

The anti-viral effects of Azithromycin and other novel macrolides

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Abstract

Asthma exacerbations are highly associated with respiratory viral infections, particularly rhinovirus (RV) infection. Currently, standard asthma medication does not include anti-viral agents despite the role viruses play. Asthmatics have also demonstrated impaired innate interferon responses to viral infection thus compounding the requirement for an anti-viral treatment. Macrolides are a class of compound often used to treat bacterial infections, Azithromycin has previously shown anti-inflammatory activity in addition to its potent antibiotic effects. In 2010, Gielen *et al* demonstrated that Azithromycin possesses anti-viral activity in an *in vitro* model of RV infection in human bronchial epithelial cells (HBECs). In this thesis we build on the findings of Gielen *et al*; we demonstrate that Azithromycin is able to augment RV-induced type I and type III interferon (IFN) and interferon stimulated gene (ISG) expression whilst reducing viral replication in HBECs cultured *ex vivo* from asthmatic donors. We also demonstrate that oral administration of Azithromycin is able to partially augment IFN production in a mouse model of RV infection. We elucidate a potential mechanism of action of Azithromycin's anti-viral activity in non-asthmatic HBECs, identifying mitochondria-associated anti-viral signalling protein (MAVS) as a potential target of Azithromycin's actions. Finally we demonstrate the development of a novel screening assay for the detection of novel macrolides with potential anti-viral activity, eventually identifying a novel macrolide (MAC5) as a compound with greater IFN augmenting ability than Azithromycin. We also identify a MAC5 analogue (MAC5K) that possesses potential anti-viral, anti-inflammatory and limited antibiotic activity.

Conference abstracts and publications arising from this thesis

Conference abstracts

- Azithromycin is able to augment IFN and ISG responses to Rhinovirus *in vitro*. James D. Porter, Jonathan Macintyre, Anny Sykes, Atul Gupta, Amelia Shoemark Cara Bossley, Jane Davies, Musa Khaitov, Sergei A. Moshkovskii, Onn Min Konn, Sejal Saglani, Andrew Bush, Sebastian L. Johnston and Michael R. Edwards *Poster Discussion Presentation* European Academy of Allergy & Immunology, Milan 2013.
- Azithromycin augments RV-induced type I IFN and type III IFNs via interaction with MAVS. James D. Porter, Musa Khaitov, Sergei Moshkovski, Lyn Jones, Lee Roberts, Kalesh Karunakaran Nair Anandamma, Ed Tate, Iain Kilty, Sebastian L. Johnston Michael R. Edwards *Oral Presentation*, XVI International Symposium on Respiratory Viral Infections Seoul, Korea 2014. <http://www.themacraefoundation.org/wp-content/uploads/2013/08/Final-Program-2.pdf>

Publications

- The role of macrolides in asthma: current evidence and future directions. Wong EHC, Porter JD, Edwards MR, Johnston SL. *Lancet Respiratory Medicine* 2014 2(8):657-70.
- Azithromycin augments type I & III interferon responses to virus infection by preventing MAVs degradation. James D. Porter, Lyn Jones, Lee Roberts, Musa Khaitov, Sergei A. Moshkovskii, Jaideep Dhariwal, Mark Almond, Ernie Wong, Ross P. Walton, Kalesh Karunakaran Nair Anandamma, Ed Tate, Iain Kilty, Sebastian L. Johnston Michael R. Edwards. *Manuscript in preparation*.
- Identification of novel macrolides with anti-bacterial, anti-inflammatory, and type I & III interferon boosting activity in airway epithelium. James D. Porter, Jennifer Watson, Lyn Jones, Lee Roberts, Jaideep Dhariwal, Mark Almond, Ernie Wong, Ross P. Walton, Iain Kilty, Sebastian L. Johnston Michael R. Edwards. *Manuscript in preparation*.

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Abbreviations

AA	Atopic asthmatic
ACK	Ammonium-chloride-potassium buffer
ACQ	Asthma control questionnaire
ANOVA	Analysis of variance
AP-1	Activator protein 1
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CARD	Caspase activation and recruitment domain
cDNA	Complementary DNA
CF	Cystic fibrosis
CMC	Carboxymethyl cellulose
COPD	Chronic obstructive pulmonary disease
dsRNA	Double stranded RNA
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FADD	Fas-associated death domain
FEV ₁	Forced expiratory volume over 1 second
GINA	Global initiative for asthma
HBEC	Human bronchial epithelial cell
ICAM	Intracellular adhesion molecule
ICS	Inhaled corticosteroids
IFN	Interferon
IFNAR	Interferon alpha receptor
Ig	Immunoglobulin
IKK	Inhibitor of NFκB kinase
IL	Interleukin
IP-10	Interferon gamma inducible protein 10
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF	Interferon stimulated gene factor
ISRE	Interferon-stimulated response element
JAK	Janus kinase
K63	Lysine-63
KC	Keratinocyte chemoattractant
LABA	Long-acting beta-2 agonists
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
Luc	Luciferase
MAVS	Mitochondria-associated anti-viral signalling protein
MDA-5	Melanoma differentiation-associated protein-5
MHC	Major histocompatibility complex

MIP	Macrophage inflammatory protein
MS	Mass spectrometry
MSD	Mesoscale discovery
NANA	Non-atopic non-asthmatic
NEMO	NF-kappa-B essential modulator
NFκB	Nuclear factor kappa B
OAS	2'-5'-oligoadenylate synthetase
OD	Optical density
PAMP	Pattern recognition receptor
PBS	Phosphate buffered saline
PC20	Provocation concentration provoking a 20% reduction in FEV ₁
PCR	Polymerase chain reaction
PEF	Peak expiratory flow
PEG	Polyethylene glycol
PKR	Protein kinase R
POLY(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
RANTES	Regulated on activation, normal T cell expressed and secreted
RIG-I	Retinoic acid inducible gene- I
RIP	Receptor-interacting protein
RLH	RIG-like helicase
RRNA	Ribosomal RNA
RSV	Respiratory syncytial virus
RV	Rhinovirus
SABA	Short-acting beta-2 agonists
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signalling
SPT	Skin prick test
ssRNA	Single stranded RNA
STAT	Signal transducers and activators of transcription
STRA	Severe therapy-resistant asthmatic
TANK	TRAF family member-associated NF-kappa-B activator
TBK	TANK-binding kinase
TCID ₅₀	Tissue culture infective dose 50%
TH	T-helper
TIR	Toll-interleukin receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNS	Trypsin-neutralising solution
TRAF	TNF receptor associated factor
TRIF	TIR-domain containing adaptor
TSLP	Thymic stromal lymphopoietin
TYK2	Tyrosine kinase 2
v/v	Volume per volume

Statement of work

I declare that the work presented here was carried out by myself unless otherwise stated. Specifically, mass spectrometry analysis of cell lysates was carried out by M. Khaitov and S. A. Moshkovskii (Institute of Biomedical Chemistry, Moscow), AZI-pull down assays were performed in collaboration with K. A. Kalesh and E. Tate (Dept. of Chemistry, Imperial College London, London) and SAR on MAC5 was performed in collaboration with J. Watson (an undergraduate student supervised by M. R. Edwards). All novel macrolides and AZI-Azide probe were provided by Pfizer Inc (Pfizer USA).

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Chapter I Introduction

I.1 Asthma definition, symptoms and epidemiology

I.1.1 Asthma definition and symptoms

Asthma is defined as a heterogeneous disease, characterised by airway inflammation, airway hyper-reactivity, reversible bronchoconstriction and variable airway obstruction that manifests symptoms such as chest tightness, wheeze, cough and shortness of breath. Diagnosis of clinical asthma is made on the presentation of one or more of the aforementioned symptoms in addition to confirmation of reduced airflow, typically measured by forced expiratory volume over one second (FEV₁) and peak expiratory flow (PEF), that is reversible within 4 weeks of therapy and in the absence of an alternative diagnosis. Symptoms may be variable in nature and may be triggered by external factors such as allergens and respiratory irritants, respiratory viral infections, changes in weather conditions and exercise [1].

I.1.2 Asthma epidemiology and global health burden

The Global Initiative for Asthma (GINA) estimates that over 300 million people suffer from asthma worldwide and the number of asthma diagnosis per year is increasing. Approximately 250,000 people die prematurely from asthma-related deaths per year. Asthma contributes to a large proportion of healthcare costs, the American Lung Association estimated that in the United States alone asthma care costs rose to approximately \$56 billion per year during the mid 2000s and Asthma UK estimate that Asthma costs are around £1 billion per year [1, 2].

1.2 Aetiology of Asthma

1.2.1 Classifications of asthma

The word “asthma” is derived from the Greek for wheeze or noisy breathing, today asthma is used as an umbrella term to describe the highly complex disease presenting with the symptoms mentioned above. GINA classifies asthmatic patients into four groups: intermittent, mild persistent, moderate persistent and severe persistent based upon the frequency and severity of symptoms [1]. Asthma can be further classified by the presence of observable phenotypes such as allergic asthma (the most common and thus most heavily studied), intrinsic asthma (that is rare and is non-atopic), adult onset asthma, exercise-induced asthma, steroid-resistant or steroid refractory asthma and asthma that is induced via a range of external stimuli (such as air pollutants and cigarette smoke). Crucially, different phenotypes are able to contribute synergistically to asthma disease. The complexity and plasticity of these endotypes has hampered our understanding of the basic aetiology of asthma to date [3, 4].

1.2.2 Immunological profile of asthma

Much of the immunology of asthma has focussed on the mucosal surface of the airways in allergic asthmatics. Bronchial biopsies and sampling of bronchoalveolar lavage (BAL) fluid and sputum have identified a number of immunological characteristics associated with asthma. Evaluation of the asthmatic airway has revealed smooth muscle hyperplasia, goblet cell hyperplasia (accounting for mucus hyper-secretion) and increased shedding of the bronchial epithelium [5]. The cytokine environment of the allergic asthmatic airway is predominantly of a T_H2 phenotype, with elevated levels of IL-4 IL-5 and IL-13 commonly found in BAL sampling from asthmatics [6]. In some patients, other cytokines are more apparent, such as $TNF\alpha$ and this is thought to be associated with steroid resistant asthma [7, 8].

High levels of serum IgE and positive allergen skin prick tests (SPTs) are used to determine the atopic status of an individual and studies have demonstrated a high degree of asthmatics are also atopic [9, 10], which is not surprising given that antibody class switching to IgE production is promoted by T_H2 cytokines IL-4 and IL-13. IgE can form and complex with allergens and binds the $Fc\epsilon R1$ on the surface of mast cells causing the release of a number of pro-inflammatory mediators including the bronchoconstrictors histamine, prostaglandins

and leukotrienes [11] Exposure to environmental allergens may account for the variable nature of bronchoconstriction observed in asthmatics, With some patients only exhibiting symptoms during the pollen season. Activated mast cells also produce IL-4, IL-5 and IL-13 further contributing IgE class switching in B cells, differentiation of Th2 T cells and production of Th2 attracting chemokines TARC/CCL17 and MDC/CCL22. These processes are thought to enhance the overall immunological profile to a T_H2 phenotype in the allergic asthmatic airway [12].

Pro-inflammatory cell influx into the airways has been highlighted as a contributing factor in asthmatic disease. In BAL and sputum samples of asthmatics, increased numbers of eosinophils and neutrophils have been identified. Eosinophilia correlated with asthma severity and was found to be reduced (in severe asthmatics) by the administration of oral corticosteroids [13, 14]. Neutrophils are present in the airways of severe asthmatics and levels of neutrophilia may correlate with lower FEV₁ scores and airway obstruction [14-16]. The presence of airway neutrophils and eosinophils is by no means exclusive; with many asthmatics found to exhibit both cell types contributing to disease [14, 15].

1.2.3 Bacterial infections in asthma

Bacterial infection as a contributor to asthma is an emerging field, with the advances in culture-independent identification techniques allowing for detailed analysis of bacterial flora within the airways, which were once thought to be sterile [17]. Analysis of the microbiome has revealed new insights into the role of bacteria, with both harmful and protective species identified. Generally speaking, bacteria of the phylum Proteobacteria, particularly *Haemophilus spp* are thought to be pathogenic [17, 18]. A number of studies have shown the presence and importance of atypical bacterial species such as *M. pneumoniae* and *C. pneumoniae* to be higher in the lung of asthmatics than healthy matched controls [19-21]. It is not clear whether the atypical bacterial colonisation seen in asthmatics is contributory to the disease or merely a factor arising from poor immune regulation or damage within the airways. Atypical bacterial infection is a well-defined risk factor in acute exacerbations of chronic obstructive pulmonary disease (COPD) and is known to increase inflammation markers within the lungs [22-24] however the association in asthma is less clear.

1.2.4 Viral infections in asthma

Asthma exacerbations are highly associated with respiratory viral infections. Studies have shown that respiratory viruses are detected in around 80% of hospital admissions for asthma exacerbations [25, 26]. Of those 80%, human rhinoviruses (RV) have been identified in as much as 60% of cases [25, 27, 28] making them the most abundant infectious agent associated with asthma exacerbations. Other respiratory viral infections have been associated with asthma and asthma exacerbations, respiratory syncytial virus (RSV) infection was shown to be associated with early onset wheeze in children [29, 30] and influenza infection (specifically the 2009 pandemic strain- H1N1) severity were shown to correlate with asthma diagnosis in a number of studies [31-33].

1.3 The role of RV infections in asthma exacerbations

1.3.1 RV lifecycle

RVs are a member of the *Picornaviridae* family of Enteroviruses, they have a single stranded, +ve sense RNA genome of approximately 7.2Kb in length and forms an icosahedral capsid approximately 30nm across [34]. RVs can be classified into three distinct species based on phylogenetic sequences, RV-As and RV-Bs of which there are over 100 different serotypes, [35, 36] and (the recently discovered) RV-Cs of which there are an estimated 60 strains (based on sequence identity) [37-39]. RV-As and RV-Bs fall into major or minor group nomenclature based upon the mechanism of host cell binding and entry. Major-group RVs binds intercellular adhesion molecule-1 (ICAM-1) on host cells [40] whilst minor-group RVs utilise the low-density lipoprotein (LDL) receptor for host cell entry [41].

Upon receptor binding, RVs are internalised into endosomal compartments, acidification of endosomes allows the release of the viral genome into the cytoplasm. The +ve sense RNA genome acts as mRNA due to the presence of a 3' poly-A tail and 5' internal ribosome entry site, the viral genome is translated into a single viral poly-protein that is cleaved into individual proteins by an RV protease (RV 3C) [42]. The +ve sense RNA genome also acts as a template for the production of –ve sense RNA (by RV RNA polymerase) that is used as the template for the production of progeny virus genomes [43-45]. Newly synthesised RV genomes and proteins assemble within the cytoplasm and are released by host cell lysis.

I.3.2 RV infection of the epithelium

The nasal and bronchial epithelial lining is considered the primary site of RV infection in humans. In human bronchial epithelial cells (HBECs) and cell lines grown *in vitro*, studies show RV is able to replicate and induce the expression of many anti-viral and pro-inflammatory cytokines including type I and III interferons (IFNs), and the pro-inflammatory cytokines IL-6 and IL-8 [46-50]. *In vivo* histological analysis shows that RV can infect the epithelial barrier in mice and produce a robust anti-viral response [51, 52]. HBECs cultured *ex vivo* from asthmatics have demonstrated the propensity for producing pro-Th2 cytokines, including IL-25, IL-33 and TSLP [53, 54], potentially promoting T_H2 adaptive responses and potentially driving the allergic environment within the asthmatic lung [55, 56]. The cellular immunology of a virus-induced asthma exacerbation is summarised in Figure 1.1.

1.3.3 RV as the cause of asthma exacerbations

RV infection is the most common infectious agent associated with asthma exacerbations, accounting for approximately 60% of all virus-associated exacerbations [25, 27, 28]. Importantly, experimental infection models in humans [58] and in mice [51] have been established and show that infection with RV can exacerbate pre-existing asthma. In healthy individuals RV causes the common cold and is rarely life threatening, however in asthmatics it is highly associated with exacerbations for reasons not yet fully understood. Interestingly, despite the high degree of association between RV infection and asthma exacerbations it has been reported that asthmatics and healthy individuals are infected with RV at the same rate, however asthmatics respond with a greater severity of disease and increased lower respiratory tract symptoms [26, 59]. This is suggestive that asthmatics may have an underlying deficiency in combating RV infection, indeed a number of studies have highlighted differences in the pathogenesis of RV infection in asthmatics compared to healthy controls (discussed below).

1.3.4 RV infection in the lower airways of asthmatics

RV infection of healthy individuals is usually limited to the upper respiratory tract, however RV have been identified in the bronchial epithelial cells taken from the lower respiratory tract of asthmatics [60-63]. RV shows no preference in infectivity towards cells isolated from the upper or lower respiratory tract [64], therefore it is likely that RV infects the lower airways of asthmatics due to inherent differences in the structure of the airways and/or differences in immune responses to viral infection.

1.3.5 Modulated RV receptor expression in asthmatics

ICAM-1, the major group RV adhesion receptor was shown to be up regulated during RV infection *in vivo* and *in vitro*. This up regulation is enhanced in HBECs from asthmatics compared to healthy controls [65, 66]. LDL receptor, the minor group RV adhesion molecule was also shown to be up regulated by RV infection however no reports to-date have shown an augmented up regulation in asthmatics [67].

