections. The question that arises is whether viruses can circumvent TLR3 signalling to gain advantage? or pathological situations that lead to aberrant/unintended activation of the receptor?

A protective role of TLR3 has been reported in infections caused by neurotropic viruses such as herpes simplex virus type 1 (HSV-1, DNA), Theiler's murine encephalomyelitis virus (TMEV, ssRNA), West Nile virus (WNV, ssRNA) and poliovirus (PV, ssRNA). The highly prevalent HSV-1 can cause herpes simplex encephalitis (HSE) in children with inborn errors of TLR3 immunity. The defective TLR3 signalling was found to impair the production of type I IFN that offer protection in neurons and oligodendrocytes (Guo et al., 2011; Lafaille et al., 2012). TLR3 signal transduction reduces the viral burden and modulates the immune responses thereby protects against TMEV demyelinating disease (Abston et al., 2012). A protective role of TLR3 against WNV has been also reported where TLR3-deficiency has elevated the viral load in neurons and increased mortality in mice (Daffis et al., 2008). In addition, TLR3 gene mutation has resulted in development encephalopathy in IAV-infected patients (Hidaka et al., 2006). TLR3 mediated protection against PV infection in transgenic mice expressing the PV receptor (Abe et al., 2012). The TLR3-mediated response has been found to be protective in viral infections of the heart such as myocarditis caused by coxsackieviruses B (CVB3 and CVB4, ssRNA). Mice lacking TLR3 when infected with CVB3 or CVB4 had lower survival rates compared with controls due the amplified viral load and inflammation in the heart (Negishi et al., 2008; Richer et al., 2009; Abston et al., 2012).

A controversial role of TLR3 has been reported in Human immunodeficiency virus type 1 (HIV-1, ssRNA) infection, where activation of the TLR3 signalling cascade with poly(I:C) triggers an antiviral response that limits HIV-1 infection in cell culture, suggesting a therapeutic potential for TLR3 ligands against HIV-1 infection in humans (Rivieccio et al., 2006; Swaminathan et al., 2012). *In vivo*, immunizing mice with purified recombinant HIV-1 envelope gp120 in addition to poly(I:C) has activated TLR3 signalling pathway that enhanced the induction of HIV-specific $CD8^+$ cytotoxic T lymphocytes (Fujimoto et al., 2004). However, elevated TLR3 mRNA levels were detected in biopsy specimens from patients with HIV-myopathies compared to nonmyopathic controls. Immunohistochemistry has detected the main localization of TLR3 in areas with close proximity to damaged or necrotic muscle fibres (Schreiner et al., 2005). In the liver, activation of TLR3 signal transduction was detrimental in the pathogenesis of Punta Toro virus (PTV, ssRNA). TLR3 deficiency in mice has lowered the serum viral load, weakened the pro-inflammatory responses in liver and increased survival of mice infected with PTV (Gowen et al., 2006).

The role of TLR3-mediated response was deleterious in the context of respiratory infections caused by RSV (negative-sense ssRNA), RV type1B (positive-sense ssRNA), IAV (negative-sense ssRNA) and VACV (DNA). In RV infection, TLR3 has been shown to initiate proinflammatory responses leading to lung inflammation and airway hyperresponsiveness (Wang et al., 2011). In IAV-infected mice, the absence of TLR3, although elevated the viral load, has increased the survival rates that correlated to the significant reduction of the proinflammatory mediators and lowering in a number of $CD8^+$ T cytotoxic lymphocytes in the bronchoalveolar airspace (Goffic et al., 2006). Similarly, TLR3 has shown a detrimental role in RSV pathogenesis. The RSV dsRNA/TLR-3 signalling has been proved to impair the alveolar fluid clearance thereby causing lung oedema (Aeffner et al., 2011). TLR3 deficiency was also beneficial in VACV infection where it substantially reduced the lung inflammation, lowered the viral burden and limited its dissemination to other organs (Hutchens et al., 2008).

Taken together, the literature suggests that the protective versus the pathogenic role of TLR3 in viral infections exhibit a differential trend that is tissue-specific and not dependent on the type of viral genome (ssRNA, dsRNA or DNA) viruses. The fine-tuning between the TLR3-mediated antiviral and immunoregulatory pathways affects protective versus detrimental outcomes.

4.2 Outline and aims of the chapter

Several studies have reported the differential role TLR3 plays in the innate immunity against RSV infections and gave clues that correlate the RSV-induced TLR3 activation to the more severe clinical syndrome of viral infection (Rudd et al., 2005; Groskreutz et al., 2006; Rudd et al., 2006; Dou et al., 2013). Complementing with these studies, we suggested a possible role for TLR3 in the poor response of RSV-induced asthma exacerbations to glucocorticoid therapy. In this chapter, we systematically examined the impact of TLR3 antagonism to ascertain its role in RSV-induced GC insensitivity. We initially investigated how RSV infection impacts the TLR3 expression in an immortalized bronchial epithelial cell line. Next, *in vitro* models of the human bronchial epithelium and small interfering RNA gene silencing method were employed to identify the role of TLR3 in RSV-triggered proinflammatory cytokines expression. Finally, we examined whether antagonizing TLR3 can restore the epithelium responsiveness to glucocorticoids.

4.3 Methods

4.3.1 TLR3 siRNA transient transfection of BEAS-2B cells

As illustrated in Figure 4.2, Cells were transfected with predesigned Silencer® select siRNA oligonucleotides targeting TLR3 (s236, sense: 5′-CCGAUGAUCUACCCACAAAtt-3′, antisense: 5′-UUUGUGGGUAGAUCAUCGGgt-3′) and silencer® select negative control no. 1 (4390843) as previously detailed in Section 2.5. Twenty-four hours post-transfection, cells were either inoculated with RSV at MOI of 0.1 as described in Section 3.3.3 for 48 h or challenged with poly I:C (10 µg/ml) for 24 h prior to treatment with dexamethasone or Vehicle (0.1% DMSO) add for the last 4 h. Total RNA was then extracted using TRIzol reagent (Invitrogen) (Section 2.6.1) and reverse-transcribed into cDNA (Section 2.6.2). Endogenous gene expression was then determined by RT-qPCR (Section 2.6.3) using specific primers listed in Table 2.1.

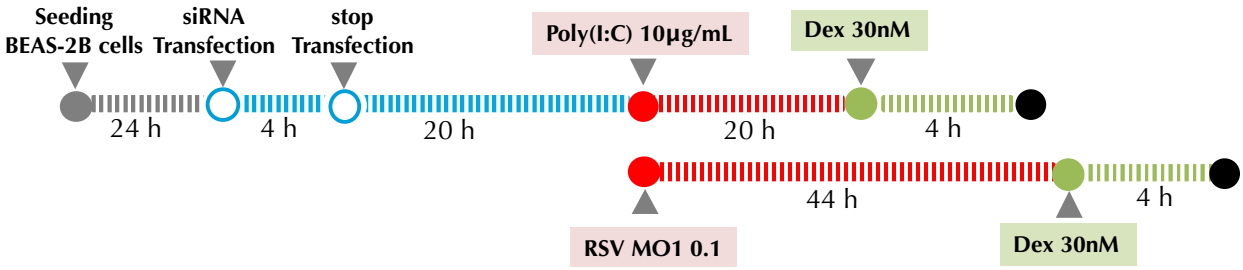


Figure 4.2| Experimental protocol.

BEAS-2B cells were transfected with either Scrambled control (Scr Ct) or TLR3 siRNA. On the following day, cells were either challenged with poly (I:C) for 24 h or inoculated with RSV at MOI of 0.1 for 48 h with Dexamethasone (Dex) (30 nM) added for the last 4 h.

4.4 Results

4.4.1 Expression of TLR3 is upregulated in BEAS-2B cells following RSV infection and poly I:C exposure

TLR3 have been recognized as important PRRs in viral infection, but its role in RSV-infected epithelial cells is not fully established. First, we thought to explore the impact of RSV infection and poly I:C on the basal expression of TLR3. As indicated in Figure 4.3, the levels of TLR3 mRNA were markedly upregulated in cells inoculated with RSV compared to Mock control, suggesting that RSV primes bronchial airway epithelial cells for boosted responsiveness to subsequent exposure to dsRNA. We also noted that poly I:C (10 µg/ml) induced a modest increase in its own putative receptor; TLR3, expression in BEAS-2B cells.

Dexamethasone is a potent modulator of the innate immune system by shifting the TLR system response pattern from an inflammatory to an anti-inflammatory state (Broering et al., 2011). However, modulation of TLR expression by glucocorticoids has not been established yet. Hence, we sought to explore the impact of dexamethasone on TLR3 expression level in BEAS-2B cells. As demonstrated in Figure 4.3, treatment with dexamethasone had no detectable effect on *TLR3* mRNA level in either baseline conditions or in RSV-infected or poly I:C-exposed cells.

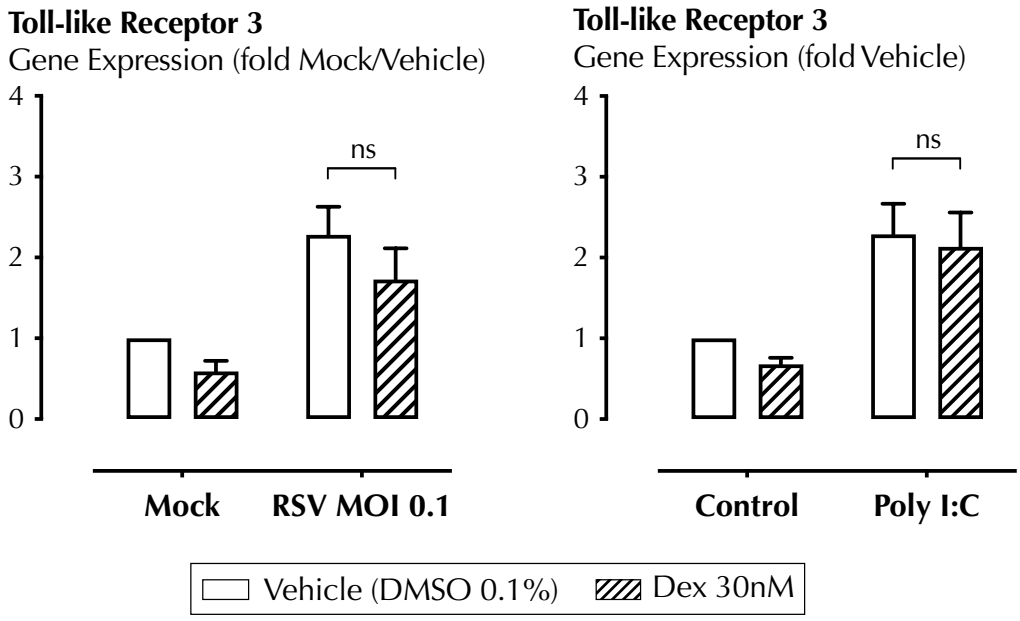


Figure 4.3| Impact of RSV infection and poly I:C upregulate the basal expression of TLR3 in BEAS-2B cells.

BEAS-2B cells were either inoculated with RSV at MOI of 0.1 for 48 h with Dexamethasone (Dex) (30 nM) added for the last 4 h. RNA was extracted, and gene expressions were determined by RT-qPCR. Data are expressed as fold change from Scrambled control (Scr Ct) and presented as mean±SEM for A, n = 3 – 4, and B, n = 4 independent experiments. Statistical significance was determined by Ordinary two-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: *p<0.05, ns, non-significant.

4.4.2 TLR3 knockdown using siRNA successfully blocked TLR3 mRNA expression while it did not affect other TLRs mRNA expression

We employed the siRNA interference approach to knock down the endogenous TLR3. Successful TLR3 ablation was assessed by measuring the TLR3 mRNA level using RT-PCR. Our data showed that transfection of BEAS-2B cells with specific TLR3 siRNA has effectively reduced the expression of TLR3 by 84% after 24 h of transfection when

compared to cells transfected with scrambled control. The TLR3 ablation has persisted for the following 48 and 72 h post-transfection where the TLR3 mRNA level has been reduced by 69.6% and 74.2%, respectively (Figure 4.4 A). The specificity of silencing TLR3 was determined by assessing the effect that silencing TLR3 has on mRNA expression level of other TLRs by RT-qPCR. Notably, the expression of other TLRs including TLR1, TLR2, TLR4, TLR5 and TLR6 was not modulated following TLR3 silencing (Figure 4.4 B). This suggests that TLR3 knockdown was achieved while avoiding a non-specific inhibition of other TLRs in response to the introduced siRNA.

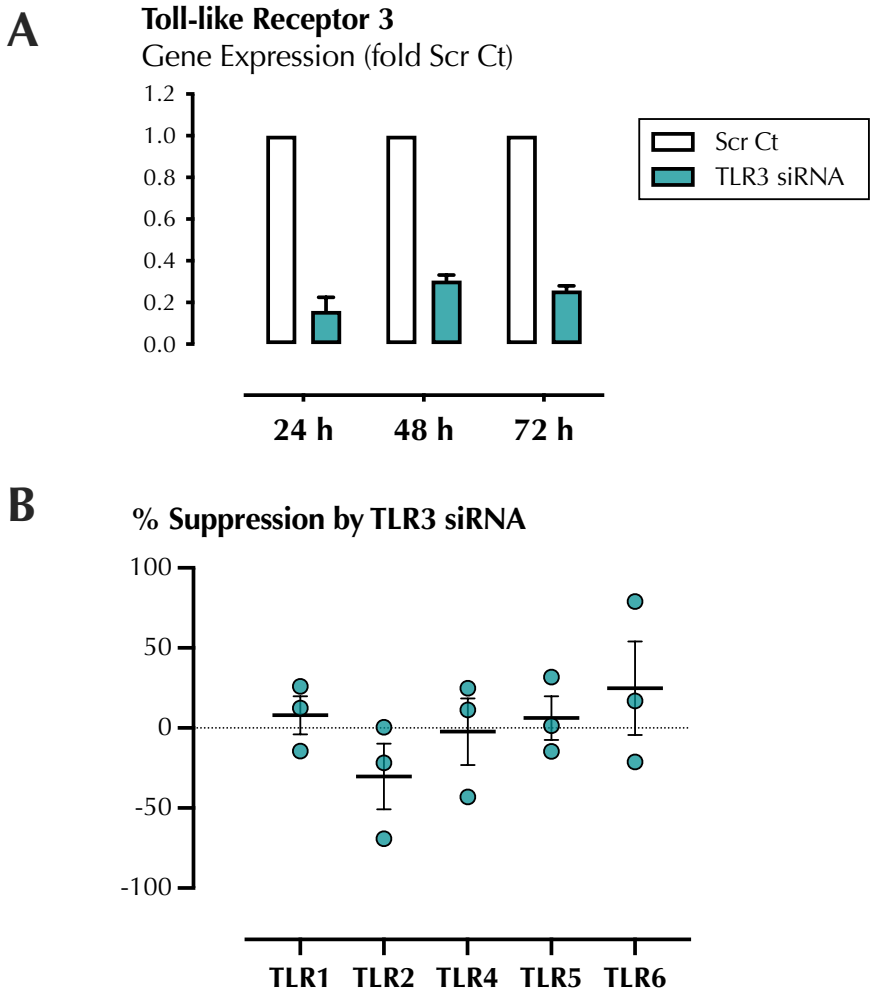


Figure 4.4| TLR3 knockdown using siRNA transfection robustly inhibited TLR3 expression, while it did not affect other TLRs mRNA expression.

BEAS-2B cells were transfected with either Scrambled control (Scr Ct) or TLR3 siRNA. RNA was extracted and gene expressions were determined by RT-qPCR. Data are expressed as fold change from Scrambled control (Scr Ct) and presented as mean±SEM for A, n = 3 – 5 and B, n = 3 independent experiments. Statistical significance was determined by Ordinary two-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: ***p<0.001, ns, non-significant.

4.4.3 Silencing TLR3 subdues the inflammatory response to poly I:C stimulation, however, does not affect RSV-induced pro-inflammatory cytokine expression

Since the airway epithelium possesses different PRRs that sense the dsRNA including TLR3, RIG-I, MDA5, PKR, we thought to explore the relative contribution of TLR3 signalling to the poly I:C-triggered inflammatory response in immortalized normal human bronchial epithelial cells. We quantified changes in the expression of selected cytokines; IL-6 and IL-8 in BEAS-2B cells challenged with poly I:C after TLR3 knockdown. Compared to cells transfected with scrambled siRNA, TLR3 siRNA-transfectants demonstrated limited inflammatory responses to poly I:C challenging with significant 36% ($p = 0.0161$) and 31% ($p = 0.0011$) reduction in IL-6 and IL-8 mRNA levels, respectively, relative to the control siRNA transfected cells (Figure 4.5 B). This observation suggests that despite the presence of multiple dsRNA sensors, TLR3 contributes significantly to the inflammatory response induced by poly I:C in the airway epithelium.

We next investigated the relative contribution of TLR3 in the proinflammatory cytokines expression triggered upon RSV infection. No obvious change in RSV-dependent *IL-6* and *IL-8* expression was observed between controls versus TLR3 silenced BEAS-2B cells (Figure 4.5 A). These results indicated that the recognition of viral PAMPs other than dsRNA allows the epithelial cells to produced pro-inflammatory cytokines in responses to RSV infection downstream signalling of other PRRs.

4.4.4 Influence of TLR3 knockdown on GC-regulated genes transactivation

To ascertain the role of TLR3 in ablating GC activity, BEAS-2B cells were transfected with TLR3 or control siRNA and subsequently either challenged with TLR3 ligand; poly I:C for 20 h or infected with RSV for 48 h. RT-PCR was used to measure the expression level of *TGF-β1* and its responsive gene *PAI-1*. At the conclusion of the experiment, TLR3-deficient cells showed significant inhibition of *TGF-β1* and *PAI-1* in BEAS-2B cells challenged by poly I:C (Figure 4.6 A). However, *TGF-β1* expression in RSV-infected cells showed a decreasing trend, albeit non-significant, following TLR3 knockdown (Figure 4.7 A). It is of primary importance to overcome the variability among data points by performing more experiments in order to reach a definitive conclusion. We next treated cells with dexamethasone and measured the expression of a panel of GC-inducible genes to examine the restoration of steroid activity in these cell cohorts. Dexamethasone-induced expression of *GILZ, ENaCα, PLZF* and *CDKN1C* was significantly impaired following poly I:C exposure (Figure 4.6 B) or RSV infection (Figure 4.7 B) in control siRNA-transfected cells. TLR3

silencing has significantly enhanced the expression of GC-responsive genes following dexamethasone treatment in cells challenged with poly I:C (Figure 4.6 B). Similarly, the expression level of dexamethasone-responsive *GILZ* and *ENaCα* were both increased in the TLR3-deficient cells compared to the control siRNA transfectants following RSV infection. However, the differences in levels of *PLZF* and *CDKN1C* expression between TLR3-silenced cells and control cells were non-significant (Figure 4.7 B). These results strengthen our assertion of the potential contribution of TLR3 in the virus-induced steroid insensitivity.

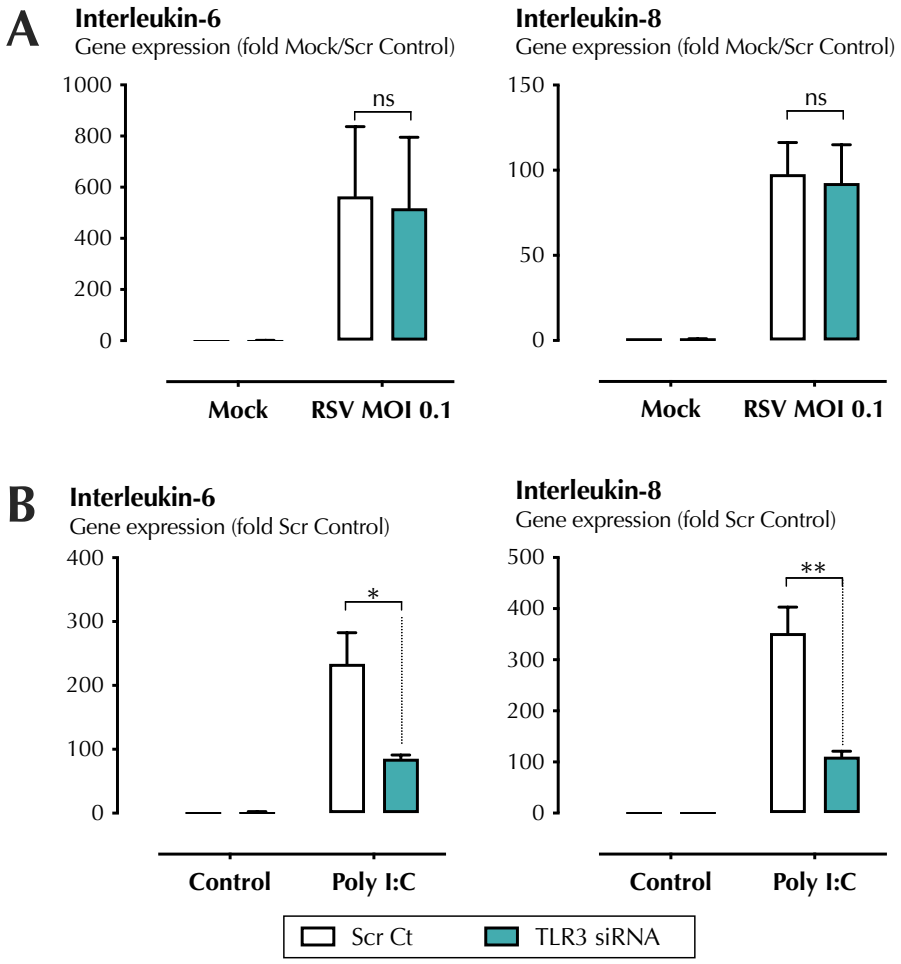


Figure 4.5| Impact of TLR3 knockdown on pro-inflammatory cytokines expression provoked by RSV infection or TLR3 activation in bronchial epithelial cells.
BEAS-2B cells were transfected with either Scrambled control (Scr Ct) or TLR3 siRNA. On the following day, cells were (A) inoculated with RSV at MOI of 0.1 for 48 h or (B) stimulated with poly I:C (10 µg/ml) for 24 h. RNA was extracted, and gene expressions were determined by RT-qPCR. Data are expressed as fold change from Scrambled control (Scr Ct) and presented as mean±SEM for n = 3 independent experiments. Statistical significance was determined by Ordinary two-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: *p<0.05, **p<0.01, ***p<0.001, ns, non-significant.

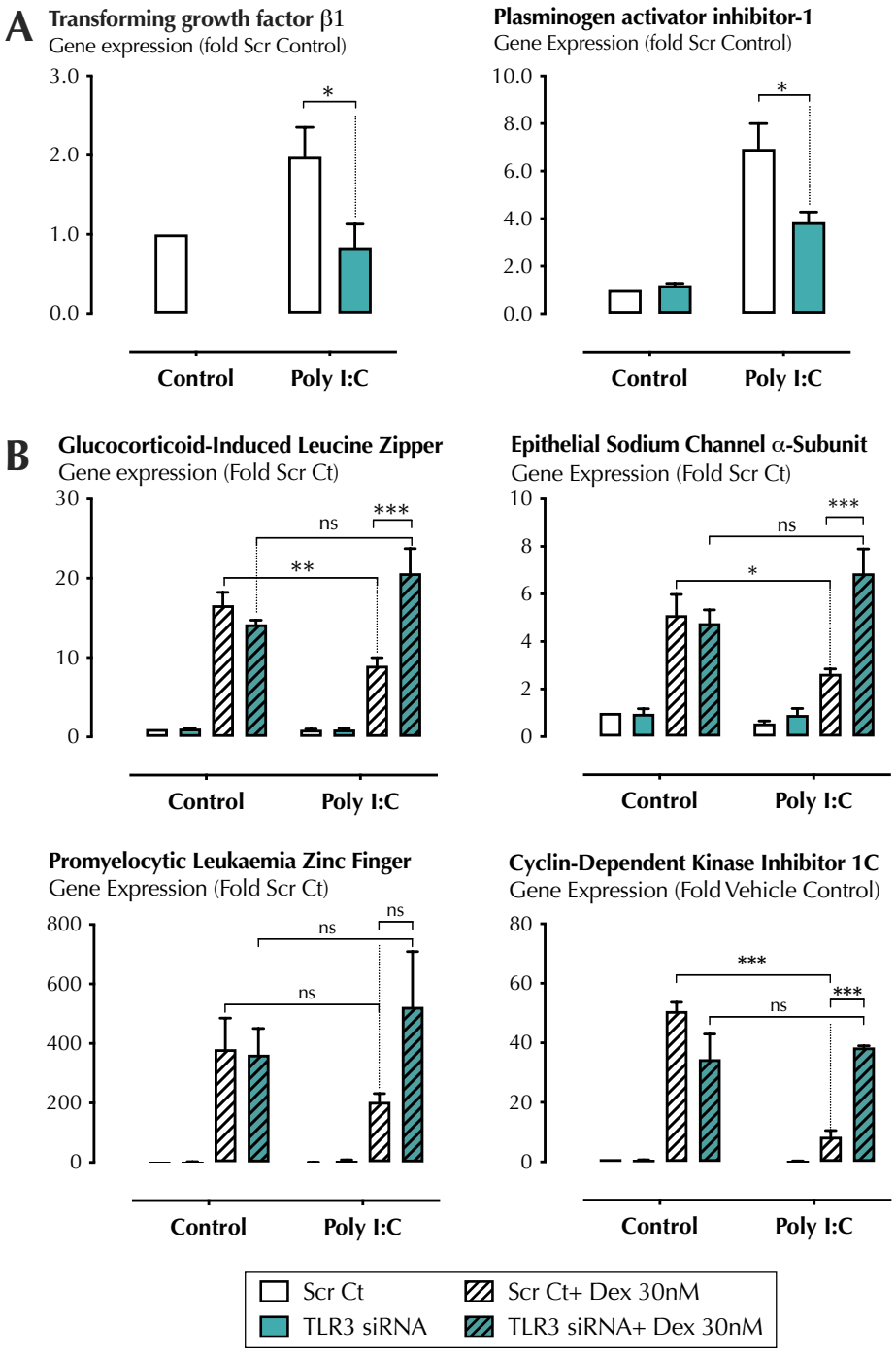


Figure 4.6| TLR3 knockdown attenuates TGF-β activity and glucocorticoid insensitivity induced by TLR3 activation in bronchial epithelial cells.

BEAS-2B cells were transfected with either Scrambled control (Scr Ct) or TLR3 siRNA. On the following day, cells were stimulated with poly I:C (10 µg/ml) for 24 h with Dexamethasone (Dex) (30 nM) added for the last 4 h. RNA was extracted and gene expressions were determined by RT-qPCR. Data are expressed as fold change from Scrambled control (Scr Ct) and presented as mean±SEM for A, n = 4 - 5, B, n = 3 -6 independent experiments. Statistical significance was determined by Ordinary two-way ANOVA, Bonferroni's multiple comparisons post-hoc test except for panel A (left) where Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test was applied and is represented as follows: *p<0.05, **p<0.01, ***p<0.001, ns, non-significant.

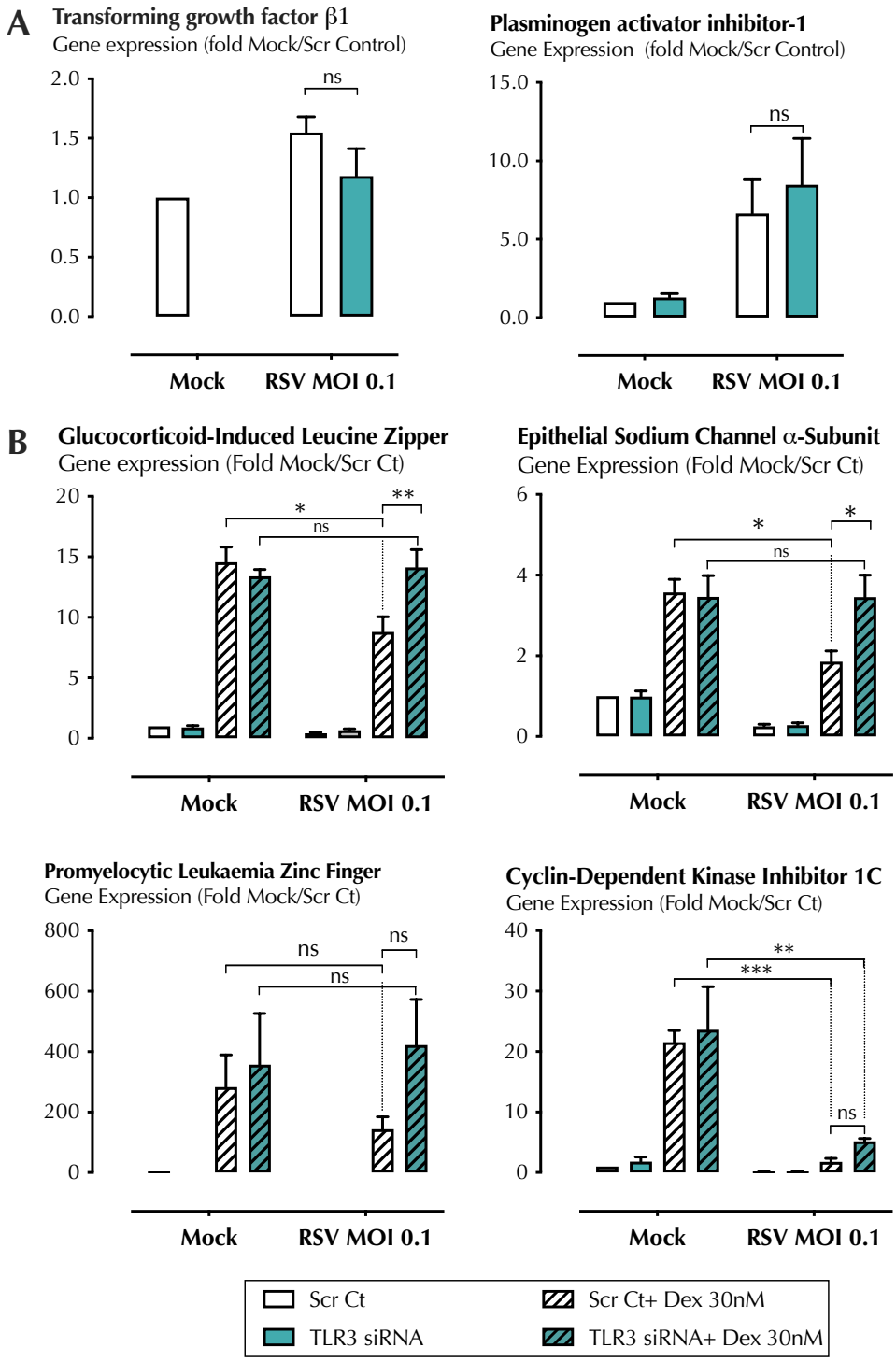


Figure 4.7| TLR3 knockdown attenuates TGF-β activity (A) and glucocorticoid insensitivity (B) in RSV-infected bronchial epithelial cells.

BEAS-2B cells were transfected with either Scrambled control (Scr Ct) or TLR3 siRNA. On the following day, cells were inoculated with RSV at MOI of 0.1 for 48 h with Dexamethasone (Dex) (30 nM) added for the last 4 h. RNA was extracted and gene expressions were determined by RT-qPCR. Data are expressed as fold change from Scrambled control (Scr Ct) and presented as mean±SEM for A, n = 3- 5 and B, n = 3 – 6 independent experiments. Statistical significance was determined by Ordinary two-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: *p<0.05, **p<0.01, ***p<0.001, ns, non-significant.

4.5 Discussion

The severity of respiratory tract inflammation in viral infections is correlated to both the cytopathic effect of the virus itself and the immune responses the host elicit against this pathogen (Rudd et al., 2006). Accumulating pieces of evidence suggest a larger role of the host immune responses in the degree of inflammation and disease severity than the virus-induced damage. To date, numerous TLR3 deficiency studies have demonstrated the pathogenic role of TLR3-mediated immune and inflammatory factors in many viral infections including Punta Toro virus (Gowen et al., 2006), influenza virus (Le Goffic et al., 2006) and vaccinia virus (Hutchens et al., 2008). Mice lacking TLR3 have developed weaker inflammatory responses that have contributed to a less severe disease phenotype when compared to the wild type counterparts. The pathogenic role of TLR3 has been reported in related human studies. For instance, a functional TLR3 is a risk factor for the predisposition to tick-borne encephalitis by TBEV (Kindberg et al., 2011; Barkhash et al., 2013). Expression of *TLR2*, *TLR3*, and *TLR4* had been shown to increase in asthmatic patients who ultimately die, suggesting their crucial role in the development of severe or even fatal asthmatic exacerbations (Kim and Rhee, 2010; Ferreira et al., 2012). Moreover, TLR3 may play a detrimental role in acute exacerbations of chronic obstructive pulmonary disease (AECOPD) patients, possibly due to TLR3 activation by viral and bacterial infection. TLR3 also enhanced the inflammatory response when in an antiviral state, leading to lung damage (Liu et al., 2018).

In the context of RSV infections, several studies have provided evidence for the important contribution of RSV-induced cytokines/chemokines to deleterious pathophysiology in the lung (Lukacs et al., 2001; Tekkanat et al., 2002; John et al., 2003; Miller et al., 2003). RSV infection results in activation of NF-κB that drives chemokines production with the subsequent recruitment and activation of the cell populations responsible for airway inflammation (Tian et al., 2002). Despite the fact that the provoked inflammation following exposure to RSV aims at destroying and/or diluting the virus, it collaterally causes lung injury and may contribute to exacerbations that are refractory to the conventional therapy with antivirals and glucocorticoid. During replication, RSV generates a dsRNA intermediate that acts as a ligand for TLR3. Aeffner and colleagues reported that challenging mice with poly I:C; the synthetic analogue of dsRNA, have induced similar pulmonary dysfunctions to RSV infection where both have impaired alveolar fluid clearance leading to lung oedema (Aeffner et al., 2011). This study and others (Rudd et al., 2005; Groskreutz et al., 2006; Rudd et al., 2006; Dou et al., 2013) have

implicated TLR3 in RSV pathogenesis and suggested TLR3 as a promising target that can modulate pulmonary inflammation reported during RSV infection.

In the preceding chapters, we, for the first time, suggested that TLR3 contributed at least partially to RSV-induced impairment of glucocorticoid responsiveness in the bronchial airway epithelial cells. This finding adds to the therapeutic value of targeting TLR3 in attempt to better control RSV-induced asthma exacerbations. So, we have moved next to evaluate the impact of TLR3 antagonism on RSV-induced inflammatory mediators' production and finally ascertain the potential of targeting TLR3 to resensitize the RSV-infected airway epithelial cells to the glucocorticoid treatment.

Using the *in vitro* model for RSV infection developed in the previous chapter, we have first assessed the impact of RSV infection on the basal expression of TLR3 in this model. TLR3 recognizes dsRNA that is generated in great quantities during RSV replication (Groskreutz et al., 2006). Our analysis of TLR3 mRNA level has shown that challenging of BEAS-2B cells with either RSV or poly I:C has increased the TLR3 expression above untreated cells implying a crucial role of epithelium-expressed TLR3 in RSV infection (Figure 4.3). Several studies have reported the TLR3 upregulation in A549 cells and lungs of BALB/c mice following RSV infection or poly I:C treatment (Groskreutz et al., 2006; Rudd et al., 2006; Huang et al., 2009; Dou et al., 2013). Another study has reported the same observation in the cell line employed in our study; BEAS-2B cells (Li et al., 2012). This corroborates our *in vitro* observations and provides evidence for the crucial role of epithelium-expressed TLR3 in RSV infection. The observed increase in TLR3 expression following RSV infection may be explained by the more dsRNA produced upon replication. However, a recent report has correlated the RSV-induced TLR3 upregulation to the induction of oxidative stress (Wang et al., 2018). Oxidative stress occurs due to either excessive generation of reactive oxygen species (ROS) or diminished antioxidant capacity, (Akira et al., 2001). Previous studies have detected oxidant overload in human airway epithelial cells in response to RSV infection (Lund et al., 2004). RSV infection *in vivo* has generated ROS that causes oxidative damage in the lung (Akira et al., 2001; Gern et al., 2003). Wang et al. have employed the oxidative stress agonist H_2O_2 and antioxidant N-acetyl-L-cysteine (NAC) to test the impact of oxidative stress modulation on TLR3 expression and TLR3-mediated responses in RSV-infected A549 cells. RSV infection has elicited the hydroxyl free radical (·OH) and nitric oxide (NO) production and reduced the superoxide dismutase (SOD) activity. Concomitantly, TLR3 and NF-κB were upregulated at the mRNA and protein levels. Pre-treatment of A549 cells with H_2O_2 has augmented the

increased TLR3 and NF-κB expression after RSV infection, however, NAC pre-treatment abrogated these effects. These observations suggested a critical regulatory role of oxidative stress on RSV-induced TLR3 expression and hinted that oxidative stress might potentiate the RSV-induced upregulation of TLR3 expression together with its associated signalling pathway such as NF-κB cascade (Wang et al., 2018). The upregulated TLR3 expression in the airway epithelium increases the sensitivity of these cells to the exposure to other viral dsRNA thereby amplifying the provoked inflammatory responses that may lead to viral exacerbations (Groskreutz et al., 2006; Resch et al., 2007).