1.3.6 RV-induced expression of pro-inflammatory mediators

RV infection in humans causes an increase in levels of airway pro-inflammatory cells (predominantly neutrophils and lymphocytes), mucus production, and a plethora of pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-10, IL-6 and TNF- α , and chemokines such as the neutrophil and lymphocyte chemokines IL-8 and RANTES (a.k.a. CXCL8 and CCL5 respectively [68-71]. Mouse models of RV infection have revealed similar virus-induced effects including airway inflammatory cell influx, mucus hyper-secretion, expression of IL-6 and induction of chemokines CXCL2/MIP-2, KC and CCL5/RANTES [51]. The expression profiles of pro-inflammatory mediators have been validated in human RV experimental infection studies [72]. Experimental RV infection studies in asthmatics have revealed increased production of RV-induced pro-inflammatory IL-8 and histamine in addition to enhanced cellular infiltrate compared to healthy controls that may contribute to airway inflammation [28, 73, 74].

1.3.7 Imbalanced T_H responses

Typically, viral infections are characterised by a T_H1 phenotypic adaptive immune response, consistent with raised levels of IFN γ and activation of CD8+ T cells. During RV infection in humans, a T_H1 phenotype seems to be protective against viral replication and lower respiratory tract symptoms whereas a T_H2 phenotype seems to be associated with increased lower-respiratory symptoms [58]. Asthmatics have shown to exhibit a T_H2 dominant antiviral response to RV infection in PBMCs with increased levels of IL-4 and decreased levels of IFN γ and IL-12 compared to healthy controls [75, 76]. These studies suggest that asthmatics may mount a T_H2-biased response to RV infection that may contribute to prolonged lower respiratory tract symptoms and contribute to asthma exacerbation pathogenesis.

1.3.8 Interferon deficiency in asthma

Innate anti-viral IFNs can be divided into two types; Type I and III IFN consisting of IFN α/β and IFN λ respectively. There are 13 subtypes of IFN α (1, 2, 4-8, 10, 13, 14, 16, 17 and 21), the genes of which are located in chromosome 9 in humans, there is one subtype of IFN β also located on chromosome 9 [77, 78]. Humans make three subtypes of IFN λ (IFN λ 1 and IFN λ 2/3, encoded by IL-29 and IL-28A/B respectively) [79].

Type I IFNs bind the IFN α receptor (IFNAR) complex, composed of IFNAR1 and IFNAR2 chains. Type III IFNs bind a unique receptor complex composed of the IL-10R β chain and the IFN λ R1a chain [80], Type I IFNs are capable of inducing the expression of a number of other genes including IFN-stimulated genes (ISGs) that may have direct anti-viral activity. IFNs have been extensively studied in RV infection in the context of asthma. RV infection in HBECs initiates the robust expression of IFN β , IFN λ 1 and IFN λ 2/3 [81-83]. In mouse models of RV infection IFN α , IFN β and IFN λ 2/3 are robustly expressed [51]. A number of studies have demonstrated that RV-infected HBECs from asthmatic donors (both children and adults) are deficient in both type I and type III IFN production compared to healthy controls [84-87]. Cells cultured *ex vivo* from bronchoalveolar lavage (BAL) and infected with RV also showed a deficiency in IFN production [46]. The mechanism behind the apparent IFN deficiency is not clearly understood, however preliminary studies indicate that suppressors of cytokine signalling (SOCS) proteins (negative regulators of IFN) may be increased in cells from asthmatic donors and directly suppress RV-induced IFNs (unpublished observations by Gielen, Edwards *et al*).

1.4 Interferon production in RV infection

1.4.1 IFN production via two distinct mechanisms

IFNs are some of the first cytokines produced in response to viral infection by host cells and are known to orchestrate the innate host defence to infection [88-90]. Humans produce three distinct types of IFN; type I (including IFN α s and IFN β), type II (IFN γ) [78, 91] and type III (IFN λ s) [79]. Typically, type I and type III IFNs are produced in the innate response to viral infection with IFN γ promoting T_H1-mediated adaptive immunity [92, 93].

Typically, there are two distinct pathways involved in the generation of IFNs by infected host cells, IFN production can be “virus-induced” where the detection of viruses within the cell leads to the *de novo* transcription of IFNs, or “IFN-induced” where initial IFN expression from the former mechanism is amplified via a positive feedback mechanism utilising the IFN receptor complex signalling. The various signalling pathways important for IFN production and IFN signalling producing further IFN and interferon stimulated genes (ISGs) are summarised in Figure 1.2. In both instances, type I and III IFNs are under tight transcriptional regulation and are expressed via the activation of IFN regulatory factors (IRFs) -3 and/or -7 and IRF-9. Activated IRF-3/7 bind to IFN-stimulated response elements (IRF sequences or ISREs- conserved nucleotide sequences within the IFN promoters) and initiate transcription [94-97]. Activated IRF-3/7 are also capable of complexing with activated Nuclear Factor κ B (NF- κ B/p65) and p300 to form an ‘enhanceosome’ promoting the transcription of a number of genes including IFN β and pro-inflammatory cytokines [98, 99].

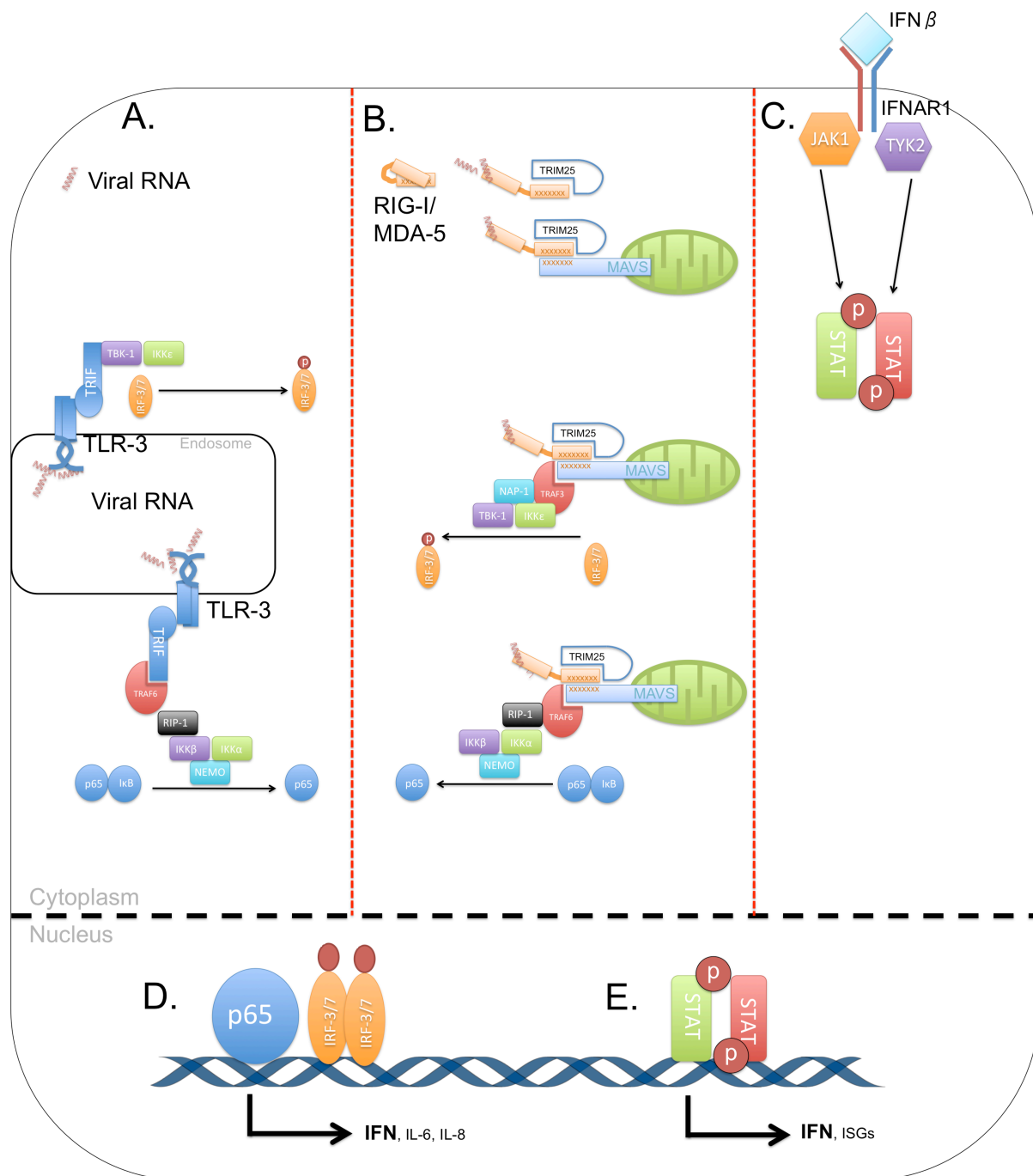


Figure 1.2 Virus infection leads to the generation of IFNs via distinct mechanisms.

IFNs can be generated via the detection of viral RNA by PRRs and by signalling through the IFN receptor complex. RV utilises TLR-3 (A) that binds viral RNA in endosomes and signals through TRIF, activating TBK1 and RIP-1 that leads to the activation of IRF3/7 and NFκB p65 (respectfully). RV can also be detected by the cytoplasmic RIG-I or MDA-5 (B) that signal through the adaptor MAVS through the interaction of CARD domains (marked with xxx), leading to TBK1 and RIP-1 activation and the subsequent activation of IRF3/7 and NFκB p65. Activated IRF3/7 and p65 translocate to the nucleus and initiate the transcription of IFNs and pro-inflammatory cytokines (D). Secreted IFNs can signal through the IFN signalling complex (C) leading to the activation of STATs that translocate to the nucleus and induce the expression of further IFN and ISGs (E).

1.4.2 Virus-induced IFN

Virus-induced IFN production via activation of IRF-3/7 and NF κ B (p65 and p50 subunits) can occur through a number of different virus-sensing pathways. Initially, viruses are detected via pattern recognition receptors (PRRs) that are able to differentiate between self and non-self structures and bind pathogen-associated molecular patterns (PAMPs) originating from virus RNA or DNA. Double-stranded RNA (dsRNA) is a typical PAMP originating from viruses (either from dsRNA viral genomes or during the replication cycle of ssRNA genomes). dsRNA is recognised by a number of different PRRs such as Toll-like receptors (TLR) -3, [100-103] and RIG-like helicases (RLHs) such as retinoic acid-inducible gene -I (RIG-I) and melanoma-differentiation associated protein -5 (MDA-5) [104, 105]. TLR and RLH signalling operate by distinct mechanisms however both result in the co-ordinated activation of IRF-3/7 and NF κ B and induction of IFN and pro-inflammatory cytokine transcription.

1.4.3 RLHs

RLHs such as RIG-I and MDA-5 are able to bind viral RNA in the cytosol. RIG-I was initially identified as a dsRNA binding helicase and was later confirmed to bind short dsRNA structures present in 5'-triphosphorylated ssRNA [106-109]. MDA5 also binds dsRNA, but of longer conformations the details of which are yet to be elucidated [110]. RNA binding occurs via a C-terminal helicase domain, the binding of which brings about a conformational change exposing an N-terminal caspase activation and recruitment domain (CARD) domain that becomes (K63-linked) poly-ubiquitinated by the E3 ubiquitin ligase TRIM25 [111]. The K63-linked ubiquitination allows for the interaction of RIG-I/MDA-5 with the putative adaptor protein mitochondria-associated anti-viral signalling protein (MAVS) [112, 113]. MAVS contains its own CARD domain that can be (K63-linked) poly-ubiquitinated by the E3 ubiquitin ligase TNF-receptor associated factor (TRAF-3) [114, 115]. Once recruited to MAVS, TRAF3 is able to recruit TANK-binding kinase 1 (TBK-1) and inducible I κ B kinase (IKK ϵ) as well as TRAF-family member associated NF- κ B activator (TANK) and NAK-associated protein 1 (NAP-1), together this complex is able to phosphorylate (and thereby activate) IRF-3 and IRF-7 that translocate to the nucleus and form homo/hetero dimers before binding to IRF/ISREs sites. [116-118].

Activated RIG-I and MDA-5 are also able to activate NF κ B. Upon binding of dsRNA and subsequent TRIM-25- mediated activation of MAVS, FAS-Associated Death Domain (FADD) and receptor interacting protein (RIP-1) are recruited via the presence of a CARD domain

that is able to interact directly with MAVS [119]. RIP-1 is able to complex with TRAF-6 and causes the activation of IKK α /IKK β /NEMO complex through K63-linked ubiquitination [119-121]. Activation of IKK α /IKK β /NEMO complex phosphorylates I κ B, causing its ubiquitination via Lys48-linked polyubiquitin chains. I κ B then undergoes subsequent degradation by the proteasome thereby activating NF κ B and allowing nuclear translocation [122, 123].

RLHs have been shown to be crucial for the effective production of RV-induced IFN *in vitro* [50, 82]. Knock-down experiments using siRNA in RV-infected HBECs demonstrated that MDA-5 expression is essential for robust IFN β and IFN λ expression. RIG-I knock-down resulted in a decrease in IFN β however no effects on IFN λ expression. Highlighting the coordinated responses of the RLHs. RLHs were also up regulated in the absence of IFNAR1 signalling *in vivo*, suggesting that they are not obligate ISGs [124].

1.4.4 TLR-3

TLR-3 is expressed primarily within the endosomes but also on the surface of certain cell types within the human body. TLR-3 is able to bind to (dsRNA) upon which it dimerises and signals through it's cytosolic Toll-IL-1 Resistance (TIR) domain, recruiting other TIR domain-containing proteins such as TIR domain-containing adaptor protein inducing IFN β (TRIF). TRIF is able to recruit RIP-1 leading to the activation of TRAF-6 and ultimately NF κ B via IKK α /IKK β /NEMO, [125] and TBK-1, leading to the activation of IRF-3/7 via TRAF-3 and IKK ϵ [122, 123, 126]

In the context of RV infection, knock-down experiments using siRNA showed TLR-3 signalling is essential for the production of RV-induced IFN and pro-inflammatory cytokines such as IL-8 and RANTES/CCL5 [50, 124]. It is also suggested that proper TLR-3 signalling is required for the RV-induced up regulation of the RLHs RIG-I and MDA-5 [124].

1.4.5 Regulation of MAVs in RLH mediated IFN production

Although many PRRs are able to detect virus PAMPs and initiate the expression of IFNs and pro-inflammatory cytokines, the signalling pathways converge at the points of TRAF-3 (for IRF activation and IFN expression) and TRAF-6 (for NF κ B activation and pro-inflammatory cytokine expression). Upstream of these points TLR-3 and the RLHs utilise the distinct adaptor proteins TRIF and MAVS, respectively.

MAVS is an essential intermediate in the RLH signalling pathways and is not thought to contribute to TLR-3 mediated virus recognition. MAVS is also a common target for viral inhibition of innate host defences, RSV and influenza are both known inhibit anti-viral responses at the point of MAVS signalling [127-129]. MAVS is predicted to be a 72kDa protein that is often localised to mitochondria where it facilitates RLH-mediated signal transduction through the interaction of CARD domains [113]. MAVS has been shown to be degraded in response to RNA stimulation within the cell, it is thought that this may constitute a regulatory mechanism of IFN production post-viral infection [130]. The degradation of MAVS has been the subject of a number of studies that have identified a range of host signalling proteins implicated in proteosomal-mediated MAVS degradation [130-134]. Interestingly, antibodies to MAVS that have been used in the characterisation of the protein have detected two bands by Western blot analysis; one at 75kDa and the other at 50 kDa. Recently it has been proposed that this smaller band represents a truncated variant of MAVS caused by bicistronic translation of the MAVS mRNA. This mini-MAVS variant was also shown to inhibit IFN production by full-length MAVS and may act as a negative regulator of IFN production [135].

1.5.6 IFN-induced IFN and IFN stimulated genes

Secreted IFN α / β and λ proteins are able to bind receptors (IFNAR1&2 complex in the case of IFN α s and IFN β , and IL-10R β & IFN λ R1 complex in the case of IFN λ s) on the surface of the host cell or neighbouring cells and initiate an auto-feedback loop resulting in the amplified expression of IFNs [80, 136]. Specifically, IFNAR1/2 and IL-10R β /IFN λ R1 are trans-membrane receptors that assemble as complexes upon ligation of IFN, this formation leads to the activation of the intracellular signalling molecules Janus kinases (JAKs), tyrosine kinase -2 (TYK-2) and signal transducers and activators of transcription (STATs). Once associated with activated receptors, JAK1 and TYK-2 auto-activate, phosphorylating STAT1 and STAT2 [137], that translocate to the nucleus and form hetero-dimers that are able to complex with IRF-9 to form the IFN-stimulated gene factor (ISGF) -3 [138]. ISGF-3 binds to ISRE sites and initiates transcription of a number of genes including other IFNs, ISGs and IRF-7 (thus amplifying the IFN response) [139].

IFN signalling also has the potential to enhance IFN production indirectly by up regulating signalling intermediates of the virus recognition pathways, for example RIG-I and MDA5 are

both ISGs and IFN-induced RIG-I and MDA-5 expression is able to further amplify IFN production via enhancing virus recognition [124].

In the context of RV infection, signalling via the IFNAR1 complex has been shown to be crucial for the robust response to RV *in vitro* and *in vivo*. Chen *et al* demonstrated deficient IFN and pro-inflammatory gene expression upon RV infection of HBECs treated with an IFN β neutralising antibody [140]. Bartlett *et al* demonstrated similar observations in *IFNAR1*^{-/-} mice versus wild-type controls [52].