We next employed the siRNA-based gene silencing method to knockdown *TLR3* expression in BEAS-2B cells thereby enabling us to investigate its role in RSV-induced GC resistance. siRNAs are synthetic, short (approximately 21 base pairs), double-stranded RNA that target specific genes in a sequence-specific manner (Castel and Martienssen, 2013). As off-target and non-specific effects are more likely to occur, a negative control (scrambled) sequence was used. The hydrophilic siRNAs were delivered using non-viral lipid-based carriers to avoid the high risk of triggering immune responses in the cell when using viral carriers. In our experiments, the TLR3 siRNAs have significantly downregulated the gene expression in BEAS-2B cells compared to the negative control siRNA. Since siRNAs are not permanently incorporated into the cellular genome, the resultant genotypic changes persist only for a limited time period (Kim and Eberwine, 2010). In our experiments, TLR3 siRNA has persistently reduced TLR3 expression at least until 72 h later which corresponds to the entire experiment timeline (Figure 4.4 A). One limitation to the siRNA gene silencing method is the off-target effects, which are the consequence of targeting sequences similar to the target of interest (Dorsett and Tuschl, 2004). The cross-reactivity with other TLRs was excluded since their basal expression levels have not changed significantly following TLR3 siRNA treatment (Figure 4.4 B). This suggests that TLR3 knockdown was achieved while avoiding a non-specific inhibition of other TLRs in response to the introduced siRNA.

A common signalling event following PRRs challenging is the activation of the canonical NF-κB pathway, which is responsible for transcriptional induction of pro-inflammatory cytokines, and chemokines including IL-6 and IL-8 (Ghosh and Karin, 2002; Hayden and Ghosh, 2011; Sun et al., 2013). Depletion of TLR3 has effectively attenuated the poly I:C-induced the IL-6 and IL-8 production, but not to the basal level in unstimulated cells (Figure 4.5B). This suggests that while poly I:C-induced inflammatory responses depend largely on TLR3, they also appeared to be mediated by cytosolic pattern

recognition receptors RIG-1 and MDA5. In the context of whole RSV virions, TLR3 ablation has not subdued the provoked inflammatory response in the bronchial epithelial cells (Figure 4.5 A). This can be attributed to the variety of ligands that a single viral pathogen can bear which are capable of engaging with multiple PRRs in the epithelium thereby triggering downstream production of the inflammatory cytokines. Moreover, our findings suggested that TLR3 is not the major driver of the RSV-mediated inflammatory response.

In addition to activation of the canonical NF-κB pathway, binding of dsRNA to TLR3 ultimately results in activation of IRF-3 and the downstream release of IFN-β and IFN response genes such as *CCL5*, *CXCL10* and *CCL12*, implying a pivotal role of TLR3 in the antiviral immunity (Yamamoto et al., 2003). Nevertheless, several studies have suggested that TLR3 ablation does not affect RSV replication. Rudd and his colleagues have reported equivalent RSV kinetics in RSV-infected HEK 293 cells stably expressing TLR3 versus vector controls (2005). This finding comes in line with another study showing that a comparable RSV pulmonary titers were recovered from the $TLR3^{-/-}$ animals and wild type controls during infection (Rudd et al., 2006). Similarly, TLR3-deficient mice have shown no alteration in the clearance of other RNA viruses including VSV, reovirus, lymphocytic choriomeningitis virus (LCMV) and murine cytomegalovirus (MCMV) (Edelmann et al., 2004). The poor sensitivity of RSV to IFNs-α/β gives logical reasoning for this observation (Roberts et al., 1992; Atreya and Kulkarni, 1999). A study by Durbin et al. has shown that RSV clearance has been only slightly delayed by IFNs-α/β *in vivo* (Durbin et al., 2002). Our findings together with previous reports suggest that TLR3 does not generate effective antiviral responses against RSV infections since lack of TLR3 does not alter either the viral clearance or impair the generation of proinflammatory cytokines in the airway epithelium (Rudd et al., 2005; Hillyer et al., 2018).

The synthetic dsRNA analogue poly I:C has been reported to activate latent TGF-β1 in immortalized human bronchial epithelial cells (iHBEC). The dsRNA-mediated TGF-β1 activation was inhibited by chloroquine; an inhibitor of endosomal acidification indicating the involvement of the endosomal receptor TLR3 rather than the cytosolic RIG-1 and MDA5 (Jolly et al., 2014). This finding has been reproduced in the current results. Challenging the bronchial epithelial cells with poly I:C has upregulated TGF-β expression by approximate 2-folds compared to unstimulated cells. Poly I:C treatment has also stimulated TGF-β-dependent expression of well-known TGF-β inducible genes, *PAI-1*. TLR3 silencing has abrogated the poly I:C-induced TGF-β1 expression and its responsive gene *PAI-1* (Figure 4.6 A).

In the context of RSV, we have found that TGF-β1 expression tended to decrease upon TLR3 ablation, albeit not significantly (Figure 4.7 A). Literature has reported that TGF-β is expressed during RSV infection of lung epithelial cells (McCann and Imani, 2007; Gibbs et al., 2009; Mgbemena et al., 2011; Bakre et al., 2015) and macrophage (Pokharel et al., 2016), however, the mechanism that elicits TGF-β1 expression during viral infection is still unclear. Pokharel *et al*. have proposed a role of autophagy in eliciting TGF-β1 release from macrophages following RSV infection. Autophagy is a crucial cellular process which maintains homeostasis for normal physiological and biological functions (Shibutani et al., 2015). RSV has been reported previously to induce autophagy in macrophages and dendritic cells in the respiratory tract of RSV-infected mice (Morris et al., 2011; Reed et al., 2013; Owczarczyk et al., 2015). Treatment of macrophages with the autophagy inhibitor 3 MA has decreased TGF-β1 release in response to RSV infection. Since TLR3 activation has been proved to induce autophagy in RSV-infected macrophages (Delgado et al., 2008; Tsai et al., 2015), it has been assumed that activation of TLR3 by the viral dsRNA elicit autophagy that in turn trigger the expression of TGF-β1 in macrophages. This assumption is supported by the striking similarity of lung cytokine profile and pathogenesis severity in TLR3 knockout mice and autophagy-deficient mice (i.e., beclin-1 deficient mice) following RSV infection (Reed et al., 2013). Additionally, TLR7 activation by viral ssRNA can induce autophagy (Sanjuan et al., 2007; Delgado et al., 2008). We suggested that dsRNA originating from RSV might in part bind to TLR3 and activates ERK1/2 pathway to induce TGF-β expression. It has been reported that ERK/MAPK signalling cascade exerts a strong stimulatory effect on autophagy (Martinez-Lopez et al., 2013; Sui et al., 2014). The cytotoxic drug Derrone causes sustained ERK phosphorylation leading to autophagic cell death in A549 cells (Kang et al., 2019). Although not fully investigated, it is tempting to postulate that RSV or poly I:C activates the TLR3/ERK/Autophagy axis to induce the TGF-β expression that mediates the steroid activity impairment in the bronchial epithelium.

We finally tested whether the decrease in TGF-β1 expression upon TLR3 silencing was able to reverse the GC insensitivity in the bronchial epithelium. Our study showed for the first time that TLR3 ablation was able to restore the dexamethasone activity as evident by the increase in the expression of GC-inducible genes following either poly I:C treatment (Figure 4.6 B) or RSV infection (Figure 4.7 B). Altogether, our data strengthen what we proposed previously that dsRNA-TLR3 signalling cascade is among the mechanisms by which RSV infections contribute to asthma exacerbations and the steroid insensitivity. Therefore, modulation of TLR3 activation offers an attractive therapeutic target not only for

attenuating the RSV-induced inflammatory damage in the lung but also for restoring the efficacy of glucocorticoids in the management of asthma exacerbations.

4.6 Limitations of the study

The current study has utilised siRNA gene silencing to knockdown TLR3 expression. Despite its efficacy *in vitro*, this method has several limitations (Unniyampurath et al., 2016). These include non-specific effects due to induction of genes mediating interferon response (Pebernard and Iggo, 2004) and the off-target effects due to the sequence similarity to the targeted gene (Dorsett and Tuschl, 2004). While the gene expression levels of all TLRs were examined in the current study as possible off-target effects of TLR3 siRNA, the expression of other poly IC/RSV sensing receptors, such as PKR, RIG-1 and MDA5 were not examined as possible off-targets following TLR3 silencing. Additionally, transient siRNA transfection does not result in stable integration into the cellular genome. Hence, this approach allows the analysis of the resulting genotypic and phenotypic changes only for a limited period of time (Kim and Eberwine, 2010). A limitation specific to the current study is the use of only one siRNA sequence for TLR3 silencing. However, identified phenotypes following siRNA gene knockdown should be confirmed with different siRNAs targeting the same transcript (Dorsett and Tuschl, 2004). A limitation specific to the current study is the use of only one siRNA sequence for TLR3 silencing. However, identified phenotypes following siRNA gene knockdown should be confirmed with different siRNAs targeting the same transcript (Dorsett and Tuschl, 2004).

We recommend the use of a stable transfected human bronchial epithelial cell line to confirm the results observed with transient siRNA transfections. The recent emergence of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 system for gene editing may take the analysis one step further, by introducing the heritable TLR3 deletions in the genome of the primary human bronchial epithelial cells, both submerged and differentiated into air-liquid interface cultures (Chu et al., 2015).

We also observed that TLR3 knockdown had abolished poly I:C-induced TGF-β expression while the impact on RSV-mediated TGF-β expression was marginal due to the variability in RSV data sets. Hence, more biological replicates need to be added to confirm the observation. Lastly, PAI-1 mRNA expression and phosphorylation of Smad2 need to be measured to further prove the role of TLR3 in TGF-β expression in response to RSV infection.

4.7 Conclusions from chapter 4

Data presented in the current chapter suggested TLR3 inhibition as a possible strategy for targeted management of RSV-induced respiratory diseases that respond poorly to steroids. We reached this conclusion for the following reasons: 1) TLR3-induced inflammatory responses are deleterious to RSV infection pathogenesis as demonstrated previously; 2) RSV infection upregulated TLR3 expression thereby aggravating the damaging effect of the induced inflammatory responses; 3) TLR3 knockdown has at least partially repressed the RSV-induced expression of TGF-β1; the main driver of steroid insensitivity; 4) TLR3 deficiency has restored the responsiveness of bronchial epithelium to glucocorticoids that remains to date the mainstay in the management of respiratory diseases.

CHAPTER 5

RSV Infection and Double-stranded RNA: Comparison of *In vivo* Responses

5 RSV infection and double-stranded RNA: comparison of *in vivo* responses

5.1 Introduction

In the preceding chapter, we presented the evidence that RSV impairs bronchial epithelial cells response to glucocorticoids. The aforementioned findings (Chapter 3) suggested that impairment of GCs sensitivity may be partially triggered by TGF-β since TGF-β inhibitors have been shown to restore the apparent glucocorticoid insensitivity *in vitro*. We have also implicated dsRNA/TLR3 as the driving downstream pathway subserving RSV-detrimental effects on glucocorticoid sensitivity. However, the complex disease processes that trigger chronic respiratory diseases, like asthma and COPD, can neither be modelled using cell culture techniques alone as there are many cell types implicated in the lung diseases pathophysiology including the recruitment of inflammatory cells and the remodelling of structural cells, which are a key component to the disease. Hence, it is essential to examine these relationships *in vivo*, where there might be more influences from cytokines and other inflammatory mediators involved in the pathophysiology of the disease. Hence in the current chapter, we investigated whether aforementioned *in vitro* findings could be replicated in a whole-body system with multiple cell types.

5.1.1 Experimental Models of RSV infection

5.1.1.1 Nonhuman primate models of hRSV

Humans are the natural hosts for RSV, but chimpanzees in contact with humans are readily infected and do display clinical signs similar to that observed in humans. Experimental infection of chimpanzees results in upper respiratory tract illness, nevertheless, no signs of lower airways illness has been identified (Belshe et al., 1977). The extent of attenuation of live-attenuated RSV vaccine candidates was assessed in chimpanzees, but the results have not always predicted the degree of attenuation in infants accurately (Cormier et al., 2010). A cold-passaged, temperature-sensitive live-attenuated RSV vaccine candidate developed at the National Institute of Allergy and Infectious Diseases, for example, was strongly attenuated in chimpanzees, yet induced significant nasal congestion in infants that interfered with eating and sleeping (Wright et al., 1976). Inconsistent prediction of attenuation and efficacy of vaccine candidates and ethical considerations make chimpanzees a seldom-used and inappropriate model for RSV infection and pre-clinical RSV vaccine development.

In addition to chimpanzees, several other nonhuman primate species were harnessed for studying RSV infection and evaluating vaccine candidates. African green monkeys, the most heavily utilized nonhuman primate species, are semi-permissive to RSV infection. This species has been used to model the enhanced disease observed following vaccinating infants with a formalin-inactivated RSV vaccine (Kakuk et al., 1993). Rhesus macaques were also employed for studying RSV disease, but the virus replicates poorly in this species, so PCR to detect viral replication products is typically required (De Swart et al., 2002). Considering the ethical and economic cost, nonhuman primates are not sufficiently sensitive to RSV infection to warrant study of this disease in these species.

5.1.1.2 Rodent Models for hRSV Disease

A major roadblock in elucidating the mechanism of RSV pathogenesis is the lack of a small animal model that accurately recapitulates human disease. Starting from the late 1970s, RSV disease has been extensively studied in small animals each of which has benefits and limitations (Cormier et al., 2010). Examples of these animal RSV models includes ferrets (Prince and Porter, 1976), guinea pigs (Hegele et al., 1993; Dakhama et al., 1997), cotton rats (Prince et al., 1978) and inbred mice (Prince et al., 1979). When interpreting data from rodent models, one should keep in mind that using highly inbred, or even outbred, rodents will not capture all of the potential responses of a genetically heterogeneous human population.

RSV-infected guinea pigs usually develop acute bronchiolitis with no clinical symptoms and weight loss (Hegele et al., 1993). Viral antigens are predominantly present in the epithelium of the airways in addition to alveolar macrophage. Intriguingly, RSV virions were reported in the lungs of infected guinea pigs for up to 60 days (Dakhama et al., 1997). RSV infection is found to upregulate cytokine expression and increase eosinophils, neutrophils and lymphocytes as well as airway hyperresponsiveness in naïve or OVA-sensitized guinea pigs (Robinson et al., 1997; Bramley et al., 2003). Ferrets infected with RSV affects the upper and lower respiratory tracts and elicits the production of IL-1α, IL-1 β, IFN- γ, TNF- α, IL-17 and various chemokines (Prince and Porter, 1976; Chan et al., 2018) and requires 9 days before the full clearance of RSV (Chan et al., 2018). Analogous to guinea pigs, RSV-infected ferrets develop no clinical symptoms (Prince and Porter, 1976). RSV replicates in infected ferrets lungs is an age-dependent manner with highest titers in the youngest animal (Prince and Porter, 1976).

Also, the cotton rats are susceptible to human RSV with analogous viral growth kinetics to mice with a peak viral titer on day 4 that falls to undetectable levels on day 7. RSV replicates in the lung of the cotton rat in an age-independent manner unlike in ferrets. The virions predominantly present in the epithelium of bronchi and bronchioles, yet absent in trachea and alveolar cells (Prince et al., 1978). The cotton rat model is standard for evaluating vaccines, neutralizing antibodies and antivirals (Cullen et al., 2015; Stobart et al., 2016).

The human RSV mouse model has been broadly employed to study viral pathogenesis and vaccine development compared to other small animal models (Prince et al., 1979; Taylor et al., 1984; Graham et al., 1988; Graham et al., 1991; Graham et al., 1993; Connors et al., 1994; Boelen et al., 2000; Jafri et al., 2004; Mukherjee et al., 2011b; Stokes et al., 2011; Openshaw, 2013; Rameix-Welti et al., 2014; Knudson et al., 2015). This is grounded on the wide pool of accessible immunological reagents as well as commercially available knockout and transgenic mice. The strengths of the mouse model stem from the low cost to acquire and house this species, the availability of immunological reagents and the ease with which this species is genetically modified.

The mouse model of RSV infection, however, has some limitations. First, mice have lower susceptibility to human RSV infection and, in contrast to human patients, primarily the lower, not the upper airway is infected (Stark et al., 2002; Stokes et al., 2011). Second, the mouse upper airway is quite resistant to RSV infection and a large volume, high virus titre inoculum is required to induce lower airway virus replication and generates viral titers systematically lower than that inoculated (Jafri et al., 2004; Miller et al., 2004; Bem et al., 2011). Finally, virus replication takes place in mouse alveolar lining cells and to a lesser extent bronchial lining cells, the cell type targeted by RSV in human infection (Johnson et al., 2007; Taylor, 2017; Altamirano-Lagos et al., 2019). This likely has some consequences regarding the pulmonary response to RSV infection, as the early window for innate and adaptive immune responses that occur during the upper airway phase of the disease are not allowed to develop. Additionally, RSV tends to produce a large number of replication-deficient antigen particles that would be confined to the upper airway in natural disease but contribute to the immense antigen load encountered by the pulmonary parenchyma when viral inoculums are delivered directly to the lung. Overall, using the mouse model of RSV disease to mimic the human disease is rather counterintuitive but has successfully elucidated certain mechanisms of RSV pathogenesis, including viral antagonism of the interferon pathway, and to evaluate the contribution of certain cellular subsets and

cytokines to the disease process (Bem et al., 2011; Yang et al., 2015; Hijano et al., 2019; Stephens and Varga, 2020).

The BALB/c mouse is the most frequent strain reported in human RSV-infected mouse models. The standard laboratory RSV strains used for mice infection are A2 (Taylor et al., 1984) and long strains (Prince et al., 1979). RSV titers peak in the mouse lung on days 4-6 post-infection before falling to undetectable levels on day 11 (Taylor et al., 1984; Anderson et al., 1990). RSV A2 strain replicates in the older mice lungs significantly greater than in younger mice lungs (Taylor et al., 1984; Graham et al., 1988; Ruckwardt et al., 2011), although in newborn mice, RSV infection exhibits similar viral kinetics to that in adults where the viral titer peaks on day 4 post-infection (Cormier et al., 2010; Empey et al., 2012). Gender does not appear to alter the susceptibility to RSV infection or viral replication (Taylor et al., 1984). Hence, we have harnessed the BALB/c mouse strain in the current chapter to test whether we can replicate the aforementioned *in vitro* results *in vivo* in an acute model of RSV disease.

Although the RSV mouse model of RSV infection has provided much of our understanding regarding allergic responses after presensitization (Openshaw, 1995; Graham, 1996), RSV is not a natural pathogen of rodents, hence *in vivo* viral replication *is* limited. Additionally, the clinical and pathophysiologic sequela in mice do not mimic those of severe RSV infection in humans. Therefore, acute respiratory infection mouse model was developed using pneumonia virus of mice (PVM) (Horsfall and Hahn 1940). PVM is a pneumovirus and natural pathogen of rodent and the closest phylogenetic relative of RSV (Easton et al., 2004; Rima et al., 2019). Mice inoculation with as few as 10 pfu of PVM results in acute inflammatory response that progress to respiratory failure and death at lung viral titers more than 10^6 pfu/g of lung tissue (Domachowske et al., 2001). The pathogenesis of PVM infection in mice closely imitates the signs and symptoms of severe human RSV infection. First, PVM is localized to the bronchiolar epithelium (Bonville et al., 2006), parallel to the distribution observed for RSV in human post-mortem specimens (Welliver et al., 2007). Second, profound inflammation of the lungs is evident by the recruitment of lymphocytes, neutrophils, and eosinophils and severe pulmonary oedema. Third, viral replication in the mouse lung tissue results in local production of proinflammatory mediators including MIP-1α, MIP-2, and MCP-1, IFNγ (Bonville et al., 2006), consistent with those detected in nasal washings and lung of human infants with RSV triggered bronchiolitis (Domachowske et al., 2004; Easton et al., 2004).

Although PVM model mimics some features that clearly conform to human pathophysiology, others do not (Dyer et al., 2012). For instance, PVM infection induce little to no overt inflammation in neonatal mice (Bonville et al., 2007). Likewise, a distinct pattern of infection cannot be established in aged mice (Bonville et al., 2010). Another drawback of PVM model include the high genetic distance between PVM and hRSV ranging from 10 to 60%, and lack of direct cross-reactivity (Krempl et al., 2005; Dyer et al., 2012).

In addition to the above-mentioned limitations, minimal virus inoculum results in robust virus replication *in situ* progressing to marked morbidity (hunching, fur ruffling), weight loss, and mortality in PVM-infected mice (Domachowske et al., 2000a; Domachowske et al., 2000b; Anh et al., 2006; Dyer et al., 2012). Furthermore, the current study involved administration of glucocorticoids that were previously reported to enhance viral replication and accelerate mortality in PVM-infected mice due to the added immunosuppression (Domachowske et al., 2001). However, RSV infection of mice does not lead to any measurable degree of morbidity, induces a mild mononuclear cell infiltration to the lung instead of a profuse granulocytic bronchiolitis, and never progresses to acute respiratory distress syndrome (ARDS) (Anh et al., 2006). For these reasons, we selected RSV A2 strain to pursue the current study.

Mouse models of human RSV infection employ different subsets of immune cells in disease development. Acute RSV infection triggers cytotoxic responses in the lung where epithelial cells release innate chemokines that attract innate immune cells to the infection site. The airway epithelium also includes resident dendritic cells that respond to allergens, pathogens and damage signals. In addition to resident dendritic cells, exudative macrophages (also named inflammatory monocytes) and innate lymphoid cells have been shown to be activated following viral infection. Epithelial-derived innate cytokines (IL-25, IL-33 and TSLP) play a crucial role where they activate type 2 innate lymphoid cells (ILC2s) and type 2 helper T (Th2) cells that release type 2 cytokines (IL-4, IL-5 and IL-13) which in turn leads to mucus metaplasia. Hence, under certain circumstances, allergen exposure is not essential to initiate type 2 inflammation by viral infection (Figure 5.1) (Han et al., 2018; van Erp et al., 2019).

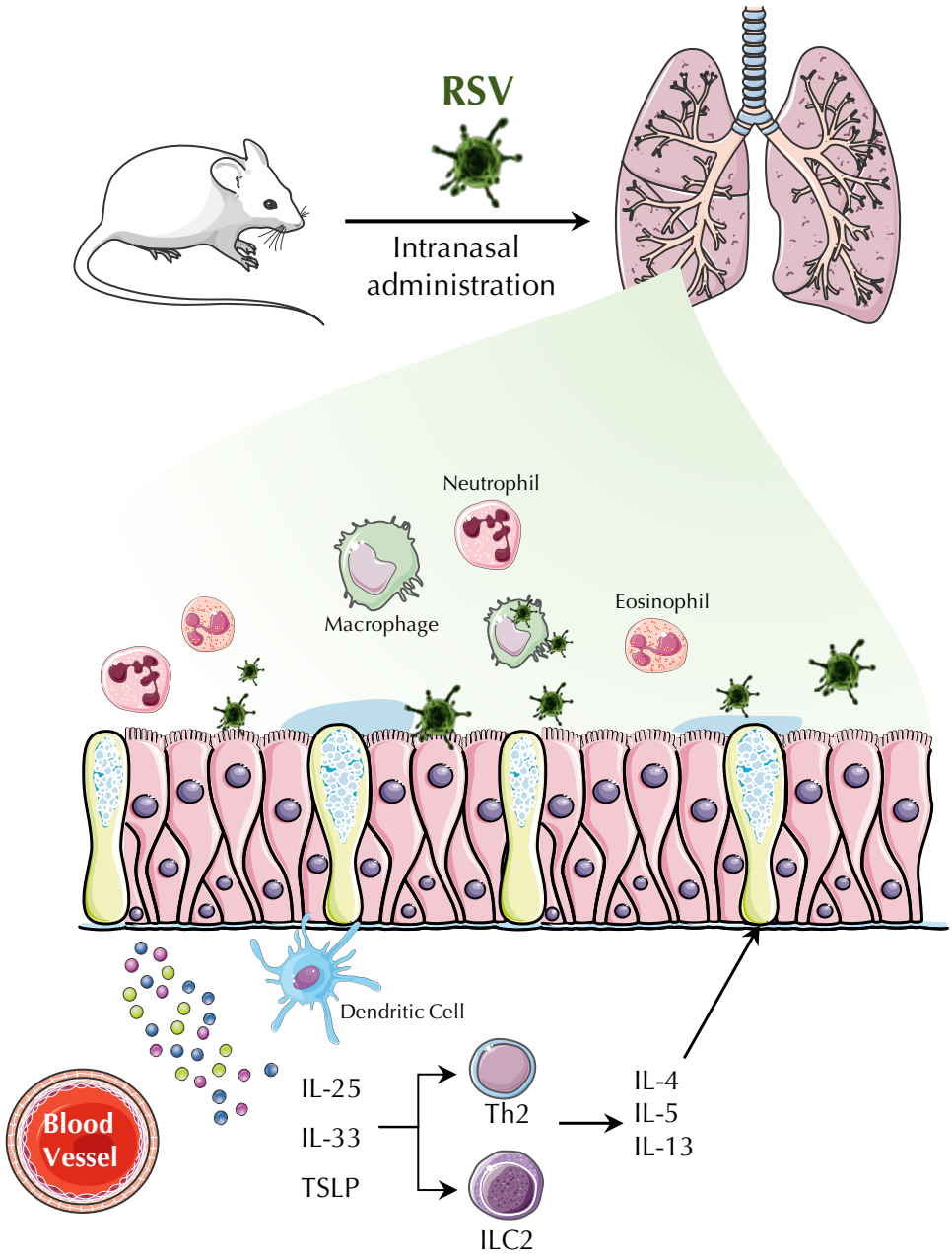


Figure 5.1 | Array of airway cells involved in RSV pathogenesis in mice.

5.2 Aims

The perturbed genes expression that occurs following the epithelial cell line RSV infection truly indicates an oversimplification of the complex pathology occurring *in vivo*. Peripheral blood monocytes, lymphocytes, dendritic cells and macrophages directly respond to viral infection or the cytokines and chemokines released by other infected cells, and these interactions were not part of *in vitro* experiments.

The present chapter aimed at determining whether poly(I:C) can trigger lung inflammation in BALB/c mice, as well as characterizing the degree of concordances between the effects of poly(I:C) treatment and RSV infection to help unravel potential common pathways of action. Moreover, by comparing RSV and poly I:C responses, might also determine the extent to which other sensing PRRs might be involved. This may in part help to determine if it is the replication of live virus which causes lung inflammation and GC insensitivity or if it is merely the signalling caused by recognition of the virus. This may lead to changes in the approach to therapy and drugs which did not alter symptoms before, but reduced replication may have a profound impact down-stream if steroids are prescribed for future illness or exacerbation. Additionally, we have explored whether the beneficial impact of CK1δ/ε inhibition observed *in vitro* could be replicated *in vivo*.

5.3 Methods

5.3.1 Animals and ethics

Eight- to ten-week-old female BALB/c mice (Animal Resources Centre, Perth, WA, Australia) were housed at in the Biomedical Sciences Animal Facility (the University of Melbourne) in a temperature-controlled room (22°C±3°C) under normal 12:12 h light: dark cycle. Food (Purina mouse chow) and water were provided to mice *ad libitum*. Mice were allowed to acclimatize for at least 5-7 days before the studies commencement. Mice were weighed daily and monitored during the experimental period for any signs of distress such as inactivity, laboured breathing, lethargy, loss of appetite and piloerection, ruffled fur, and huddling behaviour. Animals that had experienced any of these signs or had lost 15% of their original body weight were euthanized by an overdose of anaesthetic.

All experimental protocols were performed in compliance with the Animal Ethics Committee of the University of Melbourne (Ethics #1413340, #1413405) and the guidelines of the National Health and Medical Research Council (NHMRC) Australian Code of Practice for animal experimentation.

5.3.2 Drug preparation and administration

Dexamethasone (Sigma-Aldrich, Castle Hill, Australia), PF670462 (Abcam, Australia) or GW788388 (GlaxoSmithkline, Australia) was dissolved in DMSO (10%v/v; Ajax finechem) before being suspended in Arachis oil (90% v/v; Sigma Aldrich). Drugs were stored at -20°C, then thawed and vigorously mixed prior to use to ensure that DMSO and Arachis oil phases were properly mixed. For study groups receiving both drugs, Dexamethasone and PF670462 or GW788388 were co-formulated in appropriate doses

using the same vehicle (90% Arachis oil and 10% DMSO). The dose of dexamethasone used was either 0.1mg/kg or 1mg/kg whilst PF670462 and GW788388 doses used were 30 mg/kg and 10 mg/kg respectively, based on the average weight of mice in the study. Drugs were administered by intraperitoneal injection to conscious mice in a volume of 100 µl using a 27-gauge needle on a 1 ml syringe. Control mice received vehicle (90% Arachis oil and 10% DMSO) to ensure that there were no non-specific interactions with the vehicle used for drug delivery.

5.3.3 Mouse model for RSV infection

Human RSV, A2 strain (Human RSV, A2 strain (ATCC® VR-1540P™), was used in the current study. Female BALB/c mice were assigned to eight weight-matched study groups (6 mice/group). Mice were lightly anaesthetised with the inhalation anaesthetic isoflurane vaporised in O_2. A mouse was placed in an airtight plastic induction chamber with a flow rate of O_2 between 1.0-2.0 L/min containing 2-3 % isoflurane. Once the righting reflex was lost as tested by rocking the tub gently, the animal was appropriately anaesthetised and ready for the procedure. Unconscious mice were intranasally inoculated with 2,000,000 $TCID_{50}$ units of RSV in a volume of 50 µl of diluent (VP-SFM). The infective dose chosen in the current study was reported previously to develop infection that could be readily detected in lung homogenates and was accompanied by pronounced pulmonary pathology as indicated by pulmonary cellular influx and pro-inflammatory cytokine and chemokine levels (Muñoz et al., 1991; van Schaik et al., 1998; van Erp et al., 2019). Mice were then allowed to recover on a heating pad until they were active before being returned to their home cages. RSV infection causes a mild to moderate airway inflammation, but the mice remain asymptomatic with no overt disease. Mice were euthanised 5 days post RSV inoculation. Control animals were inoculated intranasally with an equal volume of mock prepared as previously described in Section 2.2 and diluted with VP-SFM at the same concentration as the stock virus.

One day after RSV infection or mock inoculation, each study group received a daily intraperitoneal injection of either vehicle (90% Arachis oil and 10% DMSO), dexamethasone (1 mg/kg), PF670462 (30 mg/kg), the TGF-β receptor kinase inhibitor, GW788388 (10 mg/kg) or a co-formulation of dexamethasone with either GW788388 or PF670462 as outlined in the experimental design in Figure 5.2. On day 5, mice were euthanized four hours after daily dosing.

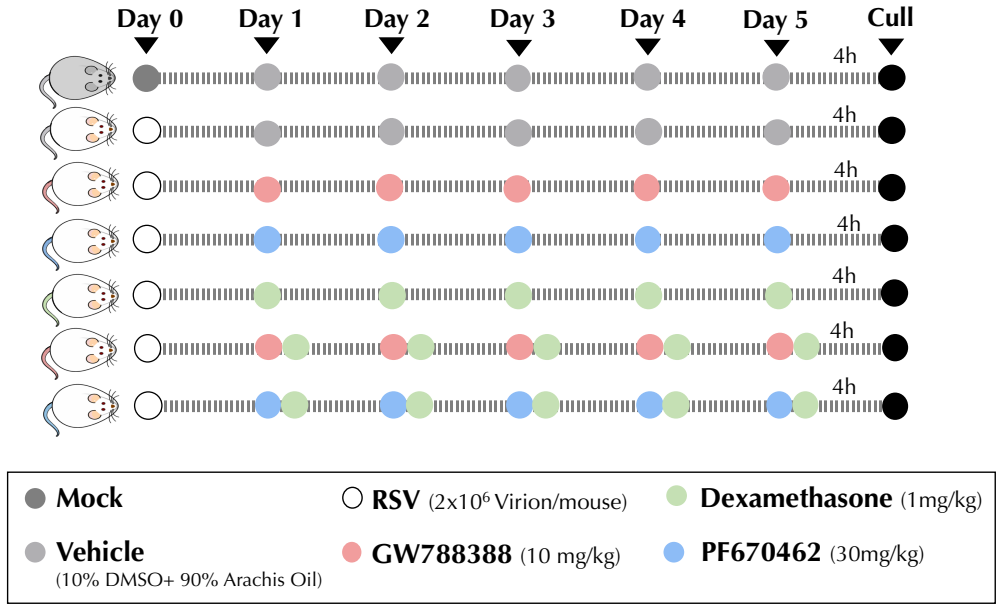


Figure 5.2| Schematic outline of Experimental design for RSV study.
Eight- to ten-week-old female BALB/c mice were intranasally inoculated with either Mock or 2 million RSV virions under anaesthesia with 2-3% isoflurane. Starting from day 1, mice received a daily intraperitoneal injection of either vehicle (90% Arachis oil and 10% DMSO), dexamethasone (1 mg/kg), PF670462 (30 mg/kg), GW788388 (10 mg/kg) or a co-formulation of dexamethasone with either GW788388 or PF670462. On day 5, Mice were euthanized.

5.3.4 Mouse model of Poly I:C acute lung injury

Female BALB/c mice were assigned to 5 weight-matched study groups (6 mice/group). Mice were lightly anaesthetised with the inhalation anaesthetic isoflurane vaporised in O_2. A mouse was placed in an airtight plastic induction chamber with a flow rate of O_2 between 1.0-2.0 L/min containing 2-3 % isoflurane. Once the righting reflex was lost as tested by rocking the tub gently, the animal was appropriately anaesthetised and ready for the procedure. Unconscious mice were intranasally administered 100 µg of poly I:C in a volume of 50 µl. Mice were then be allowed to recover on a heating pad until they are active before being returned to their home cages. Saline/vehicle control mice received 50 µl of saline only. The poly I:C stimulus causes a mild to moderate airway inflammation, but the mice remain asymptomatic. An experimental study was carried out according to the design outlined in Figure 5.3. Briefly, on day 0, all study groups received an intraperitoneal injection of either PF670462 (30 mg/kg) or vehicle (100 µl; 90% Arachis oil and 10% DMSO). On day 1 and prior to poly I:C instillation, mice were injected with either vehicle, dexamethasone (0.1 mg/kg), PF670462 (30 mg/kg) or co-formulation of both dexamethasone (0.1 mg/kg) and PF670462 (30 mg/kg). Twenty-four hours after Poly I:C instillation, mice were culled.

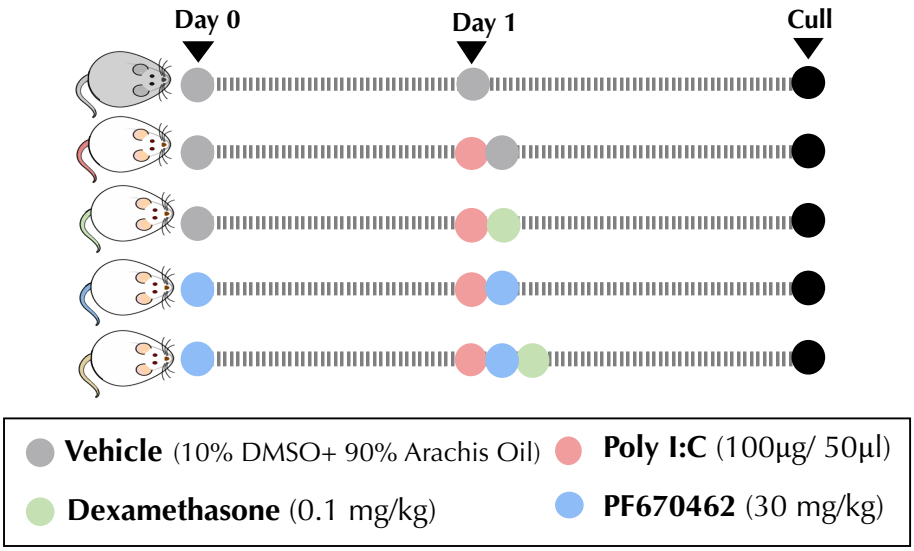


Figure 5.3| Schematic outline of Experimental design of poly I:C acute lung injury study. Mice received an intraperitoneal injection of either PF670462 (30 mg/kg) or vehicle (100 µl; 90% Arachis oil and 10% DMSO). On day later and before poly I:C instillation, mice were injected with either vehicle, dexamethasone (0.1 mg/kg), PF670462 (30 mg/kg) or co-formulation of both dexamethasone and PF670462. Twenty-four hours after challenging by intranasal poly I:C instillation, mice were culled.