1.4.7 Anti-viral ISGs

There are over 380 known ISGs [141]. Aside from inducing the up regulation of IFNs themselves, type I and III IFN signalling causes the expression of many other IFN stimulated genes (ISGs). Many ISG protein products are directly anti-viral and convey an anti-viral state upon host cells and potential host cells in the vicinity. ISG proteins such as MxA, Viperin, oligoadenylate synthetase (OAS) and protein kinase R (PKR) all act as direct antivirals and are all up regulated in models of RV infection [51, 142, 143]. These antiviral ISGs are known to act by various mechanisms; OAS is an enzyme that degrades viral RNA [144] and PKR is able to inhibit translation of viral mRNA into protein via phosphorylating the α -subunit of eukaryotic protein synthesis initiation factor 2 (eIF2 α). [145]. The mechanisms of action of Viperin and MxA are less clear, Viperin is thought to act upon viral protein trafficking and host lipid trafficking within the cell, and MxA is a small GTPase that localises to the ER and inhibits viral replication however no definitive mechanisms of actions have been agreed upon [146, 147].

In the context of cellular and adaptive immunity, IFNs are also able to induce the up regulation of the major histocompatibility complex (MHC)-1 on infected cells for increased presentation of viral antigen to CD8+ T-cells [148] and induce the activation of natural killer cells, promoting cytotoxicity of infected cells [149].

1.4.8 Negative regulation of IFN production

The regulation of IFN production by viral infection is an essential process that inhibits the over-production of potential damaging anti-viral responses. Both PRR signalling pathways leading to IFN production, and IFN signalling pathways leading to ISG induction are subject to diverse negative regulation. Suppressors of cytokine signalling (SOCS) proteins act as

suppressors of JAK/STAT signalling and thus are implicated in the suppression of IFN action and ISGs transcription. SOCS proteins are induced as ISGs themselves and therefore perpetuate a negative feedback loop upon IFN stimulation. Briefly, SOCS can act to recruit E3 ubiquitin ligases that are able to target signalling intermediates for proteasomal degradation [150]. For example, SOCS1 & SOCS3 are able to bind directly to receptors blocking STAT binding, inhibiting signal transduction from the IFNAR1 or IFN λ R1 complexes [151] and they have also been shown to act as JAK pseudosubstrates, again inhibiting STAT-mediated signal transduction [152]. As virus-sensing molecules such as RIG-I are ISGs, the action of SOCS are able to restrict the amount of virus-sensing signalling proteins available to perpetuate the anti-viral response.

Other than SOCS, the auto-repression of certain molecules is able to dampen the IFN response of host cells, the RLHs are auto-repressive and LPG2 (a invariant form of RLH similar to RIG-I and MDA-5) is thought to act as a suppressor of RIG-I and MDA-5 signalling [153, 154]. Other important negative regulators of IFN production include SIKE, an inhibitor of IKKe/TBK1, Pin 1 which targets IRF3 and optineurin which inhibits TBK1/TRAF3 complexes [155-157]. The RLH adaptor protein MAVS is also a target for intense negative regulation, for example it is known to be degraded upon cell stimulation with RNA, possibly acting as an intrinsic mechanism limiting the amount of signal transduction via MAVS signalling during viral infection [130]. Important negative regulators of MAVs are summarised in Table 1.1.

Table 1.1 Known negative regulators of MAVS.

Regulator	Mechanism of negative regulation	Reference
NDFIP1	Chaperone for the E3 ubiquitin ligase SMURF that targets MAVS for proteasomal degradation	Wang <i>et al</i> 2012
PCBP1	Ribonuclear protein known to facilitate the Proteasome-mediated degradation of MAVS by the E3 ubiquitin ligase ITCH	You <i>et al</i> 2009
PCBP2	Ribonuclear protein known to facilitate the Proteasome-mediated degradation of MAVS by the E3 ubiquitin ligase ITCH	Zhou <i>et al</i> 2012
RNF125	E3 ubiquitin ligase known to cause the ubiquitination of MAVS	Arimoto <i>et al</i> 2007
ITCH	E3 ubiquitin ligase known to cause the ubiquitination of MAVS	You <i>et al</i> 2009
SMURF1	E3 ubiquitin ligase known to cause the ubiquitination of MAVS	Wang <i>et al</i> 2012
NLRX1	Mitochondrial nucleotide binding protein, it is currently unclear how NLRX1 causes MAVS degradation	Moore <i>et al</i> 2006
PSMA7	Proteasomal subunit that is known to interact with MAVS and if over expressed causes inhibited MAV-mediated antiviral immunity	Yongxia <i>et al</i> 2009

1.5 Therapies for asthma

1.5.1 Current treatments for asthma

The current mainstay of asthma treatment is inhaled corticosteroids (ICS) such as Fluticasone Propionate or Prednisolone and inhaled long acting or short acting β_2 -adrenergic receptor agonists (LABAs or SABAs) such as Salmeterol and Salbutamol (respectively). The combination of ICS and LABA/SABAs function to reduce bronchoconstriction and dampen inflammation within the airway and have demonstrated efficacy in reducing rates of asthma exacerbations in adults and children [158-160]. ICS and LABAs have been shown to reduce levels of RV-induced pro-inflammatory cytokines and chemokines (including IL-6, IL-8, RANTES/CCL5 and IP-10) in HBECs [161, 162]. Severe asthmatics are often prescribed high doses of systemic steroids however some asthmatics have been shown to be steroid resistant by mechanisms not fully understood [163-165]. Poor adherence to therapy is thought to be a major factor in asthma morbidity and mortality [166].

1.5.2 Targeting viruses in asthma treatment

The current treatment regimen for asthma does not include anti-viral agents despite the high association between respiratory viral infections and asthma exacerbations and known deficiency of asthmatics to produce type I and type III IFN. RV-specific vaccination has only proved partly effective in preventing re-infection due to the limited cross-reactivity of neutralising antibodies [167]. Agents that target the lifecycle of RV including capsid binding agents and protease inhibitors have proved partly effective in reducing cold symptoms [168, 169]. Soluble ICAM-1 has been examined as a RV specific receptor blockade in the common cold, decreasing the total cold symptom score and reduce nasal mucus production [170]. None of these potential therapies are currently considered for the treatment of RV-associated asthma.

Aside from direct inhibition of RV infection, a number of experimental studies have focussed on augmenting the anti-viral host response to viral infection through boosting intrinsic responses using biologics. Studies have examined the effects of therapeutic IFN in the context of asthma with varying outcomes. In the 1980s recombinant IFN α therapy was shown to reduce RV replication however had little effect on patients cold symptoms [171, 172]. Djukanovic *et al* recently reported the effects of inhaled recombinant IFN β therapy in a

cohort of asthmatics in a phase II clinical trial; they show that IFN β therapy reduced symptoms and recovery time post RV infection [173]. Other biologics have shown potential to augment anti-viral responses to viral infection. Intra-nasal Poly(I:C) and R-837 (synthetic TLR-3 and TLR-7 agonists respectively) have been shown to be effective in reducing virus-associated pathology and are approved for the treatment of HIV and HPV respectively [174, 175]. Gielen *et al* showed that the macrolide Azithromycin is able to augment IFN and anti-viral responses to RV infection in HBECs cultured *in vitro*. This augmentation was accompanied with a significant decrease in viral replication and has been proposed as a possible treatment option for RV-associated asthma exacerbations [142].

1.6 Macrolides

1.6.1 Macrolide structure and antibiotic function

The discovery of Erythromycin in 1949 marked the beginning of what is now one of the widely used types of antibiotic therapy [176]. Natural and synthetic derivatives such as Telithromycin, Clarithromycin, Azithromycin and Roxithromycin all share common structural properties that account for their potent antibiotic activity. Macrolides are hydrocarbon-based compounds consisting of a macro-cyclic core of 14-16 carbon atoms and sugar substituents. All macrolide antibiotics facilitate their anti-bacterial effects via a similar mechanism, which is inhibition of bacterial protein synthesis achieved via macrolide binding to the 23S ribosomal (r)RNA within the 50S subunit of the bacterial ribosome. This binding interferes with the peptidyl transferase cavity preventing nascent peptide egression [177-180].

1.6.2 The use of macrolides for the treatment of asthma and asthma exacerbations

Studies focusing on the use of macrolides in asthma have been performed with varying results [181]. Many studies have focussed on lung function and symptom scores as primary outcomes, rather than the rate or severity of asthma exacerbations. A summary of the clinical studies performed assessing the efficacy of macrolides in asthma and asthma exacerbations are shown in Table 1.2. Brusselle *et al* and Hahn *et al* showed Azithromycin was effective at reducing symptoms (as measured by the Asthma Quality of Life Questionnaire-AQLQ) and reducing the rate of asthma exacerbations in a subset of non-eosinophilic asthmatics [182, 183]. Neither study showed an effect of Azithromycin on lung function, and Cameron *et al* and Strunk *et al* reported conflicting results with regards to symptom scores [184, 185]. Clarithromycin was demonstrated to have a beneficial effect on symptom scores in a subset of non-eosinophilic asthmatics and showed improved PC20 scores in a number of studies [186-188]. Troleandomycin was shown to act as a steroid-sparing agent when examining symptoms, FEV₁ and PEF in asthmatics [189].

Table 1.2 Summary of clinical studies of macrolides in asthma.

Study outline	Findings	Reference
Azithromycin in adults with persistent asthma	Improved symptom scores	Hahn <i>et al</i> 2012
Azithromycin in asthmatic smokers	No effects on lung function.	Cameron <i>et al</i> 2013
Azithromycin in asthmatics with recent exacerbations	Decreased exacerbation rate in non-eosinophilic subgroup. Improved symptom scores. No effects on lung function.	Brusselle <i>et al</i> 2013
Azithromycin in patients with persistent asthma	Improvement in total asthma symptoms	Hahn <i>et al</i> 2006
Azithromycin in poorly controlled asthmatics	Reduced PC20. No effects on sputum neutrophils.	Piacentini <i>et al</i> 2007
Azithromycin in poorly controlled asthmatics in high dose ICS	No effects on symptoms or lung function.	Strunk <i>et al</i> 2008
Clarithromycin in asthmatics on high dose ICS	Improved PC20. No effect on lung function.	Kostadima <i>et al</i> 2004
Clarithromycin in asthmatics with recent exacerbations	Reduction in TNF α , IL-1 β and IL-10	Fonseca-Aten <i>et al</i> 2006
Clarithromycin in mild asthma	Reduced exacerbation rate. Reduced duration of exacerbations. No effect on lung function.	Koutsoubari <i>et al</i> 2012
Clarithromycin in patients with refractory asthma	Improvement in patient symptoms. No effects on lung function.	Simpson <i>et al</i> 2008
Clarithromycin in poorly controlled asthmatics	Improved PC20. No effects on symptom score or exacerbation rate.	Sutherland <i>et al</i> 2010
Clarithromycin in poorly controlled asthmatics	Improved FEV1. No effects on BAL cytokines.	Kraft <i>et al</i> 2002
Clarithromycin in poorly controlled asthmatics	Improved PC20. Improved symptom scores. No effects on sputum eosinophils or lung function.	Amayasu <i>et al</i> 2000
Roxithromycin in poorly controlled asthma	Improvement in PEF (morning only). No effects of symptom scores.	Black <i>et al</i> 2001
Roxithromycin in poorly controlled asthma	Improved symptom scores. Reduction in blood/sputum eosinophils. No effects on lung function.	Shoji <i>et al</i> 1999
Telithromycin in asthmatics with recent exacerbations	Improvement in symptom scores and FEV1	Johnston <i>et al</i> 2006
Troleandomycin in poorly controlled asthmatics on high dose ICS.	Steroid tapering effects observed.	Kamada <i>et al</i> 1993

Studies that have examined the effects of macrolides specifically on naturally occurring acute asthma exacerbations are fewer in number. Telithromycin, given within 24 hours of asthma exacerbations requiring hospitalisation or medical attendance improved symptom scores and was associated with increased FEV₁ at 10 days post-exacerbation [190]. Two studies by Koustoubari *et al* and Fonseca-Aten *et al* demonstrated that Clarithromycin in children post-exacerbation increased the number of asthma symptom-free days whilst reducing the duration of exacerbations, and reduced levels of TNF α , IL-1 β and IL-10 in nasal aspirate (respectively) [191, 192].

The role of pathogenic bacteria in asthma exacerbations is not fully understood. Unlike COPD, where acute exacerbations are strongly linked with atypical bacterial infection thus warranting the use of antibiotics [193]; the link between bacteria and disease pathogenesis is less clear in asthma exacerbations. Bacterial burdens have been shown to be higher and more atypical species such as *Haemophilus spp* are more frequently identified in asthmatic patients than healthy controls [17, 194]. The emerging association between atypical bacterial infection and asthma may warrant the use of antibiotics including macrolides in the future.

1.6.3 Immuno-modulatory properties of macrolides

Azithromycin is the most well studied macrolide antibiotic, and is known to have a range of immuno-modulatory effects in addition to its primary role as an antibiotic. The majority of studies investigating these effects have been restricted to *in vitro* cell-culture systems. Azithromycin has been shown to inhibit the activation of NF κ B and activator protein 1 (AP-1) in HBECs and thus reduce expression of pro-inflammatory IL-6, IL-8 and T_H2-associated thymic stromal lymphopoietin (TSLP) [195-197]. Azithromycin was shown to enhance phagocytosis whilst decreasing expression of IL-8 and TNF α in macrophages from COPD patients [198]. Macrolides such as Clarithromycin have shown partial efficacy in reducing MUC5AC and MUCB gene expression through the inhibition of TNF α and IL-13 production in airway goblet cells [203, 204] and thus may be an effective add-on treatment to standard therapy.

Azithromycin has also shown immunomodulatory effects on monocytes, reducing the levels of the typical pro-inflammatory cytokines IL-1 β , TNF α , IL-8 and RANTES/CCL5 in Azithromycin-treated neutrophils from sputum samples [199]. In mice, Azithromycin has been shown to promote the M2 (T_H2) phenotype of macrophages, decreasing the expression of the M1 (T_H1) associated marker; CCR7 and expression of IL-1 β , IL-6 and IL-12 whilst up regulating the M2 marker CD23 and the anti-inflammatory IL-10 in response to IFN γ and lipopolysaccharide (LPS) [200-202].

Although still in its infancy, the field of macrolides as anti-viral agents has shown some promising results. In 2010, Gielen *et al* published the effects of Azithromycin on RV-infected HBECs from healthy donors. The study observed that Azithromycin pre-treatment of HBEC cultures augmented RV induced IFN β , IFN λ (IL-28 and IL-29), RIG-I, OAS, Viperin and MxA

by varying degrees, an up to 600% in the case of IL-28 and Viperin. Consistent with this, Azithromycin caused a decrease in both RV16 and RV1b replication by up to 50% in HBECs. Erythromycin and Telithromycin however, did not exhibit similar effects [142]. In other studies with other macrolides, Erythromycin and Clarithromycin reduced levels of the major-group RV receptor ICAM-1 with reduced viral titre, however this was not observed with the minor-group (LDL) receptor [205, 206]. In other respiratory viral models, Clarithromycin inhibited the replication of RSV *in vitro* (by inhibiting the expression of RhoA) and had an inhibitory effect on influenza replication *in vitro* [207, 208]. In *in vivo* infection studies of healthy individuals, results are promising but far from consistent. In experimental infection studies with RV, Clarithromycin had little or no effect on nasal inflammation [209]. In cases of naturally occurring infant RSV-bronchiolitis, clarithromycin reduced wheeze, oxygen requirement and length of hospital stay [210].

1.6.4 Azithromycin structure, pharmacokinetics and tissue retention

Azithromycin is a synthetic, second-generation macrolide. Its structure contains a 15-membered macro-cyclic ring, and differs from Erythromycin due to a nitrogen atom substituting for one of the carbon atoms at position nine. Figure 1.3 shows the structure of Azithromycin and Erythromycin. Azithromycin shares the same antibiotic mechanism as the other macrolides, inhibiting protein synthesis via the blocking of nascent peptide egression within the 50S subunit of the bacterial ribosome [177].

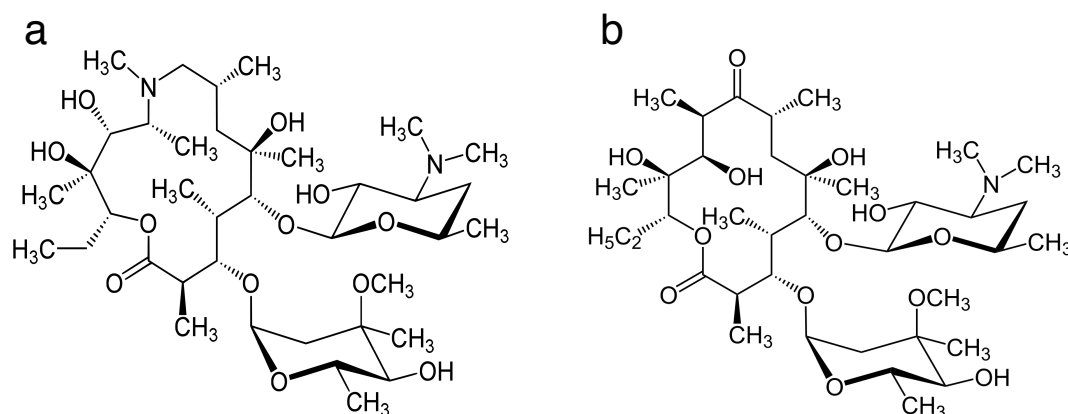


Figure 1.3 Chemical structures of Azithromycin and Erythromycin.

Azithromycin (a) and Erythromycin (b) share similar molecular structures. Azithromycin possesses a Nitrogen atom within the macrocyclic ring.

Azithromycin has relatively high tissue retention and relatively low serum concentrations after oral dosing [211] and is not actively metabolised with much of the compound eliminated unchanged in faeces [212-214]. In a study by Foulds *et al*, Azithromycin was found to be at relatively high concentrations in tissue samples (including: lung, prostate, kidney, liver, tonsil, muscle, fat and bone) compared to serum concentrations after a single 500mg oral dose. An Azithromycin lung tissue concentration was observed to be approximately 8mg/kg 24 hours post a single oral dosing (500mg) and persisted at this dose for at least 3 days compared to a serum concentration of approximately 0.8mg/kg [215]. In mice, comparable results have been obtained with regards to tissue concentrations, in mice gavaged orally with 50mg/kg Azithromycin, lung tissue concentrations were found to be 13mg/kg compared to serum concentrations of 4mg/kg 24 hours post gavage [216].

Unlike other macrolides, Azithromycin is known to accumulate at high levels at the site of inflammation [217, 218]. It is thought that this phenomenon occurs via the preferential uptake of Azithromycin by phagocytes, where Azithromycin is able to resist acidification of phagosomes (due to the presence of the nitrogen at position nine of the macrolide ring) and in bacterial infections is effectively carried to the site of infection via phagocyte chemotaxis-mediated migration, perhaps in part accounting for the anti-bacterial properties of this compound [202, 219-221]. Azithromycin also shows high levels of tissue retention in other cells including epithelial cells, lymphocytes and fibroblasts. The latter, due to its distribution, may act as an Azithromycin reservoir, slowly releasing it at the sites of infection [222-226]. It is thought that the high cellular uptake of Azithromycin is the reason behind its relatively high tissue: serum concentration ratio and allows for a shorter therapeutic course of administration.

1.7 Rationale, hypotheses and outline

1.7.1 Rationale of thesis

The association between RV infection and asthma exacerbations is well established however the current asthma treatment guidelines do not include any anti-viral agents. Gielen *et al* showed that Azithromycin may be capable of augmenting RV-induced IFN, while other studies suggest that Azithromycin is capable of decreasing pro-inflammatory cytokine expression. Therefore Azithromycin may prove to be an effective agent for the treatment of virally associated asthma exacerbations. Previous studies are limited to *in vitro* infection models using healthy HBECs and do not assess the effects of Azithromycin on cells from asthmatics, nor do they investigate Azithromycin in a mouse model of infection. The mechanism of action of Azithromycin-mediated IFN augmentation has yet to be studied and may reveal a potential therapeutic target for future drug interactions. Novel macrolide libraries that have yet to be examined for any anti-viral activity may yield compounds with superior IFN-augmenting abilities to Azithromycin, in addition to possible anti-inflammatory and anti-bacterial activity.

1.7.2 Hypotheses

- Azithromycin is able to augment anti-viral activity in HBECs cultured *ex vivo* from asthmatic donors.
- Azithromycin mediates its IFN-augmenting effects via direct or indirect interaction with one or more of the virus recognition pathways in RV-infected HBECs.
- Azithromycin is able to augment anti-viral activity in a mouse model of RV infection.
- Novel macrolides are capable of augmenting RV-induced IFN expression in HBECs in a superior manner to that observed with Azithromycin.

1.7.3 Aims

- To assess the effects of Azithromycin on anti-viral responses to RV infection in HBECs cultures *ex vivo* from asthmatic donors.
- To determine the effects of Azithromycin on the response to RV infection *in vivo*.
- To determine a potential mechanism of action of Azithromycin-mediated anti-viral augmentation.

- To identify novel macrolides with the ability to augment anti-viral responses with great efficacy to that of Azithromycin.

1.7.4 Outline of thesis

Chapter one is an in-depth introduction to the subject material covering clinical asthma exacerbations, aetiology of the disease including the association with RV infection, RV infection and signalling pathways leading to the expression of IFN and ISGs and finally an introduction to the structure and chemistry of macrolides with a particular focus on Azithromycin. Chapter two outlines all materials and experimental protocols used throughout these investigations. Chapters three, four, five and six are results chapters addressing the four hypotheses listed above respectively; the effects of Azithromycin in RV-infected HBECs from asthmatics, the possible mechanism of action of Azithromycin, the effects of Azithromycin in a mouse model of RV infection and the identification and characterisation of novel macrolides with IFN-augmenting ability. Finally, chapter seven will be a discussion and conclusion about the data obtained, implications for the field of asthma and the treatment of virus-induced asthma exacerbations and will outline possible future directions.

Chapter 2 Materials and Methods

2.1 Buffers and reagents

Table 2.1 Buffers and reagents

Buffers and reagents		
Name	Composition	Supplier
5'ppp-dsRNA	5' tri-phosphate double stranded RNA w/<0.001EU/ μ g	Invivogen- UK
5'ppp-dsRNA control	19mer blunt-end dsRNA without a 5'triphosphate	Invivogen- UK
Ammonium- Chloride- Potassium lysis buffer (ACK). NH_4Cl .	0.15M Ammonium Chloride, 1mM Potassium Hydrogen Carbonate, 0.1mM Disodium EDTA in 500mL in ddH ₂ O. Filtered.	Sigma Aldrich- USA, Invitrogen- UK.
Ampicillin (100mg/mL)	Provided in 70% (v/v) ethanol	Sigma Aldrich- USA
Azithromycin	Anhydrous powder	Sigma Aldrich- USA
Bovine Serum Albumin (BSA)	1% BSA in 0.15M NaCl pH 7.0	Sigma Aldrich- USA
Bronchoalviolar lavage (BAL) fluid	1% v/v 0.5M EDTA, 0.25% w/v Lidocaine hydrochloride monohydrate in D-PBS	Life technologies- UK, Sigma Aldrich- USA, PAA- Austria
CMC gavage fluid	1% w/v Carboxymethyl cellulose (low viscosity), 0.01%w/v Methyl cellulose (15 centipoise), 0.1% v/v Tween 80, 0.9% w/v NaCl. In ddH ₂ O. Autoclaved.	Sigma Aldrich- USA
Dulbecco's- Phosphate Buffered Saline (D-PBS)	1.4mM KH_2PO_4 , 8mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 2.6mM KCl, 137mM NaCl	PAA- Austria
ELISA (Enzyme Linked ImmunoSorbant Assay) Diluent	1%w/v BSA in D-PBS	Sigma Aldrich- USA, PAA- Austria
ELISA Stop solution	2M H_2SO_4	Sigma Aldrich- USA
ELISA wash buffer	0.05% Tween20 in D-PBS	Sigma Aldrich- USA, PAA- Austria
Ethanol (EtOH)	99.60%	VWR- UK
HEPES	1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Gibco- USA
HEPES buffered saline solution (for HBECs)	30mM HEPES in D-PBS	Lonza- USA
Lipofectamine®	Lipofectamine® 2000 (no other information supplied)	Life technologies- UK
LyoVec	Combination of DTCPTA and DiPPE lipids (no other information supplied)	Invivogen- UK

Methanol	99.80%	VWR- UK
NuPAGE Anti-oxidant	No product information available	Invitrogen- UK
NuPAGE Mops running buffer	50mL of 50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7, 500µL anti-oxidant made to 1L with ddH ₂ O	Invitrogen- UK
NuPAGE transfer buffer	Per gel: 50mL NuPAGE transfer buffer, 10% methanol (v/v), 500µL anti-oxidant made to 1L with ddH ₂ O	Invitrogen- UK
Poly(Ethylene glycol) (PEG)	PEG 6,000 MW	Sigma Aldrich- USA
Poly(I:C) (High molecular weight)	High molecular weight Polyinosinic-polycytidylic acid	Invivogen- UK
Poly(I:C) LyoVec	High molecular weight Polyinosinic-polycytidylic acid w/ LyoVec 1:6 ratio (w/w)	Invivogen- UK
Quantitect probe polymerase chain reaction (PCR) master mix	HotStarTaq DNA polymerase, quantitect probe PCR buffer (Tris-HCl, KCl, (NH ₄) ₂ SO ₄ , 8mM MgCl ₂ pH 8.7, dNTP mix, ROX passive reference dye	Qiagen- UK
Random Hexamers	Random hexadeoxynucleotides (0.5µg/mL)	Promega- USA
SeeBlue Plus2 pre-stained standard	Tris-HCl, formamide, sodium dodecyl sulphate, phenol red (no quantities provided by supplier)	Invitrogen- UK
Sodium Pentobarbitone	'Pentoject' 20%w/v Sodium Pentobarbitone	AnimalCare Ltd- UK
Streptavidin-Horseradish Peroxidase (HRP)	ELISA grade streptavidin- HRP conjugate	Sigma Aldrich- USA
Superfect transfection reagent	3mg/mL Superfect	Qiagen- UK
TMB substrate	3,3',5,5' Tetramethyl benzidine chromogen	Invitrogen- UK
Tris-Glycine SDS sample buffer (2X)	0.5M Tris-HCL (pH 6.8), glycerol, 10% (w/v) SDS, 0.1% bromophenol blue in ddH ₂ O	Invitrogen- UK
Trypan Blue 0.4%	0.4% C ₃₄ H ₂₄ N ₆ Na ₄ O ₁₄ S ₄ sodium chloride and 0.06% potassium phosphate	Sigma Aldrich- USA
Trypsin Neutralising Solution (TNS)	No information provided	Lonza- USA
Trypsin-EDTA (for BEAS-2B and HeLa cells)	0.5% trypsin, 5.3mM EDTA	Invitrogen- UK
Trypsin-EDTA (For HBECs)	0.25% trypsin, 0.01% EDTA	Lonza- USA
Tween20 (Polyoxethylene-sorbitan	2 ethylene oxide units, 1 sorbitol unit, 1 lauric acid unit	Sigma Aldrich- USA

monolaureate)		
Western Blot blocking solution	5% skimmed milk in D-PBS with 10% Tween20	Marvel- UK, PAA- Austria, Sigma Aldrich- USA
Western Blot stripping buffer	2% SDS, 62.5% Tris-HCL (pH 6.7), 100mM β_2 ME	Sigma Aldrich- USA
Western washing solution	D-PBS with 1% Tween20	PAA- Austria, Sigma Aldrich- USA
β -mercaptoethanol (β_2 ME)	14.3M solution	Sigma Aldrich- USA

2.2 Commercially available kits and reagents

Table 2.2 Commercially available kits and reagents

Commercially available kits and reagents		
Name	Components	Supplier
Dual-Luciferase Reporter Assay System	Luciferase Assay Buffer II, Luciferase Assay Substrate (lyophilized product), Stop & Glo buffer, 50X Stop & Glo Substrate, 5X Passive Lysis Buffer.	Promega- USA
ECL Western Blotting Substrate	Luminol/Enhancer, stable peroxide buffer 1:1 ratio	Thermo- USA
Human IFN β ELISA kit	Capture Ab pre-coated 96-well plate, Human IFN β Standard (100,000pg/mL), sample diluent, Ab concentrate, HRP conjugate concentrate, concentrate diluent, TMB substrate solution, stop solution.	Interferon Source- USA
Human IL-28/IL-29 ELISA kit	Capture Ab (rat anti-human IL-28AB), detection Ab (biotinylated goat anti-human IL-28AB), standard (recombinant Human IL-28AB), Streptavidin-HRP	R&D systems- USA
Human IL-6 ELISA kit	Capture Ab (rat anti-human IL-6), detection Ab (biotinylated goat anti-human IL-6), standard (recombinant Human IL-6), Streptavidin-HRP	R&D systems- USA
Human IL-8 ELISA kit	Capture Ab (rat anti-human IL-8), detection Ab (biotinylated goat anti-human IL-8), standard (recombinant Human IL-8), Streptavidin-HRP	R&D systems- USA
Maxiprep Kit	Buffer P1, Buffer P2, Buffer P3, Buffer QBT, Buffer QC, Buffer QF, Qiagen-tip 500	Qiagen- UK
MSD® IFN β Single PLEX kit	Adivin-coated 96-well plate, Biotinylated-IFN β Capture Ab, Sulfo-Tag™ detection Ab	Meso Scale Discovery- USA
MSD® IFN λ V-PLEX kit	96 well plate, IFN λ Capture Ab, Sulfo-Tag™ detection Ab	Meso Scale Discovery- USA
Omniscript RT (reverse Transcriptase) kit	dATP, dCTP, dGTP, dTTP (10mM each in H ₂ O), 10X Buffer RT, Omniscript reverse	Qiagen- UK

	transcriptase, RNase-free H ₂ O	
RNAase-free Dnase kit	1500 units RNase-free DNase I, RNase-free buffer RDD	Qiagen- UK
RNeasy mini kit	Buffer RLT, Buffer RW1, Buffer RPE, RNase-free H ₂ O, RNeasy mini spin columns, collection tubes	Qiagen- UK
TOPO TA cloning kit (with pCR 2.1- TOPO vector)	pCR 2.1 TOPO (10ng/μL) in 50% glycerol, 50mM Tris-HCL (pH 7.4), 1mM EDTA, 1mM DTT, 0.1% triton X-100, 100μg/mL BSA, phenol red, salt solution (1.2M NaCl, 0.06M MgCl ₂), One Shot Chemically Competent TOP10 <i>E. coli</i>	Invitrogen- UK

2.3 Clinical methods

2.3.1 Bronchoscopy

Bronchoscopies were performed at St. Mary's Hospital London or The Royal Brompton Hospital London under BTS guidelines using a Keymed BF260 bronchoscope (Olympus UK). Participants received 0.5mg Salbutamol and 500μg Ipratropium bromide prior to bronchoscopy. 4-6 brushings from the same lung lobe were taken and brushes were submerged *ex vivo* in pre-warmed BEGM complete medium at the point of removal from the lung. Ethics for obtaining bronchial brushings from children were granted from the Royal Brompton NHS Trust (02/302, Prof A. Bush). Research in adults has been approved by St Mary's NHS Trust (12/LO/1278 , 08/H0712/39 & 07/Q0403/20, Prof S.L. Johnston).

2.4 Cell culture

2.4.1 Cell types used in in vitro experiments

Normal Human Bronchial Epithelial Cells (HBECs) were procured from Lonza Inc. (USA), and are referred to as normal HBECs, asthmatic and non-asthmatic HBECs (grown in house) were obtained from bronchial brushings of asthmatic patients. HeLa Ohio, HeLa H1 and BEAS-2B cells were obtained from ATCC (USA). All cell cultures were maintained as 'clean' free of bacteria and/or respiratory viruses (unless otherwise stated), all work was performed in category II tissue culture hoods using sterile instruments. Mycoplasma testing was performed every 6-12 months.

2.4.2 Maintenance of cell cultures

All cell cultures were maintained in a humidified 37°C incubator with 5% CO₂ (New Brunswick Inc- USA). Culture medium was formulated as in Table 2.3. HBECs were maintained in fully supplemented BEGM medium (Lonza). Culture medium was aspirated and replaced with fresh medium every 48 hours. HeLa cells were maintained in DMEM medium supplemented with 10% heat inactivated foetal bovine serum (FBS, PAA- UK), 2% 1M HEPES (Gibco-USA) and 1% NaHCO₃ (Gibco). BEAS-2B cells were maintained in RPMI medium supplemented with 10% heat inactivated foetal bovine serum, 2% HEPES and 1% NaHCO₃.

2.4.3 Initial culture of asthmatic and non- asthmatic HBECs from bronchial brushings

Bronchial brushings were transported to the lab within 5 min, under sterile conditions brushes were passed through modified 1mL pipette tips into a petri dish containing complete BEGM medium to detach cells. Brushes were then rinsed with complete BEGM medium to ensure maximum recovery of cells. The resulting cell suspension was then centrifuged at 1200rpm for 6 min at room temperature to pellet cells. The resulting cell pellet was thoroughly re-suspended in complete BEGM medium, an aliquot of cell were counted using a haemocytometer and the remaining cell solution was diluted into a total of 6ml complete BEGM medium and incubated in a 25cm² flask for 48 hours at 37°C with 5% CO₂ for the cells to adhere. All brushings were processed within 30 min of sampling. Typical cell yields were $\leq 1 \times 10^8$ total cells (including erythrocytes) and approximately 1×10^5 ciliated epithelial cells per patient. Approximately 85% of cultures were successfully grown.

2.4.4 Thawing and initial culture of commercially derived, normal HBECs

Frozen cell suspensions were thawed at 37°C in a water bath for no longer than 2 min before being added to 20mL of fully supplemented BEGM medium and split into 2 x 75cm² culture flasks (Nunc-USA). The flasks were agitated gently to ensure even cell dispersal and incubated at 37°C (5%CO₂). After 24 hours the medium (containing non-adherent cells) was removed and replaced with fresh fully-supplemented BEGM (10mL/flask). Medium was replaced every 2 days until the flasks were approximately 90% confluent.