5.3.5 Experimental study endpoint

At the conclusion of the experiment, mice were euthanized by an intraperitoneal overdose of pentobarbitone (150 mg/kg) buffered in PBS using a 27-gauge needle. Animal Death was confirmed by stopping of breathing, loss of footpad colour and reflex. Death was further confirmed through collecting blood from posterior vena cava using 1ml syringe with 21-gauge needle. The mice tracheas were exposed surgically and cannulated for bronchoalveolar lavage (BAL) using a trimmed sterile 18-gauge intravenous catheter inserted caudally into the lumen. Lungs were lavaged by 300 µl of 0.9 % saline (Pfizer Pty Ltd., WA, Australia) three times and the wash fluid was collected and kept on ice until further analysis. Following lavage, the lungs were dissected, weighed and snap-frozen in liquid nitrogen then stored at -80°C until further analysis. Other organs such as thymus, heart, liver, spleen, kidney and uterus were dissected and weighed to determine any drug-induced weight changes.

For gene expression analyses, snap-frozen lungs were pulverised in an RNase-free mortar and pestle under liquid nitrogen and homogenised in an appropriate volume of RLT buffer/ 1% β-mercaptoethanol, by passing the tissue through a 23-gauge needle with 1 ml sterile syringe attached 5–10 times. Total RNA was isolated from a portion of the homogenised tissue using Qiagen RNeasy Mini kits, according to the manufacturer's instructions.

5.3.6 Total and differential cell counts in bronchoalveolar lavage

Cellular inflammation was assessed by total and differential cell counts in bronchoalveolar lavage fluid (BALF). Total viable cell count was determined using the fluorescent viability stain ethidium bromide acridine orange (Molecular Probes., Invitrogen). A small aliquot (10 – 20 µl) of BAL was incubation on ice for 30 s with an equal volume of viability stain (acridine orange/ethidium bromide/saline) and counted on a standard Neubauer haemocytometer under fluorescent light on an epifluorescence microscope (Zeiss Axioshop, Germany). In this method, viable cells incorporate acridine orange and appear bright green in colour.

To determine differential cell types within BAL samples, cytospins of cells were prepared by centrifuging 200 µl of the sample at 400 rpm for 10 min. Cytospins were allowed to air-dry before staining with DiffQuik reagents (Dade Behring, USA); fixative (triarylmethane dye & methanol, 5 mins), followed by xanthene dye (red; 10 min), thiazine dye (blue; 2 min) and finally excess dyes were gently washed away with running H_2O. Once dry, glass coverslips were adhered to the cytospins with entellan (Merck, Australia) and differential cell counts assessed by bright field microscopy. Macrophages, neutrophils, eosinophils and lymphocytes were identified and counted according to standard morphological criteria, (lymphocyte; small, dark purple, macrophage; dark purple nucleus with pale cytoplasm, neutrophil; irregular shaped nuclear body with bluish cytoplasm, eosinophil; red cytoplasm) with a total of 500 cells counted per slide under oil immersion at 40X magnification (Olympus BX52, Olympus Optical, Japan).

5.3.7 Collection of bronchoalveolar lavage cells and supernatant

After cell counts had been performed, BAL fluid was pelleted (10,000 rpm, 10 min) to eliminate any remaining cells. The supernatant was collected, transferred to 1.5 ml Eppendorf tubes and stored at -20°C for subsequent total protein concentrations measurement using the Bio-Rad protein assay method, as described in Section 2.7.2. The remaining cell pellet was lysed in 350 µl RLT buffer/ 1% β-mercaptoethanol using a 23-gauge needle with 1 ml sterile syringe attached. The lysed cell pellet was snap-frozen prior to storage at -80°C for later molecular analysis. Total RNA was purified QIAGEN RNeasy Mini-kits (Qiagen Inc., Valencia, CA) according to manufacturer's instructions.

5.4 Results

5.4.1 Change in body weight in female 9-week-old BALB/c mice

Weight loss is usually indicative of RSV illness severity in the BALB/c mouse model. Over the course of the study, mice were observed on a daily basis for any bodyweight changes. Individual mouse's weight was calculated as a percentage of its weight on day 0, such that any weight loss would be below 100%. At all-time points, control animals maintained a mean weight of more than 100%. On the other hand, BALB/c mice infected with 2,000,000 virions showed the first signs of weight drop on day 2 post-infection, becoming significantly different compared to the control group treated with mock-conditioned media on day 3 post-infection. (Figure 5.4). Mice receiving dexamethasone showed a significant earlier decline in body weight starting on day 2 post-infection and continued till the end of the study. Treatment with PF670462 or GW788388 failed to attenuate weight loss in RSV-infected mice.

Conversely, in a murine model of poly I:C acute lung injury, mice in all groups did not experience any significant change in body weight compared to control group over the course of the study (Figure 5.5).

5.4.2 Change in organs weight in female 9-week-old BALB/c mice

Immediately after the mice were euthanized, organs including lung, thymus, liver, kidney, spleen, thymus and uterus, were dissected from the mice, examined macroscopically, and weighed. The percentage of organ weights relative to the final body weight was then calculated.

As demonstrated in Table 5.1, RSV inoculation produced a substantial reduction in the thymus weight, while it did not affect the weight of any other organs. Treatment of RSV-infected mice with dexamethasone or PF670462 on a daily basis had caused a further decline in the thymus weight and a significant reduction in spleen weight compared to mock-infected mice. Furthermore, neither Dexamethasone nor PF670462 had any effect on the weights of liver, kidney or uterus. GW788388 administration caused a further fall in the thymus weight and significantly decreased the liver weight, while no notable changes were observed in the spleen, kidney and uterus weights.

Intranasal instillation of poly I:C had no notable change in any of the organs weighed compared to saline-treated mice. Likewise, mice treated with dexamethasone, PF670462 or co-formulation of both did not show any change in organs weight (Table 5.2).

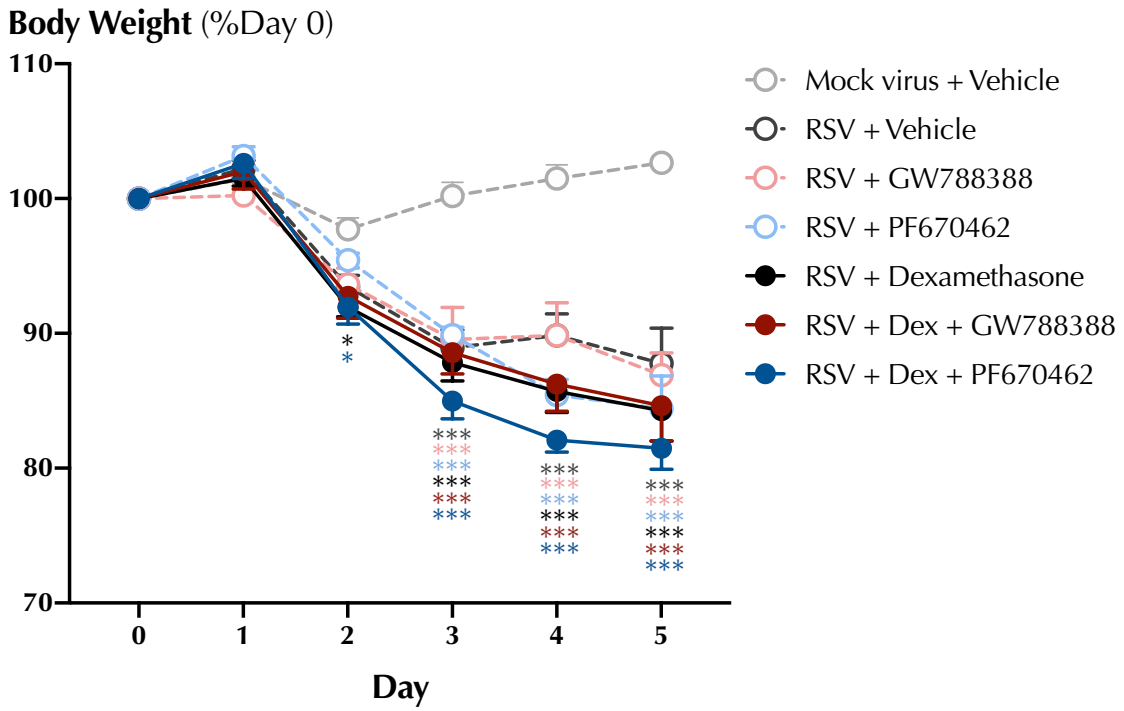


Figure 5.4| Change in body weight in RSV infected female BALB/c mice.
Eight- to ten-week-old female BALB/c mice were intranasally inoculated with either Mock or 2 million RSV virions under anaesthesia with 2-3% isoflurane. Starting from day 1, mice received a daily intraperitoneal injection of either vehicle (90% Arachis oil and 10% DMSO), dexamethasone (1 mg/kg), PF670462 (30 mg/kg), GW788388 (10 mg/kg) or a co-formulation of dexamethasone with either GW788388 or PF670462. Mice were weighed on a daily basis for 5 days post-infection and weights were displayed as a percentage of individual mouse's weight on day 0. Data are presented as means and SEM for n=5-6 mice/treatment group. Statistical significance (relative to the time-matched Mock control at each time point) was determined by Ordinary two-way ANOVA, Dunnett's multiple comparisons post-hoc test. *P*-values lower than 0.05 were represented as *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$.

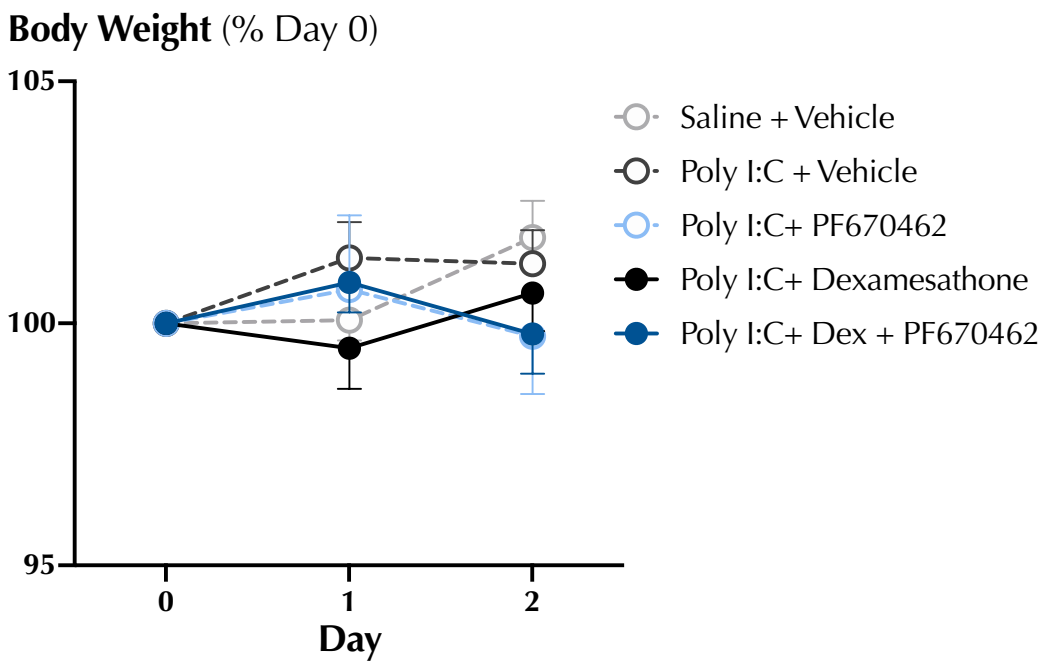


Figure 5.5 | Change in body weight of female BALB/c mice in poly I:C acute lung injury study.
Mice received an intraperitoneal injection of either PF670462 (30 mg/kg) or vehicle (100 µl; 90% Arachis oil and 10% DMSO). One day later and before poly I:C instillation, mice were injected with either vehicle, dexamethasone (0.1 mg/kg), PF670462 (30 mg/kg) or co-formulation of both dexamethasone and PF670462. Twenty-four hours following challenging by intranasal poly I:C instillation, mice were culled. Mice were weighed daily, and weights were displayed as a percentage of each mouse's weight on day 0. Data are presented as means and SEM for n=6 mice/treatment group. Statistical significance (relative to the time-matched control at each time point) was determined by Ordinary two-way ANOVA, Dunnett's multiple comparisons post-hoc test.

Table 5.1| Organ weight in female BALB/c mice in 5-days model of RSV infection.

Treatment Group	Thymus	Liver	Kidney	Spleen	Uterus
	Weight (% body weight)				
Mock virus + Vehicle	0.17± 0.008	4.43±0.112	0.602±0.0189	0.55±0.02	0.488±0.0696
RSV + Vehicle	0.09± 0.006 **	4.06±0.137	0.615±0.0181	0.54±0.03	0.499±0.105
RSV + GW788388	0.047±0.01 **	3.76±0.127 *	0.613±0.0159	0.52±0.02	0.423±0.0301
RSV + PF670462	0.059± 0.01 ***	4.24±0.154	0.635±0.0248	0.31±0.01 ***	0.346±0.0135
RSV + Dexamethasone	0.010± 0.01 ***	4.59±0.134	0.605±0.0118	0.28±0.01 ***	0.46±0.0775
RSV + Dex + GW788388	0.031± 0.01 ***	4.45±0.115	0.634±0.0204	0.29±0.02 ***	0.302±0.0364
RSV + Dex + PF670462	0.051± 0.01 ***, ##	4.96±0.114 ###	0.612±0.0228	0.22±0.01 ***	0.399±0.0432

Eight- to ten-week-old female BALB/c mice were intranasally inoculated with either Mock or 2 million RSV virions under anaesthesia with 2-3% isoflurane. Starting from day 1, mice received a daily intraperitoneal injection of either vehicle (90% Arachis oil and 10% DMSO), dexamethasone (1 mg/kg), PF670462 (30 mg/kg), GW788388 (10 mg/kg) or a co-formulation of dexamethasone with either GW788388 or PF670462. On day 5, mice were euthanized four hours after daily dosing, and thymus, liver, kidney, spleen and uterus were dissected and weighed. Organ weights are expressed as % of the body weight. Data are presented as means and SEM for n=5-6 mice/treatment group. Statistical significance (relative to * Mock control group or # RSV-infected group) was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is denoted as follows: * or #; $p<0.05$, ** or ##; $p<0.01$, *** or ###; $p<0.001$.

Table 5.2| Change in organs weight in female BALB/c mice in poly I:C acute lung injury study.

Treatment Group	Thymus	Liver	Kidney	Spleen	Uterus
	Weight (% body weight)				
Saline + Vehicle	0.239±0.029	4.41±0.12	0.595± 0.02	0.377±0.01	0.208± 0.03
Poly I:C + Vehicle	0.234±0.025	4.23±0.01	0.602± 0.02	0.376±0.02	0.171±0.03
Poly I:C + PF670462	0.231±0.014	4.51±0.16	0.575± 0.02	0.393±0.04	0.217± 0.03
Poly I:C + Dexamethasone	0.247±0.021	4.37±0.18	0.573± 0.02	0.433±0.03	0.221±0.05
Poly I:C + Dex + PF670462	0.192±0.018	4.45±0.17	0.591± 0.01	0.384±0.02	0.192± 0.02

Mice received an intraperitoneal injection of either PF670462 (30 mg/kg) or vehicle (100 µl; 90% Arachis oil and 10% DMSO). On day later and before poly I:C instillation, mice were injected with either vehicle, dexamethasone (0.1 mg/kg), PF670462 (30 mg/kg) or co-formulation of both dexamethasone and PF670462. Twenty-four hours following poly I:C instillation, mice were culled, and thymus, liver, kidney, spleen and uterus were dissected and weighed. Organ weights were expressed as % of the body weight. Data are displayed as means and SEM for n=6 mice/treatment group. Statistical significance was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test.

5.4.3 Indices of pulmonary injury in 5-days murine model of RSV infection.

To characterize the pulmonary pathogenesis following RSV infection, lung weight and BALF protein and cellular content were measured as indices of pulmonary injury. As demonstrated in Figure 5.6, inoculation of RSV in BALB/c mice induced a substantial increase in lung weight, bronchoalveolar lavage fluid protein as well as cellular content.

As demonstrated in Figure 5.6 C, treatment with either dexamethasone or PF670462 alone directly reduced cell recruitment following RSV infection, as did the combination which was associated with BAL cell count decreasing to baseline levels. However, treatment of RSV-infected mice with GW788388 alone had no remarkable change in BAL cells counts. The profound reduction achieved by dexamethasone alone was maintained in dexamethasone plus GW788388-treated mice. None of the treatments; dexamethasone, PF670462, GW788388 or dexamethasone co-formulated with PF670462 or GW788388 had altered the increase in lung weight or BAL cell protein levels in RSV-infected mice (Figure 5.6 A and B).

In order to assess the relative populations of inflammatory cells implicated, cells recovered from lung lavages and differential analysis was done on prepared cytospins of the BAL cells. Figure 5.7 shows the numbers of neutrophils, monocytes, eosinophils and lymphocytes in the lungs of infected and control mice. RSV infection markedly increased the recruitment of neutrophils and lymphocytes as evident by increased cell counts. However, monocytes number in BAL following RSV infection was not changed. The combination of dexamethasone and PF670462 reduced lymphocytes counts in BALF, whereas neither compound alone had an effect. Such synergistic interaction seen in dexamethasone and PF670462 was not reproduced in dexamethasone plus GW788388-treated mice. Additionally, none of the treatments used had any detectable effect on neutrophils counts in RSV-infected mice.

As demonstrated in Figure 5.8, RSV infection triggered the expression of intracellular adhesion molecule 1 (*ICAM-1*), albeit non-significantly ($p= 0.0692$). Treatment of RSV-infected mice with dexamethasone alone or concurrently with either PF670462 or GW788388 had no detectable effect on *ICAM-1* expression, suggesting insensitivity to tested compounds.

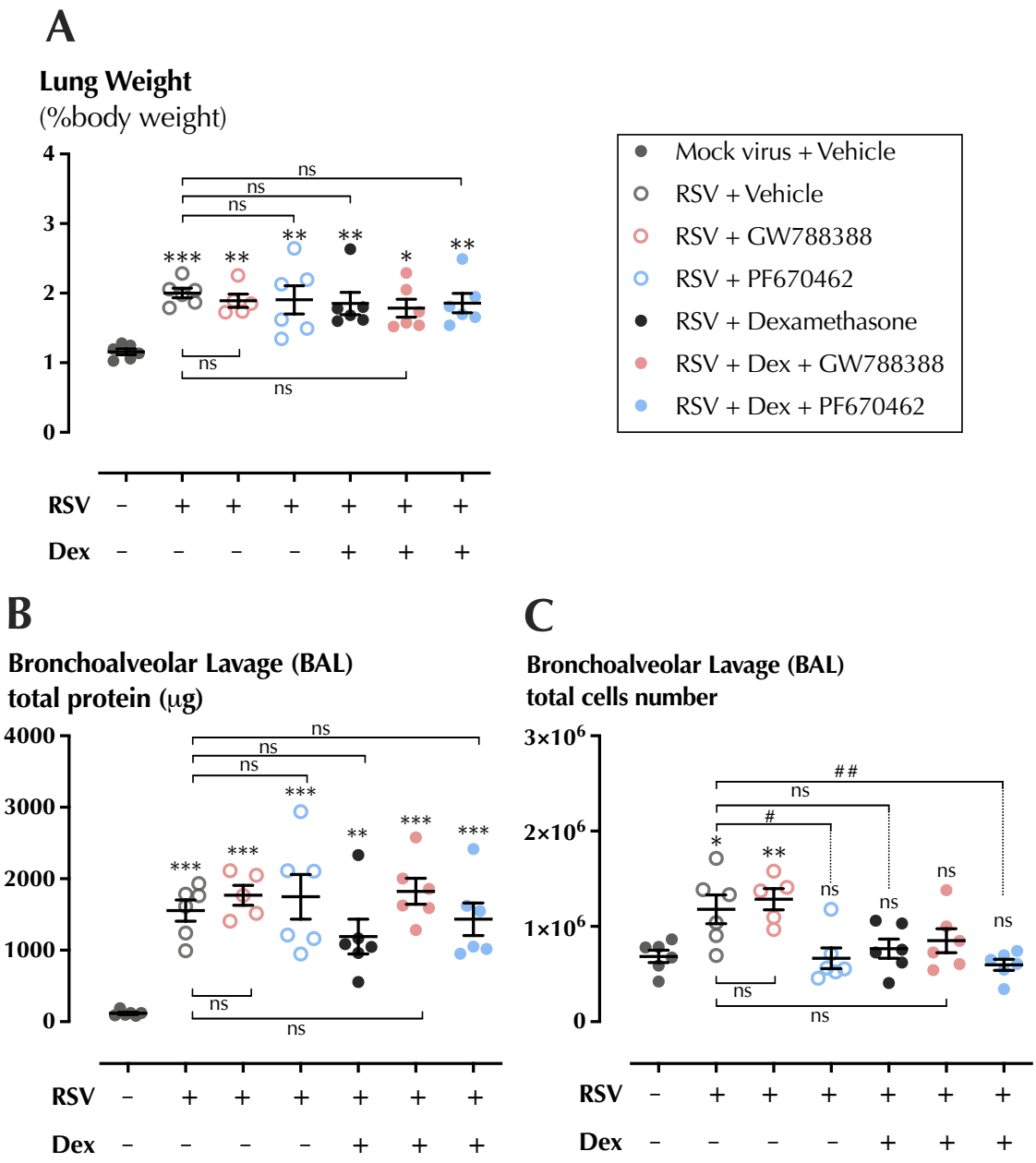


Figure 5.6| Indices of pulmonary injury in 5-days murine model of RSV infection.
Eight- to ten-week-old female BALB/c mice were intranasally inoculated with either Mock or 2 million RSV virions under anaesthesia with 2-3% isoflurane. Starting from day 1, mice received a daily intraperitoneal injection of either vehicle (90% Arachis oil and 10% DMSO), dexamethasone (1 mg/kg), PF670462 (30 mg/kg), GW788388 (10 mg/kg) or a co-formulation of dexamethasone with either GW788388 or PF670462. On day 5, mice were euthanized four hours after daily dosing. A, Lungs were dissected and weighed. Lung weight is expressed as a % of the body weight. Bronchoalveolar lavage was collected for subsequent analysis to determine total proteins (B) and total cell number (C). Data are presented as means and SEM for n=5-6 mice/treatment group. Statistical significance (* *c.f.* Mock control group or # *c.f.* RSV-infected group) was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: * or #; p<0.05, **or ##; p<0.01, ***; p<0.001, ns; non-significant.

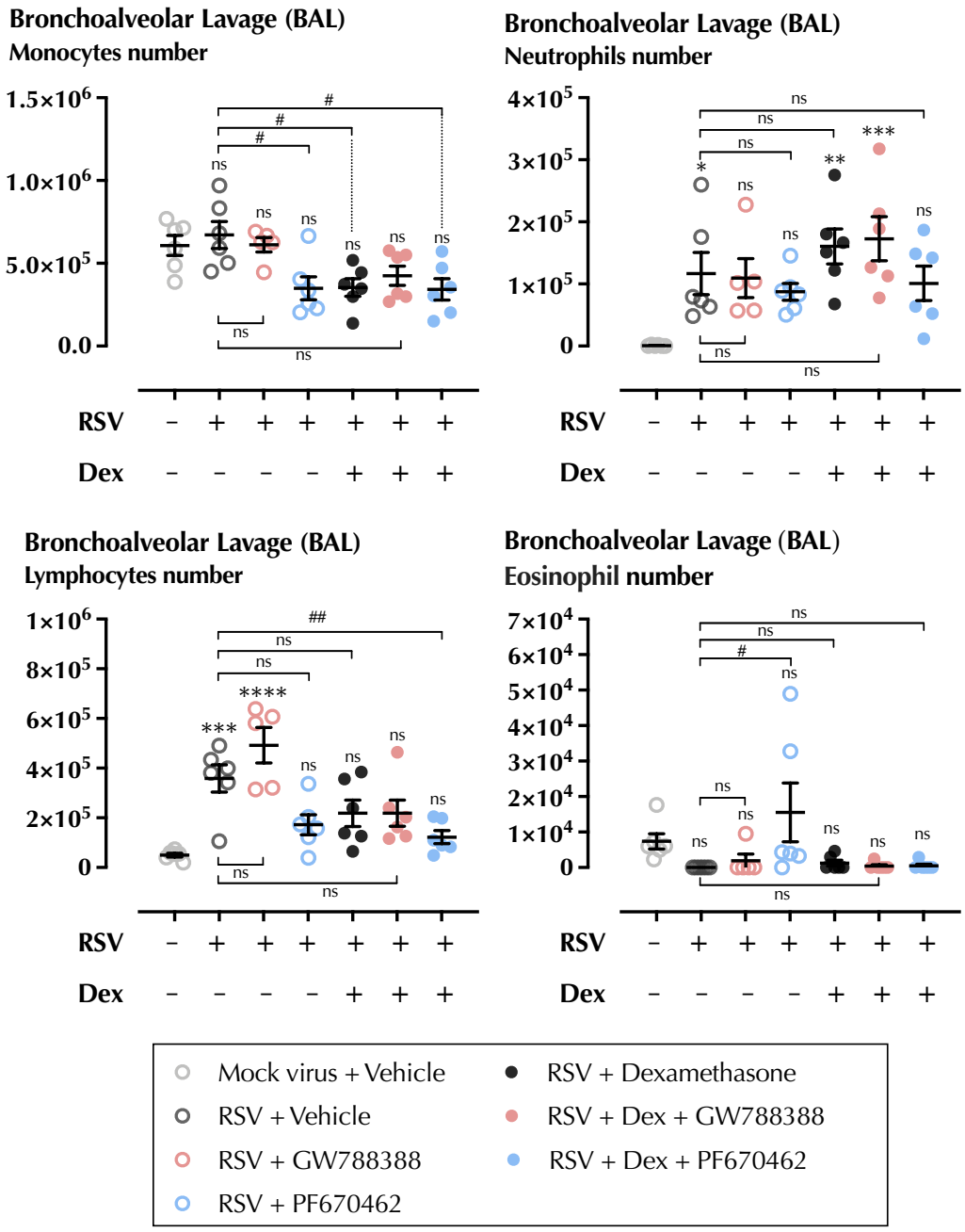


Figure 5.7| Differential cell count of bronchoalveolar lavage in 5-days murine model of RSV infection.

Eight- to ten-week-old female BALB/c mice were intranasally inoculated with either Mock or 2 million RSV virions under anaesthesia with 2-3% isoflurane. Starting from day 1, mice received a daily intraperitoneal injection of either vehicle (90% Arachis oil and 10% DMSO), dexamethasone (1 mg/kg), PF670462 (30 mg/kg), GW788388 (10 mg/kg) or a co-formulation of dexamethasone with either GW788388 or PF670462. On day 5, mice were euthanized four hours after daily dosing. Lungs were dissected and bronchoalveolar lavage was collected and used to determine differential cell count. Data are presented as means and SEM for n=5-6 mice/treatment group. Statistical significance (* *c.f.* Mock control group or # *c.f.* RSV-infected group) was determined by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: * or #; $p<0.05$, **or ##; $p<0.01$, ***; $p<0.001$, ns; non-significant.

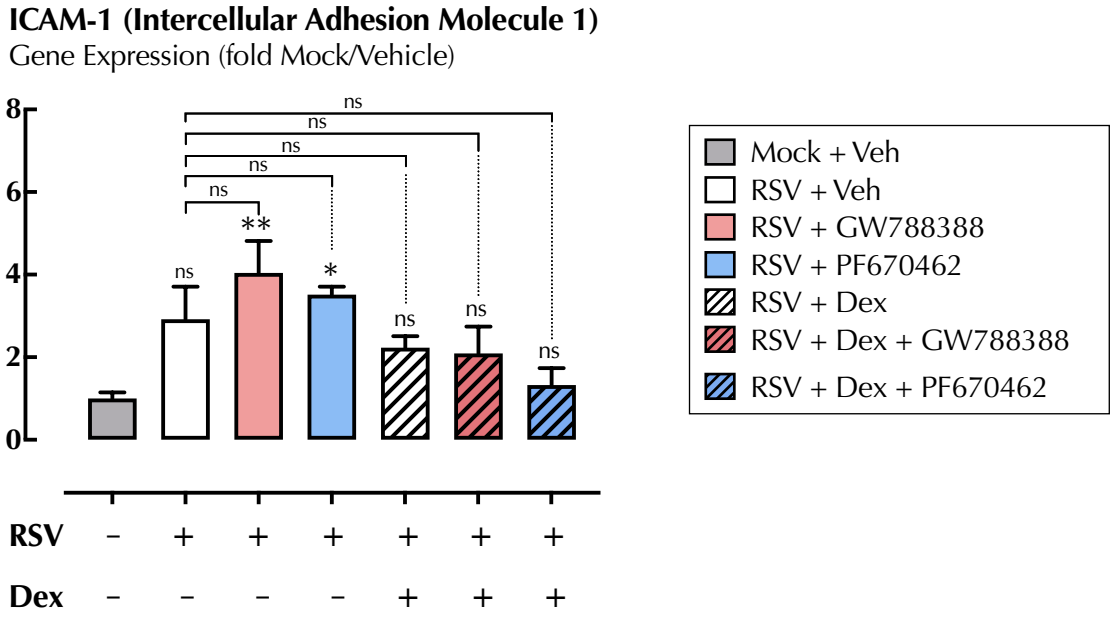


Figure 5.8| Intracellular adhesion molecule 1 expression in lung tissue following RSV infection.

Eight- to ten-week-old female BALB/c mice were intranasally inoculated with either Mock or 2 million RSV virions under anaesthesia with 2-3% isoflurane. Starting from day 1, mice received a daily intraperitoneal injection of either vehicle (90% Arachis oil and 10% DMSO), dexamethasone (1 mg/kg), PF670462 (30 mg/kg), GW788388 (10 mg/kg) or a co-formulation of dexamethasone with either GW788388 or PF670462. On day 5, mice were euthanized four hours after daily dosing, and lungs were dissected. Lung tissue was subjected to RNA extraction after bronchoalveolar lavage and gene expression was assessed by RT-qPCR. Results are displayed as fold change from Mock/vehicle control. Data are presented as means and SEM for n=5-6 mice/treatment group. Statistical significance (* *c.f.* Mock control group or # *c.f.* RSV-infected group) was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post hoc test, and is represented as follows: *; $p<0.05$, **; $p<0.01$, ns, non-significant.

5.4.4 Intranasal exposure to poly I:C triggers mild lung injury in normal BALB/c mice on day 2.

Intranasal exposure to poly I:C; the double-stranded RNA analogue, triggered mild lung injury in normal BALB/c mice on day 2. No mortality was observed following intranasal exposure to either saline or 100 µg/mouse poly I:C. Nevertheless, exposure to poly I:C, yet not saline, led to a significant increase in lung weight on day 2 and was indistinguishable in the extent to that observed in RSV-infected mice (Figure 5.9 A). Intranasal poly I:C exposure led to a moderate increase in epithelial permeability on day 2, as evident by the increased protein concentration in the bronchoalveolar lavage fluid (Figure 5.9 B). Similarly, a significant increase in BAL cellular content was detected in poly I:C-treated mice, indicative of cell recruitment following inflammation (Figure 5.9 C). Differential cell counts of the BAL cells in mice challenged with poly I:C showed a significant increase in neutrophils counts but not other leukocyte subsets (Figure 5.10). None of the treatments either alone or in combination had subsided neutrophil recruitment to the lung following poly I:C exposure. Lung weight, BAL protein and BAL cellularity increases in response to intranasal poly I:C exposure were not affected by dexamethasone, PF670462 or the combination of both treatments as was the increase in neutrophils count (Figure 5.9 and Figure 5.10).

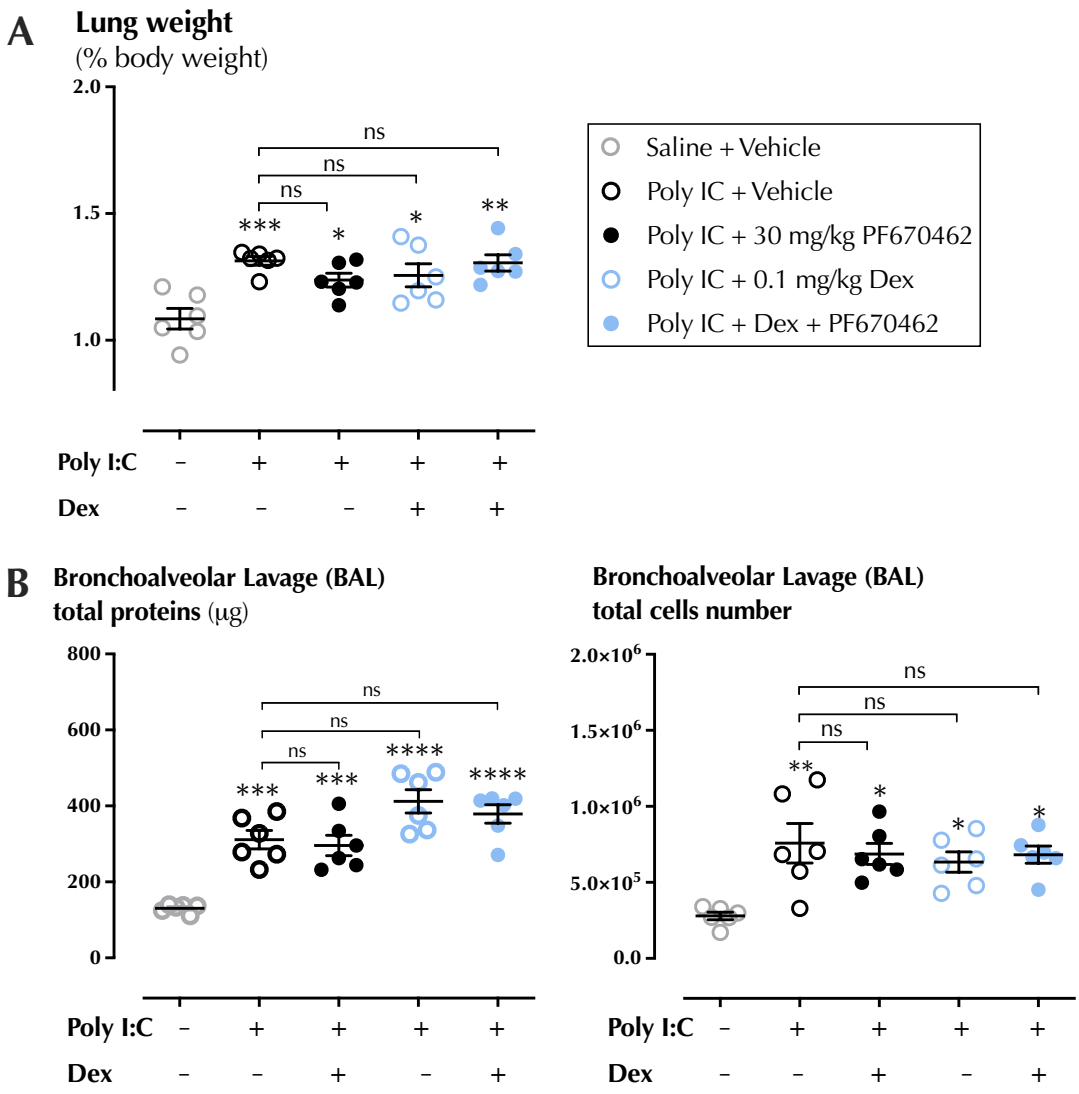


Figure 5.9| Intranasal exposure to poly I:C; double-stranded RNA analogue, triggers mild lung injury on day 2 in normal BALB/c mice.

Mice received an intraperitoneal injection of either PF670462 (30 mg/kg) or vehicle (100 µl; 90% Arachis oil and 10% DMSO). On day later and before poly I:C instillation, mice were injected with either vehicle, dexamethasone (0.1 mg/kg), PF670462 (30 mg/kg) or co-formulation of both dexamethasone and PF670462. Twenty-four hours following poly I:C instillation, mice were culled. A, Lungs were dissected and weighed. Lung weight is expressed as a % of the body weight. B, bronchoalveolar lavage was collected for subsequent analysis to determine total proteins (lift) and total cell number (right). Data are presented as means and SEM for n=6 mice/treatment group. Statistical significance was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, non-significant.