Table 2.3 Cell culture medium and supplements

Cell culture medium and supplements		
Name	Composition	Supplier
BEAS-2B cell growth medium	50mL (10%) FCS, 10mL (2%) HEPES, 5mL (1%) NaHCO ₃ in 500mL RPMI medium	PAA- Austria, Invitrogen-USA
BEAS-2B cell infection medium	10mL (2%) FCS, 10mL (2%) HEPES, 5mL (1%) NaHCO ₃ in 500mL RPMI medium	PAA- Austria, Invitrogen-USA
BEGM SingleQuot™ additives	13g/L bovine pituitary extract, 5g/L insulin, 50g/L gentamicin, 0.1mg/L retinoic acid, 10g/L transferrin, 6.5mg/L triiodothyronine, 0.5g/L hydrocortisone & epinephrine, 0.5mg/L human epidermal growth factor	Lonza- USA
Dulbeccos Modified Eagles Medium (DMEM)	With high glucose 4.5g/L with L- glutamine	PAA- Austria
Foetal Calf Serum (FCS)	Heat inactivated foetal calf serum	Invitrogen- USA
HBEC growth medium	BEBM with BEGM SingleQuot™ additives	Lonza- USA
HBEC infection medium	BEBM	Lonza- USA
HeLa cell growth medium	50mL (10%) FCS, 10mL (2%) HEPES, 5mL (1%) NaHCO ₃ in 500mL DMEM medium	PAA- Austria, Invitrogen-USA
HeLa cell growth medium for titrations	20mL (4%) FCS, 10mL (2%) HEPES, 5mL (1%) NaHCO ₃ in 500mL DMEM medium	PAA- Austria, Invitrogen-USA
HeLa cell infection medium	10mL (4%) FCS, 10mL (2%) HEPES, 5mL (1%) NaHCO ₃ in 500mL RPMI medium	PAA- Austria, Invitrogen-USA
HEPES buffer	1M HEPES Na salt	Gibco Life Technologies- USA
Opti-MEM® transfection medium	Reduced serum medium w/Phenol Red	Life technologies- UK
Penicillin/ Streptomycin	10,000units/mL Penicillin, 10,000µg/mL streptomycin	PAA- Austria
Roswell Park Memorial Institute (RPMI) medium	With L-glutamine and phenol red	PAA- Austria
Sodium Bicarbonate (NaHCO ₃)	7.5% NaHCO ₃ solution	Invitrogen- USA

2.4.5 HBEC subculture and seeding

When cells reached approximately 90% confluency the culture medium was removed and the cell monolayer was rinsed twice with 4mL/flask HEPES Buffered Saline Solution (HBSS- Lonza), the final rinse was completely aspirated to ensure total removal of cell culture medium. 2mL of Trypsin/EDTA (Lonza) was added to each flask and flasks were agitated by

hand to ensure complete coverage of the cell monolayer, flasks were then incubated at 37°C (5%CO₂) for 3minutes. Flasks were tapped by hand firmly and detached cells were removed and added directly to 4mL/flask of Trypsin Neutralizing Solution (TNS-Lonza). A further 1mL of Trypsin/EDTA was added to flasks to remove stubbornly adherent cells and returned to the incubator for 1min, after which each flask was thoroughly rinsed with 4mL TNS and the cell solution pooled. The cell solution was then centrifuged at 1200rpm for 6minutes and the supernatant was discarded. The cells were thoroughly re-suspended in 10mL fully supplemented BEGM, this solution was then split between fresh 75cm² flasks and the total volume was made up to 10mL/flask. 2 flasks (from thawing) were split into 9 and grown to 90% confluency, 3 of those flasks were split into 9 more while cells from the remaining 6 flasks were counted and seeded onto culture plates at a density of 0.5x10⁵ cells/mL in fully-supplemented BEGM for experiments. Cells were not grown beyond 6 passages.

2.4.6 Thawing and initial culture of HeLa and BEAS-2B cells

Frozen cell suspensions of HeLa and BEAS-2B cells were thawed at 37°C in a water bath for no longer than 2min before being added to 25mL of fully supplemented DMEM or RPMI medium (respectively) and placed in 125cm² culture flasks (Nunc-USA). The flasks were agitated gently to ensure even cell dispersal and incubated at 37°C (5% CO₂). Cell culture medium was replaced after 24 hours to remove DMSO. The flasks were split when approximately 90% confluent.

2.4.7 HeLa and BEAS-2B Subculture and seeding

Cells were washed 2X in D-PBS and 1X Trypsin-EDTA (PAA) was added to each flask, BEAS-2B cultures were incubated at room temperature for approximately 30 seconds (until the cells had detached), HeLa cells were incubated at 37°C for 1-2 minutes (until the cells had detached). Trypsin was quenched with complete medium. For seeding, cells were counted and seeded at 1.7x10⁵ cells/mL into culture plates in complete medium.

2.4.8 Cell quantification

All cell types used were quantified using the same method; 20μL of fully suspended cells was added to 20μL 0.1% Trypan Blue (Gibco), a Neubauer haemocytometer (Marienfeld, Germany) was filled with the solution and viewed under a light microscope, viable cells were counted and cells/mL was calculated.

2.5 RV propagation

Stocks of RV (both RV16 and RV1b) were cultured for use in *in vitro* and *in vivo* experiments, details for generating those stocks are listed below.

2.5.1 RV propagation for *in vitro* use

HeLa Ohio cells (ATCC- USA) were cultured as detailed in section 2.4.7, 2X 125cm² flasks (at 90% confluent) were inoculated with stock RV1b or RV16 (ATCC) by diluting the virus into 50mL DMEM w/ 2% FBS 2% 1M HEPES and 1% NaHCO₃ and placing on cells (25mL/flask) for 1 hour at room temperature (with agitation), then incubating for 24 hours at 37°C (5% CO₂). After 24 hours infected cells were detached from the flasks by hitting the outside of the flask and/or by scraping the cells with a cell scraper, the contents of the 2 flasks were pooled and freeze/thawed twice at -80°C. This crude inoculum was centrifuged at 300xg for 15 minutes at 4°C to pellet cell debris and the virus-containing supernatant was filtered using a 0.2µm filter (Pall Life Sciences- Uk), aliquoted and frozen at 80°C until use.

2.5.2 Propagation and purification of RV for *in vivo* use

Virus was grown as described in section 2.5.1, the 500mL of filtered virus was used to infect a further 25X 125cm² H1 cultures (90% confluent) by distributing the filtered inoculum evenly to the flasks and incubating for 1 hour at room temperature (with agitation), then over night at 37°C (5% CO₂). Before substantial cytopathic effects (CPE- as determined by disruption of the monolayer) was observed, cells were scraped off the flasks using a cell scraper and washed 3X in D-PBS. Cell pellets were frozen at -80°C and freeze/thawed twice. RV was purified by first centrifuging cells at 300xg for 15 mins to pellet cellular debris, supernatants were then incubated in 5mL of 5M sodium chloride containing 0.7g/mL polyethylene glycol (Sigma) for 1 hour at room temperature to precipitate virus out of solution. This precipitate was isolated by centrifugation and dissolved in PBS and centrifuged again to remove any remaining cellular debris. The supernatant was filtered through a 0.2µM filter into a 15mL Amicon Ultra centrifugal filtration device (Millipore, USA) and centrifuged according to the manufacturers instructions. Pure RV was re-suspended in 2.5mL PBS and frozen before use in experiments.

2.6 Titration of RV and TCID₅₀ calculation

Supernatants from cell cultures infected with RV were serially diluted 1:10 at a range of 10^{-1} - 10^{-6} in serum-free DMEM, the dilutions were vortexed and aliquoted onto a 96-well culture plate (Nunc) in replicates of 8. HeLa Ohio cells were co-incubated with supernatant dilutions at 0.5×10^5 cells/mL in DMEM supplemented with 4% FCS, 2% HEPES and 1% NaHCO₃, control wells containing no supernatant were also included on the plate (see section 2.4.7 for counting and subculture) for 4 days at 37 °C with 5% CO₂. Cytopathic effects (CPE) on HeLa cells were determined as disruption of the cell monolayer as viewed by light microscopy, wells were scored as either positive or negative for CPE and a tally generated, TCID₅₀ was then calculated by the Spearman-Kärber method [227].

2.7 RSV and influenza virus

Stocks of RSV-2A and stocks of influenza (pandemic H1N1- A/Eng/159 and H3N2- A/Eng/691 2010) were a kind gift from Prof. Peter Openshaw and Prof. Wendy Barclay, respectively.

2.8 Virus infection of cell cultures

Prior to infection all cells were placed in infection medium (HBECs: supplement-free BEBM, BEAS-2Bs: RPMI with 2% FBS 2% HEPES and 1% NaHCO₃, HeLa: DMEM with 2% FBS 2% HEPES and 1% NaHCO₃) for 24 hours (with or without macrolides as stated). Stock RV1b, RV16 (see section 2.5), RSV-2a (ATCC), H1N1 or H3N2 was diluted in the relevant infection medium prior to application to cell cultures to give an MOI of 1, 200µL per well of a 12-well plate (24-well plate: 100µL, 48-well plate: 50µL), was added to cells and cultures were incubated at room temperature (RV16 or RV1b) or at 37°C (RSV and Flu) for 1 hour with agitation. Virus was then removed and the cultures were washed 3X with infection medium. Cultures were placed in infection medium and incubated for a specified amount of time at 37°C (5% CO₂).

2.9 Macrolide treatment of cell cultures

Azithromycin and all novel macrolide compounds were treated in the same manner unless stated otherwise. A stock concentration of 0.1M was generated by adding the appropriate amount of 70% EtOH to anhydrous powdered macrolide, solutions were vortexed for a

minimum of 10 seconds and sonicated in a water bath for to up to 3 minutes to ensure complete dissolution. The stock solutions were kept at -20°C. Working concentrations of 50µM and 10µM were generated by thoroughly thawing the stock solutions in a 37°C water bath for 10 minutes, vortexing for 10 seconds and diluting the stock into the appropriate volume of serum/supplement-free medium. Working solutions were then vortexed and added to cell culture wells. As a control, a volume of 70% EtOH equal to that of the 50µM macrolide solution was diluted in serum/supplement-free medium used as a “vehicle control”.

2.10 Treatment of cells with Pattern Recognition Receptor (PRR) agonists

2.10.1 Treatment of normal HBECs with poly(I:C)

Normal HBECs grown in 12-well culture plates (Nunc) were treated topically with 1µg/well of the TLR3 agonist Poly(I:C) (high molecular weight) (Invivogen, USA) diluted in supplement-free BEBM medium and incubated for 8 hours at 37°C (5% CO₂).

2.10.2 Treatment of normal HBECs with R848

Normal HBECs grown in 12-well culture plates (Nunc) were treated topically with 1µM of the TLR7/8 agonist R848 (Sigma Aldrich) diluted in supplement-free BEBM medium and incubated for 8 hours at 37°C (5% CO₂).

2.10.3 Transfection of normal HBECs with Poly (I:C)

Normal HBECs were grown in 12-well culture plates (Nunc) were transfected with 0.25µg/well of the MDA-5 agonist Poly(I:C)/LyoVec (Invivogen). Poly(I:C)/LyoVec was diluted in supplement-free BEBM medium, vortexed thoroughly and added to HBEC cultures for 8 and 24 hours at 37°C (5% CO₂). LyoVec (Invivogen) at a concentration of 0.25µg/well was used as a transfection control.

2.10.4 Transfection of normal HBECs with 5'ppp-dsRNA

Normal HBECs were transfected with 0.5µg/well of the RIG-I agonist 5'ppp-dsRNA (Invivogen). 0.5µg/well of 5'ppp-dsRNA was mixed with 5µg/well LyoVec and incubated at room temperature for 15 minutes to allow complexes to form. This mixture was then diluted

in 890 μ L/well of a 1:3 mixture of complete BEGM and supplement free BEBM medium as per the manufacturer's instructions and vortexed thoroughly. 1mL/well (of a 12-well plate) was added to each well and plates were incubated at 37°C (5% CO₂) for 8 or 24 hours. dsRNA lacking the 5' tri-phosphate (Invivogen) was used at the same concentration as a negative control.

2.11 Treatment of normal HBECs with recombinant IFN β

Normal HBECs grown in 12-well culture plates (Nunc) were treated topically with 2.5ng/well recombinant IFN β (PBL Interferon Source USA) diluted in supplement-free BEBM medium and incubated for 8 hours at 37°C (5% CO₂).

2.12 siRNA suppression of MAVS and MAVS-regulating proteins in normal HBECs

Normal HBECs were grown in 24-well plates and transfected with 1 μ g/mL of ON-TARGETplus siRNA oligos (Dharmacon, USA) targeted against MAVS, known MAVS regulators or non-targeting control (4 sequences were targeted per pool Table 2.4). Briefly, siRNA oligos were diluted in Opti-MEM low serum medium (Life-Technologies-USA) and diluted with Lipofectamine (Invitrogen) and incubated for 20 minutes at room temperature. After this incubation siRNA/Lipofectamine complexes were further diluted in supplement-free BEBM and added to cells. Cells were then incubated at 37°C (5% CO₂) for 5 hours and washed 1X with PBS before the addition 1:2 fully-supplemented BEGM: supplement-free BEBM containing Azithromycin or the appropriate control.

Table 2.4 siRNA SMARTpool sequences.

siRNA oligonucleotide sequences	
Gene targeted	Target sequences 5'-3'
Non-targeting control	uaaggcuauagaagagauac
	auguauuggccugauuuag
	augaacgugaauugcucaa
	ugguuuacaugucgacuaa
MAVS	aaguauaucugccgcauuu
	cauccaaagugccuacuag
	gcaauguggacguuguaga
	cauccaaaugcccauaa
NDFIP1	cuuuuacuggauuggguuu
	ucucagaggauuuaucaau
	guaaggagaggauuaggua
	acuguagauuagugcuuaa
PCBP1	cuacucgauucaaggacaa
	caaagagaucgcgagagu
	caacuuuaucauccgcuaa
	ucgcuaugaucaucgacaa
PCBP2	gaaaggagaaucaaguuaag
	ggaaagggauguuggcagua
	gauugaagguggauuuuuu
	cgaauugauuuggcugcaua
RNF125	uuuauuacccgaugagaa
	gcagcaagguguguauugc
	uuagaccggucacuucuu
	gaaaugagggcacauuuu
ITCH	cgaagacguuuguggguga
	gaaauuaagagucaugauc
	caacauugggacguuuuuu
	guugggaacugcugcauuu
SMURF1	aagaauacguccgguugua
	gaucgacauuccaccuuu
	ggaggagaccugcggguuu
	gcacuaugaucuauauguu
NLRX1	gugcuggguuugcgcaaga
	ccacaccagcucagcguga
	ucgucaaccugcugcgcaa
	gcacauuuccgucgggau
PSMA7	caucguggguuucgacuuu
	gaagagacauuguuguucu
	gcacauaccaugccuggaa
	gaaagaacagugcggaaga

2.13 Promoter activity reporter assay in BEAS-2Bs

Promoter activity in BEAS-2B cell cultures was assessed using a Dual-Luciferase kit (Promega) as per the manufacturers description, detailed below.

2.13.1 Transfection of BEAS-2Bs with reporter constructs

Transfections were performed on 12 or 48 well culture plates (Nunc). Stock plasmids were at 1µg/mL, and stored at -80° C. For 12 well plates, an initial master mix of reporter plasmid (IFNβ-Luc or IFNλ1-Luc grown in house) was diluted in serum-free RPMI (0.8µg/well) with an internal control vector (Renilla- 0.2µg/well) making a total of 1µg/well DNA in 75µL/well, this was vortexed thoroughly and incubated at room temperature for 2 minutes. 3µL/well of Superfect (Qiagen-UK) was added to the master mix, the mix was vortexed thoroughly and incubated at room temperature for 15 minutes to allow complexes to form. This master mix was diluted 1:5 with serum-free RPMI. Cell cultures were washed 1X with D-PBS and 475µL/well of diluted master mix was added to each well. Cultures were incubated for 3 hours at 37°C (5% CO₂) after which transfection medium was removed and the cells were washed 3X with RPMI containing 2% FBS 2% HEPES and 1% NaHCO₃. For transfection on BEAS-2B cells in a 48-well format, these values were divided by 4 (but using an identical protocol as above).

2.13.2 Dual luciferase detection of reporter activity

Relative Light Units (RLU) were determined using a Promega (USA) Dual Luciferase kit according to the manufacturers instructions and a FLUOstar Omega (BMG Labtech) fluorescence detector. Relative promoter activity was calculated by dividing the RLU of the reporter plasmid (IFNβ-Luc or IFNλ-Luc) over the RLU of the internal control vector (Renilla). To assess any augmentation of promoter activity, relative promoter activity was then expressed as a percentage of RV-induced promoter activity (RV induction being 100%).

2.14 Bacterial culture and macrolide anti-bacterial assay

E. coli (one-shot, competent cells, Invitrogen) were grown in liquid culture in the presence or absence of macrolide compounds, details of the specific growth medium are listed in Table 2.5. Initially bacteria from frozen glycerol stocks (Invitrogen) was plated out using aseptic techniques onto agar plates and incubated at 37°C for 24 hours. An individual colony was picked and grown in liquid broth over night at 37°C with agitation to allow the bacteria to reach log-phase growth, from this starter colony a 1:100 dilution was made into fresh medium containing macrolides and incubated for up to 12 hours at 37°C with agitation. Sequential sampling of cultures was performed at specific time points and analysed by spectrometry to give an optical density reading at 600nm (OD₆₀₀).

Table 2.5 Bacterial culture medium

Bacterial culture medium		
Name	Composition	Supplier
Luria-Bertani (LB) broth	10g tryptone, 5g yeast extract, 5g NaCl in 1L ddH ₂ O adjusted to pH 7.0	Sigma Aldrich USA
LB agar	14g agarose in 1L LB broth	Sigma Aldrich USA

2.15 Quantitative PCR

2.15.1 RNA sample collection and isolation

Supernatants from cell cultures were removed and stored at -80°C. 350µL RLT (Qiagen) buffer was added to each culture well. Cells were scraped thoroughly with a pipette tip to ensure complete disruption of the monolayer, transferred to eppendorf tubes and stored at -80°C. All RNA extractions were performed using RNeasy (Qiagen) kits according to the manufacturers instructions. RLT samples were thawed at room temperature and 350µL 70% EtOH added to each sample and mixed thoroughly. Samples were transferred to RNeasy spin columns and centrifuged at 8,000xg for 1 min. Flow-through was discarded and 350µL of RW1 buffer was added to each column and columns were centrifuged at 8,000xg for 1 min. Flow-through was discarded and 70µL of DNase solution (Qiagen) was added to the membrane of each column and incubated at room temperature for 15minutes. 350µL of RW1 buffer was added to each column and columns were centrifuged at 8,000xg for 1 min. Flow-through was discarded and the columns were transferred to fresh collection tubes, 500µL of

RPE buffer was added to each column and columns were centrifuged at 8,000xg for 1 min, flow-through was discarded and this step was repeated, centrifuging 8,000xg for 2 minutes. After the second spin with RPE buffer, flow-through was discarded and the columns were centrifuged 'dry' for 1 min to ensure complete removal of buffer. Columns were transferred to fresh collection tubes and RNA was eluted by adding either 30 μ L or 42 μ L (for larger cDNA preparations) RNA-free H₂O and centrifuging at 8,000xg for 1 min.

2.15.2 Reverse transcription of RNA to cDNA

RNA was converted to cDNA using an Omniscript RT kit (Qiagen) according to the manufacturers instructions. 14 μ L of RNA was incubated with 1 μ L Omniscript Reverse Transcriptase, 1 μ L Random Hexamers (Promega USA), 2 μ L 10X RT buffer and 2 μ L dNTP mix (5mM each dNTP) in a total reaction volume of 20 μ L. For larger cDNA preparations, 42 μ L of RNA was used and each reagent was added in triple volume, giving a total reaction volume of 60 μ L per sample. Each mixture was incubated at 37°C for 60 minutes and frozen at -80°C until used.

2.15.3 Design of PCR primers and probes

Real-time PCR primers and probes specific for gene of interest (including 18S rRNA reference gene-Table 2.6) were designed using PrimerExpress (Life Technologies- USA) software coupled with either NCBI PubMed or Ensembl.org genome sequences and verified by PrimerBlast (NCBI). Where possible primers were designed to bind (and amplicons to extend over) exon-exon boundaries of cDNA sequences (as not to amplify genomic DNA). Optimum concentrations of forward and reverse primers were assessed prior to experiments and No Template Controls (NTCs) were used to assess primer-dimer formation. Probes used the combination of a 5' FAM fluorophore and a 3' TAM quencher.

Table 2.6 Primer and probe sequences

Primer and probe sequences	
Primers	Sequence (5'-3')
18S rRNA Forward (300nM)	cgccgctagaggtgaaattct
18S rRNA Probe (100nM)	accggcgcaagacggaccaga
18S rRNA Reverse (300nM)	cattcttggcaaattgcttctg
hIFN β Forward (300nM)	cgccgcattgaccatcta
hIFN β Probe (100nM)	tcagacaagattcatctagcactggctgga
hIFN β Reverse (900nM)	ttagccaggaggttctcaacaatagtgtca
hIL-28 Forward (300nM)	ctgccacatagcccagttca
hIL-28 Probe (100nM)	tctccacaggagctgcaggccttta
hIL-28 Reverse (900nM)	agaagcgactcttctaaggcatctt
hIL-29 Forward (900nM)	ggacgccttgaagagtcact
hIL-29 Probe (100nM)	agttgcagctctcctgtcttccc
hIL-29 Reverse (300nM)	agaagcctcaggtcccaattc
hIL-6 Forward (300nM)	cccagggagaaggcaactg
hIL-6 Probe (100nM)	ccttctccacaagcgccttcggt
hIL-6 Reverse (900nM)	ccttctccacaagcgccttcggt
hIL-8 Forward (300nM)	ctggccgtggctctcttg
hIL-8 Probe (100nM)	cagccttcctgatttctgcagctctgtgt
hIL-8 Reverse (900nM)	ccttggcaaaactgcacctt
hITCH Forward (900nM)	atgatgatggctccagatcca
hITCH Probe (100nM)	atgaaacaagagtgcagacaa
hITCH Reverse (900nM)	catcttcagggtcatctgatcca
hMAVS Forward (300nM)	cccacgggatgggtgtga
hMAVS Probe (100nM)	agtagccacacagcaggtgcgacctc
hMAVS Reverse (900nM)	catgaggagaaggacacagaactg
hMxA Forward (900nM)	cagcacctgatggcctatcac
hMxA Probe (100nM)	aggccagcaagcgcatctccag
hMxA Reverse (900nM)	catgaactggatgatcaaagg
hNDFIP1 Probe (100nM)	aagaagagtctggagaacc
hNDFIP1 Reverse (900nM)	ctgcgctctctgcagaaatg
hNDFIP1 Forward (900nM)	cggtagcagcagttgcagaa
hNLRX1 Probe (100nM)	cagcgcctctgca
hNLRX1 Reverse (900nM)	ggtagccgctgtatagcttcag
hNLRX1 Forward (900nM)	aggcatggacggctgttc
hPCBP1 Reverse (900nM)	tccgcgcgcactct
hPCBP1 Probe (100nM)	tcggttaagaggatccg
hPCBP1 Forward (900nM)	gaagcatcattgggaagaaagg
hPCBP2 Reverse (900nM)	tccatgtcgagcagtggtctg
hPCBP2 Probe (100nM)	accactcaaagtcc
hPCBP2 Forward (900nM)	cccaaccagtgaacaaagactt
hPSMA7 Probe (100nM)	ctcgggcacatacca
hPSMA7 Reverse (900nM)	ctatggcattggccttcca

hPSMA7 Forward (900nM)	gcactcctaggctctatcagactga
hRNF125 Probe (100nM)	ccgttcctgtattgct
hRNF125 Reverse (900nM)	caggtccactgttctttagact
hRNF125 Forward (900nM)	cgctgtggccacgtattct
hSMURF Reverse (900nM)	ctcataggactcatatggtggaatgt
hSMURF Probe (100nM)	cttccgaaggcccatacctgctttaacc
hSMURF Forward (900nM)	ggccccggctgttcac
hViperin Forward (900nM)	cacaaagaagtgtcctgcttgggt
hViperin Probe (100nM)	cctgaatctaaccagaagatgaaagactcc
hViperin Reverse (900nM)	aagcgcatatatttcatccagaataag
mIFN β Forward (300nM)	ccatcatgaacaggtggat
mIFN β Probe (100nM)	ctccacgctgcgttcctgctgtg
mIFN β Reverse (300nM)	gagagggctgtggtggagaa
mIFN λ 2/3 Forward (900nM)	aaaggattgccacattgctc
mIFN λ 2/3 Probe (100nM)	tccccaaaagagctgcaggc
mIFN λ 2/3 Reverse (900nM)	tcaagcagcctcttctcgat
mOAS Forward (900nM)	tcctgggtcatgttaataacttcca
mOAS Probe (100nM)	caagcctgatcccagaatctatgccatc
mOAS Reverse (900nM)	ccccagggagggtacattcct
mPKR Forward (900nM)	cggaacatcctctagcgttgtc
mPKR Probe (100nM)	agctgctggaaaagccactga
mPKR Reverse (900nM)	gggaaacaccattactgtcatagac
mViperin Forward (900nM)	cgaagacatgaatgaacacatcaa
mViperin Probe (100nM)	ccagcgcacagggctcaggg
mViperin Reverse (900nM)	aattaggaggcactggaaaacct
RV Forward (300nM)	gtgaagagccactgtgtgct
RV Probe (100nM)	tgagtcctccggcccctgaatg
RV Reverse (300nM)	gctacaggggttaagggttagcc

2.15.4 Generating standards for Quantitative PCR

Standards were generated cloning a quantitative PCR product (amplicon) into a plasmid vector, then transforming *E. coli* with this vector to produce a high copy number stock. This was performed using protocols detailed below.

2.15.4.1 PCR product purification

Amplicons were purified using a QIAquick (Qiagen) gel extraction kit following the manufacturers instructions. Briefly, amplicons were diluted 1:5 in PB1 buffer and passed through a QIAquick spin column (centrifuged at 8,000xg for 30 seconds), the column was then washed 1X with PE buffer (centrifuged at 8,000xg for 30 seconds) and eluted in 30 μ L EB buffer (centrifuged at 8,000xg for 30 seconds).

2.15.4.2 Ligation of purified amplicon into pCR 2.1-TOPO

Purified amplicons were ligated into pCR 2.1-TOPO (Invitrogen- containing Ampicillin resistance), 4 μ L (approximately 15-100ng of purified amplicon) with 1 μ L salt solution was incubated at room temperature for 15 minutes to allow for ligation. Water was used in the place of amplicon as a negative control.

2.15.4.3 Transformation of *E. coli*

50 μ L OneShot *E. coli* (Invitrogen) was transformed with 5-6 μ L pCR 2.1-TOPO ligated with amplicon (or water control). Transformation was achieved by incubating the *E. coli*-plasmid mixture on ice for 30 minutes after which the mixture was transferred to a 42°C for 45 seconds then placed back on ice for 2 minutes. 450 μ L LB Broth was then added to transformed bacteria and incubated at 37°C for 1 hour with agitation. 100 μ L of the transformation mixture was spread onto LB agar plates containing 100 μ g/mL ampicillin (as a positive selection marker) and incubated at 37°C for 24 hours. *E. coli* transformed with stock (un-ligated) plasmid was used as a negative control.

2.15.4.4 Colony screening and mini-prep analysis of plasmid DNA

Confirmation of plasmid ligation and transformation was assessed by a mini-prep protocol detailed below. Colonies were screened for their ability to grow on agar supplemented with ampicillin (indicating successful transformation of 2.1-TOPO), a single colony was picked and grown in 2mL LB agar (w/ 100 μ g/mL ampicillin) at 37°C (with agitation) for 24 hours. Bacterial cells were centrifuged at 8,000xg for 10 seconds and the resulting pellet was re-suspended in 0.2mL in buffer P1, 0.2mL buffers P2 and P3 were added sequentially and gently mixed before the resulting solution was centrifuged at 8,000xg for 10 minutes. Supernatants were taken and incubated with 1mL 100% Ethanol for 20 minutes at room temperature to precipitate the plasmid DNA into solution, the sample was then centrifuged at 8,000xg for 20 minutes and the resulting pellet was washed X1 by centrifuging in 70% Ethanol. After the wash the pellet was air-dried before being re-suspended in 30 μ L nuclease-free H₂O. Mini-prep generated products were tested for amplification using quantitative PCR primers and probes. Samples that confirmed ligation of pCR 2.1-TOPO with the correct PCR product of interest were processed by Maxi-prep described below.

2.15.4.5 Maxi prep isolation of plasmid DNA and quantification

A colony from an LB plate that was verified as containing ligated vector (from mini-prep) was picked and grown in 2mL LB agar (with 100µg/mL ampicillin) at 37°C (with agitation) for 8 hours, this starter culture was transferred to 100mL of LB broth (containing 100µg/mL ampicillin) and incubated over night at 37°C (with agitation). Plasmid DNA was precipitated with 100% isopropanol (VWR- UK) and centrifuged at 7,000xg for 1 hour, the DNA pellet was washed with 70% Ethanol and centrifuged at 7,000xg for 30 minutes. The pellet was then air-dried and re-suspended in 1300µL TE buffer. Plasmid DNA was quantified using a Nano-Drop (Thermo) photo spectrometer and diluted µL nuclease-free H₂O to the stock concentration of 10¹⁰ copies/µL.

2.15.5 Quantitative PCR assay

Quantitation of mRNA copy numbers was measured by TaqMan PCR (Applied Biosystems 7500 Fast machine (ABI, USA) or LightCycler 480 II (Roche, USA). Experiments were confined to one particular machine as to minimise discrepancies between the two technologies. A 96 or 384-well (stated) reaction plate (Applied Biosystems or Roche respectively) was used, each PCR mixture contained 6.25µL QuantiTect probe PCR Master Mix (Qiagen), sense and anti-sense template primers at an optimised ratio 175nM probe (see Table 2.6) and 1µL unknown or standard DNA. RNase-free H₂O was used in place of template cDNA as a No Template Control (NTC). Unknown samples, standards and NTCs were run in duplicate. The quantitative PCR cycling program was as follows: 50°C for 2 minutes, 94°C for 10 minutes followed by amplification cycles of 94°C for 15 seconds and 60°C for 15 seconds to a total of 45 cycles. An 8-point standard curve was used to determine mRNA copy number in unknown samples. Standards generated in section 2.15.4 were diluted in nuclease-free H₂O to the top standard of 10⁷ copies/µL, sequential 1:10 dilutions were produced (vortexing thoroughly between each standard) down to 10⁰ copies/µL.

2.15.6 Calculation of mRNA copy numbers.

Data was analysed using either ABI Prism 7500 software (ABI) or LightCycler software (Roche). Total mRNA copy numbers were calculated by deriving a cycle threshold (CT) value for each unknown sample (during the log-phase of amplification, averaged from the two replicates) and extrapolating copy numbers via a 4-parameter standard curve generated from the CT values of the standards diluted 10-fold (10⁷, -10¹ copies per µL). These copy

numbers were normalised for RNA quantity by comparing them to CT values of 18S rRNA as a reference gene ($\text{copy number} \times 2^{-(\text{CT}_{18\text{S rRNA}} - \text{CT}_{18\text{S rRNA}_{\min}})}$), mRNA copy numbers were expressed as a percentage of RV or a given stimulus (expressed as 100%).

2.16 ELISA

ELISAs used to quantify proteins from cell culture supernatants or mouse BAL fluid from *in vivo* experiments were performed using paired antibody sets and appropriate recombinant standards (R&D systems USA). 96-well microtitre plates (Nunc) were coated with capture antibody and incubated at room temperature over night. The plate was washed 3X with wash buffer and incubated with blocking solution (typically 1% Bovine Serum Albumin BSA- Sigma Aldrich) for 2 hours at room temperature. Samples, standards or negative controls (BLANK) were added in duplicate (samples were diluted in diluent as appropriate, standard were made by serially diluting a standard stock to the manufacturers instructions) and incubated at room temperature for 2 hours. The plates were then washed 3X with wash buffer and incubated with biotinylated detection antibody (to the manufacturers directions) for 1 hour at room temperature. The plates were washed 3X before addition of Streptavidin-HRP (to the manufacturers directions) for 20 minutes at room temperature after which the plates were washed 3X and TMB substrate was added to each well and the plate was incubated in the dark for up to 20 minutes at room temperature. The reaction was stopped by the addition of 2M H_2SO_4 when colour started to appear in the BLANK wells. Absorbance at 450nm was measured using a SpectraMax plus (Molecular Devices, USA) ELISA plate reader and protein levels were determined by extrapolation of a 4-parameter standard curve generated in SoftMax Pro software (Molecular Devices).

2.17 Meso Scale Discovery

Meso Scale Discovery (MSD-USA) plates (96 well) was used to quantify levels of IFN β and IFN λ protein in supernatants from HBECs cultured *ex vivo* as per the manufacturers instructions. Supernatants were analysed in singlet and were added to each well undiluted and a 7-point standards curve was run in parallel. Data was analysed using MSD discovery workbench software (MSD).

2.18 SDS-PAGE and Western Blotting

2.18.1 Sample processing

Whole cell lysates for Western blot analysis were obtained by first washing cell cultures with PBS and adding 100 μ L/well Tris-Glycine SDS sample buffer (Invitrogen) and thoroughly scraping the cells with an inverted pipette tip. Whole cell lysates were frozen at -80°C until required. Prior to SDS-PAGE separation, samples were sonicated in a water bath for 3 minutes, 1.4M β -mercaptoethanol was added and samples were heated to 95°C for 5 minutes.

2.18.2 SDS-PAGE

The outer chamber of an electrophoresis tank (BioRad, USA) was filled with approximately 800mL of Novex MOPS Running Buffer (1X) (Invitrogen), the inner chamber was filled with 200mL MOPS running buffer containing 500 μ L of NuPAGE anti-oxidant (Invitrogen). 25 μ L of processed whole cell lysates were loaded into pre-cast 8-12% Tris-Glycine (Invitrogen) gels, 15 μ L of pre-stained protein standard (Invitrogen) was loaded onto each gel as a reference. Samples were electrophoresed for 45 minutes at 180 amps.

2.18.3 Protein transfer

Gels were acclimatised at room temperature in Novex Transfer Buffer (Invitrogen) for 15 minutes and placed onto transfer buffer-soaked nitrocellulose membrane (Invitrogen). The gel/membrane was then sandwiched between pre-soaked filter paper followed by pre-soaked foam padding and placed in a transfer cell (BioRad) and run at 180 amps for 2 hours with constant agitation and cooling. The membranes were then washed for 15 minutes in PBS-T.