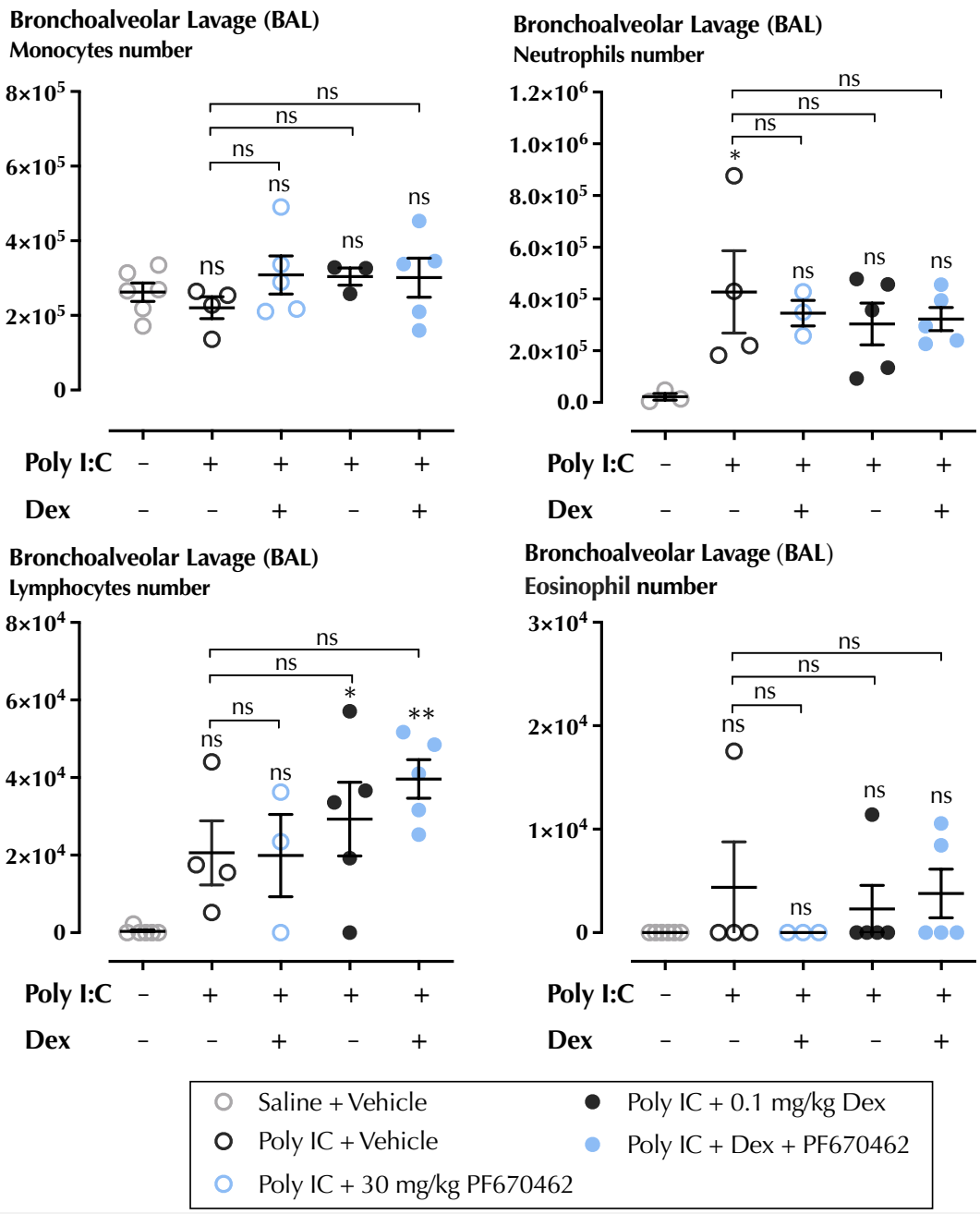


Figure 5.10| Differential cell count of bronchoalveolar lavage in poly I:C acute lung injury model.

Mice received an intraperitoneal injection of either PF670462 (30 mg/kg) or vehicle (100 µl; 90% Arachis oil and 10% DMSO). On day later and before poly I:C instillation, mice were injected with either vehicle, dexamethasone (0.1 mg/kg), PF670462 (30 mg/kg) or co-formulation of both dexamethasone and PF670462. Twenty-four hours following poly I:C instillation, mice were culled. Lungs were dissected and bronchoalveolar lavage was collected and used to determine differential cell count. Data are presented as means and SEM for n=6 mice/treatment group. Statistical significance was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is denoted as follows: *, $p<0.05$; ns, non-significant.

5.4.5 Changes in the expression of inflammatory mediators following RSV infection in BALB/c mice.

In line with the previously observed changes in inflammatory cell infiltrates, gene expression analysis showed that RSV inoculation significantly elevated the expression of pro-inflammatory cytokines, such as *TNF-α* as measured in BAL cells and lung tissue, while *IL-6* increased in lung tissue only. Dexamethasone blunted RSV-triggered expression of *TNF-α* in BAL cells, whereas it only reduced the expression of *IL-6* but not *TNF-α* in lung tissue. Co-administration with PF670462, not GW788388 synergistically decrease TNF-α in lungs of RSV-infected mice. While treatment with PF670462 alone shows remarkable anti-inflammatory effect evident in dampened *TNF-α* expression in BAL cells but not in lung tissue, GW788388 alone did not show similar potential either in BAL cells or lung tissue (Figure 5.11).

On the other hand, mice receiving intranasal instillation of poly I:C showed a significant surge in the expression of pro-inflammatory cytokines; TNF-α, IL-6 and KC (murine homolog of human IL-8). Neither dexamethasone nor PF670462 diminished the elevated pro-inflammatory cytokines expression in BAL or lung tissue of mice receiving poly I:C, as was the combination (Figure 5.12).

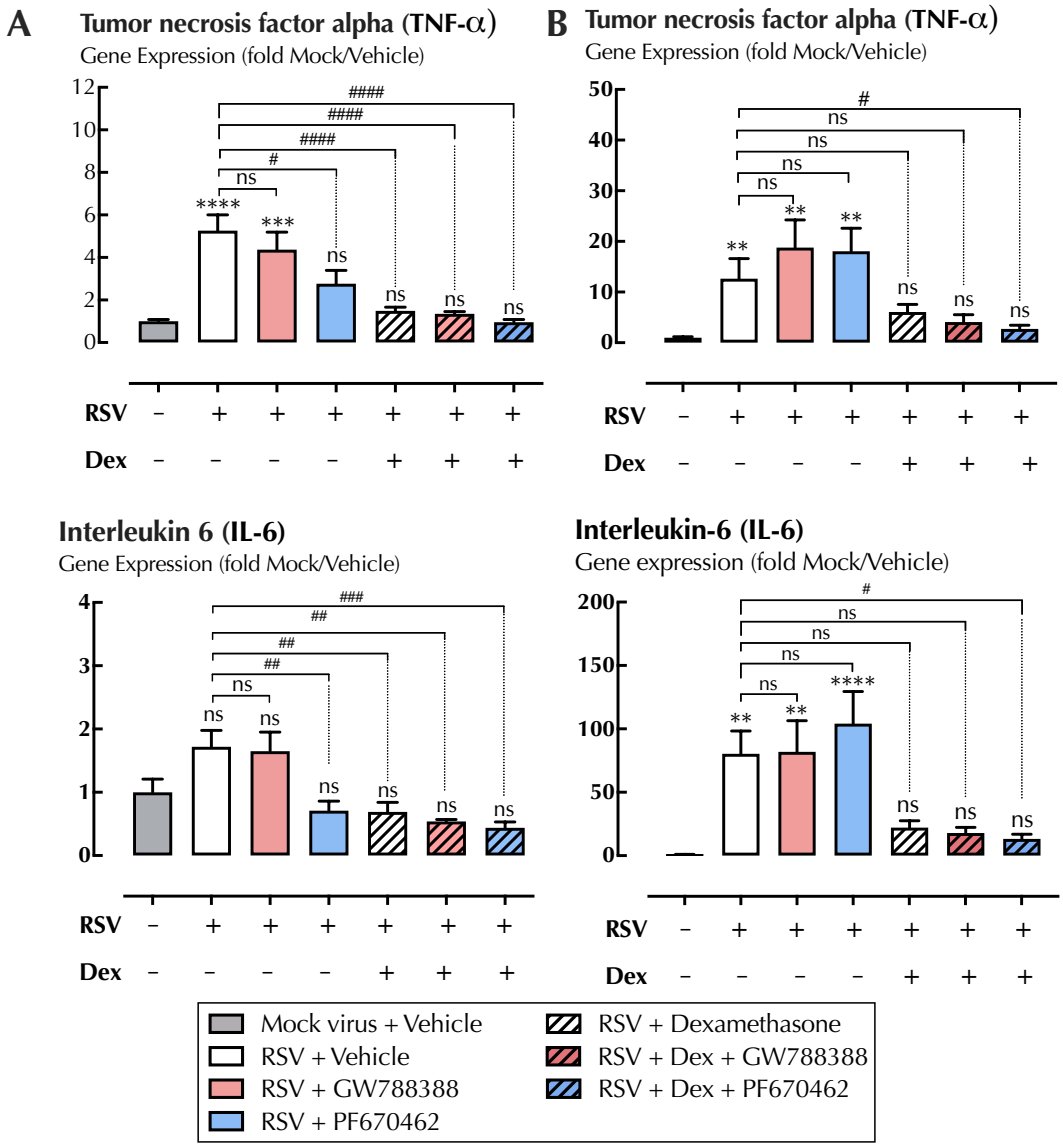


Figure 5.11| Expression of inflammatory mediators in bronchoalveolar lavage (BAL) cells (A) and lung tissue (B) following RSV infection in BALB/c mice.

Eight- to ten-week-old female BALB/c mice were intranasally inoculated with either Mock or 2 million RSV virions under anaesthesia with 2-3% isoflurane. Starting from day 1, mice received a daily intraperitoneal injection of either vehicle (90% Arachis oil and 10% DMSO), dexamethasone (1 mg/kg), PF670462 (30 mg/kg), GW788388 (10 mg/kg) or a co-formulation of dexamethasone with either GW788388 or PF670462. On day 5, mice were euthanized four hours after daily dosing, and lungs were dissected after bronchoalveolar lavage. BAL cells (A) and Lung tissue (B) were subjected to RNA extraction and gene expression was assessed by RT-qPCR. Results are displayed as fold change from Mock/vehicle control. Data are presented as means and SEM for n=5-6 mice/treatment group. Statistical significance (* *c.f.* Mock control group or # *c.f.* RSV-infected group) was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: * or #; $p<0.05$, ** or ##; $p<0.01$, ***or ###; $p<0.001$, ****; $p<0.0001$, ns; non-significant.

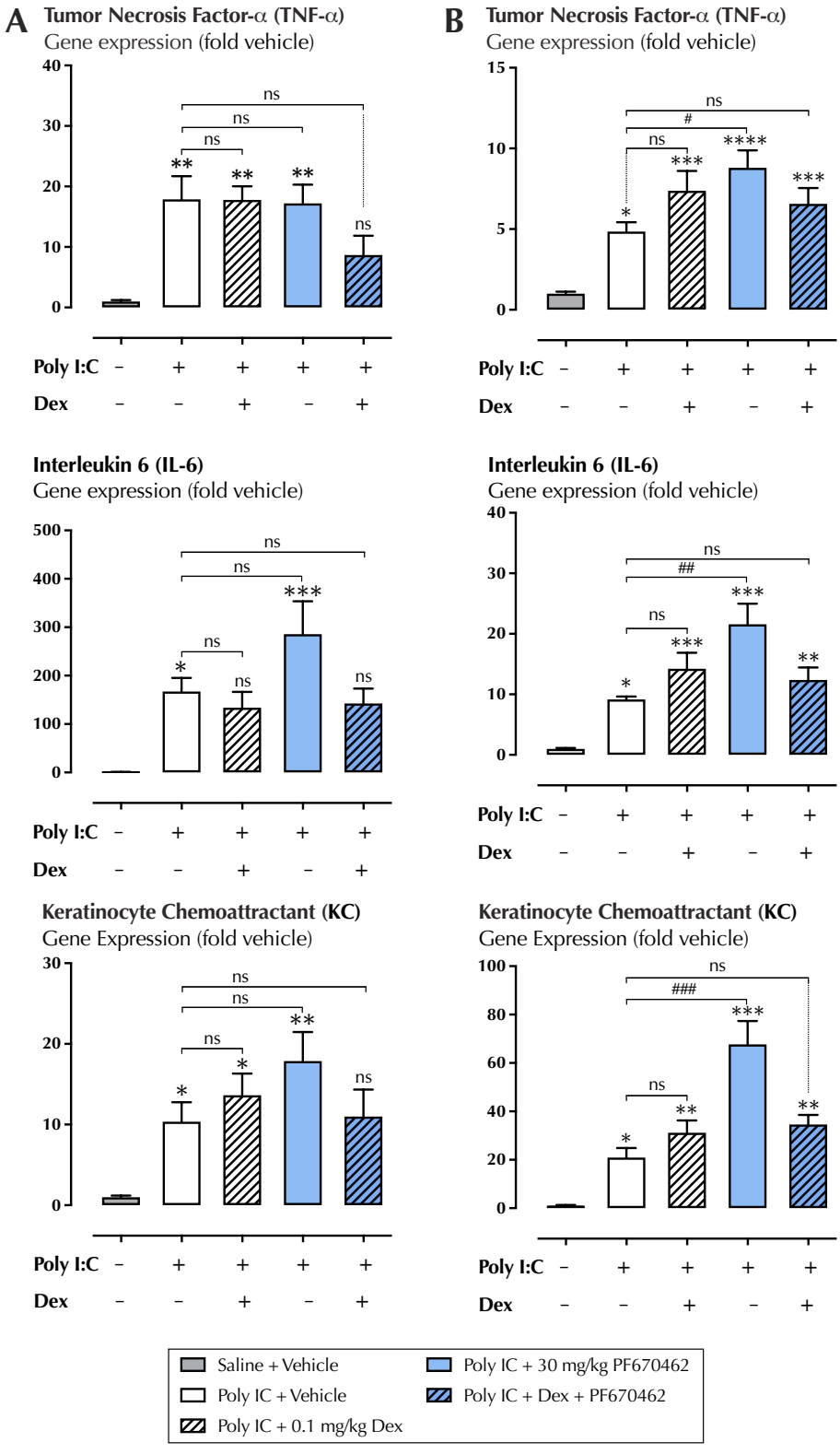


Figure 5.12| Expression of inflammatory mediators in bronchoalveolar lavage (BAL) cells (A) and lung tissue (B) in poly I:C acute lung injury model.

Mice received an intraperitoneal injection of either PF670462 (30 mg/kg) or vehicle (100 µl; 90% Arachis oil and 10% DMSO). On day later and before poly I:C instillation, mice were injected with either vehicle, dexamethasone (0.1 mg/kg), PF670462 (30 mg/kg) or co-formulation of both dexamethasone and PF670462. Twenty-four hours after poly I:C

instillation, mice were culled, and lung was dissected after bronchoalveolar lavage. Total mRNA was extracted from BAL cells (A) or lung tissue (B) and gene expression was assessed by RT-qPCR. Results are displayed as fold change from vehicle control. Data are shown as means and SEM for n=6 mice/treatment group. Statistical significance (* *c.f.* saline control group or # *c.f.* poly I:C-treated group) was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is denoted as follows: * or #, $p<0.05$; *** or ##, $p<0.001$; ***or ###; $p<0.001$, ns, non-significant.

5.4.6 Antiviral genes expression following RSV infection in normal BALB/c mice

We have previously shown that RSV infection mediated upregulation of proinflammatory genes in BALB/c mice on day 5 post-infection (Figure 5.11). So, we thought to evaluate the immune response in the lung following RSV infection. The expression pattern of selected anti-viral genes, including, *IFNα, IFNβ, IFNγ, IFNλ2* and *IFNλ3* was measured in RSV infection model described in Section 05.3.3.

As shown in Figure 5.13, gene expression analysis by real-time RT-PCR has shown that while the expression of *IFNα* remained unchanged in lung tissue following RSV infection, the mRNA expression of *IFNβ*, *IFNγ*, *IFNλ2* and *IFNλ3* increased, albeit non-significantly, compared to animals receiving Mock-conditioned media. Such slight increase in INF expression remained unchanged following dexamethasone treatment.

5.4.7 Antiviral genes expression following intranasal exposure to poly(I:C) in normal BALB/c mice

To assess the biological activity of poly I:C in the mouse model described in Section 5.3.4, type I, II and III IFNs induction was assessed by measuring mRNA expression of *IFN-α/β, IFN-γ, IFNλ2/3* respectively in lung tissue and BAL cells by RT-qPCR. As demonstrated in Figure 5.14 and Figure 5.15, intranasal instillation of the double-stranded RNA analogue poly I:C in normal BALB/c mice induced interferon expression, including *IFNβ* in BAL cells and *IFNλ2* in lung tissue. Treatment with dexamethasone did not affect the upregulation of *IFN β* or *IFN λ 2* expression in mice receiving poly I:C compared with animals receiving saline. Interestingly, PF670462 administration markedly augmented poly I:C mediated upregulation of *INF-β* expression in BAL cells, whereas it had no detectable effects on other INF expression.

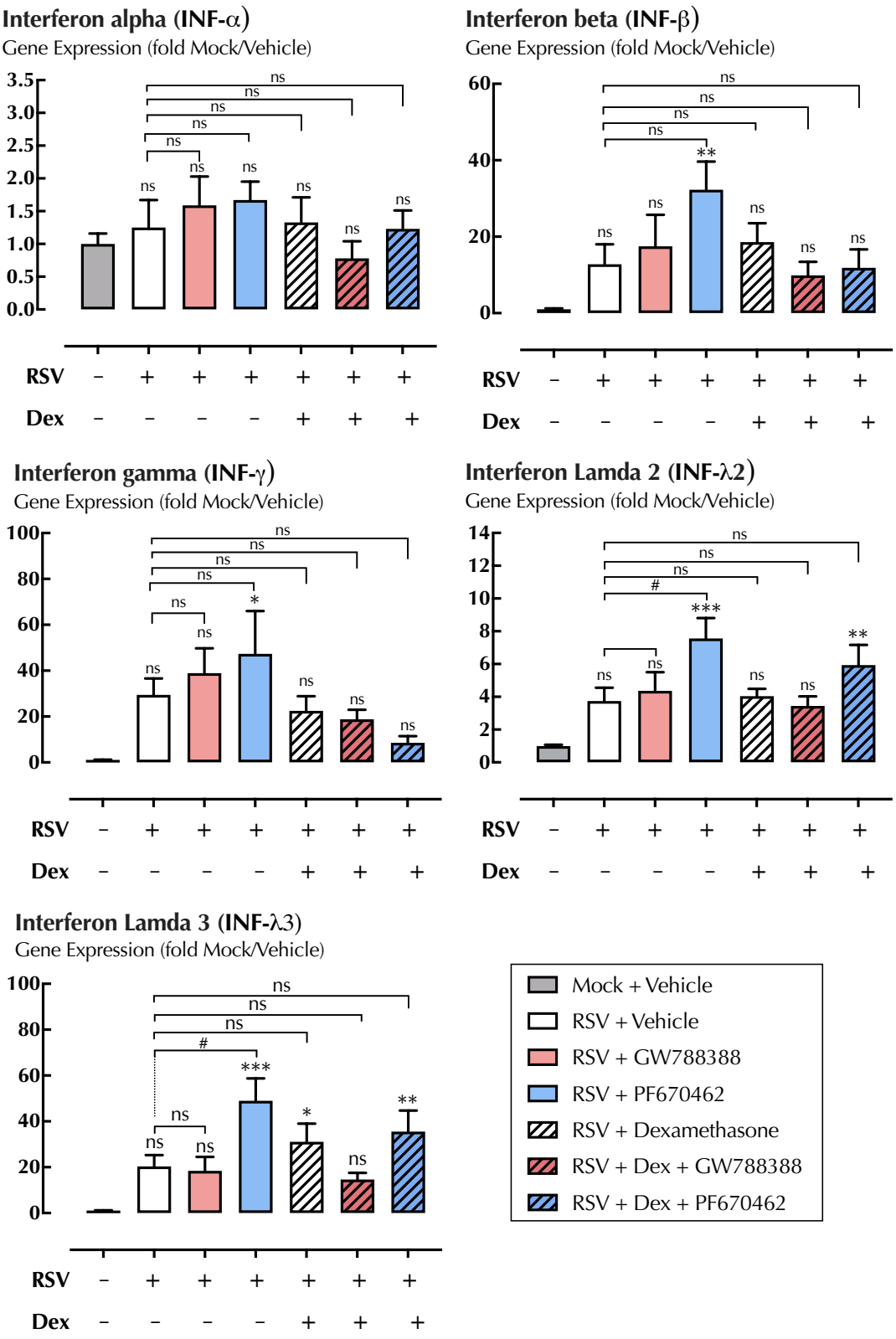


Figure 5.13| Interferons expression as measured in lung tissue after RSV infection in female BALB/c mice.

Eight- to ten-week-old female BALB/c mice were intranasally inoculated with either Mock or 2 million RSV virions under anaesthesia with 2-3% isoflurane. Starting from day 1, mice received a daily intraperitoneal injection of either vehicle (90% Arachis oil and 10% DMSO), dexamethasone (1 mg/kg), PF670462 (30 mg/kg), GW788388 (10 mg/kg) or a co-formulation of dexamethasone with either GW788388 or PF670462. On day 5, mice were euthanized four hours after daily dosing, and lungs were dissected. Lung tissue was subjected to RNA extraction after bronchoalveolar lavage and gene expression was assessed by RT-qPCR. Results are displayed as fold change from Mock/vehicle control. Data are presented as means and SEM for n=5-6 mice/treatment group. Statistical significance (* *c.f.* Mock control group or # *c.f.* RSV-infected group) was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: * or #; $p<0.05$, **; $p<0.01$, ***; $p<0.001$.

5.4.8 Impact of RSV infection on T-cell responses in BALB/c mice

To establish the impact of different treatments on T-cells responses, the expression of *TBX21* (T-Box Transcription Factor 21), *CTLA4* (Cytotoxic T-Lymphocyte Antigen 4) and *FOXP3* (forkhead box P3) were measured in lung tissue.

TBX21 (T-Box Transcription Factor 21) is a gene encoding for transcription factor T-bet that is responsible for the induction of T helper (Th)1 cell and the repression of Th2 cells from naïve T lymphocytes and has been implicated in asthma pathogenesis. *FOXP3* (forkhead box P3) is crucial for the formation and normal functioning of regulatory T cells, that are essential to prevent autoimmunity (Fontenot et al., 2003). It acts also as a transcription activator for many genes including Cytotoxic T-Lymphocyte Antigen 4 (*CTLA4*); that encodes for a transcription factor that is a major negative regulator of T-cell responses (Togashi et al., 2019).

As shown in Figure 5.16, RSV infection markedly promoted the expression of *TBX21* and *CTLA4* as compared to mock-infected mice, while *FOXP3* expression increased, albeit non-significantly. Treatment of RSV-infected mice with dexamethasone alone had no detectable effect on *TBX21* and *CTLA4* expression as did dexamethasone coformulated with GW788388. Likewise, dexamethasone coformuled with PF670462 did not show any significant effect on *CTLA4* and *FOXP3* expression in RSV-infected mice, however it attenuated RSV-mediated upregulation of *TBX21*. Intriguely, GW788388 alone, but not PF670462, had upregulated the expression of *FOXP3* and *CLTA4* as compared to RSV-infected mice.

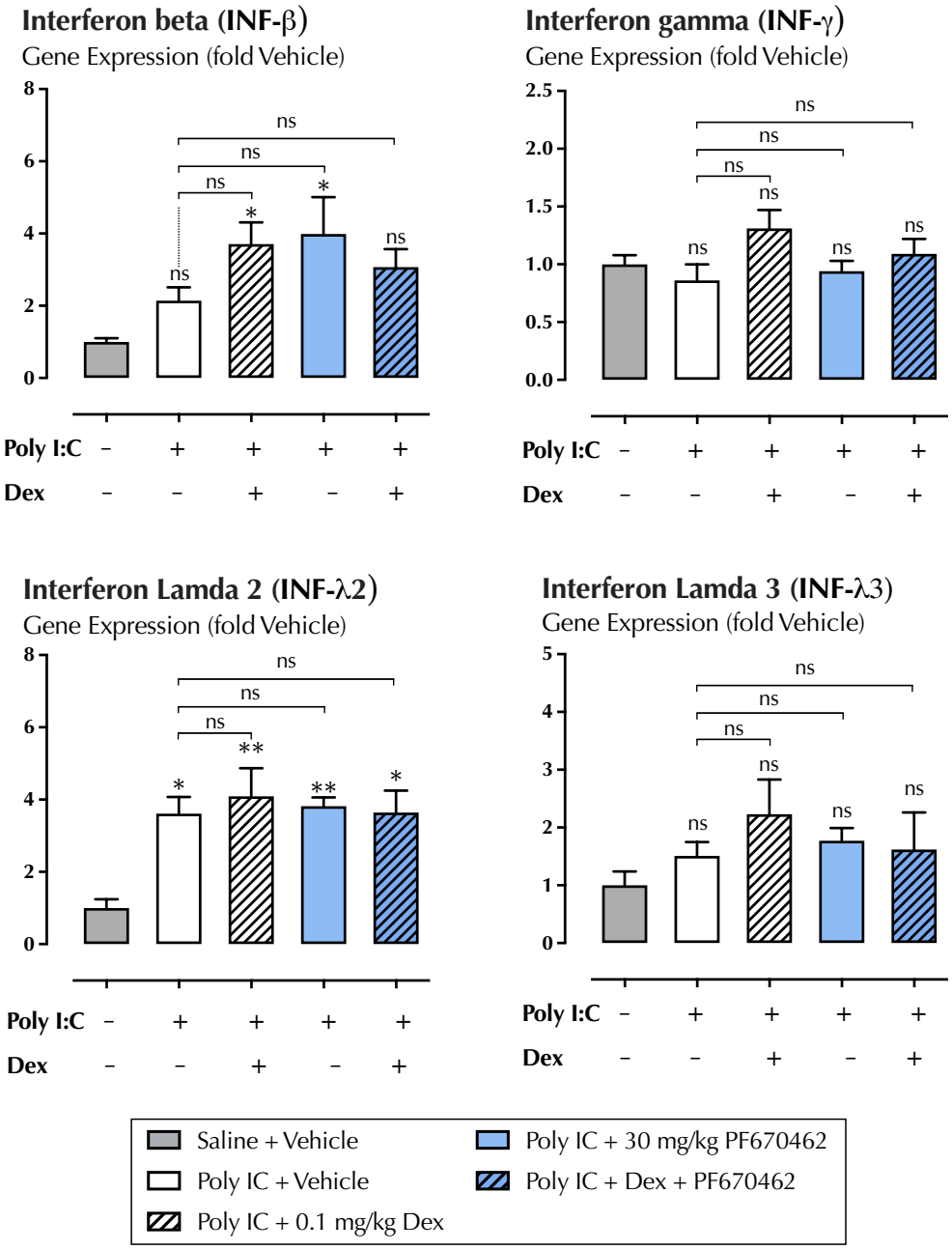


Figure 5.14| Interferons expression as measured in lung tissue following poly I:C instillation in BALB/c mice.

Mice received an intraperitoneal injection of either PF670462 (30 mg/kg) or vehicle (100 µl; 90% Arachis oil and 10% DMSO). On day later and prior to poly I:C instillation, mice were injected with either vehicle, dexamethasone (0.1 mg/kg), PF670462 (30 mg/kg) or co-formulation of both dexamethasone (0.1 mg/kg) and PF670462 (30 mg/kg). Twenty-four hours after poly I:C instillation, mice were culled, and lungs were dissected. Lung tissue was subjected to RNA extraction after bronchoalveolar lavage and gene expression was assessed by RT-qPCR. Results are presented as fold change from vehicle control. Data are shown as means and SEM for n=6 mice/treatment group. Statistical significance (* *c.f.* saline control group or # *c.f.* poly I:C-treated group) was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: *; $p<0.05$, **; $p<0.01$

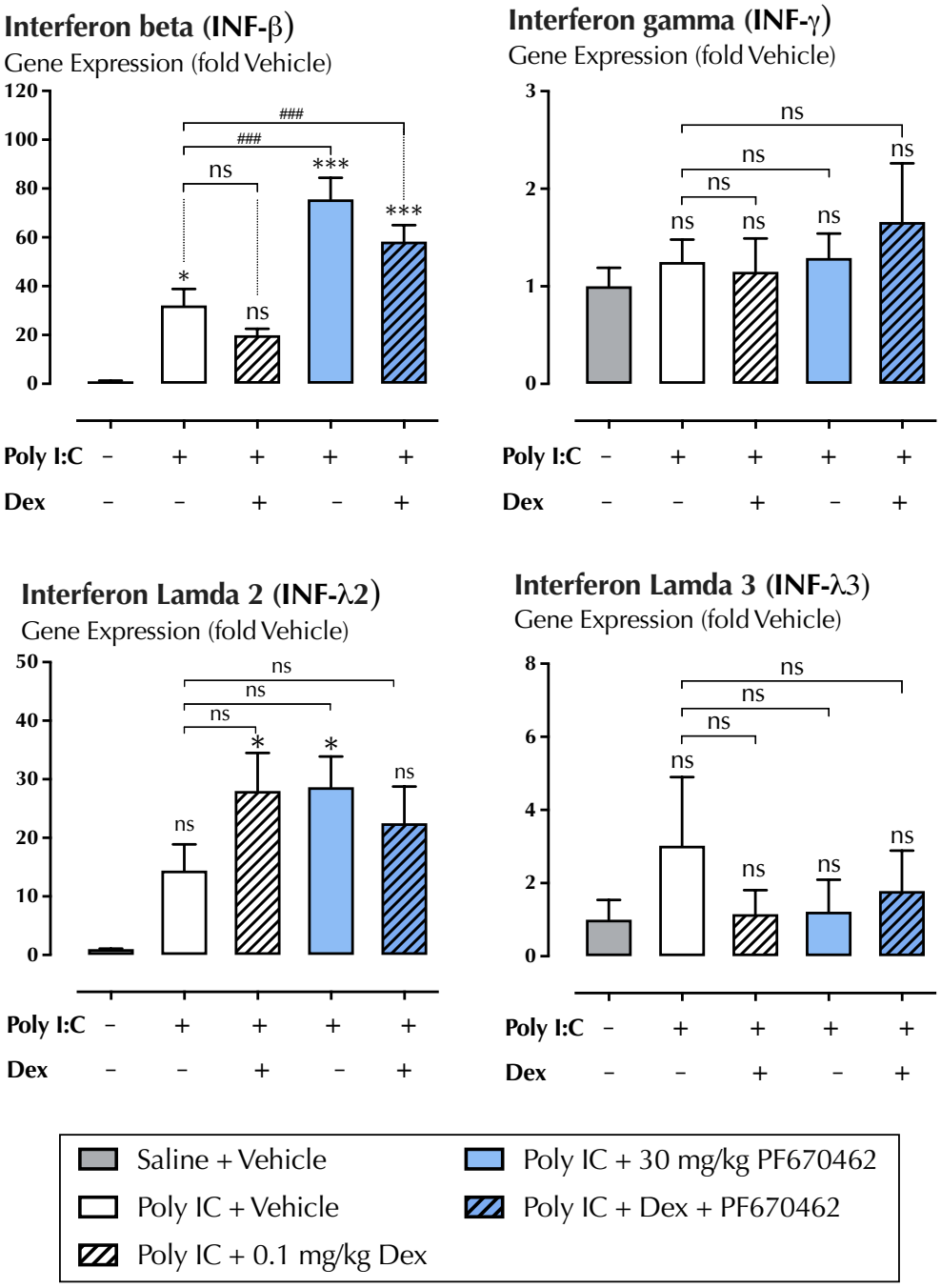


Figure 5.15| Interferons expression as measured in bronchoalveolar lavage (BAL), following poly I:C instillation in BALB/c mice.

Mice received an intraperitoneal injection of either PF670462 (30 mg/kg) or vehicle (100 µl; 90% Arachis oil and 10% DMSO). On day later and before poly I:C instillation, mice were injected with either vehicle, dexamethasone (0.1 mg/kg), PF670462 (30 mg/kg) or co-formulation of both dexamethasone and PF670462. Twenty-four hours after poly I:C instillation, mice were culled. Total mRNA was extracted from BAL cells and gene expression was assessed by RT-qPCR. Results are displayed as fold change from vehicle control. Data are shown as means and SEM for n=6 mice/treatment group. Statistical significance (* *c.f.* Saline control group or # *c.f.* poly I:C-treated group) was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: *, $p<0.05$; *** or ###, $p<0.001$; ns, non-significant.

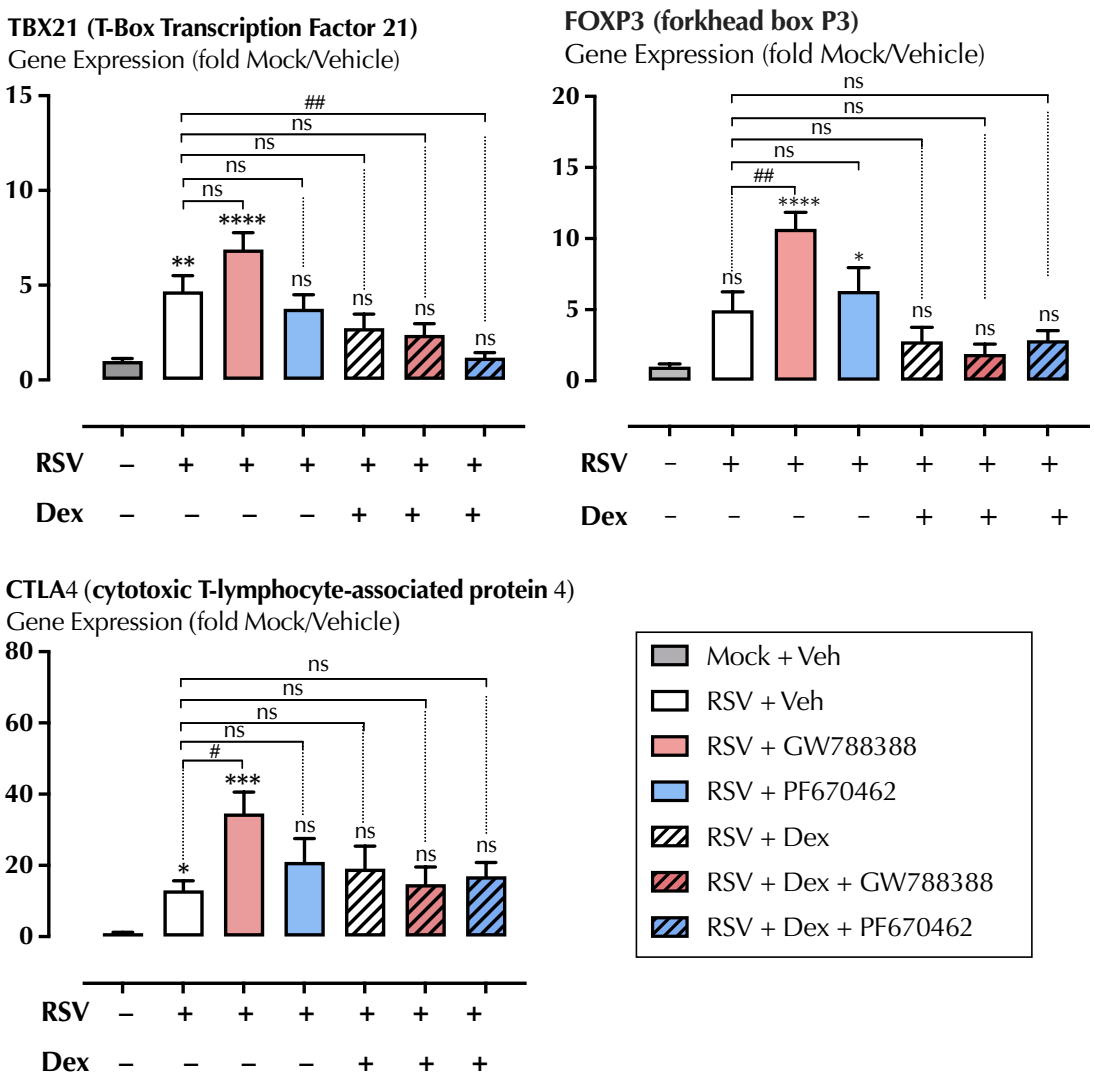


Figure 5.16| Impact of RSV infection on T cell responses as measured in lung tissue in female BALB/c mice.

Eight- to ten-week-old female BALB/c mice were intranasally inoculated with either Mock or 2 million RSV virions under anaesthesia with 2-3% isoflurane. Starting from day 1, mice received a daily intraperitoneal injection of either vehicle (90% Arachis oil and 10% DMSO), dexamethasone (1 mg/kg), PF670462 (30 mg/kg), GW788388 (10 mg/kg) or a co-formulation of dexamethasone with either GW788388 or PF670462. On day 5, mice were euthanized four hours after daily dosing, and lungs were dissected. Lung tissue was subjected to RNA extraction after bronchoalveolar lavage and gene expression was assessed by RT-qPCR. Results are presented as fold change from Mock/vehicle control. Data are shown as means and SEM for n=5-6 mice/treatment group. Statistical significance (* *c.f.* Mock control group or # *c.f.* RSV-infected group) was assessed by Ordinary one-way ANOVA, Bonferroni's multiple comparisons post-hoc test, and is represented as follows: *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$.