2.18.4 Western Blotting

Membranes were blocked in 5% w/v skimmed milk powder (Marvel) in PBS-T (blocking solution) for 2 hours at room temperature with constant agitation. The blocking solution was removed and replaced with fresh blocking solution containing primary antibody (see Table 2.7 for Abs and dilutions), membranes were incubated at 4°C overnight with agitation. Primary Ab solution was removed and the membranes were washed three times in PBS-T

before being incubated with Secondary Ab in fresh blocking solution for 1 hour at room temperature with agitation. Secondary Ab solution was removed and the membranes were washed three times in PBS-T. Protein bands were detected using ECL substrate (Thermo) with X-ray film (GE healthcare UK) and an X-ray developer.

Table 2.7 Antibodies for Western blotting

Antibodies for Western Blot			
Name	Isotype	Working concentration	Supplier
MxA	Rabbit IgG	200ng/mL	Santa Cruz-USA
RSAD2 (Viperin)	Rabbit IgG	600ng/mL	Proteintech Europe- UK
MAVS	Rabbit IgG	1000ng/mL	AbCam- UK
β Actin	Rabbit IgG	200ng/mL	Cambridge Bioscience- UK
Anti-rabbit secondary	Sheep IgG-HRP	20 μ g/mL	AbD serotech-UK

2.18.5 Membrane stripping

Nitrocellulose membranes were washed three times in PBS-T prior to incubation with 2%w/v SDS, 62.5mM Tris-HCL, 100mM β -mercaptoethanol (stripping buffer) for 30 minutes at 55°C with agitation. Membranes were washed three times in PBS-T before being re-blotted (section 2.18.4)

2.18.6 Densitometry analysis of protein levels

Semi-quantitative analysis of Western blots was performed by measuring the relative density of bands using a Chemi-Lab bio imaging system software (UVP, USA). Bands were normalized to β -actin levels and data was expressed as 'relative density'.

2.19 Proteomic screening

Whole protein lysates from normal HBECs treated with 50 μ M Azithromycin or equivalent 70% EtOH vehicle were assessed for changes in protein production as detailed below.

2.19.1 Sample preparation

Normal HBECs were grown to 90% confluency in 21.5cm² culture dishes (Nunc) in complete BEGM medium (Lonza). Cultures were treated with BEBM (Lonza) supplemented with 50µM Azithromycin or Vehicle for 4 or 24 hours at 37°C 5% CO₂. After this incubation culture dishes were placed on ice for 10 minutes and washed 1X with ice cold PBS, 8mL/ dish of PBS supplemented with DTT, EDTA and protease inhibitor cocktail (Roche) was then added to the cultures and the cells were scraped using a cell scraper. Cell cultures were placed into pre-cooled falcon tubes and centrifuged at 300xg for 6 minutes (at 4°C), the supernatant was carefully removed and resulting cell pellet was snap-frozen in liquid N₂.

2.19.2 Mass spectrometry

This stage of the experiment was performed by M. Khaitov and S. A. Moshkovskii (Institute of Biomedical Chemistry, Moscow). Samples were trypsonised and assessed using liquid chromatography mass spectrometry with an LTQ-OrbiTrap mass spectrometer (Thermo). Samples were analysed using MaxQuant and Persues software (Max Plank Institute, Germany).

2.20 Identification of Azithromycin binding partners by mass spectrometry

A pull-down assay was performed to identify any potential Azithromycin-protein binding partners. Briefly, a Azithromycin-Azide probe was bound to agarose-Alkyne beads and incubated in the presence of total protein lysates from normal HBECs. Potential Azithromycin binding proteins were then separated from whole cell lysates via centrifugation.

2.20.1 Design and validation of Azithromycin-Azide probe

An Azithromycin molecule containing a free Azide residue was synthesised by Lyn Jones and Lee Roberts at Pfizer Inc. The probe differed from Azithromycin by replacing the CH₃ group on the N residue of the lactone ring with the Azide-containing “arm” C₇H₁₃N₃O₂ (Figure 2.1). To ensure that the Azide probe’s “arm” didn’t affect the augmenting properties of this molecule, the probe was assessed for its ability to augment IFN and ISG expression in HBECs infected with RV (at 50µM, as per the Azithromycin model).

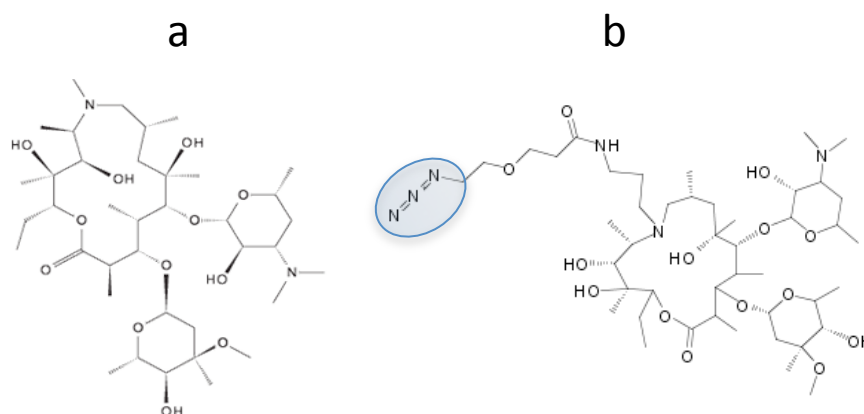


Figure 2.1 Azithromycin-Azide probe

Comparison of azithromycin (a) with the Azithromycin-Azide probe (b) with the azide residue highlighted.

2.20.2 Bead preparation

Alkyne-agarose beads were prepared using Click-iT[®] Protein Enrichment Kit (Molecular Probes, USA) and bound to AZI-Azide probe according to the manufactures instructions.

2.20.3 Sample preparation

Whole protein lysates from HBECs were generated by growing cultures in dishes (Nunc) until 90% confluent, medium was then removed and the cultures were then placed on ice and washed 3X with ice-cold lysis buffer (D-PBS containing DTT (1mM) and protease inhibitor cocktail (1:1000 dilution, Active Motif-USA). Cells were scraped from the dishes using a cell scraper in 5mL lysis buffer and centrifuged at 300xg for 6 minutes at 4°C. Supernatants were discarded and cells were re-suspended in 2mL of ice-cold lysis buffer and placed in a sonication bath for 10 minutes. To ensure complete lysis, cells were passed several times through a .45mm needle on ice. A Bradford assay assessed the total protein content within the samples and samples were diluted to 1mg/mL and snap frozen in liquid N₂.

2.20.4 Competition pull-down assay with alkyne beads

This stage of the experiment was performed by K. A. Kalesh (Dept. of Chemistry, Imperial College London). AZI-Azide-Alkyne-Agarose mixture was washed three times in ice-cold PBS before being incubated with the HBEC protein lysates (0.5mg/mL diluted in PBS with 2.5% Ethanol) for 1 hour at room temperature. For competition, lysates were incubated with a 5 molar excess of Azithromycin for 30 mins at room temperature. Samples were centrifuged at 1000 x g for 5 mins at 4°C and the supernatants were discarded. The beads were washed four times by centrifugation with PBS after which the beads were suspended in 50mM (filtered) ammonium bicarbonate and treated with trypsin at 37°C over night to dissociate the proteins from the beads. The samples were vortexed thoroughly and centrifuged at 5000 x g and the supernatant (peptide mixture) was retained, the pelleted beads were washed in deionized H₂O with 0.1% acetic acid and re-spun to acquire the all of the peptide mixture (that was added together).

The samples were subjected to “stage tipping” prior to analysis by mass spectroscopy. Briefly, samples were passed through a pre-washed C18 membrane (Sigma) by centrifugation (2000 x g for 5 mins) to isolate peptides, membranes were flushed with deionized H₂O (again by centrifugation) to remove salts from the peptides. Peptides were eluted from the membrane by adding 79% acetonitrile (Sigma) and centrifuging (2000 x g for 5 mins). The peptide mixture was then dried using a speed vacuum before being analysed by MS.

2.20.5 Mass spectrometry

This stage of the experiment was performed by K. A. Kalesh. Samples were analysed in an LTQ-Orbitrap-Velos-ETD (Thermo) FT mass spectrometer at the Imperial College Mass Spectrometry Facility (Imperial College London). Readings were analysed using MaxQuant and Persues software.

2.2 | *In vivo* methods

2.21.1 Mice

7 week old, female BALB/c were obtained from Charles River UK and were housed in specific pathogen free conditions in the Central Biomedical Services facility at Imperial

College London St. Mary's Campus. Mice were given free access to food and water prior to and during all experiments.

2.2.1.2 Preparation of Azithromycin for oral administration

AZI or equivalent volumes of EtOH vehicle were suspended in Carboxy-Methyl Cellulose (CMC) gavage fluid and sonicated in a water bath for 20 minutes. This suspension was vortexed vigorously prior to administration to ensure an even suspension was administered to each animal.

2.2.1.3 Dosing of Azithromycin in a mouse model of RV infection

Mice were gavaged orally with 200µL per mouse with either 50mg/kg or 25mg/kg of body weight of AZI or VEH or PBS in CMC gavage fluid twice 24 hours apart. 24 hours following the final dosing, mice were infected intranasally (i.n) with 50µL of RV1b (5×10^6 TCID₅₀) or mock (PBS) control. Mice were euthanized 8 and 24 hours post viral inoculation by intraperitoneal injection of 200µL of Sodium Pentobarbital (AnimalCare Ltd, UK), as shown in Figure 2.2.

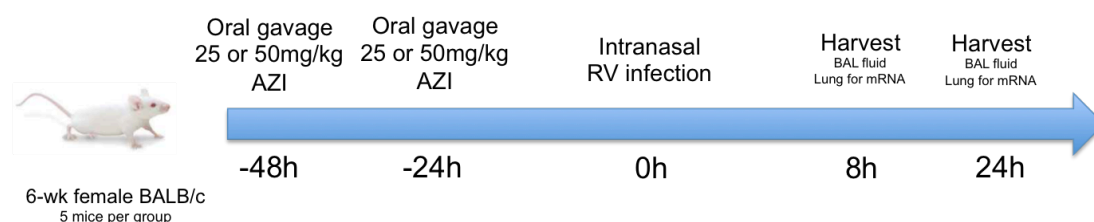


Figure 2.2 Azithromycin in a mouse model of RV infection; experimental design.

2.2.1.4 Sample harvesting

Bronchoalviolar lavage (BAL) was performed on euthanized mice by the administration (and subsequent recovery) of 1.5mL of BAL fluid (PBS containing 0.5M EDTA and 12mM Lidocaine hydrochloride monohydrate, Sigma) intratracheally into the lungs. BAL fluid was centrifuged at 300xg for 1 minute to pellet BAL cells, the supernatant was then frozen in LN for protein analysis by ELISA, as in section 2.16. The BAL cell pellet was resuspended in ACK buffer for one minute to lyse any erythrocytes and the ACK was neutralised by addition of RPMI supplemented with 10% FCS. BAL cells were then pelleted by centrifugation and

resuspended in 1 mL of RPMI for cytopsin analysis. The right apical lung lobe was removed (post-BAL), rinsed in PBS and frozen in 600 μ L of RNAlater (Qiagen) for qPCR analysis, as in section 2.15.

2.21.5 Cytopsin and Differential BAL cell counts

100 μ L of BAL cell suspension was centrifuged using Shandon cytoslides (Shandon USA). Slides were left to air dry before being fixed in 90% methanol. Slides were then stained with Diff-quick (Thermo USA) xanthene and thiazine and differential cell counts were performed under light microscopy counting neutrophils, macrophages and lymphocytes. A total of 300 cells per slide were counted to determine differential cell counts.

2.22 Statistical analysis

For data pertaining to gene or protein expression by cultured normal HBECs the mean and SEM (standard error of the mean) was displayed. For data pertaining to gene or protein expression from HBECs cultured *ex vivo* the median values were given. Gene and protein expression data was normalised to percentage over induction by positive control (where the positive control was expressed as 100%). As the data was not normally distributed, a One-way Kruskal-Wallis non-parametric analysis, with Dunn's comparison was used to assess significance between groups. Data from time course experiments was analysed by two-way Analysis of variance (ANOVA) with Bonferroni's post-test. Data from dose response assays was analysed using non-linear regression on a sigmoidal dose response to generate EC₅₀ and R² values. Two-tailed Spearman correlation analysis was employed where stated. For bacterial growth curves, area under the curve (AUC) was calculated and analysed by ANOVA with Bonferroni's post-test. All statistical analysis employed a confidence interval of 95%. p values of <0.05 were considered statistically significant.

Chapter 3 Azithromycin augments IFN responses in asthmatic and non-asthmatic cultured bronchial epithelial cells.

3.1 Introduction

Numerous studies have demonstrated the high association between asthma exacerbations and RV infection [25, 59, 228]. Whilst the common treatment regimen of inhaled corticosteroids and β_2 -adrenergic receptor agonists aims to address the pro-inflammatory aspects of the disease, the current treatment guidelines for asthma do not include any anti-viral agents. Yearly analysis of asthma exacerbations demonstrated a correlation between asthma exacerbations and the peak incidences of RV infections [26]. Furthermore, human experimental RV challenge studies have shown that changes in airway hyper-responsiveness and lower airway symptom score (post challenge) are related to increased virus load [58]. It would therefore seem pertinent that effective anti-viral agents against RV be included in the treatment regimen for asthma.

While there is a high degree of association between asthma exacerbations and RV infection, crucially it has also been shown that although asthmatics and healthy individuals are infected with RV at the same rate, asthmatics respond with a greater severity of disease and increased lower respiratory tract symptoms [59]. This is suggestive that asthmatics may have an impaired immune response to RV infection compared to non-asthmatics. This proposed impairment in anti-viral immunity in the asthmatic airway has been the subject of a number of studies, Wark *et al* and Contoli *et al* initially described deficiencies in type I and type III IFN production by RV-infected HBECs cultured from asthmatics compared to non-asthmatics [84, 85]. Sykes *et al* demonstrated BAL cells (but not PBMCs) from atopic asthmatic adults were deficient in IFN α and IFN β expression when infected *ex vivo* with RV [46]. Baraldo *et al* in 2012 and Edwards *et al* in 2013 published similar observations of reduced IFN β and IFN λ expression in RV-infected HBECs from asthmatic children and severe therapy-resistant asthmatic (STRA) children (respectively) compared to non-asthmatic controls. Interestingly, this deficiency was

observed when cells were stimulated with the TLR3 agonist Poly(I:C) in the absence of infection indicating that it is not a RV-specific deficiency [86, 87].

Macrolides are commonly prescribed for the treatment of chronic airway inflammatory diseases such as COPD, CF and panbronchiolitis, these diseases are often associated with pathogenic bacterial infection justifying the use of anti-microbial macrolides. Many macrolides are known to have immunomodulatory effects in addition to their well-established anti-bacterial efficacy [196, 197] and clinical studies of macrolides in asthma have demonstrated partial efficacy. Telithromycin has been shown to improve FEV₁ and decrease asthma symptoms in asthmatics when administered at exacerbation however it had no significant effect on peak expiratory flow [190]. Recently, Brusselle *et al* demonstrated that Azithromycin was effective in reducing asthma exacerbations in a subset of patients with severe non-eosinophilic asthma [182]. Clarithromycin was also shown to reduce levels of CXCL8 secretion in sputum of patients with non-eosinophilic severe refractory asthma [187]. The studies of macrolides in asthma to date are unable to differentiate between the effects caused by anti-bacterial activity or immunomodulatory activity of the macrolides. Clarithromycin, Telithromycin and Azithromycin are likely to alter the bacterial flora of the airway, the effects of which in the context of asthma pathogenesis are as yet unknown.

Macrolides as anti-viral agents is an emerging field, a number of studies have demonstrated possible anti-viral activity *in vitro*. In 2008, Miyamoto *et al* demonstrated that Clarithromycin is able to partially inhibit the replication of influenza virus *in vitro* [207]. Gielen *et al* reported that Azithromycin is able to augment IFN and ISG expression in primary HBECs from healthy donors infected with RV16 or RV1b *in vitro*, this augmentation was accompanied with a decrease in viral replication [142]. It has also been shown that Clarithromycin is able to suppress the expression of RSV-induced Platelet Activating Factor (PAF) [229]. It is worth noting that although the multiple clinical studies of macrolides in asthma did not address the effects on RV infection, the anti-viral activity of macrolides may have contributed to the effects observed. Further investigation into the antiviral effects of macrolides is warranted if they are to be considered as a therapeutic option for asthma. Importantly, whether or not macrolides such as azithromycin can restore or augment deficient IFN levels in cells from asthmatic patients has not yet been evaluated.

We used a similar experimental model to that used by Gielen *et al* to examine the effects of Azithromycin on augmenting anti-viral effects in RV-infected HBECs from asthmatics.

Initially, we confirmed that pre-treating commercially available (normal) HBECs with Azithromycin prior to infection with RV1b could augment gene expression of IFN β , IL-28, IL-29, MxA and Viperin whilst decreasing RV replication. We then applied this model to HBECs cultured from bronchial brushings of non-asthmatic adults, mild/moderate atopic asthmatic adults and STRA children, we showed that Azithromycin was able to augment IFN and ISG expression to RV infection and decrease viral replication in HBECs cultured *ex vivo* regardless of clinical status. Consistent with IFN/ISG gene augmentation, asthmatic cells treated with Azithromycin showed decreased RV replication.

3.2 Hypothesis

- Azithromycin is able to augment antiviral responses to RV infection in HBECs from both asthmatics and non-asthmatics.