5.5 Discussion

The proceeding chapters have focused on the airway epithelium, demonstrating that RSV infection attenuates GC transactivation responses in the airway epithelial cells, consequently limiting anti-inflammatory GR-mediated actions. The apparent resistance was partly down-stream of TLR3 activation. Additionally, we found that CK1 δ/ε inhibitor; PF670462 attenuate RSV-induced glucocorticoid insensitivity. Although airway epithelial cells are the major site for RSV infection and replication and contribute to the inflammation of the airways following viral infection, the complex interaction between the inflammatory, immune, epithelial and other structural cells essentially underlie the persistent chronic inflammatory response, particularly observed in asthma and COPD (Barnes, 2008). So, we thought to further validate the *in vitro* findings in murine models of RSV infection and poly I:C acute lung injury.

In the preceding chapters, we have shown that poly(I:C); the synthetic dsRNA analogue, had recapitulated RSV-induced GC insensitivity in respiratory epithelial cells *in vitro*. Therefore, it is readily conceivable to characterize the degree of concordance between the impact of poly I:C and RSV on murine lung. This may help unravel the relative contribution of TLR3 activation to the pathogenesis of lung dysfunction following RSV infection.

RSV infection can lead to noticeable illness in mice manifested by reduced activity, ruffled fur and weight loss. These parameters were reported to be closely correlated with lung pathology in experimental infection of mice (Morris et al., 2019). Several studies have reported that the A2 strain of RSV caused weight loss one-week post-infection (Graham et al., 1988; Peebles et al., 2001; Moore et al., 2007). This finding was reproduced in the current results, where intranasal insufflation of RSV (2 million virions) to mice caused a significant weight loss starting on day 3 post-infection. Previous studies had related the weight loss observed to the secretion of proinflammatory cytokines including TNF-α and IL-1α which are known to cause discomfort and reduce appetite, leading to weight loss (Kelley et al., 2003; Borchers et al., 2013). On the other hand, poly I:C exposure did not replicate this aspect of RSV pathology where no remarkable change in body weight was observed during the study course. The dissimilarity in findings may be attributed to the difference in the exposure period, where mice were exposed to RSV for 5 days while poly I:C treated mice were exposed to the stimulus for only 24 h.

Despite dampening the inflammatory response to RSV infection in the lung, dexamethasone failed to ameliorate body weight loss in RSV-infected mice. On the contrary, RSV-infected mice treated with dexamethasone showed earlier (on day 2) and more, however non-significant, body weight loss compared to the vehicle-treated group. The decrease in the body weight caused by the treatment with dexamethasone is generally a common side effect, considered to be the result of the potent catabolic effects of glucocorticoids, particularly on the muscle tissue (Schakman et al., 2013).

Five days post RSV infection, a marked decline was observed in thymus weight, indicative of thymus involution in mice. Treatment of RSV-infected mice with dexamethasone caused a further decrease in the thymus and significant decline in spleen weights which was in line with previous findings (Xavier-Elsas et al., 2015). As glucocorticoids are known inducers of T lymphocyte apoptosis (Keenan et al., 2015), the involution of the main lymphoid organs, including thymus and spleen, is an indicator of their immunosuppressive activity.

Next, we thought to explore the indices of pulmonary inflammation including the change in 1) the lung weight relative to body weight, 2) the protein content of the BAL fluid, and 3) cellular content and differential cell population in the BAL fluid (Lax et al., 2014). The leakage of protein into the airspaces normally reflects plasma leakage from inflamed blood vessels, its accumulation in the interstitium and subsequently passage through a compromised epithelial barrier. BAL also contains cells, including those that are present under physiological circumstances (mainly macrophages) and those that are recruited following injury infection or allergic inflammation. The composition of the BAL cell infiltrate in these different types of inflammation varies, as it does over the time course of induction to the period of resolution (Uchida et al., 2006). Reductions in either or both of BAL cells and protein is considered to be evidence of a protective effect in various pulmonary pathologies (Su et al., 2007).

RSV infection has been reported to increase the gross weight of the lungs and elevated protein content in BALF in BALB/c mice (Bolger et al., 2005) and infants and studies of certain infants infected with RSV (Brandenburg et al., 2000; Hornsleth et al., 2001). These corroborate our observation that RSV infection elicited a remarkable pulmonary pathology as evident by the substantial increase in lung weight, bronchoalveolar lavage fluid protein as well as cellular content. Additionally, exposure to poly I:C elicited a significant increase in lung weight that was indistinguishable in degree to that observed in RSV-infected mice. Intranasal exposure to 100 µg/mouse poly I:C also

resulted in a moderate elevation in BALF protein and cell counts in the lung parenchyma at day 2 secondary to increased epithelial permeability. These findings are consistent with previous studies utilizing comparable poly I:C doses (Aeffner et al., 2011). The increase in lung weight occurs secondary to pulmonary hypertrophy, inflammation, and oedema reported in infected lungs. Most of the protein in BALF is likely attributed to the release of inflammatory cytokines such as TNF-α that increase the vascular permeability leading to the extravasation of plasma proteins (Hussell et al., 2001).

While treatment of RSV-infected mice with dexamethasone alone partially reduced the cellularity of BAL fluid, PF670462, interestingly, abolished the recruitment of inflammatory cells to the airways. Synergy was evident in the regulation of BALF cellular content when dexamethasone was co-administered with PF670462 in RSV-treated mice. The potential seen with PF670462 was absent in GW788388-treated mice. These data suggest that PF670462 has superior ceiling effectiveness in regulating RSV-induced lung inflammation to dexamethasone or to the global blockade of TGF-β signalling by the ALK5 inhibitor, GW788388.

Although infiltration of immune cells plays a crucial role in viral clearance, it also contributes to tissue damage. Following RSV infection, neutrophils, monocytes, eosinophils and NK cells were the main peripheral immune cells recruited to the lung (Glaser et al., 2019). This corroborates our observation that RSV infection markedly increased the recruitment of neutrophils and lymphocytes in BAL fluid. Dexamethasone treatment dampened monocytes influx in RSV-infected mice, however, synergy was observed in alleviation of lymphocytes infiltration in RSV-infected mice treated with dexamethasone plus PF670462, where neither compound alone had an effect.

On contrary to other recruited immune cells, the neutrophils influx following RSV-infection was not affected by dexamethasone or any of the other treatments tested. Similarly, poly I:C exposure induced neutrophils recruitment that does not respond adequately to dexamethasone alone or in combination with compounds tested. Given that the GC-resistant asthma phenotype is considered to be neutrophil-predominant (Chambers et al., 2015), these findings highlight the potential pathophysiological relevance of RSV infection through TLR3 activation to the contribution of GC insensitivity in the patients with severe GC-resistant asthma exacerbations.

Neutrophils are the main cell population in the airways lumen of children suffering severe RSV bronchiolitis (Everard et al., 1994; McNamara et al., 2003; Kerrin et al., 2017).

It is not definite yet whether they are detrimental or beneficial in resolving viral infections (Openshaw et al., 2017; Takamura, 2018). Following RSV infection, neutrophils migrate across the epithelium. The transepithelial migration was predicted to involve the expression of intracellular adhesion molecule-1 (ICAM-1) on epithelial cells and its interaction with CD11a and CD11b on the surface of neutrophils (Iijima and Iwasaki, 2014; Bergsbaken et al., 2017). This may in part explain lack of beneficial effect of dexamethasone on RSV-induced neutrophils recruitment, where RSV infection augments the expression of ICAM-1 hence facilitate transepithelial migration of neutrophils and such an increase in ICAM-1 expression is irresponsive to dexamethasone treatment.

In line with the changes in inflammatory cell infiltrates, RSV inoculation significantly provoked the mRNA expression of pro-inflammatory cytokine including *TNF-α* and *IL-6*. Dexamethasone blunted RSV-triggered expression of *TNF-α* and *IL-6* in BAL cells, whereas it only reduced the expression of *IL-6* but not *TNF-α* in lung tissue. Co-administration with PF670462, not GW788388 dampened *TNF-α* mRNA expression in lungs of RSV-infected mice suggesting synergistic interaction. On contrary to PF670462 that had shown remarkable anti-inflammatory effect when administered alone, GW788388 did not show similar potential either in BAL cells or lung tissue. Mice receiving intranasal instillation of poly I:C showed a significant surge in the mRNA expression of pro-inflammatory cytokines; TNF-α, IL-6 and KC expression that was irresponsive to either dexamethasone, PF670462 or the combination. Altogether, we have provided a consistent suite of findings indicating that the CK1δ/ε inhibitor, PF670462 was effective either alone or in combination with dexamethasone in reducing some aspects of lung inflammation associated with RSV infection in BALB/c mice. This signifies CK1δ/ε as a therapeutic target for effective management of pulmonary inflammation partially controlled by glucocorticoids.

RSV infection increased the mRNA expression of *IFNβ*, *IFNγ*, *IFNλ2* and *IFNλ3* as measured in lung tissue compared to animals receiving Mock-conditioned media. Such an increase in INF expression remained unchanged following dexamethasone treatment. This finding comes in line with the results of McAllister et al. (2019; 2020), who found that dexamethasone has increased the viral titre *in vitro* and delayed RSV clearance in mice despite the pronounced reduction in RSV-driven mucus overproduction. This was accompanied by a reduction in the expression levels of several immune-response genes and decreased the *in vivo* lymphocyte infiltration. However, dexamethasone has not altered the antiviral interferon signalling. Recent studies suggested that while the

immunosuppressive activity of dexamethasone has favourably inhibited the RSV-induced mucus production, it has diminished the immune defence activities against RSV infection leading to delayed viral clearance (McAllister et al., 2019; 2020). These findings provide plausible reasoning for the inefficacy of glucocorticoid in RSV-infected patients.

Poly I:C, as mentioned earlier, can be recognized by TLR3 and MDA5 and activates IRF3- or NF-κB-dependent signalling pathways, resulting in the induction of type I IFN and proinflammatory cytokines including TNF-α and IL-6. Intranasal exposure to poly I:C had triggered the expression of antiviral genes including *IFN-β*, and *IFN-λ2*. Similar to what we have observed in RSV-infected mice, treatment with dexamethasone did not affect the upregulation of INF expression, while PF670462 administration markedly augmented poly I:C mediated upregulation of INF- β expression in both lung tissue and BAL cells.

The cytokine TGF-β is involved in critical cellular processes, such as proliferation, differentiation and cellular morphogenesis that are involved in main features of tissue homeostasis including wound healing and immunomodulation. Moreover, TGF-β has crucial roles in inflammation, tissue remodelling and immunity (Lee et al., 2006; Ferrari et al., 2009). Therefore, blanket inhibition of TGF-β compromises its beneficial pleiotropic effects leading to serious adverse effects, such as cardiac valve defects and autoimmune colitis (Anderson et al., 1990; Akhurst and Hata, 2012). For TGF-β modulators to have acceptable safety profiles, they will need to selectively block TGF-β signalling pathways linked only to the targeted pathology. Unlike ALK5 inhibitor GW788388 that cause global TGF-β inhibition, the CK1 δ/ε inhibitor, PF670462 administration has not revealed any evidence of auto-immune responses in the respiratory tract verified by lack of changes in the expression of the TGF-β-dependent Treg transcription factor, FoxP3. Thus, we believe that PF670462; CK1δ/ε inhibitor provides a safer adjuvant to glucocorticoids than the ALK5 inhibitor, GW788388.

5.6 Limitations of the study

Although similarities are evident, the critical fact remains that human RSV has no animal reservoir and has evolved to infect humans as its natural host, not the commonly used rodent models, which require supraphysiological infection doses of hRSV (Russell et al., 2017). In addition, the key differences in immune responses to RSV infection between human and mice. In human, the neutrophilic inflammation has a significant contribution to RSV pathogenesis where neutrophils comprise up to 85% of BALF count compared to 15 to 20% of cells in mice (McNamara et al., 2013a). The robust and reliable Th1 (IFN-γ)

responses in mice are variable in humans (Dakhama et al., 2005; Borchers et al., 2013). While eosinophils play an important role in the RSV pathogenesis in human, there is no evidence for the involvement of these cells in the immune response to RSV in mice (Borchers et al., 2013; Knudson et al., 2015). The current study was performed in female mice only while several reports showed that the immune response differ between the different ovarian cycle phases (Krzych et al., 1978; Schwartz et al., 2004).

Another limitation is that most pathological and immunological parameters were only examined at one time-point post RSV infection. However, the findings might change due to variations in the kinetics of virus replication and host response at different time-points. Notably, in the current study, we only examined the short-term consequences of RSV infection. It remains a possibility that long-term immune responses, including humoral and cellular memory responses, are affected in a different manner following RSV infection. Also, the effects of pharmacological inhibitors weren't examined on control animals not exposed to RSV. The absence of a group treated with dexamethasone only has confined our ability to confirm that poly IC and RSV promote GC resistance *in vivo* and determine whether inhibition of TGF-β or CK1 restores or improves GC responsiveness. We recommend first identifying relevant GC-resistant endpoints (e.g., neutrophilic inflammation) at a given dose of dexamethasone then determine whether treatment with the inhibitor of interest (e.g., GW or PF compound) restores/improves the anti-inflammatory effects of dexamethasone. Since RSV and dsRNA both engage multiple receptor pathways, the use of TLR3 knockout mice is also crucial before having any solid conclusions about the role of TLR3 in glucocorticoid resistance in mice and RSV pathogenesis.

5.7 Conclusions from chapter 5

In conclusion, compared to RSV infection, this chapter demonstrated that challenging BALB/c mice with poly I:C elicits pulmonary pathology analogous to those triggered by RSV A2 strain. Poly I:C treatment has particularly induced a moderate increase in lung weight, bronchoalveolar lavage fluid proteins and neutrophils influx in airways compartments, all of which are evident in mice following RSV infection. These data suggested that dsRNA/TLR3 interaction through TLR3 ligand exposure may at least partially recapitulate the pathophysiological effects of RSV infection in BALB/c mice. When tested *in vivo*, the CK1 δ/ε inhibitor, PF670462 has not only proven beneficial in the management of several aspects of lung injury following RSV infection but also possess anti-viral activity. Moreover, PF670462 has not induced any auto-immune responses seen with the global blockade of TGF-β.

CHAPTER 6

General Discussion and Conclusions

6 General Discussion

6.1 Key findings and their significance

6.1.1 Introduction

Respiratory syncytial virus is among the viruses causing acute lower respiratory tract infections in children around the world. Around 40% of infants that have been hospitalized due to severe RSV bronchiolitis usually develop asthma in adulthood (Sigurs et al., 2010). Decades of research have failed to produce a reliable vaccine to RSV and treatment with the recombinant monoclonal antibody palivizumab is still of high cost (Power, 2008). Despite the proven efficacy of glucocorticoids in the management of chronic inflammatory diseases, this drug class offers no significant benefit for patients with viral-induced exacerbations of chronic airway inflammatory diseases; including asthma and COPD (Ermers et al., 2009; Fernandes et al., 2013). *In vitro* and *in vivo* studies have shown that RSV compromises the glucocorticoids therapeutic potential through the viral interference with GC signalling via mechanisms that remains unclear (Hinzey et al., 2011; Webster Marketon et al., 2014). Better insight into the mechanisms underlying GC insensitivity during RSV infection may offer anchor points for new therapeutic strategies.

Airway epithelium regulates the interface with the environment where it maintains the microbial defence and has well-defined immune-modulatory functions that shape a proper immune response in the lung. Alterations in airway epithelial functions can significantly contribute to inflammation in chronic respiratory diseases, including asthma and COPD (Lambrecht and Hammad, 2012). The airway epithelium is the prime locus for viral infection and replication and the crucial target for glucocorticoid therapy. Emerging evidence suggests that TGF-β1 is a potent inducer of GC insensitivity in the airway epithelium, which prompts the question of whether TGF-β1 is the driving cytokine of RSV-mediated repression of CGs responsiveness.

This thesis examined the contribution of TGF-β1 in RSV-induced GC resistance in the airway epithelium and pinpointed which RSV-sensing PRRs is involved in GC irresponsiveness using *in vitro* experimental methods. This thesis also focused on characterizing TLR3 silencing and the CK1δ/ε inhibition as a potential modifier of GC resistance in the epithelium. Finally, the thesis has evaluated the impact of the CK1δ/ε inhibitor; PF670462, on airway inflammation in murine models of RSV infection and poly I:C acute lung injury.

6.1.2 Evidence that RSV limits GC responsiveness in the human airway epithelium

Glucocorticoids exert their anti-inflammatory effects in the airway epithelium either by transactivation of genes or through inhibition of pro-inflammatory cytokines release, including GM-CSF (Keenan et al., 2015). Evidence has also been presented indicating that GC-mediated gene transactivation was compromised by RSV infection in the BEAS-2B human bronchial epithelial cell line. Data presented herein has provided the first evidence that RSV ablates GC-induced transcription of several genes in the BEAS-2B human bronchial epithelial cell line, consequently limiting anti-inflammatory therapeutic potential. These findings are significant for a number of reasons. Firstly, through impairment of GC-transactivation of anti-inflammatory factors, e.g., *GILZ*, RSV may interfere with the GC-inhibition of NF-κB-signalling pathways, leading to the release of pro-inflammatory mediators. Furthermore, RSV limits GC-induction of the mRNA of *ENaCα* that plays a key role in luminal sodium reabsorption through the apical membrane of epithelial cells and hence interfere with airway surface liquid clearance (Song et al., 2009; Kong et al., 2013; Kaskinen et al., 2020). RSV, also, profoundly represses GC-induction of the *CDKN1C*; a negative regulator of cell cycle that accounts for the anti-proliferative actions of glucocorticoids (Dapat and Oshitani, 2016).

6.1.3 Evidence that targeting TLR3 can reverse RSV-induced GC resistance in the bronchial epithelium

The airway epithelium expresses a repertoire of PRRs that directly activate innate immunity following the recognition of the viral PAMPs. Of these PRRs, RSV can be only recognised by Toll-like receptors (TLR2/6, TLR3, TLR4 and TLR7) and RNA helicases (RIG-I and MDA-5) (Kato et al., 2005). Among all the TLRs agonists tested in this thesis, only the synthetic dsRNA analogue; poly I:C was able to recapitulate the RSV-induced impairment of GC actions in the epithelium implying that dsRNA/TLR3 interactions may contribute significantly to the poor response of epithelium to GC treatment during the course of RSV infection. TLR3 ablation using the siRNA interference approach was able to restore the epithelium sensitivity to glucocorticoids medicament. So, we identified TLR3 as a possible target to circumvent glucocorticoid resistance during RSV infection. Our results add more therapeutic value to TLR3 antagonism since it has been proved previously that TLR3-induced inflammatory responses are deleterious to RSV pathogenesis while RSV is insensitive to the TLR3-induced antiviral responses (Alexopoulou et al., 2001; Kitazawa and Villena, 2014; Wang et al., 2018).

Since dsRNA originates from other respiratory viruses, our group tested whether this finding is expandable to other viral infections. Rhinovirus and Influenza A virus are among the viruses detected in patients with asthma and COPD exacerbations (Kurai et al., 2013; Jamieson et al., 2015; Leigh and Proud, 2015). RV infection has been reported to impair GC transactivation in bronchial and alveolar cells in addition to submerged primary bronchial epithelial cells (Papi et al., 2013; Rider et al., 2013). Although out of the scope of this thesis, we have found that TLR3 silencing is able to circumvent the RV-induced impairment of glucocorticoids actions in bronchial epithelium (Xia et al., 2017).

TLR3 inhibition is achievable through disrupting the contact between the TIR domain of TLR3 and the viral dsRNA. Cheng *et al*. have successfully designed a small molecule; CU CPT 4a, that acts as a high-affinity competitive inhibitor that interferes with dsRNA binding to TLR3 and hence selectively antagonizes stimulated TLR3 signalling and repress the downstream production of IL-1β, TNF-α and NO in Poly(I:C)-induced macrophages (Cheng et al., 2011). In the context of this thesis, we have tested the TLR3/dsRNA complex inhibitor (thiophenecarboxamidopropionic acid, molecular formula $C_{18}H_{13}ClFNO_3S$) and found that this compound has effectively attenuated the RSV and poly I:C-mediated repression of GC actions on GC-responsive genes (data not shown). In this view, the dsRNA/TLR3 pathway may be novel treatment targets of virus-induced exacerbation of airway inflammatory diseases.

6.1.4 Casein kinase 1δ/ε (CK1δ/ε) : a novel therapeutic target for effective management of viral-induced exacerbations of inflammatory respiratory diseases

Data presented in this thesis have provided the first evidence that RSV infection or poly I:C impairs glucocorticoid activity in airway epithelial cells in a transforming growth factor-β1 (TGF-β1)-mediated manner. The TGF-β type I receptor kinase (ALK5) inhibitor; SB431542 has effectively attenuated TGF-β1 suppression of glucocorticoid-induced functional endogenous gene expression in RSV-infected bronchial epithelial cells. Although successful, the ban blockade of TGF-β1 activity by ALK5 inhibitors has limited or no clinical feasibility. Inhibiting ALK5 is known to induce auto-immune diseases, increase tumorigenesis (Hahm et al., 2001; Akhurst and Hata, 2012; Oyanagi et al., 2014) and cause heart valve lesions (Anderton et al., 2011). These possible toxicities and adverse effects are only acceptable in high-mortality diseases. However, this approach is not clinically tractable in chronic inflammatory diseases such as asthma and COPD.

Using hypothesis-free approaches, including proteomics and transcriptomics, our group have provided the first evidence that CK1δ/ε is implicated in TGF-β1 signalling pathway suggesting a promising target for management of inflammatory pulmonary diseases with TGF-β1 involvement (Li et al., 2019). Elevated levels of CK1δ has been detected in the airway wall of severe asthmatic patients compared to well-controlled asthmatics and non-asthmatic controls (Waddell et al., 2004; Zhang et al., 2015). We have shown for the first time that pharmacological inhibition of CK1δ/ε with PF670462 has inhibited the expression and activity of TGF-β1 and subsequently prevented the GC impairment provoked by either RSV infection or poly I:C. Additionally, PF670462 significantly subdued RSV-induced expression of pro-inflammatory gene encoding IL-6, suggesting a potential for additional anti-inflammatory activities for PF670462. This potential was further confirmed *in vivo*, where we have provided evidence of an interaction between PF670462 and Dexamethasone to achieve greater control of the pulmonary inflammation following RSV infection.

Two more features of PF670462 favour its clinical use for TGF-β modulation. Firstly, PF670462 is highly selective for CK1δ/ε inhibition compared to the ALK5 inhibitor; SB431542. The high selectivity of PF670462 avoids compromisation of TGF-β dependent regulatory T cells (Tregs) populations. The global TGF-β inhibition results in the loss of these cell population leading to gut autoimmune colitis (Lachapelle et al., 2018). Secondly, the high potency of PF670462 allows its delivery by inhalation thus limiting the adverse effects associated with the systemic administration including the risk of cardiac or gut autoimmune defects and disruption to central circadian rhythm (Keenan et al., 2018). Taken together, our findings support the possible use of PF670462 for the prevention and/or management of GC resistance during RSV infection-triggered bronchiolitis or asthma and COPD exacerbations. Moreover, the interventions aiming to modulate the activity of CK1δ/ε for the treatment of respiratory diseases have been patented (Patent No. WO2016149756A1).

6.2 Future Directions

6.2.1 Further evidence using a better in vitro model of the airway epithelium

In the current thesis, we have employed the immortalized human bronchial epithelial cell lines. It is a simple model that allows successful investigation of the roles of the specific targets in the cells. Data obtained from experiments using transient transfections and siRNAs to knockdown TLR3 in the immortalized BEAS-2B cells suggest

that TLR3 partly mediates impairment of GC-actions during RSV infection. BEAS-2B cell line is generated from normal human bronchial epithelial cells and proven to be responsive to glucocorticoids. However, the limitation of this cell line is that it does not replicate many of the characteristics of differentiated cells, such as development of beating cilia and mucus production *in vitro*. Additionally, since it is derived from a single donor, it does not reflect the natural biological variation observed between individuals. The different response patterns seen between cell lines and primary epithelial cells may also be attributed to the immortalization process, polarization, differentiation or because they originate from different regions of the bronchial tree.

Accordingly, it is still of paramount importance to verify our findings using a more physiologically relevant model with ciliated and goblet epithelial cells. Our group had established that findings in RSV-mediated GC insensitivity were paralleled by similar observations when the air-liquid interface culture of HBECs was subjected to RSV infection (Xia et al., 2017). Although, It is acknowledged that mucocilliary-differentiated primary HBECs are the most relevant *in vitro* model, there are many disadvantages limiting the broad use of these cells for research, including their limited life span, high cost of culture, high inter-individual and inter-passage variability, and difficulty of genetic manipulation (Stewart et al., 2012). To overcome limited life span of primary HBECs, basal cell immortalized-nonsmoker 1.1 (BCi-NS1.1) cell line can be used where it retains characteristics of the original primary cells and maintain a multipotent differentiation capacity for over 40 passages (Walters et al., 2013). Future experiments should focus on exploring our findings using BCi-NS1.1 cell line in air-liquid interface settings.

Asthma and COPD have been proved to alter the normal airway epithelium (De Rose et al., 2018; Gohy et al., 2019; Calvén et al., 2020). This fact prompts the question of whether findings using primary HBECs derived from healthy donors in air-liquid interface culture could be extrapolated to asthma and COPD.

Children in their first 2 years of life comprise the major risk group for RSV severe disease, with a peak in infants approximately 3 months old, after which the incidence gradually declines with age (Bianchini et al., 2020). This highlights the significance of using paediatric-derived primary cells to test our hypothesis in more clinically relevant model.

6.2.2 Further evidence using other gene silencing techniques

Gene silencing using siRNAs are transient (Kim and Eberwine, 2010), and the off-target and non-specific effects of the siRNAs generally complicate the interpretation of the results (Persengiev et al., 2004; Jackson and Linsley, 2010). Hence, other gene silencing techniques, including CRISPR/Cas9 system, should be considered to stably knock out TLR3 in ALI culture of primary HBECs. Finally, given that the airway epithelium is known to be altered in the asthmatic and COPD lung (Hiemstra et al., 2015), introducing heritable TLR3 deletions via CRISPR/Cas9 system into primary HBECs from asthmatic or COPD donors and culturing them at air-liquid interface would certainly give us further insights into physiological and pathophysiological roles of TLR3 in the airway epithelium.

6.2.3 Investigating other cells contributions

In this thesis we focused on the airway epithelium, however, the complex interaction between the inflammatory, immune, epithelial and other structural cells essentially underlie the persistent chronic inflammatory response, particularly observed in asthma and COPD (Barnes, 2008). Glucocorticoids affect almost all cell types in the airway. For example, Glucocorticoids inhibit the production and release of pro-inflammatory cytokines from monocytes and macrophages (Valledor and Ricote, 2004). Furthermore, airway smooth muscle cells are a prominent target of inhaled GCs, which not only act to inhibit their proliferation and migration (Stewart et al., 1995), but also their production of cytokines (Hirst and Lee, 1998). Moreover, other cells including neutrophils, eosinophils, monocytes, and NK cells are recruited to the lung following RSV infection and contribute to RSV pathogenesis. Our findings, therefore, highlight the need to further investigate whether the effect of RSV in limiting GC actions, is unique to the airway epithelial cells or expands to other GC-responsive cell types in the airways. This may be achieved using either these cell types alone or co-cultured with bronchial epithelial cells.

Since Mice often respond to experimental interventions in ways that differ from humans (Seok et al., 2013), we strongly believe that the human cell organotypic models are more definitive and relevant to the human tissue *in situ*. One of the most recent *in vitro* models that recreate both the mechanical and biochemical sides of the *in vivo* coin is the lung-on-a-chip (Shrestha et al., 2020). This device represents a 3D model of a living, breathing human lung on a microchip generated from human lung and blood vessel cells. The advantage of this model is that it can mimic the inflammatory response triggered by microbial pathogens as well as predict absorption of airborne nanoparticles.

6.2.4 *In vivo* Studies: future directions

The *in vivo* studies performed herein revealed some intriguing findings suggesting that PF670462 either alone or through synergistic interaction with dexamethasone attenuated RSV infection-induced lung inflammation. Hence, we believe that it is of value to comprehensively investigate this potential in allergen-induced lung inflammation and allergic responses in the ovalbumin, house dust mite (HDM) or cockroach allergen-induced asthma models as well as the cigarette smoke exposure model of COPD, with or without viral exacerbation. Because the function of the airways is closely coupled to its structure, an altered airway function may reflect alterations in airway structure due to inflammation, tissue damage, or abnormal tissue remodelling. Thus, airway function is an important feature to monitor when evaluating the impacts of infection on respiratory health and the efficacy of therapeutic interventions.

In the *in vivo* studies presented in the thesis, PF670462 were administered to mice through intraperitoneal injection. Minimizing the systemic adverse effects of TGF-β modulation is crucial in PF670462 development. The administration of small molecule kinase inhibitors CK1δ/ε by inhalation will limit their inevitable off-target effects reported with the systemic use including disruption to central circadian rhythm (Badura et al., 2007; Meng et al., 2010). Additionally, the inhalation route will overcome the low oral bioavailability of PF670462 due to the fast first-pass hepatic metabolism (Wager et al., 2014). For the above-mentioned reasons, future studies are needed to evaluate the therapeutic potential of aerosolized PF670462. Moreover, dose-escalation and safety studies should be performed, and phase 2 clinical trial started, aiming to translate these *in vivo* findings into a novel therapeutic for management of virus-induced asthma exacerbation that respond poorly to steroid.

We identified TLR3 as a possible target to circumvent glucocorticoid resistance during RSV infection. Our results add more therapeutic value to TLR3 antagonism since it has been proved previously that TLR3-induced inflammatory responses are deleterious to RSV pathogenesis while RSV is insensitive to the TLR3-induced antiviral responses (Alexopoulou et al., 2001; Kitazawa and Villena, 2014; Wang et al., 2018). Future studies should, therefore, be focused on assessing GC responsiveness in viral-induced exacerbations od asthma or COPD models using TLR3 knockout mice. These studies may identify approaches to improve GC activity in RSV-infected patients.

6.2.5 Human model of experimental RSV Infection

Human experimental infection studies represent the interface between pre-clinical models and observational studies of patients and thus help mitigate the risks inherent in progressing to the late-stage trials. The first RSV experimental infection study was done by Mills et al. (1971) who have challenged healthy males with RSV Long strain. The human model used in this study allowed studying RSV infection under controlled conditions and permitted comparison of symptoms, airway physiology, lung function and airway inflammation (Mills V et al., 1971). Its evolution to include atopic individuals and mild asthmatic volunteers has increased our understanding of virus-induced exacerbations and their underlying processes (Hall et al., 1981; McKay et al., 1988; Higgins et al., 1990; Watt et al., 1990; Hall et al., 1991a; Lee et al., 2004; DeVincenzo et al., 2010; DeVincenzo et al., 2014; Kim et al., 2014; DeVincenzo et al., 2015; Habibi et al., 2015). In addition, the human challenge studies have been instrumental in early proof-of-concept clinical trials to test the efficacy of candidate RSV vaccines and therapeutics (Habibi et al., 2015). Hence, we recommend future testing of our findings using healthy or asthmatic volunteers to help evaluate the therapeutic value of interfering with TLR3 and CK1δ/ε activity to circumvent RSV-induced GC resistance.

6.3 General conclusions

Glucocorticoid insensitivity remains a challenging clinical problem in the management of viral-induced exacerbation of chronic airway inflammatory diseases. Thus, it is crucial to unravel the mechanisms underlying the apparent impairment of GC actions and responsiveness *in situ*. In this thesis, we demonstrated that RSV infection ablates certain actions of glucocorticoids in the airway epithelium contributing to glucocorticoid insensitivity. The observed GC insensitivity may occur partly through TLR3 activation with subsequent release of TGF-β1, a potent mediator of glucocorticoid resistance in the airway epithelium. We also implicated CK1δ/ε in the apparent resistance. In conclusion, the findings presented in this thesis have provided anchor points for novel therapeutic strategies to restore GC sensitivity during RSV infection-induced bronchiolitis or exacerbations of chronic airway inflammatory diseases, including asthma and COPD (Figure 6.1).

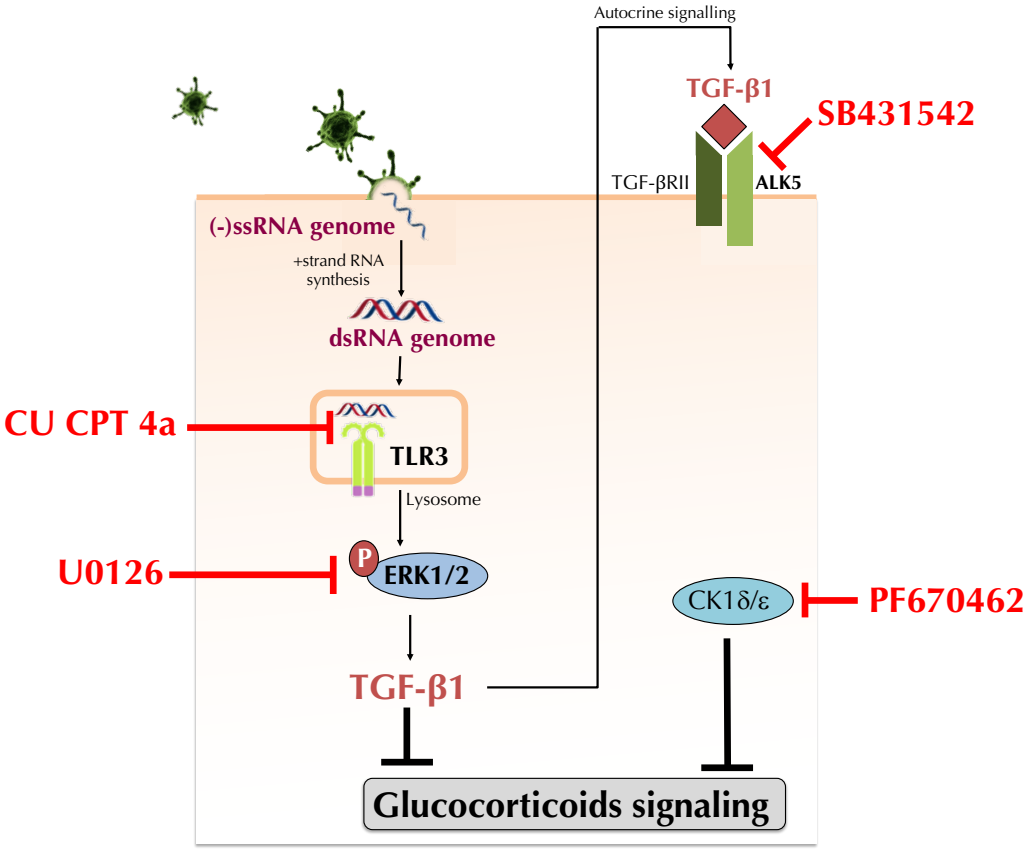


Figure 6.1 | The summary of thesis findings.

Viral PAMPs, most importantly dsRNA, activate TLR3 that in turn signals via the ERK1/2-dependent pathway to promote TGF-β activity in airway epithelium. Interrupting dsRNA/TLR3 interaction or inhibition of Casein kinase 1δ/ε (CK1δ/ε) are plausible novel therapeutics to reverse GC insensitivity induced by viral infection.

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APPENDIX

Table for reporting responses to reviewers' comments

Examiner's comments	Student's response	Change made to thesis (if any)	Page reference
I would suggest adding few sentences in each of the results chapter reflecting on the limitations of the present work (*i.e.,* use of female animals only, effects of pharmacological inhibitors on control animals not exposed to RSV, etc...)	Accepted	Section 3.6, 4.6 and 5.6 were added to each result chapter to include limitations of the study as follows: • **3.6 Limitations of the study** The morphology and differentiation of the airway epithelium impact susceptibility to viral infection and the ensuing immune response. Thus, while studies using BEAS-2B cell line may provide an appropriate and feasible approach to investigate RSV responses, it has some limitations. First, the initial infection of RSV is typically ciliated airway epithelium and the BEAS-2B cell line does not show normal epithelial differentiation with respect to ciliated, goblet and club cells and cultured as a submerged monolayer rather than at air-liquid interface. Second, BEAS-2B cells are derived from single donor, so they cannot capture all of the potential responses of the genetically heterogeneous human population. Third, the dichotomy between adult and paediatric-derived primary cells highlights the difficulty in interpreting observations made with regard to childhood airway repair. Moreover, BEAS-2B cells are relatively normal and not diseased, hence extrapolating findings to asthma is problematic due to intrinsic differences between non-asthmatic and asthmatic airway epithelial cells, i.e the baseline expression levels of genes/proteins of interest. In the current chapter, we only examined the gene expression of inflammatory cytokines at mRNA level. However, changes in mRNA transcript levels do not always correspond to the changes in protein abundance due to post-transcriptional mechanisms controlling the rate of protein translation, different half-lives of mRNAs and the corresponding protein products, and differences in the intracellular locations of expressed proteins (Gygi et al., 1999). Hence, assessing the observed expressional changes at the protein level using ELISA or western blotting should be the focus of future experiments. Lastly, we employed various small molecule kinase inhibitors including SB431542, PF670462, and U0126. While pharmacological inhibition can be immediate, it might have non-specific, "off-target" effects that can affect the cellular physiology, hence altering the experimental outcomes. Due to the potential non-specific, "off-target" effects, we recommend the combined use of two or more unrelated methodological	107-108 127

		approaches, such as siRNA gene silencing, expression of dominant-negative mutants, or gene knockouts. • **4.6 Limitations of the study** The current study has utilised siRNA gene silencing to knockdown TLR3 expression. Despite its efficacy in vitro, this method has several limitations (Unniyampurath et al., 2016). These include non-specific effects due to induction of genes mediating interferon response (Pebernard and Iggo, 2004) and the off-target effects due to the sequence similarity to the targeted gene (Dorsett and Tuschl, 2004). While the gene expression levels of all TLRs were examined In the current study as possible off-target effects of TLR3 siRNA, the expression of other poly IC/RSV sensing receptors, such as PKR, RIG-1 and MDA5 were not examined as possible off-targets following TLR3 silencing. Additionally, transient siRNA transfection does not result in stable integration into the cellular genome. Hence, this approach allows the analysis of the resulting genotypic and phenotypic changes only for a limited period of time (Kim and Eberwine, 2010). A limitation specific to the current study is the use of only one siRNA sequence for TLR3 silencing. However, identified phenotypes following siRNA gene knockdown should be confirmed with different siRNAs targeting the same transcript (Dorsett and Tuschl, 2004). A limitation specific to the current study is the use of only one siRNA sequence for TLR3 silencing. However, identified phenotypes following siRNA gene knockdown should be confirmed with different siRNAs targeting the same transcript (Dorsett and Tuschl, 2004). We recommend the use of a stable transfected human bronchial epithelial cell line to confirm the results observed with transient siRNA transfections. The recent emergence of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 system for gene editing may take the analysis one step further, by introducing the heritable TLR3 deletions in the genome of the primary human bronchial epithelial cells, both submerged and differentiated into air-liquid interface cultures (Chu et al., 2015). We also observed that TLR3 knockdown had abolished poly I:C-induced TGF-β expression while the impact on RSV-mediated TGF-β expression was marginal due to the variability in RSV data sets. Hence, more biological replicates need to be added to confirm the observation. Lastly, PAI-1 mRNA expression and phosphorylation of Smad2 need to be measured to further prove the role of TLR3 in TGF-β expression in response to RSV infection. • **5.6. Limitations of the study** Although similarities are evident, the critical fact remains that human RSV has no animal reservoir and has evolved to infect humans as its natural host, not the commonly	167

		used rodent models, which require supraphysiological infection doses of hRSV (Russell et al., 2017). In addition, the key differences in immune responses to RSV infection between human and mice. In human, the neutrophilic inflammation has a significant contribution to RSV pathogenesis where neutrophils comprise up to 85% of BALf count compared to 15 to 20% of cells in mice (McNamara et al., 2013a). The robust and reliable Th1 (IFN-γ) responses in mice are variable in humans (Dakhama et al., 2005; Borchers et al., 2013). While eosinophils play an important role in the RSV pathogenesis in human, there is no evidence for the involvement of these cells in the immune response to RSV in mice (Borchers et al., 2013; Knudson et al., 2015). The current study was performed in female mice only while several reports showed that the immune response differ between the different ovarian cycle phases (Krzych et al., 1978; Schwartz et al., 2004). Another limitation is that most pathological and immunological parameters were only examined at one time-point post RSV infection. However, the findings might change due to variations in the kinetics of virus replication and host response at different time-points. Notably, in the current study, we only examined the short-term consequences of RSV infection. It remains a possibility that long-term immune responses, including humoral and cellular memory responses, are affected in a different manner following RSV infection. Also, the effects of pharmacological inhibitors weren't examined on control animals not exposed to RSV. The absence of a group treated with dexamethasone only has confined our ability to confirm that poly IC and RSV promote GC resistance in vivo and determine whether inhibition of TGF-β or CK1 restores or improves GC responsiveness. We recommend first identifying relevant GC-resistant endpoints (e.g. neutrophilic inflammation) at a given dose of dexamethasone then determine whether treatment with the inhibitor of interest (e.g. GW or PF compound) restores/improves the anti-inflammatory effects of dexamethasone. Since RSV and dsRNA both engage multiple receptor pathways, the use of TLR3 knockout mice is also crucial before having any solid conclusions about the role of TLR3 in glucocorticoid resistance in mice and RSV pathogenesis.	
I would also refrain from overstating, as seen in some part of the PhD thesis, on the clinical impact of the present experimental work no validation was done in a real model of virus-induced exacerbation (i.e., page 160, the candidate stated, "*we are the first to identify TLR3 as a novel target to improve the therapeutic outcomes of glucocorticoids in*	Edited	In the amended thesis, the following sentences were rephrased as follow: • "we have successfully pinpointed TLR3 and to a lesser extent TLR4 as the major contributor to this detrimental effect." —> Our data suggested that out of all PRRs tested, TLR3 and to a lesser extent TLR4 contributes, at least partially, to RSV-mediated GC resistance. • "we are the first to identify TLR3 as a novel target to improve the therapeutic outcomes of glucocorticoids in RSV-induced exacerbations of respiratory diseases that	110 171

RSV-induced exacerbations of respiratory diseases that respond poorly to steroids".		respond poorly to steroids" was edited as "we identified TLR3 as a possible target to circumvent glucocorticoid resistance during RSV infection.".	
I would also go back and check the statistical analysis of some of the data where two-way ANOVA was used on normalized data but compared to controls expressed a 1 or 100 (which has therefore no variance).	Edited	We chosed the comparator to be the steroid induced response and compare relevant GRE responses to that one (avoiding comparing to the baseline that has no variance), thus we do not violate the assumptions of ANOVA and retain power of parametric analysis. The following figures were amended accordingly: • In page 76, Figure 3.3 A was amended. • In page 78, Figure 3.4 B was amended. • In page 80, Figure 3.5 was amended. • In page 84, Figure 3.8 was amended. • In page 85, Figure 3.9 was amended. • In page 86, Figure 3.10 was amended. • In page 90, Figure 3.13 was amended. • In page 91, Figure 3.14 was amended. • In page 92, Figure 3.15 was amended. • In page 93, Figure 3.16 was amended. • In page 95, Figure 3.18 was amended. • In page 97, Figure 3.19 was amended. • In page 99, Figure 3.20 was amended. • In page 116, Figure 4.3 was amended. • In page 117, Figure 4.4 was amended. • In page 119, Figure 4.5 was amended. • In page 120, Figure 4.6 was amended. In page 121, Figure 4.7 was amended.	Same pages as comments

Examiner's comments	Student's response	Change made to thesis (if any)	Page reference
• The candidate has in correctively presented her in vitro work as MEAN ± SEM, based on the one triplicate values. With an n=3, there is not enough power in the generated data to be presented as such. Rather SDEV is required to illustrate the range of data generated. The data should be presented with SDEV not SEM and the associated statistics repeated since the lower number will limit the type of analysis permitted. Use appropriate statistics to account for the low n value (n=3 stated in all the figure legends). The differences observed were so significant one would think that with the adjusted analysis the outcomes would still be the same. • There needs to be additional clarity on how many replicates and repeats were performed in the in vitro experiments.	Commentary/ Edited	• n represent number of biological replicates not technical replicates. • In the amended thesis, specific number of biological replicates were stated in each figure ligand in chapter 3 and 4.	
There was numerous sentence that required citations that were not included and these need to be addressed.	Accepted	• In page 1, (Health and Welfare, 2020) was added. • In page 4, (Johnston et al., 1995; Guibas and Papadopoulos, 2017) were added. • In page 4, (Pecchini et al., 2015) was added. • In page 6, (Sin et al., 2004; Tamm et al., 2012) were added. • In page 8, (Henderson et al., 2020) was added. • In page 16, (Tam et al., 2011) was added. • In page 19, (Ueno et al., 2008; Ballester et al., 2021) were added. • In page 24, (Kim and Lee, 2014) was added. • In page 101, (Luo et al., 2012; Tatematsu et al., 2013; Tatematsu et al., 2018) were added.	Same pages as comments

		• In page 123, (Prince et al., 1979; Taylor et al., 1984; Graham et al., 1988; Graham et al., 1991; Graham et al., 1993; Connors et al., 1994; Boelen et al., 2000; Jafri et al., 2004; Mukherjee et al., 2011b; Stokes et al., 2011; Openshaw, 2013; Rameix-Welti et al., 2014; Knudson et al., 2015) were added. • In page 123, (Stark et al., 2002; Stokes et al., 2011) were added. • In page 123, (Jafri et al., 2004; Miller et al., 2004; Bem et al., 2011) were added. • In page 123, (Bem et al., 2011; Yang et al., 2015; Hijano et al., 2019; Stephens and Varga, 2020) were added. • In page 124, (Han et al., 2018; van Erp et al., 2019) were added. • In page 160, (Song et al., 2009; Kong et al., 2013; Kaskinen et al., 2020) were added. • In page 160, (Dapat and Oshitani, 2016) was added. • In page 161, (Alexopoulou et al., 2001; Kitazawa and Villena, 2014; Wang et al., 2018) were added. • In page 164, (Shrestha et al., 2020) was added.	
This is a published abstract and should be cited as such (Similar to TSANZ is published in Respirology and was cited as such above).	Edited	• In the amended thesis, C22 AIRWAY EPITHELIUM: MOLECULAR REGULATION OF INFLAMMATION. American Thoracic Society, pp A4638-A4638 was replaced by Am J Respir Crit Care Med 193, A4638-A4638.	x
ATS published citation as mentioned above.	Edited	• In the amended thesis, C72 Epithelial Biology. American Thoracic Society, pp A5886-A5886 was replaced by Am J Respir Crit Care Med 193, A5886-A5886.	x
The use of abbreviations needs to be revisited as there were a number omitted from the abbreviation list at the start of the dissertation and not unform consistency in the implementation of these in the thesis.	Accepted/ Edited	• List of abbreviations were updated to include missed terms. • Implementation of the abbreviations were corrected throughout thesis.	xxii- xxvii
Such as? different viruses are known to induce exacerbation so there should be numerous citations here	Accepted	The following citations were added "(Johnston et al., 1995; Freymuth et al., 1999; Rakes et al., 1999; Kotaniemi-Syrjänen et al., 2003; Thumerelle et al., 2003; Heymann et al., 2004; Jartti et al., 2004; Johnston et al., 2005; Lemanske et al., 2005; Kusel et al., 2006; Allander et al., 2007; Chung et al., 2007; Khetsuriani et al., 2007; Kusel et al., 2007; Bosis et al., 2008; Jackson et al., 2008; Papadopoulos et al., 2011; Turunen et al., 2014; Christensen et al., 2019)".	3
Change "hyperresponsiveness of airways" to "airway hyperresponsiveness"	Edited	In the amended thesis, "hyperresponsiveness of airways" was changed to "airway hyperresponsiveness".	4

sentence appears incomplete?	Accepted	In the amended thesis, the sentence was deleted.	
citation illustrating the poor compliance as this is in fact a major continuing issue.	Accepted	The following citations were added "(Lindsay and Heaney, 2013; Gaude et al., 2014; Engelkes et al., 2015)."	7
there are more recent references that can be used in conjunction. Evidenced in various animal models.	Accepted	The following references were added: • Alangari AA (2014) Corticosteroids in the treatment of acute asthma. Annals of thoracic medicine 9(4):187-192. doi:10.4103/1817-1737.140120 • Hasegawa, K., Craig, S.S., Teach, S.J., and Camargo, C.A. (2021). Management of Asthma Exacerbations in the Emergency Department. J Allergy Clin Immunol *Pract*. In press. • Hadjigol S, Netto KG, Maltby S, Tay HL, Nguyen TH, Hansbro NG, Eyers F, Hansbro PM, Yang M,Foster PS (2020) Lipopolysaccharide induces steroid-resistant exacerbations in a mouse model of allergic airway disease collectively through IL-13 and pulmonary macrophage activation. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology 50(1):82-94. doi:10.1111/cea.13505 • Ueda K, Nishimoto Y, Kimura G, et al. Repeated lipopolysaccharide exposure causes corticosteroid insensitive airway inflammation via activation of phosphoinositide-3-kinase δ pathway. Biochem Biophys Rep. 2016;7:367-373. Published 2016 Jul 28. doi:10.1016/j.bbrep.2016.07.020	7
Important to showcase that results were not overly positive and in a subpopulation that in fact it worsened outcomes.	Accepted	In the amended thesis, the following paragraph was added "Djukanovic and colleagues had reported potential deleterious effects of inhaled IFN-β in subgroup of patients with mild asthma, where asthma symptoms have increased, although non-significantly compared to placebo group (Djukanovic et al., 2014). Epithelial cell derived from patients with mild asthma have normal IFN production (Sykes et al., 2014). It is possible that the addition of exogenous IFN-β in this subgroup could lead to greater inflammation and subsequent worsening of symptoms (Djukanović et al., 2014; Jackson, 2014). Watson et al. recently demonstrated that intermittent prophylactic doses of exogenous IFN-β could modulate viral infection in an in vitro model. Moreover, they reported that chronic dosing with IFN-β was more effective than dosing post viral infection while it was not associated with inflammatory mediators production (Watson et al., 2019)."	7
This is now 9 years old. What are the outcomes??? Surely, data is suggesting an outcome either way.	Accepted	The outcomes were added as follow "Although capsid binders are attractive and potent early-stage inhibitors of RV replication in vitro, in vivo results were disappointing due to problems with pharmacodynamics, in vivo efficacy, and	9

		resistance development (Ortega et al., 2020). Similarly, 3C protease inhibitors are effective in vitro, while ineffective in clinical trials (Glanville et al., 2013)."	
inappropriate term. RSV disease.... edit.	Accepted	RSV disease —> RSV-induced airway disease	14
There needs to be a sentence or two to illustrate that RSV preferentially infected ciliated cells in the airway. https://doi.org/10.1016/j.virol.2015.05.014	Accepted	In the amended thesis the following paragraph was added "Early reports demonstrated that airway ciliated epithelial cells were the major locus for RSV replication (Henderson et al., 1978; Wright et al., 1997). RSV replication in human airway epithelial cells occurs at the apical cell surface of airway ciliated epithelial (Zhang et al., 2002; Wright et al., 2005). Furthermore, recent observations indicated that cilia might be a primary site of RSV morphogenesis (Smith et al., 2014; Jumat et al., 2015). In addition to preferential infection of ciliated cells via the apical membrane, RSV was shed exclusively from the apical surface and spread by the motion of the cilial beat to neighboring epithelial cells (Zhang et al., 2002)."	18
Authors also found apical expression of TLR 7. Edit sentences to include this.	Edited	Sentence was edited as follows "TLR4, TLR5, TLR7, TLR9, and TLR10 were weakly expressed in the apical surface"	21
luminal not the optimal work use in sentence. APICAL expression is a more appropriate term.	Edited	luminal —> apical	21
This figure showcases ACE culture expression, this section refers to in vivo expression.	Edited	Cross-reference "(Figure 1.7)" was deleted.	21
edit to update TL7 expression. TLR7 shown to be expressed in AEC... (Ioannidis et al. 2013)	Edited	AEC was added to Table 1.1	23
• Ioannidis et al., looked at expression in the human airway epithelium and then primary AEC cultures. The images shown in this figure is the expression in AEC cultures and not in in human airway which the section is conveying.	Edited	Figure 1.7 was updated to include TLR expression in the airway epithelium of the human trachea and primary AEC cultures. The figure ligand was amended as follows "Figure 1.7| Pattern of TLRs expression and localization in the human airway epithelium. A, A a schematic diagram illustrating the different TLRs in the airway epithelium, the size of TLRs is proportional to their abundance in the epithelial cells. TLR distribution in the airway epithelium of the human trachea (B) and human primary airway epithelial cells (AEC) cultures (C). Different TLRs are visualized by immunofluorescence utilizing anti-human TLR-specific antibodies. The apical surface is on the top (Ioannidis et al., 2013)."	24
I think the differences in expression should be discussed in this section.	Accepted	In the amended thesis, the following paragraphs were added.	21

1: expression invivo 2: expression in primary cultures 3: highlight differences.		"They reported that each TLR had its unique expression pattern and distribution on tracheal epithelial cells which is likely to contribute to the regulation of AECs innate response to commensal and pathogenic microorganisms in the pulmonary tract. As shown in Figure 1.7 B, TLR1 and TLR3 were detected on the surfaces and in the cytoplasm of epithelial cells throughout the whole width of the pseudostratified epithelium. TLR1 expression was more conspicuous on the apical surface of the tracheal epithelium. TLR3 was mainly expressed on the apical and basal mucosal surface. TLR2 and TLR6 showed a predominant basolateral distribution that may limit their interactions with PAMPs unless the epithelial barrier integrity is compromised. Although TLR2 was weakly expressed, TLR6 showed an intense basolateral staining. TLR4, TLR5, TLR7, TLR9, and TLR10 were weakly expressed in the apical surface of the tracheal mucosa; terminal plates and cilia. While, TLR3, TLR7, and TLR9 are commonly expressed in the endosomal compartments of dendritic cells (Caramori et al., 2006; Blasius and Beutler, 2010), they were expressed both intracellularly and on the cell surfaces of AECs. These findings imply a tissue-specific distribution of TLRs. As demonstrated in Figure 1.7 C, in hAEC cultures, TLR1 and TLR3 demonstrated both apical and basolateral expression on the cell surface and in the cytoplasm. TLR2 and TLR6 were predominantly localized in the basolateral surface with only some apical expression. Expression of TLR4, TLR5, TLR7, TLR9, and TLR10 was mainly observed on the surface of the apical cells, especially in the terminal bars or in the cilia. No TLR8 expression was detected. Loannidis and coworkers suggested that the expression pattern of all TLRs in hAEC cultures corroborated findings from the trachea (Ioannidis et al., 2013). "	
"TLR7 is expressed mainly in lung" this is not seen the table 1.1 and then the figure.	Accepted	AECs was added to Table 1.1 and Figure 1.7 A was updated.	23, 24
Phipps et al. 2007??	Accepted	More recent references were added as follows "(Kvarnhammar and Cardell, 2012; Flores-Torres et al., 2019)."	29
add the word 'assay' after immunoprecipitation	Accepted	In the amended thesis, the word assay was added	29
Kicic et al., 2006 looked at TGFB protein production in asthmatic and non-asthmatic AEC.	Accepted	The following paragraph was added: "A study investigating the intrinsic differences between the paediatric healthy and asthmatic bronchial epithelium has detected the markedly diminished production of TGF- β in Asthmatic epithelial cells compared to healthy nonatopic samples. This difference was consistent over several passages indicating the independency on an in vivo environment (Kicic et	41

Following study looked at expression post airway injury. These studies are worth including in this section. https://doi.org/10.1164/rccm.200603-392OC DOI: 10.1111/resp.12810		al., 2006). Given the crucial function of TGF-β1 in pAEC wound repair in vitro, the blunted production of TGF-β1 by paediatric asthmatic epithelium subsequently contributes to the dysregulated airway epithelial repair leading ultimately to a defective airway epithelium; one of the fundamental factors of the development and persistence of asthma (Ling et al., 2016)."	
under aseptic conditions.	Edited	"in sterile" was replaced by "under aseptic conditions"	45
You interchange between British and American spelling styles for many words. keep consistent	Edited	• fetal —> foetal • normalised —> normalized	45 56
FBC or FCS?? Keep consistent	Edited	FBS was changed to FCS throughout the thesis	45, 46, 49
Justification for this as housekeeping gene of choice?	Accepted	The following explanation was added in the amended thesis "18S rRNA was chosen as a housekeeping gene since, first, it has a low turnover rate, while the large 18S rRNA pool is less prone to substantial changes from physiological perturbations. Second, its use has been validated by numerous studies that showed its invariant expression across various organisms, tissues, developmental stages, and treatments (Butte et al., 2001; Kuchipudi et al., 2012; Hounkpe et al., 2020). ".	56, 57
Justification for the use of 2 housekeeping proteins not clearly provided	Accepted	The following explanation was added in the amended thesis "The housekeeping control was chosen based on the size of the protein(s) of interest (i.e., they should have substantially different molecular weights) to ensure that the detection bands of loading controls do not interfere with those of the protein(s) of interest. Hence, β-actin (45 KDa) was chosen as loading control for Smad2/3 (60, 52 KDa), while GAPDH (37 KDa) was chosen as loading control for p44/42 MAPK (Erk1/2) (42, 44 KDa)".	62
Stronger justification is need for the infection MOI used in the in vitro studies (MOI 0.1 or 1)	Accepted	• The following clarification was added to Section 3.3.3 "The number of virions that infect each cell at different MOI, is described by the Poisson distribution: $P(k) = e^{-m}m^k/k$ where, P(k) is the fraction of cells infected by k virus particles, and m is the MOI. Natural RSV infection occurs through inhalation of aerosolized droplets that contain viral particles, resulting in infection of small foci of apical respiratory epithelial cells (Johnson et al., 2007; González-Parra and Dobrovolny, 2018). Hence, we have chosen lower MOI (0.1) to simulate the natural infection and allow for multiple cycles of replication. Based on Poisson distribution, high MOI (1) will infect around	70

		65% of BEAS-2B cells, however lower MOI (0.1) will only infect 9.5% of BEAS-2B cells which is more physiologically relevant."	
Justify the end points chosen	Accepted	• The following Justification was added to Section 3.3.3 "It is known that the clinical manifestation of RSV infection typically appears after four to six days after exposure to the virus (Piedimonte and Perez, 2014). Therefore, we chosed 48 hours viral infection. Moreover, the RSV infection at MOI 0.1 longer than 48 hours induces apparent cytopathic effects, which impacts the cell viability, therefore, maximum of 48 hours viral infection had been conducted. Our group had conducted preliminary experiments to examine 24 hours RSV infection, in parallel to 48 hours RSV infection, with 4 hours dexamethasone treatment in BEAS-2B cells. The expression of GC-responsive genes was also decreased, however non-significantly, with 24 h RSV infection. Moreover, our group also found that TGF-β mRNA at 24 h time point was higher than the expression at 48 h time point. Previous data suggests that TGF-β effect can be detected within 8 hours. Therefore, we could expect to see the secreted TGF-β-induced effect by 48 hours after viral infection. Expression of PAI-1 mRNA was used as a consistent marker for TGF-β activity, as expected; the expression level of PAI-1 was significantly increased with 48 hours viral infection." • The following sentence was added to section 3.3.6 "The 4-hour time point for mRNA expression of the GC-inducible genes was determined to be suitable by our group based on time-course evaluations (Salem et al., 2012; Keenan et al., 2014)". • The inhibitors used were added 30 min prior to the RSV infection or poly I:C to examine whether inhibition of TGF-β activity would prevent the RSV infection or poly I:C -impaired GC actions.	71 72-73
but they are not derived from asthmatic so the response may differ. you need to acknowledge this.	Accepted	• The following sentence was added "However, BEAS-2B cells are derived from healthy doner, so extrapolating findings to asthma is problematic due to intrinsic differences between non-asthmatic and asthmatic airway epithelial cells, i.e the baseline expression levels of PRRs." • Added as a limitation in Section 3.6	101
"similar characteristics" like?	Accepted	In the amended thesis, the characteristics were detailed as follows: BEAS-2B cells have been proved to share some characteristics of primary bronchial epithelial cells. For instance, BEAS-2B cells maintain the typical epithelial morphology and the same epithelial functional characteristics, however, they lack the individual variability observed in hAECs (Stewart et al., 2012). They also	100

		exhibit almost identical intracellular signalling pathways to that present in primary bronchial epithelial cells (Sauty et al., 1999; Schwarze et al., 2004) and show a similar responsiveness spectrum to numerous stimuli and pathogens (Thomas et al., 1998; Pichavant et al., 2003; Ieki et al., 2004; Schwarze et al., 2004). Moreover, BEAS-2B cells express all types of TLRs with the same spatial localization in the differentiated human AECs (Ioannidis et al., 2013), hence these cells present a satisfactory platform to test different TLRs ligands.	
TLR expression in diseased airway epithelial cells. Baseline differences?	Accepted	The following text was added to Section 3.5 "However, BEAS-2B cells are derived from healthy donor, so extrapolating findings to asthma is problematic due to intrinsic differences between non-asthmatic and asthmatic airway epithelial cells, i.e the baseline expression levels of PRRs. A study has examined baseline expression of PRRs; TLR3, MDA5 and RIG-I in cultured pBEC from asthmatic and healthy subjects and found similar mRNA expression levels in the two subject groups. Similar pattern was observed in an in vivo setting by measuring the TLR3, RIG-I and MDA5 mRNA levels from endobronchial biopsies obtained from these subjects (Parsons et al., 2014). However, cultured pBECs from asthmatics released significantly reduced levels of types I and III IFNs when exposed to RV infection indicating the defective signalling through TLR3 and MDA5 in asthmatic pBEC, despite the normal expression and induction of these PRR (Wark et al., 2005; Contoli et al., 2006; Parsons et al., 2014). Another study has investigated the expression of TLR2, TLR3 & TLR4 in large and small airways of fatal asthmatics, mild asthmatic and deceased control subjects. The immunohistochemistry has shown higher TLR2 expression in the epithelial and outer layers of large and small airways of fatal asthmatic patients compared with deceased controls. Fatal asthmatic patients had greater TLR3 expression in the outer layer of large airways and no significant differences in the small airways. A greater TLR4 expression were detected in the outer layer of small airways of fatal asthmatics when compared to deceased controls. No bacterial DNA was detected in both groups. QRT-PCR had detected only a small difference in TLR3 expression between mild asthma patients and healthy controls (Ferreira et al., 2012a; Ferreira et al., 2012b). Upregulation of epithelial gene expression for TLR2 and TLR4, but not TLR5, was observed by examining the epithelial brushings obtained from the large airways at bronchoscopy from asthmatic and healthy subjects (Cottey et al., 2011). On the contrary, targeted gene expression arrays of asthmatic and non-asthmatic airway wall samples have examined the expression patterns of candidate	101

		genes hypothesized to be involved in the asthma pathophysiology. Of all TLRs tested, only TLR7 was down-regulated (Pascoe et al., 2017)."	
Only gene expression for cytokines and should be corroborated at protein production levels via ELISA	Accepted	The following sentence was added "The observed downregulation of pro-inflammatory cytokines (IL-6 and IL-8) would need to be validated at protein level."	103
cite appropriately (Hinzy et al. 2011).	Edited	Citation was corrected as follows (Hinzy et al. 2011).	103
The issue that initial infection of RSV is typically ciliated airway epithelium and the cell line chosen isn't	Accepted	Added as a limitation in Section 3.6	107-108
no limitations to this chapter identified and conveyed.	Accepted	Section 3.6 was added to discuss limitations of the study.	107-108
This is incorrect. Bronchial epithelium implies in vivo expression however this chapter was conducted on cells, so you need to be specific and say ' in an immortalized bronchial epithelial cell line"	Edited	bronchial epithelium —> an immortalized bronchial epithelial cell line	114
include the p values in the text.	Accepted	In the amended thesis, p values were added to the text. "36% (p=0.0161) and 31% (p=0.0011) reduction in IL-6 and IL-8 mRNA levels, respectively"	118
Eg RV.. and studies in asthma and COPD	Accepted	The following sentence was added "Expression of TLR2, TLR3, and TLR4 had been shown to increase in asthmatic patients who ultimately die, suggesting their crucial role in the development of severe or even fatal asthmatic exacerbations (Kim and Rhee, 2010; Ferreira et al., 2012). Moreover, TLR3 may play a detrimental role in acute exacerbations of chronic obstructive pulmonary disease (AECOPD) patients, possibly due to TLR3 activation by viral and bacterial infection. TLR3 also enhanced the inflammatory response when in an antiviral state, leading to lung damage (Liu et al., 2018)."	122
• ROS and its importance in numerous chronic respiratory diseases and other infections • ROS in vivo (humans) and connection to RSV infections	Accepted	In the amended thesis, a new section was added "section 1.5.5" **1.5.5 Induction of oxidative stress during RSV Infection** Reactive oxygen species (ROS) are ubiquitous, highly diffusible, and reactive molecules, including free radical superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydroxyl radicals ($OH^{\bullet}$), perhydroxyl radicals ($HO_2^{\bullet}$) and hydrogen peroxide (H_2O_2) (Dröge, 2002). These species are generated upon one electron reduction of molecular oxygen ($2O_2 + 2e^- \rightarrow 2O_2^{\bullet-}$) and formation of the parent ROS $O_2^{\bullet-}$ (Halliwell and Gutteridge, 1999). Due to its negative charge, the radical superoxide ($O_2^{\bullet-}$) cannot diffuse across cellular membranes	35-36

		and acts only at the site of production (Vlahos and Selemidis, 2014). One electron reduction of $O_2^{\bullet-}$ produces hydrogen peroxide ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$). H_2O_2 is uncharged so it can penetrate cell membrane (Vlahos and Selemidis, 2014). Inside the cell, ROS are generated in the mitochondria, peroxisomes and microsomes as by-products of the normal O_2 metabolism of or from dysfunctional enzymes (Turrens, 2003; Bedard and Krause, 2007; Vlahos and Selemidis, 2014). The members of NADPH oxidase (NOX) family function solely to generate ROS (Bedard and Krause, 2007; Vlahos and Selemidis, 2014). The enzyme NOX2, a major source of $O_2^{\bullet-}$, is highly abundant in all cell types of the airways and in the residing alveolar macrophages or in the phagosomes of neutrophils and macrophages recruited to the lungs upon inflammation (Bedard and Krause, 2007; Vlahos and Selemidis, 2014; Singel and Segal, 2016). ROS at low levels are crucial for regulation of redox-sensitive genes involved in proinflammatory, immune responses and apoptosis (Dröge, 2002). At excessive levels, ROS cause oxidative stress, inflammation and tissue damage (Bedard and Krause, 2007; Vlahos and Selemidis, 2014). Hence, cells are well equipped with mechanisms to keep ROS levels under control. The first mechanism aims at limiting the generation of the parent ROS $O_2^{\bullet-}$ and its precursors, however it is crucial to balance the antiviral effects of ROS versus the detrimental effects of high ROS levels (Dröge, 2002; Bedard and Krause, 2007; Vlahos and Selemidis, 2014). An alternative approach is via simple stoichiometric scavenging by antioxidant molecules, such as vitamins A, C and E or the more complex antioxidant enzyme systems including superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase, thioredoxins, and peroxiredoxins (PRDXs) (Dröge, 2002; Bedard and Krause, 2007; Vlahos and Selemidis, 2014). Oxidative stress plays a major role in the pathogenesis of RSV-associated lung inflammatory diseases, as evident by the surge of markers of oxidative damage that correlate to the disease severity in RSV-infected children (El Saleeby et al., 2011; Garofalo et al., 2013). Both transcriptomic and proteomic studies analysing host responses to RSV infection were able to detect virus-induced alterations in cell stress responses including transcriptional, ER and oxidative stress at mRNA transcript (Janssen et al., 2007; Martínez et al., 2007; Mayer et al., 2007; Mejias et al., 2013; Brand et al., 2015; Do et al., 2017; Mariani et al., 2017) and protein levels (Brasier et al., 2004; Jamaluddin et al., 2010; Munday et al., 2010a; Munday et al., 2010b; van Diepen et al., 2010; Ternette et al., 2011; Hastie et al., 2012; Wu et al., 2012; Diepen et al., 2015; Munday et al., 2015; Zhou et al., 2018). In addition, RSV infection of airway epithelial cells induced rapid production of intracellular ROS with a concomitant modification of expression and/or activities of antioxidant enzymes (Hosakote et al., 2009).	

		A study examining how RSV infection drives the rapid production of ROS suggested that macrophages are responsible for the oxidative damage to lung during RSV infection, most likely via $O_2^{\bullet-}$ production through phagocyte NOX2 (Nguyen et al., 2016). Inhibition of NOX2 activity or knocking down its expression significantly decreased ROS levels in AECs following RSV infection confirming the role of $O_2^{\bullet-}$-producing NOX2 in RSV-induced oxidative stress (Fink et al., 2008). In addition, NOX2 depletion in RSV-infected AECs also blocks IRF3 phosphorylation and its downstream antiviral responses and inhibits NF-kB activation leading to blockage of the downstream expression of proinflammatory cytokines and chemokines (Indukuri et al., 2006; Fink et al., 2008; Soucy-Faulkner et al., 2010). Hence, RSV infection causes oxidative stress due to the increased ROS production via $O_2^{\bullet-}$-producing NOX2, that activates the NF-kB and IRF3 signalling pathways producing proinflammatory and antiviral responses. Another mechanism through which RSV infection induce oxidative stress is inhibition of antioxidant enzymes activity. This is evident from the reported reduction in the expression and activity of the antioxidant enzymes SOD1/3, PRDXS6, GPX1and catalase in RSV-challenged mice and children with RSV-induced bronchiolitis (Hosakote et al., 2011). How RSV infection interferes with the expression/activity of antioxidant enzymes is believed to involve the nuclear factor erythroid 2 (NFE2)-related factor 2 (NRF2) (Cho et al., 2009). NRF2 is a transcription activator that binds to the response element in promoter region of the antioxidant enzymes (Jaiswal, 2004). Under basal conditions, NRF2 is cytoplasmic and bounded kelch-like enoyl-CoA hydratase (ECH)-associated protein 1 (KEAP1) (Jaiswal, 2004). KEAP1 acts as an adaptor between NRF2 and cullin-3/RING box protein 1 (CUL3/RBX1), a ubiquitination ligase that ubiquitinates NRF2 and promotes its proteasomal degradation (Jaiswal, 2004). Oxidative stress promotes KEAP1 degradation and hence release of NRF2 from the KEAP2/ CUL3/RBX1 complex (Jaiswal, 2004; Kaspar et al., 2009). NRF2 then translocates to the nucleus and activates the transcription of antioxidant enzymes to protect the cells from oxidative stress (Jaiswal, 2004; Kaspar et al., 2009; Taguchi et al., 2011; Ma and He, 2012). RSV this mechanism via significantly increasing the expression and activity of histone deacetylase (HDAC) 2, which then deacetylases the nuclear NRF2 and targets it for proteasomal degradation. Dissociation of NRF2 from the response element sites of the antioxidant enzymes genes supress their transcription (Jamaluddin et al., 2005; Choudhary et al., 2009; Komaravelli et al., 2015; Feng et al., 2016). Indeed, treatment of RSV-infected airway epithelium with HDAC2 inhibitors restored NRF2 nuclear localisation and mRNA levels of antioxidant enzymes (Komaravelli et al., 2015). In summary, RSV infection results in oxidative stress via increased ROS production with the concomitant inhibition of antioxidant enzymes transcription.	

Again no limitations identified from this chapter as to what future experiments should be addressing.	Accepted	A new section "Section 4.6" was added to discuss the study limitations in addition to the suggested experiment to address these limitations.	127
needs citation if have been shown or previously shown	Edited	• "have been shown" refers to the results of chapter 3 that is why no citation was added. For further clarification, the sentence was edited as follows "The aforementioned findings suggested that". • "previously shown" refers to results presented in Figure 5.11. For further clarification, (Figure 5.11) was added to the end of sentence.	130 130
Interesting comment to convey especially when the in vitro cell model used the last two chapters had the same limitation yet was still advocated for.	Accepted	Added as a limitation in section 3.6	107-108
formation is inappropriate term./... replace with 'production'	Edited	formation —> production	131
This contradicts the last sentence on p122, as it was clearly conveyed that infection was age-dependent in ferrets	Edited	It was a typing error. age-dependent manner —> age-independent	132
you need to include various independent studies in these citations to illustrate the globally accepted limitation of this animal model of RSV infection and not just cite Johnson et al 2007	Accepted	The following citations were added (Taylor, 2017; Altamirano-Lagos et al., 2019).	132
how was the dose justified ??	Commentary	Justification was added as follows "The infective dose chosen in the current study was reported previously to develop infection that could be readily detected in lung homogenates and was accompanied by pronounced pulmonary pathology as indicated by pulmonary cellular influx and pro-inflammatory cytokine and chemokine levels (Muñoz et al., 1991; van Schaik et al., 1998; van Erp et al., 2019)."	137
folk mock vehicle??	Edited	Figure 5.11 Panel B "fold Vehicle —> fold Mock/Vehicle"	154
needs citation if previously shown	Edited	• "previously shown" refers to the data presented in (Figure 5.11). That is why no citation was added. For further clarification, "(Figure 5.11)" was added to the end of sentence.	156 162

		• "previously shown" refers to the data presented in Chapter 3 and 4. For further clarification, the sentence was edited as follows "In the preceding chapters, we have shown that"	
intranasal instillation ??	Rebuttal Commentary	No, it was actually insufflation triggered by respiratory reflex in response to fluid placed on the nares	162
part of future studies?	Edited	No, it is an explanation for the body loss observed in RSV-infected mice based on the findings of previous studies cited as follows (Kelley et al., 2003; Borchers et al., 2013). For clarification, the sentence was rephrased as follows "Previous studies had related the weight loss observed to the secretion of proinflammatory cytokines including TNF-α and IL-1α which are known to cause discomfort and reduce appetite, leading to weight loss".	162
this is gene expression, and the sentence needs to reflect this.	Edited	In the amended thesis, the sentence was edited as "In line with the changes in inflammatory cell infiltrates, RSV inoculation significantly provoked the mRNA expression of pro-inflammatory cytokine including TNF-α and IL-6".	165
accuracy of statement... observations were made only at gene level and not protein.	Edited	In the amended thesis, the sentence was edited as "Mice receiving intranasal instillation of poly I:C showed a significant surge in the mRNA expression of pro-inflammatory cytokines".	165
I don't think this was direct shown in your study, so again in future studies how are you going to test /prove this.?	Edited	"These findings" refers to results shown in two recent studies. For clarification, the sentence was rephrased as follows "Recent studies suggested that" Future experiments were discussed in Section 6.2.4	165
"we believe that PF670462; CK1e/d inhibitor provides a safer adjuvant to glucocorticoids" so, going forward how would you specifically test this.	Accepted	Future directions were discussed in Section 6.2.4 as follows **6.2.4 In vivo Studies: future directions** The in vivo studies performed herein revealed some intriguing findings suggesting that PF670462 either alone or through synergistic interaction with dexamethasone attenuated RSV infection-induced lung inflammation. Hence, we believe that it is of value to comprehensively investigate this potential in allergen-induced lung inflammation and allergic responses in the ovalbumin, house dust mite (HDM) or cockroach allergen-induced asthma models as well as the cigarette smoke exposure model of COPD, with or without viral exacerbation. Because the function of the airways is closely coupled to its structure, an altered airway function may reflect alterations in airway structure due to inflammation, tissue damage, or abnormal tissue remodelling. Thus, airway function is an important feature to monitor when evaluating the impacts of infection on respiratory health and the efficacy of therapeutic interventions.	176

		In the in vivo studies presented in the thesis, PF670462 were administered to mice through intraperitoneal injection. Minimizing the systemic adverse effects of TGF-β modulation is crucial in PF670462 development. The administration of small molecule kinase inhibitors CK1δ/ε by inhalation will limit their inevitable off-target effects reported with the systemic use including disruption to central circadian rhythm (Badura et al., 2007; Meng et al., 2010). Additionally, the inhalation route will overcome the low oral bioavailability of PF670462 due to the fast first-pass hepatic metabolism (Wager et al., 2014). For the above-mentioned reasons, future studies are needed to evaluate the therapeutic potential of aerosolized PF670462. Moreover, dose-escalation and safety studies should be performed, and phase 2 clinical trial started, aiming to translate these in vivo findings into a novel therapeutic for management of virus-induced asthma exacerbation that respond poorly to steroid. We identified TLR3 as a possible target to circumvent glucocorticoid resistance during RSV infection. Our results add more therapeutic value to TLR3 antagonism since it has been proved previously that TLR3-induced inflammatory responses are deleterious to RSV pathogenesis while RSV is insensitive to the TLR3-induced antiviral responses (Alexopoulou et al., 2001; Kitazawa and Villena, 2014; Wang et al., 2018). Future studies should, therefore, be focused on assessing GC responsiveness in viral-induced exacerbations od asthma or COPD models using TLR3 knockout mice. These studies may identify approaches to improve GC activity in RSV-infected patients.	
Study limitation? Future studies?	Accepted	Study Limitations were discussed in section 5.6 as mentioned before. Future directions were discussed in section 6.2.4 as mentioned above.	167 176
This section should have discussed the primary airway model in more detail.... Relevant diseased models of primary cells relevant to this thesis and experiments that need to be performed.	Accepted	Section 6.2.1 was amended, and a new section was added "Section 6.2.2" as follows: **6.2.1. Further evidence using a better in vitro model of the airway epithelium** In the current thesis, we have employed the immortalized human bronchial epithelial cell lines. It is a simple model that allows successful investigation of the roles of the specific targets in the cells. Data obtained from experiments using transient transfections and siRNAs to knockdown TLR3 in the immortalized BEAS-2B cells suggest that TLR3 partly mediates impairment of GC-actions during RSV infection. BEAS-2B cell line is generated from normal human bronchial epithelial cells and proven to be responsive to glucocorticoids. However, the limitation of this cell line is that it does not replicate many of the characteristics of differentiated	173-174

		cells, such as development of beating cilia and mucus production in vitro. Additionally, since it is derived from a single donor, it does not reflect the natural biological variation observed between individuals. The different response patterns seen between cell lines and primary epithelial cells may also be attributed to the immortalization process, polarization, differentiation or because they originate from different regions of the bronchial tree. Accordingly, it is still of paramount importance to verify our findings using a more physiologically relevant model with ciliated and goblet epithelial cells. Our group had replicated our findings in RSV-mediated GC insensitivity using the air-liquid interface culture of HBECs (Xia et al., 2017). Although, It is acknowledged that mucocilliary-differentiated primary HBECs are the most relevant in vitro model, there are many disadvantages limiting the broad use of these cells for research, including their limited life span, high cost of culture, high inter-individual and inter-passage variability, and difficulty of genetic manipulation (Stewart et al., 2012). To overcome limited life span of primary HBECs, basal cell immortalized-nonsmoker 1.1 (BCi-NS1.1) cell line can be used where it retains characteristics of the original primary cells and maintain a multipotent differentiation capacity for over 40 passages (Walters et al., 2013). Future experiments should focus on exploring our findings using BCi-NS1.1 cell line in air-liquid interface settings. Asthma and COPD have been proved to alter the normal airway epithelium (De Rose et al., 2018; Gohy et al., 2019; Calvén et al., 2020). This fact prompts the question of whether findings using primary HBECs derived from healthy donors in air-liquid interface culture could be extrapolated to asthma and COPD. Children in their first 2 years of life comprise the major risk group for RSV severe disease, with a peak in infants approximately 3 months old, after which the incidence gradually declines with age (Bianchini et al., 2020). This highlights the significance of using paediatric-derived primary cells to test our hypothesis in more clinically relevant model. **6.2.2 Further evidence using other gene silencing techniques** Gene silencing using siRNAs are transient (Kim and Eberwine, 2010), and the off-target and non-specific effects of the siRNAs generally complicate the interpretation of the results (Persengiev et al., 2004; Jackson and Linsley, 2010). Hence, other gene silencing techniques, including CRISPR/Cas9 system, should be considered to stably knock out TLR3 in ALI culture of primary HBECs. Finally,	175

		given that the airway epithelium is known to be altered in the asthmatic and COPD lung (Hiemstra et al., 2015), introducing heritable TLR3 deletions via CRISPR/Cas9 system into primary HBECs from asthmatic or COPD donors and culturing them at air-liquid interface would certainly give us further insights into physiological and pathophysiological roles of TLR3 in the airway epithelium.	
the potential to utilise natural human infection models to progress the field.	Accepted	A new section was added "Section 6.2.5" **6.2.5 Human model of experimental RSV Infection** Human experimental infection studies represent the interface between pre-clinical models and observational studies of patients and thus help mitigate the risks inherent in progressing to the late-stage trials. The first RSV experimental infection study was done by Mills et al. (1971) who have challenged healthy males with RSV Long strain. The human model used in this study allowed studying RSV infection under controlled conditions and permitted comparison of symptoms, airway physiology, lung function and airway inflammation (Mills V et al., 1971). Its evolution to include atopic individuals and mild asthmatic volunteers has increased our understanding of virus-induced exacerbations and their underlying processes (Hall et al., 1981; McKay et al., 1988; Higgins et al., 1990; Watt et al., 1990; Hall et al., 1991b; Lee et al., 2004; DeVincenzo et al., 2010; DeVincenzo et al., 2014; Kim et al., 2014; DeVincenzo et al., 2015; Habibi et al., 2015). In addition, the human challenge studies have been instrumental in early proof-of-concept clinical trials to test the efficacy of candidate RSV vaccines and therapeutics (Habibi et al., 2015). Hence, we recommend future testing of our findings using healthy or asthmatic volunteers to help evaluate the therapeutic value of interfering with TLR3 and CK1δ/ε activity to circumvent RSV-induced GC resistance.	177
Potential significance of mucins and mucus as a result of infection	Accepted	A new section was added; "Section 1.5.6." **1.5.6 Role of mucus secretion in RSV pathogenicity** The inner lining of the respiratory tract is covered by a layer of a viscoelastic, gel-like substance called mucus (Rose, 1992). The viscosity and acidity of mucus protect the epithelial surface from energy and inhibits microbial attachment to the underlying cells. The viscoelastic properties for mucus are attributed to mucin (MUC). Mucins are a family of high molecular mass, heavily glycosylated macromolecules that are the main components of mucus (Evans and Koo, 2009). Twenty-two MUC proteins are characterized in human and classified according to their subcellular localization into two groups. The membrane-bound mucins	38-39

		possess a transmembrane domain to attach them to the cell membranes. This group includes MUC1, MUC3A/B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC18, MUC20, MUC21, and MUC22. The second group is secreted mucins and includes MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, and MUC19. Apart from MUC6, MUC7, and MUC17, all mucins are expressed in the airways (Hauber et al., 2006; Linden et al., 2008). Mucus overproduction is often observed in inflammatory lung diseases like bronchial asthma, chronic obstructive pulmonary disease, bronchiectasis, and cystic fibrosis (Hauber et al., 2006) in addition to several malignancies like breast, gastric, colorectal, pancreatic, lung, small bowel, and ovarian cancers (Rakha et al., 2005; Chauhan et al., 2009). RSV is the leading cause of bronchiolitis and later stages of infection are characterized by the airways blockage by mucus plugs containing mucus, fibrin, cellular debris and lymphocytes (Pickles and DeVincenzo, 2015). In mice, RSV-induced lung damage has been correlated to raised neutrophil infiltration and elevated levels of TNF-α, a neutrophil recruitment factor that trigger the formation of MUC1 and MUC5AC (Hotard et al., 2015). The RSV G protein has been linked to mucus secretion in RSV-infected mice (Boyoglu-Barnum et al., 2013). This is believed to be triggered by the expression of type 2 cytokine, mainly IL-4, IL-5 and IL-13, and the chemokine MCP-1 (Zuhdi Alimam et al., 2000). In RSV-infected mice, signalling through the chemokine receptor CXCR2 has increased MUC5AC expression and in turn the mucus production (Miller et al., 2003). Other studies have highlighted the role of IL-17, a pro-inflammatory cytokine secreted by activated T cells, in mucus production in lung (Chen et al., 2003; Hashimoto et al., 2004). Lately, it has been reported that infecting A549 cells with the structurally-similar RNA viruses; RSV and human metapneumovirus (hMPV), triggered differential expression of MUC2, MUC5AC and MUC5B as well as membrane bound mucins (Baños-Lara Mdel et al., 2015). To summarize, RSV infection can change the composition of infected airway epithelium via increasing MUC5AC-secreting cells suggesting a crucial role of mucus secretion in pathogenicity (Persson et al., 2014).	
Typographical and grammatical errors			
In abstract:" Dexamethasone" should be lower case	Edited	Dexamethasone —> dexamethasone	iv

Standardise the font for the beta unit	Edited	Font of beta symbol has been adjusted in the respective pages.	Iv, 78
In Acknowledgement "DR" change to Dr	Edited	DR —> Dr	vii
Use of abbreviations in text, few places the same words are spelt out in full and abbreviated after	Edited	• rhinovirus —> RV • bronchoalveolar lavage fluid—> BALF	7, 8, 97 135, 153
is higher in "THE" obese population	Accepted	"the" was added	2
gender is repeated twice.	Accepted	"And gender" was deleted	3
Change impair to impairs	Edited	impair —> impairs	4
Change "have decreased" to have also been shown to decrease	Edited	"have decreased" —> "have also been shown to decrease"	8
Change in vitro italics	Edited	in vitro —> in vitro	8
Sentence grammar is not correct.	Edited	These sentences were rephrased as "This asthma cohort may experience poorly controlled asthma due to the few alternative anti-inflammatory therapies available. This in turn leads to disproportionate health care costs due to hospitalisations and emergency services use."	8
and none ARE related	Edited	is —> are	10
Change corticosteroid to corticosteroids	Edited	corticosteroid —> corticosteroids	10
Correct grammar	Edited	thereby hindering from interacting—> thereby hindering GRα from interacting	12
Delete 'The"	Accepted	"the" was deleted in the respective pages	12, 13
Change human to humans	Edited	human —> humans	17
Correct grammar	Edited	The sentence was deleted	19
delete receptors as R in TLR is already receptor	Accepted	"receptors" was deleted	19
that has been shown NOT that is proved….	Edited	that is proved —> that has been shown	19
include space between type and bracket	Accepted	Space was added.	27
has not yet BEEN confirmed	Edited	has not yet confirmed —> has not yet been confirmed	29

Change ascertain to ascertaining	Edited	ascertain —> ascertaining	37
Consistency of units (hour, hr and h, all used in this thesis).	Edited	In the amended thesis, hours and hr was replaced by h throughout thesis.	41, 64, 76, 77, 79, 92
Font of micro symbol. Standardize with the rest of thesis. As different font used.	Edited	Font of micro symbol has been adjusted in the respective page.	41, 82, 83
Spacing between data value and units ... others no spacing, keep consistent.	Edited	Corrections were made in the respective pages. 41-50, 53-55, 62-66, 68-72, 75-77, 79-87, 90, 92, 96, 107, 112, 113, 126-129, 130-134, 136, 138, 140,141.143, 145, 147-149, 151	Same pages as comment
Space between temperature and units and others no spacing, keep consistent.	Edited	Space between temperature and units was deleted throughout the thesis.	40, 41, 130
Use of superscript and subscript where appropriate.	Edited	• TCID50 was changed to $TCID_{50}$ and amended for the entire thesis where relevant. • MgCl2 was changed to $MgCl_2$ • 108 was changed to 10^8	xxiii, 42, 61, 127 48, 65
Change stock to stocks	Edited	stock —> stocks	45
this has been abbreviated before.	Edited	ethylene-diamine-tetra-acetic acid —> EDTA	53
add comma as you use this for our seeding densities	Edited	75000 —> 75,000	62
Add space"))"	Accepted	Space was added.	66
Moch?	Edited	Moch —> Mock	70
Gene names should always be italicized	Edited	Gene names were italicized thought the thesis.	
Grammar "On contrary to"	Edited	In the amended thesis, correction was made as follows "Exposure of BEAS-2B cells transiently transfected with GRE-SEAP expression vector to TLR2 ligands; Zymosan A (0.1, 1 and 10 µg/mL), or Lipoteichoic acid (1 or 10 µg/mL), for 24hr before 24 h of dexamethasone induction (30 nM), contrary to TLR3 and TLR4 ligands, did not attenuate dexamethasone-induced GRE activity".	75
Delete hitherto as not scientific language	Edited	The word "hitherto" was deleted	93
Need space	Accepted	Space was added	93
hypothesis not hypothesize	Edited	hypothesize —> hypothesis	95

Edit as sentence is not scientific writing.	Edited	This result brings TLR3 back into focus. —> These findings support the notion that poly I:C diminished GC actions downstream of TLR3.	97
Change proved to proven	Edited	proved —> proven	98
incriminate is not scientific writing. edit sentence.	Edited	first to incriminate TLR3 for—> suggested a possible role for TLR3	105
formatting issues here	Edited	Formatting was adjusted.	106
put figure above this.	Edited	Figure 4.2 position was adjusted.	106
Change oxidants to oxidant	Edited	oxidants —> oxidant	115
could	Edited	can —> could	121
Glucocorticoids	Edited	glucocorticoid —> glucocorticoids	121
we investigated this is a study performed and thus should be referred to in past tense not future	Edited	would like to investigate —> investigated	121
Delete 'the'	Accepted	"The" was deleted	122
increase	Edited	increases—> increase	122
Infection is found to upregulate.....	Edited	RSV infection often upregulates —> RSV infection is found to upregulate	122
makes no grammatical sense	Edited	before the full RSV clearance RSV —> the full clearance of RSV	122
kinetics	Edited	kinetic —> kinetics	123
levels	Edited	level —> levels	123
this sentence needs grammatical attention to flow from the previous sentence.	Edited	In the amended thesis, corrections were made as follows "Acute RSV infection triggers cytotoxic responses in the lung where epithelial cells release innate chemokines that attract innate immune cells to the infection site. The airway epithelium also includes resident dendritic cells that respond to allergens, pathogens and damage signals. In addition to resident dendritic cells, exudative macrophages (also named inflammatory monocytes) and innate lymphoid cells have been shown to be activated following viral infection. Epithelial-derived innate cytokines (IL-25, IL-33 and TSLP) play a crucial role where they activate type 2 innate lymphoid cells (ILC2s) and type 2 helper T (Th2) cells that release type 2	124

		cytokines (IL-4, IL-5 and IL-13) which in turn leads to mucus metaplasia. Hence, under certain circumstances, allergen exposure is not essential to initiate type 2 inflammation by viral infection".	
replace with 'could be replicated in vivo"	Edited	would replicate in vivo —> could be replicated in vivo	125
insert "the" before BALB/c	Accepted	"The" was added	125
delete word "be"	Accepted	be was deleted	127
were	Edited	are —> were	127
Grammatically incorrect... "At the conclusion of the experiment'	Edited	"At the experiment conclusion" was changed to "At the conclusion of the experiment"	129
Grammar... use 'receiving'	Edited	received —> receiving	130
showed	Edited	have shown —> showed	130
the minus sign is much larger than others seen below for -80.... keep consistent.	Edited	–20°C was changed to -20°C	130
delete "has"	Edited	has led to —> led to	139
neutrophil	Edited	neutrophils —> neutrophil	139
triggered. Again, refer to this study in the past tense.	Edited	triggers —> triggered	139
that characterization of the extent of concordances. grammar. edit text.	Edited	In the amended thesis, corrections were made as follows "Therefore, it is readily conceivable to characterize of the degree of concordance between the impact of poly I:C and RSV on murine lung. This may help unravel the relative contribution of TLR3 activation to the pathogenesis of lung dysfunction following RSV infection".	152
grammar	Edited	In 5-days model of RSV infection —> Five days post RSV infection, a marked decline was observed in thymus weight, indicative of thymus involution in mice.	153
incorrect spelling	Edited	thaught—> thought	153
Delete "the"	Accepted	"the" was deleted	159
suggests	Edited	suggest —> suggests	159
gene not genes	Edited	genes —> gene	160
spelling	Edited	Int —> in	164
Mice lowercase	Edited	Mice—> mice	164

Examiner's comments	Student's response	Change made to thesis (if any)	Page reference
I note that the major findings reported in Chapters 3 and 4 have previously been published in an excellent manuscript co-authored by the candidate (Xia, Radwan et al. PloS Pathogens 2017). However, the data sets in the manuscript are not the same as those that appear in the thesis. The relationship between the published experiments in the manuscript and experiments which appear in the thesis needs to be clarified, as this raises questions about novelty of the data in the thesis and possibly duplication of work. If the data sets presented in the thesis have been published, they must be appropriately referenced.	Commentary	• For RSV infection, two sets of experiments were performed using two different glucocorticoids: budesonide and dexamethasone. For the manuscript, we have selected the data sets done using budesonide rather than dexamethasone since budesonide is more clinically relevant. However, for the thesis I have selected to include dexamethasone data sets to keep consistent with the rest of experiments presented throughout the thesis. • Figure 6 in the manuscript is identical to Figure 3.19 in the thesis. • S7 Fig.in the manuscript is identical to Figure 3.10 in the thesis. • S6 Fig. in the manuscript is equivalent to Figure 3.20 A, but with extra n added to the data displayed in thesis. • Figure 7A in the manuscript is identical to data presented in Figure 4.4 A (48 h) • Figure 7B (left) in the manuscript is identical to Figure 4.7 A in the thesis. • Figure 7C in the manuscript is identical to Figure 4.7 B in the thesis. • Figure 5 C (right) in the manuscript is identical to Figure 3.14 (right), • Figure 5 C (left) in the manuscript is identical to Figure 3.11 (right).	
The abstract needs to be re-written; It needs to provide a concise rationale for the thesis, clearly articulate the specific aims, the main methods used to address each of the aims, all major findings, and conclusions.	Accepted	Abstract was re-written as follows: Chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) pose a major public health burden. Hospitalization, mortality and increased health care costs owing to these diseases are amongst the consequences of exacerbations. Exacerbations are usually elicited by respiratory infections, allergens, environmental pollutants or occupational sensitizers. These factors may work in either an additive or synergistic manner. Regular management using inhaled glucocorticoids (GCs) has an unquestioned benefit in alleviating exacerbations unless the respiratory viruses were the trigger. Due to the few alternative anti-inflammatory therapies available, restoring glucocorticoid (GC) efficacy is indispensable. Among the respiratory viruses implicated in asthma/COPD exacerbations is respiratory syncytial virus (RSV); the prime respiratory virus associated with bronchiolitis and pneumonia in infants, young children, immunocompromised adults and the elderly worldwide. Despite the fact that RSV-induced pathogenesis is in part inflammatory, the beneficial effect of GCs in alleviating the symptoms or altering the course of illness is still debatable. Several reports suggested that RSV infection has a detrimental effect on GC signalling through mechanisms that are not yet defined.	iii-iv

		Being the prime locus for viral infection and replication and the crucial target for glucocorticoid therapy as well, airway epithelium has been suggested as a key cell type in which virus-induced glucocorticoid resistance may occur. The airway epithelium releases a surplus of cytokines and chemokines leading to airway inflammation and airway structural changes seen in asthma. Among these inflammatory mediators is the transforming growth factor β1 (TGF-β1). Our laboratory has identified TGF-β1 as the key mediator of glucocorticoid insensitivity in bronchial epithelial cells where it ablates glucocorticoid activity at pathophysiologically relevant picomolar concentrations. We, therefore, investigated the possible contribution of this key cytokine to RSV-induced GC resistance. Data presented within this thesis has provided the first evidence that TGF-β1 drives RSV-induced GC-insensitivity in bronchial epithelium transformed with Ad12-SV40 2B (BEAS-2B) cell line. However, blanket inhibition of TGF-β1 using TGF-β receptor (activin-like kinase 5 (ALK5/ TGF-βR1 kinase) selective inhibitor, SB431542 compromises its beneficial pleiotropic effects leading to serious adverse effects. Our group has recently identified casein kinase 1delta /epsilon (CK1δ/ε) as a downstream effector of TGFβ-induced remodelling and steroid resistance. Our data also implicated CK1δ/ε in RSV triggered impairment of GC responsiveness where pharmacological inhibition of CK1δ/ε by PF670462 has inhibited the expression and activity of TGF-β1 and subsequently prevented the GC impairment provoked by RSV infection in our in vitro model. Moreover, PF670462 has significantly subdued RSV-induced expression of pro-inflammatory gene suggesting the potential for additional beneficial anti-inflammatory activities mediated by PF670462. This potential was further confirmed in vivo, where we have provided evidence of an interaction between PF670462 and dexamethasone to achieve greater control of the pulmonary inflammation following RSV infection. Moreover, we evaluated the contribution of different epithelial pattern recognition receptors (PRRs) involved in RSV sensing in the apparent resistance using a wide array of PRRs ligands. Among the ligands tested was polyinosinic:polycytidylic acid (poly I:C); a synthetic analogue of double-stranded RNA (dsRNA) that is frequently used as a viral mimic. Our results showed that that viral pathogen-associated molecular patterns (PAMPs), most importantly double-stranded RNA (dsRNA), activates toll-like receptor 3 (TLR3), activates toll-like receptor 3 (TLR3) that in turn signals through the extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent pathway to promote TGF-β activity hence mediating GC resistance in airway epithelial cells. Genetic approaches established that targeting TLR3 may restore the responsiveness to glucocorticoids.	

		In conclusion, we demonstrated that RSV impairs glucocorticoid sensitivity in airways epithelium partly through TLR3 activation with subsequent release of TGF-β1, a potent mediator of glucocorticoid resistance. We also implicated CK11δ/ε in the apparent resistance. This thesis provided novel insights into mechanisms of glucocorticoid insensitivity in the airway epithelium. These findings offered two novel anchor points; CK11δ/ε and TLR3, for a potential treatment for the prevention/treatment of GC insensitivity occurring during RSV infection-induced bronchiolitis or asthma/ COPD exacerbations.	
CK1□/□ needs to be appropriately introduced so that the reader understands the rationale for investigating it and the significance of the finding	Accepted	The following sentences was added: "However, blanket inhibition of TGF- β1 using TGF-β receptor (activin-like kinase 5 (ALK5/ TGFβR1 kinase) selective inhibitor, SB431542 compromises its beneficial pleiotropic effects leading to serious adverse effects. Our group has recently identified casein kinase 1delta /epsilon (CK1δ/ε) as a downstream effector of TGF-β-induced remodelling and steroid resistance."	iii-iv
It is unclear how the TGF beta and CK pathways are related, this needs explanation	Accepted	The following sentence was added. "Our group has recently identified casein kinase 1delta /epsilon (CK1 δ/ε) as a downstream effector of TGF-β-induced remodelling and steroid resistance."	iv
poly I:C needs to be introduced	Accepted	In the amended thesis, the poly I:C was introduced as follows "polyinosinic:polycytidylic acid (poly I:C); a synthetic analog of double-stranded RNA (dsRNA),"	iv
Steroid-resistant asthma is relatively resistant to corticosteroids in general, not just during periods of disease exacerbation. Please revise this introduction so that rationale is clear - you are not focussed on the steroid-resistant (severe) asthma phenotype per se, rather you are concerned with viral exacerbations of asthma and their lack of response to treatment with steroids (this is a different thing).	Edited	In the amended thesis, introduction was edited as follows: "Chronic respiratory diseases are a diverse group of illnesses affecting the airways and other lung structures. The most significant of these disorders are asthma, chronic obstructive pulmonary diseases (COPD), pulmonary hypertension and idiopathic pulmonary fibrosis (IPF). Among these disorders, asthma is the most prevalent in Australia with one in nine (equivalent to 2.8 million people) has asthma based on data reported from the 2017–18 Australian Health Survey (AHS) (Health and Welfare, 2020). Despite being incurable, asthma is largely manageable except for exacerbations that respond poorly to steroids. This fact sparks our interest to explore treatment improvement for steroid-resistant asthma exacerbations. Hence, in this section, we will review the epidemiology, pathogenesis and treatment options of asthma exacerbations with a focus on exacerbations of viral aetiology."	1
Delete or	Accepted	mild or moderate or severe or life-threatening —> being mild, moderate, severe, or life-threatening.	2
This is a very limited study published 14 years ago. I suspect we have learnt more about the exacerbator phenotype since then,	Accepted	More recent references were cited.	2

perhaps include/discuss more recent papers on this?		• Suau, S.J., and Deblieux, P.M.C. (2016). Management of Acute Exacerbation of Asthma and Chronic Obstructive Pulmonary Disease in the Emergency Department. *Emergency Medicine Clinics of North America* 34, 15-37. • Kostakou, E., Kaniaris, E., Filiou, E., Vasileiadis, I., Katsaounou, P., Tzortzaki, E., Koulouris, N., Koutsoukou, A., and Rovina, N. (2019). Acute Severe Asthma in Adolescent and Adult Patients: Current Perspectives on Assessment and Management. Journal of Clinical Medicine 8, 1283.	
Delete word(s)	Accepted	The following words were deleted: • In page 3, "changes in", "and gender" • In page 4, "impaired", "the" • In page 19, "intracellular", "receptors" • In page 22, "anti" • In page 26, "a mimic to RSV" • In page 31, "growth factors" • In page 34, "release" • In page 35, "has" • In page 114, "diluting"	Same pages as comments
Are you suggesting that factors discussed here are modifiers of viral-induced exacerbation? Or are these factors that are associated with more severe exacerbation? Please clarify	Edited	The sentence was amended as follows "Some individual characteristics have been proposed as potential modifiers of asthma exacerbations severity"	3
Write dash	Edited	bronchoconstrictor like —> bronchoconstrictor-like	4
Delete sentence "The virus and increased sensory nerve exposure to allergens."	Accepted	The sentence was deleted.	4
This is a systematic review from 20 yrs ago. Are there recent references to support this?	Accepted	More recent references were added: • Alangari AA (2014) Corticosteroids in the treatment of acute asthma. Annals of thoracic medicine 9(4):187-192. doi:10.4103/1817-1737.140120 • Hasegawa, K., Craig, S.S., Teach, S.J., and Camargo, C.A. (2021). Management of Asthma Exacerbations in the Emergency Department. J Allergy Clin Immunol *Pract*. In press.	7
Reducing risk of future exacerbation (by improving asthma control) is different to medical management of virus-induced	Edited		

exacerbations. You need to be mindful of this difference when discussing each of the therapeutic options - you have made the distinction for GCs, IFN-b and anti-IgE. • Please clarify use of anti-cytokine therapies in terms of reducing risk vs use in management of exacerbation. This is about reducing risk, not treating the exacerbation once it has occurred. Please clarify		• The sentence was edited as follows "The third approach to reduce the risk of exacerbations is to alleviate pulmonary inflammation via different anti-inflammatory strategies."	8
Again, distinguish between reducing risk and treating the exacerbation	Edited	The sentence was amended as "The last approach to reduce the risk of virus-induced asthma exacerbations is to target the virus itself via vaccination, use of antiviral drugs or preventing the entry of the virus".	9
Legend to ligands	Accepted	binding of TLRs to their legend —> TLRs ligands binding	25
Correct TL3	Accepted	TL3 —> TLR3	25
RSV-induced GC-resistance has not been specifically discussed Significant omission is the critical review of mechanisms by which RSV impairs GC activity	Accepted	RSV-induced GC-resistance was discussed in Section 3.1 Being the prime locus for viral infection and replication and the crucial target for glucocorticoid therapy as well, airway epithelium has been proposed as the main cell type where virus-induced glucocorticoid resistance may occur. RSV has been shown to potentially interfere with the anti-inflammatory activity of steroids in epithelial cells (Bonville et al., 2001; Carpenter et al., 2002), yet with undefined mechanism. Three potential pathways could contribute to RSV interference with GC signalling. These include production of autocrine factors such as cytokines; activation of intracellular signalling pathways; or via a direct viral proteins or RNA effect. Airway epithelial cells produce an array of pro- and anti-inflammatory cytokines and chemokines including the type-I and -III interferons (IFN-α/β and IFN-λ), TNFα, IL-4, IL-8, IL-13, IL-17, CCL3 and RANTES (Zeng et al., 2011). Some of these autocrine factors such as IL-2/IL-4, IL-13, TNFα, IL-6, IL-8, IFNγ and IFNα have been proved to interfere with epithelial response to GC (Rider et al., 2011; Zijlstra et al., 2012; O'Connell et al., 2015). This fact has tempted researchers to postulate that RSV-induced cytokines could impair GR function. When tested, RSV-conditioned media failed to repress expression of GC-responsive genes (Webster Marketon et al., 2014) despite being able to induce IL-8 transcription (Patel et al., 1998). These findings suggest that RSV-induced GC irresponsiveness is not triggered by a soluble autocrine factor.	65-66

		RSV infection also activates a number of host signalling pathways, such as NF-κB, phosphoinositide 3-kinase (PI3K), and MAP kinase pathways (Garofalo et al., 1996; Mastronarde et al., 1996; Thomas et al., 2002; Lindemans et al., 2006; Singh et al., 2007). Several studies have reported the mutual transcriptional antagonism between GR and these pathways, so GC resistance could be a result of the activation of these pathways (Ray and Prefontaine, 1994; Irusen et al., 2002; Adcock and Lane, 2003; Marwick et al., 2009; Ayroldi et al., 2012). However, Hinzey et al. (2011) have excluded the possible involvement of NF-κB pathway in repressing dexamethasone actions in RSV-infected lung epithelial cells. Interference with the initiation of these signalling cascades has been also tested. MyD88 is a downstream adaptor molecule of TLRs or RIG-1 signalling pathways and its knocking-down did not restore dexamethasone-induction of GILZ expression in RSV-infected A549 cells ruling out its contribution to the apparent RSV-induced GC resistance. Knockdown of MAVS, another downstream adaptor of RIG-1 signalling cascade, has significantly reduced dexamethasone induction of GILZ in mock-infected cells that further reduced in case of RSV infection, albeit non significantly (Webster Marketon et al., 2014). Hence, we cannot exclude MAVS biological role in the RSV-induced GC insensitivity. Webster Marketon et al. (2014) have continued to test the possibility of a direct viral proteins or RNA effect. It is known that NS1 and NS2 proteins of RSV inhibit the interaction between RIG-I and MAVS with subsequent suppression of type I interferon response (Ling et al., 2009; Boyapalle et al., 2012). Overexpression of NS1, but not NS2, repressed GC induction of GILZ. This observation was held true when tested using viruses with deletions in the NS1, but not NS2 genes, that prevented RSV suppression of glucocorticoid-induced GILZ (Webster Marketon et al., 2014) suggesting that nonstructural proteins mediate the RSV suppression of GR function.	
The complete omission of any review/discussion of casein kinase 1δ/ε, which is investigated in chapters 3 and 5	Accepted	The following text was added to Section 3.1 We have previously established TGF-β as a profound mediator of glucocorticoid resistance in A549 cells (Salem et al., 2012), human immortalized bronchial epithelial BEAS-2B cells, and in ALI-differentiated primary HBECs (Keenan et al., 2014). Our group has unravelled a potential link between RSV infection and TGF-β, where TGF-β functions in an autocrine manner to mediate glucocorticoid insensitivity in RSV-infected BEAS-2B and primary bronchial epithelial cells (Xia et al., 2017). Since TGF-β is crucial to diverse cellular activities (Boxall et al., 2006), broad inhibition of TGF-β is not clinically feasible due to the known risk of autoimmune colitis (Shull et al., 1992; Kulkarni et al., 1993), tumorigenesis (Hahm et al., 2001; Oyanagi et al., 2014) and mitral valve lesion (Anderton et al., 2011).	66-67

		Our group comprehensively reviewed TGF-β1 signalling pathways to define the downstream pathways by which TGF- impairs epithelial GC actions, hence identify novel targets to circumvent GC resistance (Salem et al., 2012; Keenan et al., 2014). We have found that TGF-β profoundly impairs GC responsiveness not through known non-canonical pathways, nor through Smad4-dependent signalling, suggesting that a novel non-canonical signalling mechanism. Comprehensive literature review revealed a little-known connection between TGF-β1 signalling and casein kinase (CK) activity. Of particular interest, casein kinase has also been implicated in the TGF-β1 signalling cascade in compelling studies. The immune-precipitated TβRII is reported to possess an intrinsic casein kinase activity, suggesting that casein kinase may be involved in one of the signalling cascades subserving the activity of TGF-β1 (Hall et al., 1996). Furthermore, CK1ε is able to phosphorylate the receptor-activated Smads and the TβRII in vitro. More importantly, inhibition of endogenous CK1 catalytic activity with IC261 blocked TGF-β-stimulated transcriptional activity (Waddell et al., 2004). Intriguingly, our laboratory found that inhibition of CK1δ/ε by PF670462, prevented TGF-β1-induced EMT in A549 human alveolar epithelial cells and TGF-β1-activated fibrogenic gene expression in human lung fibroblasts (Keenan et al., 2018). CK1δ/ε may therefore be an important component in the TGF-β signalling cascade. Hence, in the current study, we sought to selectively modulate TGF-β1 activity by targeting CK1δ/ε using PF670462, a dual inhibitor of CK1δ/ε activity, seeking to restore GC sensitivity in RSV-infected epithelial cells.	
"~~Thesis~~ hypothesis and ~~main~~ aims"	Edited	~~Thesis~~ hypothesis and ~~main~~ aims —> Hypothesis and aims	43
Please provide references	Accepted	The following citations were added (Hinzey et al., 2011; Webster Marketon and Corry, 2013a; b; Webster Marketon et al., 2014)	43
Please articulate aims such that they reflect the specific studies undertaken in each of the chapters.	Edited	The aims were edited as follows: • defining the contribution of TGF-β in the RSV-induced GC resistance. The results of this work are presented in Chapter 3. • systematically evaluating the impact of activation of different PRRs involved in RSV sensing by their respective ligands, on GC responsiveness in human bronchial epithelial cells. The results of this work are presented in Chapter 3. • characterizing the novel role of the casein kinase 1 δ/ε (CK1δ/ε) inhibitor; PF670462, in counteracting RSV-mediated attenuation of steroids response in the human airway epithelial cells. The results of this work are presented in Chapter 3.	43

		• confirming the intracellular signalling cascade involved in RSV-mediated ablation of GC responsiveness in human airway epithelial cells. This was done by siRNA-mediated gene silencing. The results of this work are presented in Chapter 4. • ascertaining the impact of the CK1δ/ε inhibitor; PF670462, on airway inflammation in murine models of RSV infection and poly I:C acute lung injury. The results of this work are presented in Chapter 5.	
This is a systematic review re use of chest physiotherapy in bronchiolitis, does not relate to effects of GC in acute bronchiolitis	Accepted	"Roqué i Figuls et al., 2012" and the corresponding reference was deleted.	65
RSV is not mentioned in this reference	Accepted	"Webster and Sternberg, 2004" and the corresponding reference was deleted.	65
These are abstracts. Not clear why manuscript by Yuxiu C. Xia et al is not referenced?		In the amended thesis, the following citations (Yuxiu et al., 2013; Yuxiu et al., 2014; Yuxiu et al., 2015) were replaced by (Xia et al., 2017).	66, 70, 89, 102, 172
legend to ligand	Accepted	legend —> ligand	67
"Thus, we systemically assessed the PRRs including that contribute to RSV sensing for their potential contribution to GC insensitivity following RSV infection in order to delineate the driving mechanisms of the apparent resistance." You have not investigated all PRRs, state the specific PRRs that were investigated and rationale for selecting these PRRs	Edited/ Commentary	"including TLR 2, 3, 4, 7 and RIG/MDA5" was added. They have been selected based on their role in the innate immunity to RSV as clarified in "PRRs that contribute to RSV sensing"	68
• Casein kinase 1 delta/epsilon signalling needs to be introduced		• In Section 3.1, text was added as mentioned above.	60
Is there a reason why you did not examine mRNA expression of RIG-I and MDA-5?	Commentary	The mRNA expression levels of RIG-I and MDA-5 were not measured since the respective primers where not available in the laboratory.	
Is this a typo? Data in Fig 3.2A does not suggest robust TLR5 basal expression of in BEAS-2B cells. TLRs-1, -5, -6, -9 are present, but at low levels, while the expression of TLR7, -8 and -10 appears to be negligible	Edited	The sentence was edited as follows "Based upon the basal mRNA expression ($2^{-\Delta CT}x10^{8}$), the most highly expressed TLRs were TLR2, TLR3 and TLR4, TLR 1, TLR5, TLR6 and TLR9 were also expressed but al lower levels, whereas TLR7, TLR8, and TLR10 mRNA appeared to be negligible as shown in Figure 3.2."	73

Since hypothesis is that RSV-induced TGF-b release might be responsible for RSV-mediated GC resistance, it is unclear why TGF-b release was not examined at this early stage of the investigation	Commentary	Our group assayed TGF-β released in the cell culture supernatant using BD OptEIA™ Human TFG-β1 ELISA Set. However, there was some technical difficulties with the assay sensitivity. The minimal TGF-β level could be detected was approximate 10-fold higher than the minimal level claimed on the kit technical data sheet (1ng/ml vs 0.1ng/ml). The ELISA assay absorbance values obtained from our samples were lower than the linear range of the curve, which suggested that the released TGF-β level was lower than 1ng/ml and the data was unreliable interpretation. Our group did not put too much effort on troubleshooting, as TGF-β can be produced in a latent form. TGF-β activity is regulated through the conversion of latent TGF-β to active TGF-β by a number of proteases. There is not necessarily a linear relationship between the viral infection-induced expression of TGF-β mRNA and its activity. The low TGF-β mRNA expression level does not mean low TGF-β activity. Therefore, the time-dependent expression and release of TGF-β, and the concentration response-relationship for the induction of PAI-1, were not studied.	
please give specific n numbers	Accepted	In the amended thesis, specific number of biological replicates were stated in each figure ligand.	
Please specify comparisons being made, I assume comparisons here are versus unstimulated control	Accepted	In the amended thesis, "*c.f.* unstimulated control" was added.	74
Please all results in text. You have neglected to mention that RSV does not impair Dex-induced Promyelocytic Leukaemia Zinc Finger Protein	Accepted	The following sentence was added "Moreover, mRNA expression of promyelocytic leukaemia zinc finger protein (PLZF); a transcriptional repressor that mediates antiproliferative and apoptotic actions of GCs, showed a slight decrease, however non-significantly, in RSV-infected cells.	75
Add * RSV vs RSV+Dex in GRE data	Accepted	Panel A in figure 3.3 was amended to include the required comparison.	76
• "c.f. Dex 30 nM response" this is confusing as it does not indicate which dex response... not needed, as comparisons are made in the figure. • Also, why 2 stars here and 4 stars in the figure? • Please clarify if GRE activity in Dex treated RSV infected cells is still significantly induced, i.e. is there significant induction in GRE activity compared with vehicle treated RSV infected cells. In other words, is GRE	Accepted	• "c.f. Dex 30 nM response" was deleted. • "**p<0.01" was replaced with "****p<0.0001" • In the amended thesis, the following sentence was added "Noteworthy, GRE activity was not reduced to baseline levels in RSV-infected cells treated with dexamethasone (30 nM), and there was still some residual, however significant, induction of GRE activity compared to vehicle-treated RSV infected cells (p=0.0047)".	77 77 75

activity reduced to baseline levels, or is there still some residual activity in RSV infected cells treated with Dex			
Up until this point, you have not demonstrated RSV-induced GC insensitivity - you have shown that RSV attenuates Dex-induced GRE activity and transactivation	Accepted	RSV-induced GC insensitivity —> RSV-induced attenuation of GC-induced gene transactivation and GRE activity	80
It is unclear why the candidate has used both heat-inactivated virus and UV inactivated virus to answer this question – this needs to be better explained; if heat-inactivation is not examining the impact of viral replication, then why include the data?	Commentary	The reason was added in Section 3.4.3 as follows "Heat inactivation of the virus denatures the viral proteins, hence, allows exploring the residual impact of ssRNA viral genome alone. On the contrary, UV inactivation damages the viral genome through dinucleotide crosslinking, while maintaining the viral proteins intact."	78
Rationale/purpose of these studies is unclear - they do not address the aim stated at the start of this paragraph	Edited	• A new section "Section 3.4.3 RSV-induced expression of inflammatory cytokine was largely replication-dependent" was added. • The aim was clarified as follows "We thought to explore whether virus replication was needed to induce expression of inflammatory cytokine; IL-6 and IL-8 or the surface contact with viral components was sufficient."	78 78
Please clarify it is clear from Fig 3.4 that Dex inhibits IL-6 and IL-8 expression in RSV infected cells, however, it is not clear if this is also the case in cells infected with HI or UV treated RSV. Please clarify in text and include relevant statistical comparisons in figure 3.4B	Edited	• Dexamethasone (30nM) remarkably dampened expression of pro-inflammatory cytokines; IL-6 and IL-8 in RSV-infected, HI-RSV- or UV-RSV-challenged BEAS-2B cells (Figure 3.4 B). • Figure 3.4 B was amended to include the requested statistical comparisons.	78 79
• You cannot derive this conclusion from the data presented in Fig 3.5. • To examine whether RSV-dependent inhibition of GRE activity is replication dependent, need to compare effect of replication competent RSV, HI-RSV and UV-RSV in the same experiment. • HI-mock and UV-mock is not appropriate control treatment	Edited/ Rebuttal commentary	• The sentence was edited as follows "These data support the notion that RSV-induced impairment of GC sensitivity is at least partially replication-dependent." • Text was added as follows "Although the fact that the sets of experiments using replication competent RSV, HI-RSV and UV-RSV were performed separately in different cell culture plates, which is a suboptimal experimental setup for comparison, during each experiment, the virus and mock used were originating from the same virus or mock batch. In addition, BEAS-2B cells were obtained from the same source, were of similar passage number, and were handled at the same facility. These might partly minimize the impact of confounders and make the comparison possible."	80 80 69-70

		• Mock was used as a control in the current study to ensure that the observed effects were due to the virus itself not to another RSV stock component since it is crudely prepared from infected HEp-2 cells. The following explanation was added to section 3.3.2 "Previous study reported that when cell culture medium was irradiated with UV at relevant disinfection doses, the chemical composition of the media was affected with respect to several carboxylic acids, and to a minimal extent, amino acids (Yen et al., 2014). Moreover, heat inactivation decomposes heat-liable medium components. Hence, the mock inoculum was subjected to the identical inactivation procedure either by heat or UV-irradiation to help serve as a negative control in these sets of experiments."	
Add reset of ** in IL-6 IL8 figures vs dex treatment for hirsv and uvrsv	Accepted	Figure 3.4 B was amended to include the requested statistical comparisons.	79
UV-mock and HI-mock are inappropriate controls. Need to compare response to replication competent RSV.	Rebuttal Commentary	Mock was used as a control in the current study to ensure that the observed effects were due to the virus itself not to another RSV stock component since it is crudely prepared from infected HEp-2 cells. Justification was previously mentioned explanation in Section 3.3.2	69-70
there is one star in figure A	Accepted	**—> *	81
Did RSV infection lead to complete inhibition of GRE activity? See previous comment	Accepted	In the amended thesis, the following sentence was added "Noteworthy, GRE activity was not reduced to baseline levels in RSV-infected cells treated with dexamethasone (30 nM), and there was still some residual, however significant, induction of GRE activity compared to vehicle-treated RSV infected cells (p=0.0047)" In the amended thesis, the following sentence was added "However, neither of the tested concentrations had led to complete inhibition of GRE activity."	76 80
Also, pre-incubation with TLR2 ligand zymosan seems increase GRE activity. Please include result in text.	Accepted	In the amended thesis, the following sentence was added "Noteworthy, pre-incubation with TLR2 ligand; Zymosan A at 10 µg/mL had led to approximately 50% increase in GRE activity".	83
Please include statistical comparisons in figure for better clarity (even if not statistically sig.)	Accepted	Figure 3.9 was amended to include suggested statistical comparisons.	86
It would have been useful to confirm expression of these receptors in your in vitro system, as you did for other TLRs.	Commentary	The mRNA expression levels of RIG-I and MDA-5 were not measured since the respective primers where not available in the laboratory.	
You cannot draw this conclusion from this data, as GC-insensitivity may be mediated by other mechanisms.	Accepted	The sentence was edited as follows "RLRs might not contribute to RSV-induced GC insensitivity in epithelial cells."	83

All you can say is that RIG-I/MDA-5 signalling does not impair GC induction of GR-regulated genes			
It would be helpful to the reader if at this point you clarify that RSV induced TGF-b expression appears to be dependent on the activation of TGF-b receptors. Is it possible that RSV activates TGF-beta receptors?	Accepted	• The following text was added "These findings suggested that RSV-induced TGF-β expression might be dependent on the activation of ALK5. Our group provided additional evidence of a role for ALK5 activation in RSV-induced TGF-β expression using a structurally distinct ALK5 inhibitor; GW788388 and ALK5 siRNA, where both methods had shown to ablate TGF-β expression in RSV-infected cells."	88
Cannot make this conclusion, inappropriate controls used	Rebuttal commentary	Justification for use of HI-mock and UV-mock was added to Section 3.3.2 as mentioned above.	69-70
Inappropriate controls used	Rebuttal commentary	Comment is mentioned above.	69-70
Please include statistical comparisons in figure for better clarity (even if not statistically sig.)	Accepted	Figure 3.13 was amended to include requested statistical comparisons.	91
This needs some introduction/explanation, it is unclear how CK1 relates to TGF-beta pathway How does CK1 regulate TGF-b release? Is there any mechanistic basis for this?	Accepted	New text was added to Section 3.1 as mentioned above	67
These results are more about PF and SB, rather than dex? Rationale for these studies needs to be clarified	Edited	Section 3.4.11 was amended as follows: **3.4.11 Inhibition of CK1 δ/ε or ALK5 subdues pro-inflammatory cytokine expression in RSV-infected BEAS-2B cells** The expression of genes encoding IL-6 and IL-8 are known to be regulated by glucocorticoid transrepression mechanism (King et al., 2013). Therefore, we investigated whether RSV infection impairs the regulation of IL-6 and IL-8 expression in BEAS-2B cell. As expected, RSV infection prompted the expression of IL-8 and IL-6 mRNA in BEAS-2B cells. Dexamethasone (30 nM) subdued the RSV infection-induced IL-6 and IL-8 expression. Next, we explored whether the inflammatory response triggered by RSV infection can be inhibited by targeting ALK5 or CK1δ/ε. Interestingly, pre-treatment of cells with either SB431542 (1 μM) or PF670462 (10 μM) prior to RSV inoculation had no effect on IL-8 mRNA expression, yet significantly repressed RSV-induced IL-6 expression (Figure 3.18 A and Figure 3.18 B).	95
You have not specifically demonstrated a role for TLR3 (findings with poly IC suggest	Accepted	TLR3-ERK1/2-dependent pathway —> ERK1/2-dependent pathway	97

a role for TLR3, this needs to be confirmed through specific inhibition of TLR3)			
What is rationale for focussing on the ERK pathway?	Accepted	The following sentence was added "Therefore, the involvement of these pathways was investigated. Interestingly, while RSV infection had no significant effect on phosphorylation of p38MAPK, Akt, JNK and NFκB (results not mentioned here), RSV infection triggered ERK1/2 phosphorylation in BEAS-2B cells, as demonstrated by Western blotting using corresponding antibodies and phospho-antibodies (Figure 3.19 A).	97
Given size of error bars (Fig 3.19A), I would not draw any conclusions about trends	Edited	tend to suppress —> " had no effect on the phosphorylation of ERK1/2, albeit the data were too variable to provide a confident interpretation (1951.6 ± 910.4, 1348.7 ± 543.4, p=>0.9999)."	97
As discussed in Ch1, the morphology and differentiation of the airway epithelium impact susceptibility to viral infection and the ensuing immune response. Thus, while studies in BEAS-2B monolayer cultures may provide an appropriate and feasible approach to investigate RSV responses, it is important to recognize limitation of using cell line in monolayer culture - please discuss possible limitations.	Accepted	The concern was added as a limitation in Section 3.6	108-109
You have not really done this, you have examined possible role of viral sensing PRRs in impairing GC response - it was not a comprehensive investigation based on receptor inhibition etc	Edited	we have dissected the possible role of each of RSV-sensing PRRs in ablating GC sensitivity during viral infection —> we examined the possible role of each of RSV-sensing PRRs in altering GC sensitivity during viral infection.	111
You are overstating this - data using TLR3 ligand suggests a role for TLR3, you are yet to definitively establish this	Edited	The following sentence was rephrased as "we have successfully pinpointed TLR3 and to a lesser extent TLR4 as the major contributor to this detrimental effect." —> Of all PPRs tested, we suggested that TLR3 and to a lesser extent TLR4 had partly contributed to the apparent GC resistance	111
Iminify is this a word?	Edited	minify —> diminish	113
As a major sensing of viral pathogens, it would be hard to argue it has a detrimental role? It is more about whether viruses can circumvent TLR3 signalling to gain	Edited	The question that arises is whether TLR3 functions as a defence or offence in the antiviral host immunity —> However, it is quite challenging to understand the protective versus pathogenic contribution of TLR3 in viral infections. The question that	113

advantage? Or pathological situations that lead to aberrant/unintended activation of the receptor?		arises is whether viruses can circumvent TLR3 signalling to gain advantage? or pathological situations that lead to aberrant/unintended activation of the receptor?	
Not really clear from this para what you are referring to by a detrimental role. Needs better explanation	Edited	The sentence was amended as follows "A controversial role of TLR3 has been reported in Human immunodeficiency virus type 1 (HIV-1, ssRNA) infection, where activation of the TLR3 signalling cascade with poly(I:C) triggers an antiviral response that limits HIV-1 infection in cell culture, suggesting a therapeutic potential for TLR3 ligands against HIV-1 infection in humans (Rivieccio et al., 2006; Swaminathan et al., 2012)."	114
this was not investigated	Accepted	"replication" was deleted	115
What is rationale for investigating effect of Dex on RSV/poly IC induced TLR3 expression? This is not set out as an aim	Accepted	The following text was added "Dexamethasone is a potent modulator of the innate immune system by shifting the TLR system response pattern from an inflammatory to an anti-inflammatory state (Broering et al., 2011). However, modulation of TLR expression by glucocorticoids has not been established yet. Hence, we sought to explore the impact of dexamethasone on TLR3 expression level in BEAS-2B cells."	117
Did you check impact on other viral sensing PRRs? May need to acknowledge this as a limitation	Accepted	Added as a limitation in Section 4.6	128
There was no significant inhibition of TGF-b expression	Edited	Text was amended as follows "However, TGF-β1 expression in RSV-infected cells showed a decreasing trend, albeit non-significant, following TLR3 knockdown (Figure 4.7 A)."	119
Data for PAI-1? When there is missing data, please acknowledge, perhaps provide reasons why the experiments were not done?	Accepted	PAI-1 graph was added to Figure 4.8, Panel A (right)	122
Not correct, data with dsRNA suggests a role for TLR3, which you specifically investigate here	Edited	The sentence was edited as follows "In the preceding chapters, we, for the first time, suggested that TLR3 contributed at least partially to impairment of RSV-induced glucocorticoid responsiveness in the bronchial airway epithelial cells."	124
You have not studied RSV replication?	Accepted	"replication" was deleted.	124
What about effects on other relevant (dsRNA/RSV sensing receptors, such as PKR, RIG-1 and MDA5)?? Need to acknowledge possible off-target effects re these receptors and acknowledge as limitation (as u did not confirm that they were not impacted by TLR3 siRNA)	Accepted	Added as a limitation in Section 4.7	128

This may also suggest that TLR3 is not the major driver of the RSV-mediated inflammatory response? Should discuss this possibility and implications	Accepted	The following sentence was added "Moreover, our findings suggested that TLR3 is not the major driver of the RSV-mediated inflammatory response."	126
No data re viral clearance is presented in this chapter	Edited	This refers to previous studies. For clarification, the following citations was added "(Rudd et al., 2005; Hillyer et al., 2018)"	126
Do these references show that RSV-mediated TGF is TLR3 dependent? Please clarify - I had a quick look at the references, and it does not look like they investigate role of TLR3 in RSV-mediated TGF-b release	Edited	Text was amended as follows "Literature has reported that TGF-β is expressed during RSV infection of lung epithelial cells (McCann and Imani, 2007; Gibbs et al., 2009; Mgbemena et al., 2011; Bakre et al., 2015) and macrophage (Pokharel et al., 2016), however, the mechanism that elicits TGF- β1 expression during viral infection is still unclear."	127
abrogated to induced	Edited	Abrogated —> induced	127
This is not correct	Edited	Text was amended as follows "In the context of RSV, we have found that TGF-□1 expression tended to decrease upon TLR3 ablation, albeit not significantly."	127
You did not examine the TLR3 pathway	Edited	Text was amended as follows "We suggested that dsRNA originating from RSV might in part bind to TLR3 and activates ERK1/2 pathway to induce TGF-β expression."	127
In the current study, it is tempting to postulate that RSV or poly I:C activates the TLR3/ERK/Autophagy axis to induce the TGF-β expression that mediates the steroid activity impairment in the bronchial epithelium. Data presented in this chapter does not support a role for TLR3 in RSV mediated TGF-b release	Edited	Text was amended as follows "Although not fully investigated, it is tempting to postulate that poly I:C activates the TLR3/ERK/Autophagy axis to induce the TGF-β expression that mediates the steroid activity impairment in the bronchial epithelium."	127
This conclusion cannot be drawn from the data presented.	Edited	Text was amended as follows "Data presented in the current chapter suggested TLR3 inhibition as a possible strategy for targeted management of RSV-induced respiratory diseases that respond poorly to steroids."	129
The data does not support this conclusion	Edited	Text was amended as follows "TLR3 knockdown has at least partially repressed the RSV-induced expression of TGF-β1"	129
You have not specifically examined TLR3 expression or activation in this chapter	Edited	The title of the chapter was changed as follows TLR3 Activation —> Double-stranded RNA	130- 131
Detailed review of animal models of RSV infection is not directly relevant to the question of the thesis. A concise justification		Text was added as follows "Although the RSV mouse model of RSV infection has provided much of our understanding regarding allergic responses after presensitization (Openshaw, 1995; Graham, 1996), RSV is not a natural pathogen of rodents, hence in	134 - 135

for use of mouse model is all that is needed. Also, important to discuss rationale for using RSV rather than natural mouse pathogen (PVM)		vivo viral replication is limited. Additionally, the clinical and pathophysiologic sequela in mice do not mimic those of severe RSV infection in humans. Therefore, acute respiratory infection mouse model was developed using pneumonia virus of mice (PVM) (Horsfall and Hahn 1940). PVM is a pneumovirus and natural pathogen of rodent and the closest phylogenetic relative of RSV (Easton et al., 2004; Rima et al., 2019). Mice inoculation with as few as 10 pfu of PVM results in acute inflammatory response that progress to respiratory failure and death at lung viral titers more than 106 pfu/g of lung tissue (Domachowske et al., 2001). The pathogenesis of PVM infection in mice closely imitates the signs and symptoms of severe human RSV infection. First, PVM is localized to the bronchiolar epithelium (Bonville et al., 2006), parallel to the distribution observed for RSV in human post-mortem specimens (Welliver et al., 2007). Second, profound inflammation of the lungs is evident by the recruitment of lymphocytes, neutrophils, and eosinophils and severe pulmonary edema. Third, viral replication in the mouse lung tissue results in local production of proinflammatory mediators including MIP-1α, MIP-2, and MCP-1, IFNγ (Bonville et al., 2006), consistent with those detected in nasal washings and lung of human infants with RSV triggered bronchiolitis (Domachowske et al., 2004; Easton et al., 2004). Although PVM model mimics some features that clearly conform to human pathophysiology, others do not (Dyer et al., 2012). For instance, PVM infection induce little to no overt inflammation in neonatal mice (Bonville et al., 2007). Likewise, a distinct pattern of infection cannot be established in aged mice (Bonville et al., 2010). Another drawback of PVM model include the high genetic distance between PVM and hRSV ranging from 10 to 60%, and lack of direct cross-reactivity (Krempl et al., 2005; Dyer et al., 2012). In addition to the above-mentioned limitations, minimal virus inoculum results in robust virus replication in situ progressing to marked morbidity (hunching, fur ruffling), weight loss, and mortality in PVM-infected mice (Domachowske et al., 2000a; Domachowske et al., 2000b; Anh et al., 2006; Dyer et al., 2012). Furthermore, the current study involved administration of glucocorticoids that were previously reported to enhance viral replication and accelerate mortality in PVM-infected mice due to the added immunosupression (Domachowske et al., 2001). However, RSV infection of mice does not lead to any measurable degree of morbidity, induces a mild mononuclear cell infiltration to the lung instead of a profuse granulocytic bronchiolitis, and never progresses to acute respiratory distress syndrome (ARDS) (Anh et al., 2006). For these reasons, we selected RSV A2 strain to pursue the current study."	
Isn't this a good reason to consider the use of the natural mouse pathogen from the RSV		Text was added as follows "Although PVM model mimics some features that clearly conform to human pathophysiology, others do not (Dyer et al., 2012). For instance,	153

family - pneumovirus of mouse (PVM) - as an alternative?		PVM infection induce little to no overt inflammation in neonatal mice (Bonville et al., 2007). Likewise, a distinct pattern of infection cannot be established in aged mice (Bonville et al., 2010). Another drawback of PVM model include the high genetic distance between PVM and hRSV ranging from 10 to 60%, and lack of direct cross-reactivity (Krempl et al., 2005; Dyer et al., 2012). In addition to the above-mentioned limitations, minimal virus inoculum results in robust virus replication in situ progressing to marked morbidity (hunching, fur ruffling), weight loss, and mortality in PVM-infected mice (Domachowske et al., 2000a; Domachowske et al., 2000b; Anh et al., 2006; Dyer et al., 2012). Furthermore, the current study involved administration of glucocorticoids that were previously reported to enhance viral replication and accelerate mortality in PVM-infected mice due to the added immunosupression (Domachowske et al., 2001). However, RSV infection of mice does not lead to any measurable degree of morbidity, induces a mild mononuclear cell infiltration to the lung instead of a profuse granulocytic bronchiolitis, and never progresses to acute respiratory distress syndrome (ARDS) (Anh et al., 2006). For these reasons, we selected RSV A2 strain to pursue the current study."	
References?	Accepted	The following citations were added "(Han et al., 2018; van Erp et al., 2019)".	135
Specific aims of the chapter are not clear, they need to be explicitly stated. • You have not examined role of TLR3 in these studies • By comparing RSV and PIC responses, may also determine extent to which other sensing PRRs might be involved	Accepted	Section 5.2 was amended as follows: • "TLR3-dependent" was deleted. • The following sentence was added as follows "Moreover, by comparing RSV and poly I:C responses, might also determine the extent to which other sensing PRRs might be involved. This may in part help to determine if it is the replication of live virus which causes lung inflammation and GC insensitivity or if it is merely the signalling caused by recognition of the virus."	136- 137
Delete 8	Accepted	"8" was deleted	138
on to one	Accepted	on —> one	144
• This data in Fig 5.6C is difficult to interpret. In Fig 5.6C, you indicate that there is no sig. difference between RSV and RSV + Dex group. Therefore, one interpretation is that Dex does not significantly attenuate	Edited/ Accepted	• The description of the data was amended as follows "As demonstrated in Figure 5.6 C, treatment with either dexamethasone or PF670462 alone directly reduced cell recruitment following RSV infection, as did the combination which was associated with BAL cell count decreasing to baseline levels. However, treatment of RSV-infected mice with GW788388 alone had no remarkable change in BAL cells counts.	147

RSV induced increase in total cells. However, statistical comparisons between mock and RSV + Dex group are not shown. If there is no significant difference between mock and RSV + Dex group, then it would appear that Dex reduces cell numbers back to baseline levels. On the other hand, if cell numbers in RSV + Dex group are elevated compared with mock, then it is fair to say that Dex does not significantly attenuate RSV induced increase in cell number. • It is important that you include statistical comparisons between mock and RSV + Dex so that data can be interpreted. This also applies to other drug groups, i.e. need to include statistical comparisons between mock and all RSV infected/drug treated mice.		The profound reduction achieved by dexamethasone alone was maintained in dexamethasone plus GW788388-treated mice." • Figure 5.6 was amended to display the suggested comparisons.	148
• You have neglected to discuss the data in relation to effects of GW on BAL cells • Statistical comparisons between RSV infected and RSV + GW/GW+ Dex groups are not indicated in Fig 5.6C	Accepted	• The following sentence was added "However, treatment of RSV-infected mice with GW788388 alone had no remarkable change in BAL cells counts. The profound reduction achieved by dexamethasone alone was maintained in dexamethasone plus GW788388-treated mice." • Figure 5.6 C was amended to display the suggested comparisons.	147 148
For consistency, please annotate statistical comparisons between groups in Fig 5.6 A, B (as has been done in C)	Accepted	Figure 5.6 A and B was amended to display the suggested comparisons.	148
Please be consistent in how statistical differences are annotated. In A, I assume * means sig increase versus mock virus + vehicle? In B (total protein), I assume * means sig increase versus mock virus + vehicle?	Accepted	Figure 5.6 was amended to display the suggested comparisons.	148

In B right or C (as per text), statistical comparison between selected groups is shown, however statistical differences between mock, RSV + GW, PF, Dex, Dex/GW and Dex/PF is not shown. Statistical comparison between RSV and RSV + GW is also not shown			
In the text, you refer to B (right) as C	Accepted	Figure ligand was amended as follows "Bronchoalveolar lavage was collected for subsequent analysis to determine total proteins (B) and total cell number (C)."	148
• In the text, it is stated that the number of monocytes is increased in response to RSV infection. In Fig 5.7 statistical comparison between mock and RSV infected groups are not indicated, but there does not appear to be a significant increase in monocyte numbers following RSV infection. • Does RSV infection significantly increase monocyte numbers? Please clarify. If RSV infection does not lead to significant increases in monocyte numbers, then it is difficult to interpret the impact of drug treatment (in the absence of drug only controls). • Please include statistical comparison between mock and RSV, RSV+drug treated groups. Please also include statistical comparison between RSV and RSV+GW, RSV+GW+Dex groups.	Edited	• The text was amended as follows "RSV infection markedly increased the recruitment of neutrophils and lymphocytes as evident by increased cell counts. However, monocytes number in BAL following RSV infection was not changed." • The following paragraph was deleted "Treatment with either Dexamethasone or PF670462 alone directly suppressed monocytes recruitment following RSV infection, as did the combination. However, treatment of RSV-infected mice with GW788388 alone had no remarkable change in monocytes counts. The profound reduction achieved by dexamethasone alone was maintained in dexamethasone plus GW788388-treated mice." • Figure 5.7 was amended to display the suggested statistical comparisons.	147 149
Statistical comparisons are not shown in Figure 5.7. As per Fig 5.6, please include statistical comparisons for mock vs RSV, all RSV + drug groups and RSV vs all RSV + drug groups.	Accepted	Figure 5.7 was amended to display the suggested comparisons.	149

I would suggest removing quantification of "other" cells. As the identity of the cells is not known, and there is no sig. effect of RSV infection, this does not add much.	Accepted	In Figure 5.7, the graph demonstrating BAL other cells count was deleted.	149
Please show statistical comparisons between mock control and all treatment groups and indicate whether these are significant or not	Accepted	Figure 5.9 was amended to display the suggested comparisons.	152
Please show statistical comparisons between mock control and all treatment groups and indicate whether these are statistically significant or not	Accepted	Figure 5.10 was amended to display the suggested comparisons.	153
From data presented in Fig 5.11, it appears that RSV leads to significant increase in IL-6 in tissue but not cells - please clarify.	Edited	The text was edited as follows "In line with the previously observed changes in inflammatory cell infiltrates, gene expression analysis showed that RSV inoculation significantly elevated the expression of pro-inflammatory cytokines, such as TNF-α as measured in BAL cells and lung tissue, while IL-6 increased in lung tissue only."	154
RSV does not induce sig. increase in IL-6 expression in cells, thus not possible to assess effects of drug treatments?	Edited	The text was amended as follows "Dexamethasone blunted RSV-triggered expression of TNF-α in BAL cells, whereas it only reduced the expression of IL-6 but not TNF-α in lung tissue. Co-administration with PF670462, not GW788388 synergistically decrease TNF-α in lungs of RSV-infected mice. While treatment with PF670462 alone shows remarkable anti-inflammatory effect evident in dampened TNF-α expression in BAL cells but not in lung tissue, GW788388 alone did not show similar potential either in BAL cells or lung tissue (Figure 5.11)."	154
Please show statistical comparisons between PIC and all drug treated groups (not just selected groups)	Accepted	Figure 5.12 was amended to display the suggested comparisons.	156
Please indicate if there is a statistically significant increase in the expression of each of these genes in RSV vs mock infected mice in text and figure.	Accepted	• Figure 5.13 was amended to display the suggested comparisons. • Text was amended as follows "As shown in Figure 5.13, gene expression analysis by real-time RT-PCR has shown that while the expression of IFNα remained unchanged in lung tissue following RSV infection, the mRNA expression of IFNβ, IFNγ, IFNλ2 and IFNλ3 increased, albeit non-significantly, compared to animals receiving Mock-conditioned media."	158 157
Relative to RSV infected mice, treatment with PF compound enhances lamda 2 and lamda 3 only. Cannot make comparison with mock control	Edited	The following paragraph was deleted "Intriguingly, treatment with PF670462 markedly enhanced the levels of IFNλ2 and IFNλ3 in lung tissue of RSV-infected mice. Conversely, GW788388 did not affect interferon expression triggered by RSV infection in mice. Enhancement of interferon production by PF670462 may be expected to limit the duration of RSV infection by enhancing anti-viral activity in the airway."	158

Please show statistical comparisons between mock control and all treatment groups and RSV vs all RSV+drug groups.	Accepted	Figure 5.13 was amended to display the suggested comparisons.	158
• From data presented in figures, IFN-beta appears to be sig. increased in cells but not tissue? • From data presented in figures, lamda 2 appears to be sig. increased in tissue but not in cells. • Please revise this sentence to clarify impact of Dex treatment on PIC induced IFN production. • This appears true for BAL cell but not lung tissue expression, please check.	Edited	Text was amended as follows "As demonstrated in Figure 5.14 and Figure 5.15, intranasal instillation of the double-stranded RNA analogue poly I:C in normal BALB/c mice induced interferons expression, including IFNβ in BAL cells and IFNλ2 in lung tissue. Treatment with dexamethasone did not affect the upregulation of IFNβ or IFNλ2 expression in mice receiving poly I:C compared with animals receiving saline. Interestingly, PF670462 administration markedly augmented poly I:C mediated upregulation of INF-β expression in BAL cells, whereas it had no detectable effects on other INFs expression."	157-158
Please show statistical comparisons between saline and PIC treated groups & PIC vs PIC + drug treated groups	Accepted	Figure 5.14 was amended to display the suggested comparisons.	160
Please show statistical comparisons between saline and all treatment groups and PIC vs PIC + drug groups	Accepted	Figure 5.15 was amended to display the suggested comparisons.	161
This is not an adequate scientific description of the data in the figures.	Edited	Text was amended as follows "As shown in Figure 5.16, RSV infection markedly promoted the expression of TBX21 and CTLA4 as compared to mock-infected mice, while FOXP3 expression increased, albeit non-significantly. Treatment of RSV-infected mice with dexamethasone alone had no detectable effect on TBX21 and CTLA4 expression as did dexamethasone coformulated with GW788388. Likewise, dexamethasone coformuled with PF670462 did not show any significant effect on CTLA4 and FOXP3 expression in RSV-infected mice, however it attenuated RSV-mediated upregulation of TBX21. Intriguely, GW788388 alone, but not PF670462, had upregulated the expression of FOXP3 and CLTA4 as compared to RSV-infected mice."	159
RSV and dsRNA both engage multiple receptor pathways - thus you cannot make any conclusions re role of TLR3 unless you use TLR3 KO mice or inhibit TLR3 expression in some way.	Accepted	Added this concern as study limitation in section 5.6	168

Can you provide some insight re the difference in findings? Mice were exposed to RSV for 5 days and poly IC for 24 h - could difference in exposure period be a reason?	Accepted	The following sentence was added "The dissimilarity in findings may be attributed to the difference in the exposure period, where mice were exposed to RSV for 5 days while poly I:C treated mice were exposed to the stimulus for only 24 h."	163
where is this data?	Edited	"systemic inflammatory response to RSV infection" was edited as "inflammatory response to RSV infection in the lung".	164
monocytes were not increased?	Accepted	"monocytes" was deleted.	165
Cannot draw any conclusions re TLR3 signalling in poly IC vs RSV models as poly IC and RSV engage other receptor pathways in addition to TLR3.	Edited	The sentence was edited as follows "These data suggested that dsRNA/TLR3 interaction through TLR3 ligand exposure may at least partially recapitulate the pathophysiological effects of RSV infection in BALB/c mice." and the concern was added to the limitation of the study discussed in section 5.6	169
Another introduction is not needed.	Accepted	Section 6.1 Introduction was moved to Section 6.1.1	171
You have not shown any effect on PLZF??	Accepted	"and the transcriptional repressor PLZF" was deleted.	172
This data is not included in the thesis	Accepted	"we" changed to "our group"	173
No information about this	Accepted	The sentence was edited as follows "Using hypothesis-free approaches, including proteomics and transcriptomics, our group have provided the first evidence that CK1□/□□is implicated in TGF-□1 signalling pathway suggesting a promising target for management of inflammatory pulmonary diseases with TGF-□1 involvement (Li et al., 2019)."	174
This has been done to some extent in the study by Xia et al.?	Edited	The sentence was edited as follows "Our group had established that our findings in RSV-mediated GC insensitivity were paralleled by similar observations when the air-liquid interface culture of HBECs was subjected to RSV infection (Xia et al., 2017)"	175
These references are abstracts Unclear why the paper published in PloS Pathogens 2017 is not referenced	Accepted	References were deleted and replaced with "Xia, Y.C., Radwan, A., Keenan, C.R., Langenbach, S.Y., Li, M., Radojicic, D., Londrigan, S.L., Gualano, R.C., and Stewart, A.G. (2017). Glucocorticoid Insensitivity in Virally Infected Airway Epithelial Cells Is Dependent on Transforming Growth Factor-β Activity. PLOS Pathogens 13, e1006138.".	225