3.3 Aims

- To confirm the effects first observed by Gielen *et al* showing Azithromycin augmenting IFN responses to RV infection in commercially available 'normal' HBECs.
- To replicate these effects in HBEC cultures obtained from bronchial brushings from both atopic asthmatic adults, severe therapy resistant asthmatic children and non-atopic non-asthmatic adults, demonstrating augmented IFN and ISG expression and decreased viral replication.
- To determine if any clinical observations correlate with the effectiveness of Azithromycin in augmenting the anti-viral response.

3.4 Results

3.4.1. Azithromycin augmented anti-viral responses but not pro-inflammatory responses to RV infection in normal HBECs

Gielen *et al* first demonstrated the effects of Azithromycin augmenting RV-induced IFN and ISG expression in normal HBECs *in vitro* [142], here we sought to confirm these effects and assess the dose dependent nature of Azithromycin augmenting IFN and ISG expression. Normal HBECs (procured from Lonza Inc.) were treated with 50 μ M Azithromycin (AZI) for 24 hours prior to infection with RV1b (MOI:1) for 24 hours. AZI treatment augmented IFN and ISG mRNA expression to similar levels observed by Gielen *et al* (Figure 3.1 IFN β : 471.84%, IL-28: 653.73%, IL-29: 543.27%, MxA: 220.14%, Viperin: 421.01% increase over vehicle 100%. AZI treatment in the absence of infection failed to augment levels of IFN β , IL-28, IL-29, MxA or Viperin. n=8). AZI did not augment RV1b-induced IL-6 or IL-8 mRNA expression (Figure 3.2 IL-6: 126.72%, IL-8: 87.93% increase over vehicle, 100%. n=8). AZI also reduced RV1b replication in normal HBECs (Figure 3.3 50 μ M AZI: 49.99% TCID₅₀ compared to vehicle treatment 100%. n=4).

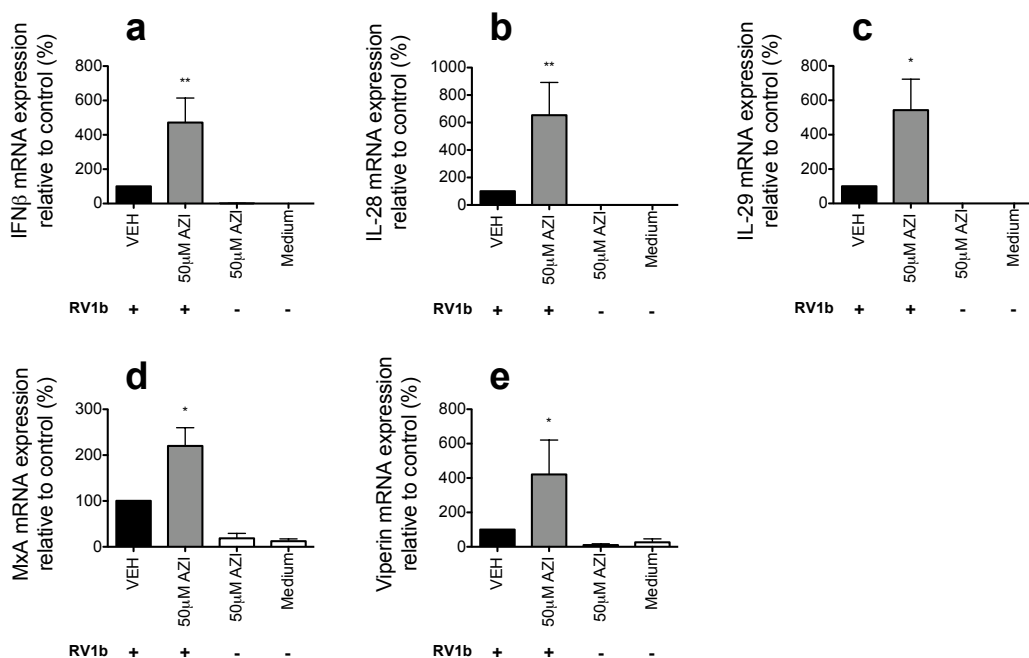


Figure 3.1 AZI augments IFN and ISG mRNA expression in normal HBECs.

Normal HBECs treated with 50 μ M Azithromycin (AZI) augmented RV1b-induced (a) IFN β (b) IL-28, (c) IL-29, (d) MxA and (e) Viperin mRNA expression. Data expressed as % augmentation compared to RV1b + Vehicle (VEH) 100%. * p <0.05, ** p <0.01. n=8.

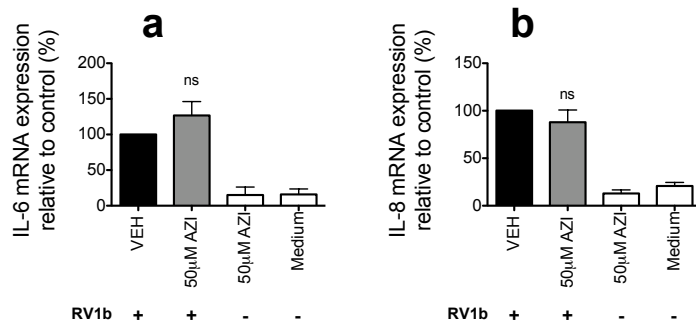


Figure 3.2 AZI did not augment RV1b-induced IL-6 mRNA or IL-8 mRNA expression in normal HBECs.

Normal HBECs treated with 50μM AZI did not augment RV1b-induced (a) IL-6 or (b) IL-8 mRNA expression. Data expressed as % augmentation compared to RV1b + Vehicle (VEH) 100%. n=8.

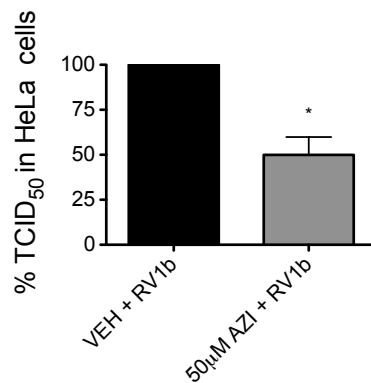


Figure 3.3 AZI reduced RV1b replication in normal HBECs.

Supernatants from normal HBECs treated with 50μM AZI showed decreased RV1b TCID₅₀ when titrated onto HeLa cells. Data expressed as % TCID₅₀ compared to vehicle (100% black bar). *p<0.05. n=4.

3.4.2 Azithromycin augmented IFN responses to RV in a dose dependent manner in normal HBECs

To assess whether the effects of AZI were dose dependent, normal HBECs were treated with serial dilutions of AZI (1:2 50 μ M-0.78 μ M) for 24 hours prior to infection with RV1b (MOI:1) for 24 hours, Figure 3.4 IFN β , IL-28, IL-29 MxA and Viperin showed a dose dependent augmentation of RV1b-induced mRNA expression when used in doses ranging from 6.25 μ M-50 μ M. All genes displayed a biphasic peak at 3.12 μ M. Viperin also showed a dose dependent augmentation with an EC₅₀ value of 35.48 μ M. Data expressed as % augmentation over RV1b + Vehicle (100%). Between 25 μ M and 12.5 μ M AZI showed a dose dependent suppression of RV1b-induced IL-6 and IL-8 however this was not observed at other doses (Figure 3.5 Data expressed as % suppression over RV1b + Vehicle 100%).

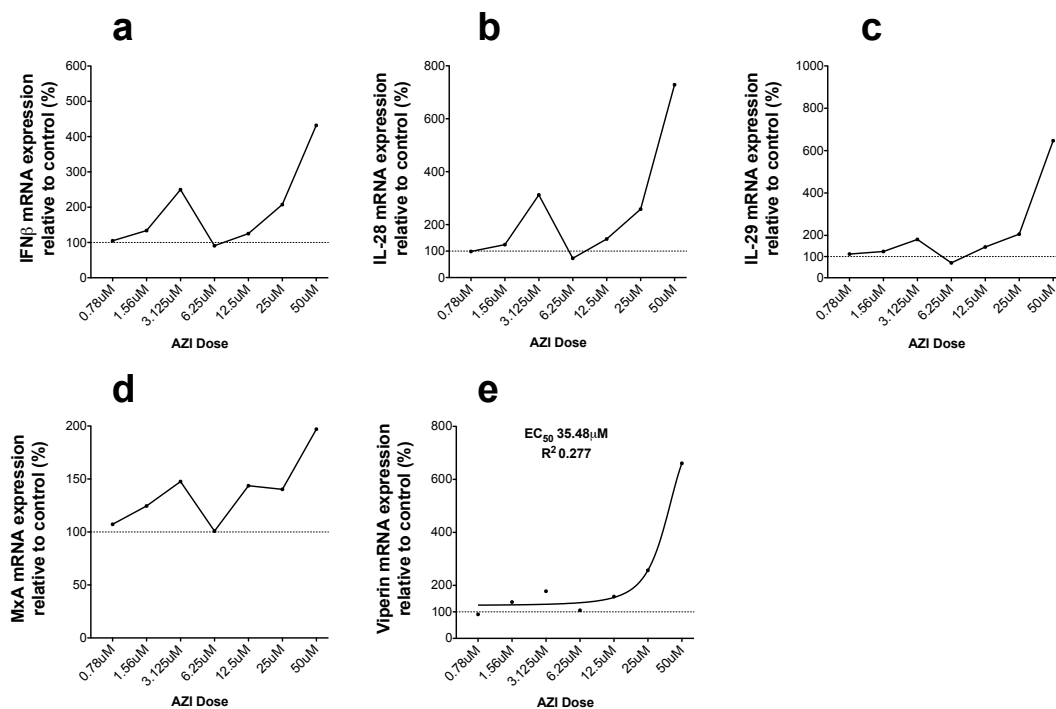


Figure 3.4 7-point dose response of AZI augmenting IFN and ISG mRNA expression in RV1b-infected normal HBECs.

Normal HBECs were treated with a 1:2 serial dilution of AZI (50 μ M-0.78 μ M) for 24 hours prior to infection with RV1b. Expression of IFN β (a), IL-28 (b), IL-29 (c), MxA (d) and Viperin (e) mRNA showed a dose dependent augmentation with AZI treatment. Data expressed as % change over RV1b + vehicle dashed line. n=3)

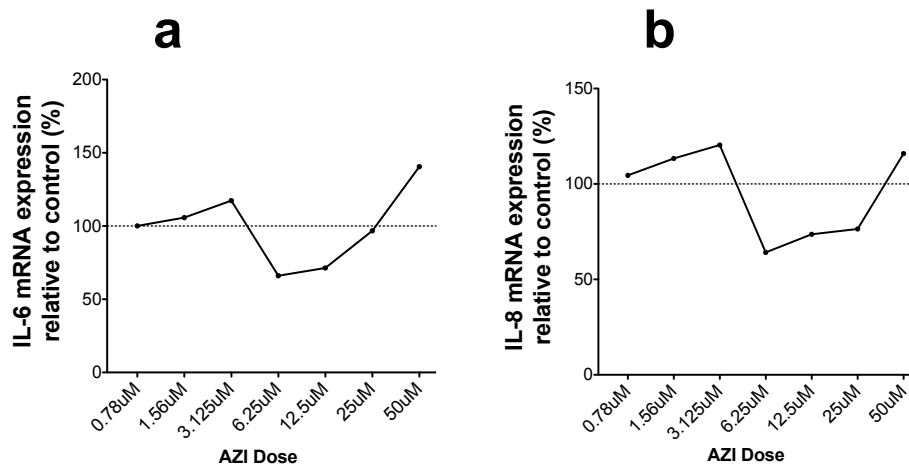


Figure 3.5 7-point dose response of AZI suppressing IL-6 and IL-8 mRNA expression in RV1b-infected normal HBECs.

Normal HBECs were treated with a 1:2 serial dilution of AZI (50μM-0.78μM) for 24 hours prior to infection with RV1b. Expression IL-6 (a) and IL-8 (b) showed a dose depended suppression with AZI treatment. Data expressed as % change over RV1b + vehicle dashed line. n=3)

3.4.3 Azithromycin augmented IFN responses but not pro-inflammatory responses in asthmatic and non-asthmatic HBECs

To determine the effects Azithromycin on epithelial cells from asthmatics, HBECs cultured from bronchial brushings of mild/moderate atopic asthmatics (AA) adults, severe therapy-resistant asthmatic (STRA) children and also non-atopic non-asthmatic adults (NANA) were grown *ex vivo* (see Table 3.1). Cultures were treated for 24 hours with 10μM or 50μM AZI or VEH prior to infection with RV16 or RV1b (MOI:1) for 24 or 48 hours. Expression levels of type I and type III IFNs and ISG mRNA and IFNβ and IFNλ protein were examined in addition to replication levels of RV16 and RV1b infection. In all instances any HBEC cultures that did not induce detectable anti-viral genes or secreted protein when infected with RV were not included.

Table 3.1 HBEC donor characteristics.

Adults		
Parameter	AA	NANA
n	13	6
% male sex	53.84	33.33
Average age (years)	28.53	36.33
Physician diagnosis of asthma	Yes	No
% predicted FEV ₁	90.23	n/a
Average ACQ	1.76	n/a
PC ₂₀ (histamine)	1.75	n/a
Serum IgE (IU/mL)	197.66	n/a
Number of +ve SPT	3	0
Children		
Parameter	STRA	NANA
n	4	1
% male sex	75	0
Average age (years)	11	14
Physician diagnosis of asthma	Yes	No
% predicted FEV ₁	75.5	n/a
Average ACT	11.25/25	n/a
Serum IgE (IU/mL)	337.25	n/a
Number of +ve RAST	4.25	n/a

n/a= Not assessed

3.4.4 Azithromycin augmented anti-viral gene mRNA expression in RV16-infected HBECs from asthmatic adults

Azithromycin pre-treatment of HBECs from AA (both 10 μ M and 50 μ M doses) significantly augmented levels of RV16-induced MxA mRNA, trends for augmentation of RV16-induced IFN β , IL-28 and Viperin mRNA were also observed (Figure 3.6 median levels of 10 μ M/ 50 μ M AZI IFN β : 286.85/ 317.87%, IL-28: 150.87/ 97.06%, IL-29: 86.72/ 87.14%, MxA: 189.15/ 212.62%, Viperin: 167.82/ 174.93% compared to vehicle treatment 100% n=8-10).

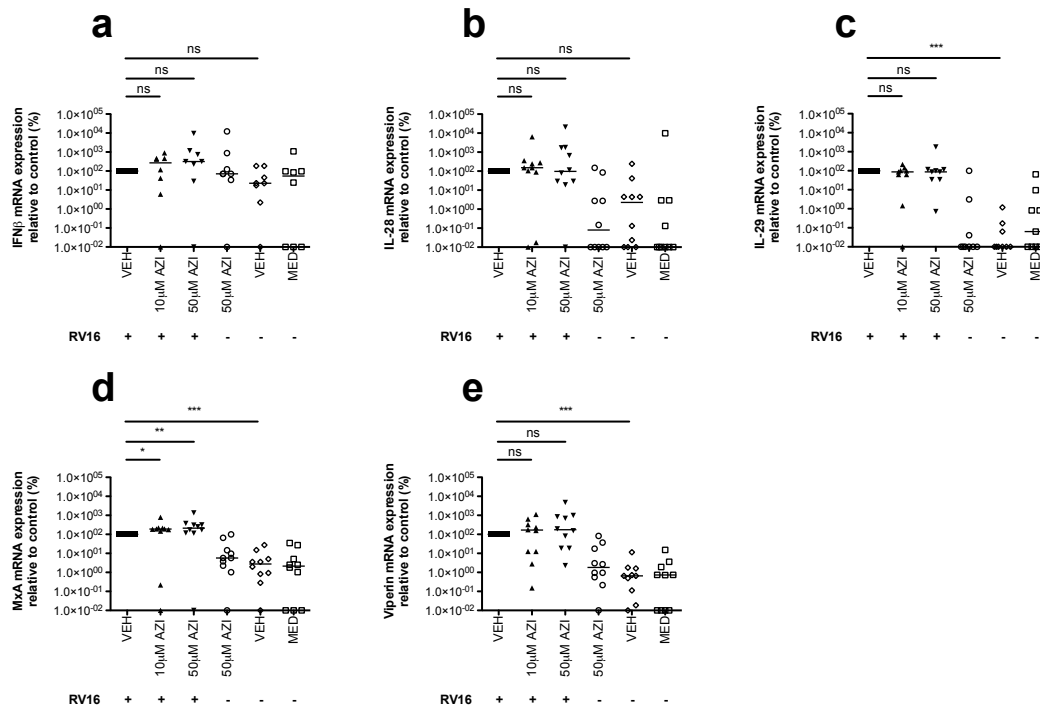


Figure 3.6 Azithromycin augmented ISG mRNA expression in RV16-infected HBECs from AA.

Azithromycin (AZI) treatment showed trends for the augmentation of RV16-induced (a) IFN β , (b) IL-28 and (e) Viperin mRNA. AZI significantly augmented levels of RV16-induced (d) MxA mRNA in HBECs cultured *ex vivo* from mild/moderate asthmatic adults. no effect was observed with IL-29 expression. Data expressed as % of vehicle treated cells (VEH 100%) * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. $n = 8-10$.

3.4.5 Azithromycin augmented anti-viral gene mRNA expression in RV1b-infected HBECs from asthmatic adults

Azithromycin pre-treatment of HBECs from asthmatic adults (with the 50 μ M dose) showed augmentation of RV1b-induced MxA mRNA expression and trends for augmentation of RV1b-induced IFN β , IL-28, IL-29 and Viperin mRNA (Figure 3.7 median levels of 10 μ M/ 50 μ M AZI IFN β : 94.84/ 157.17%, IL-28: 135.91/ 136.55%, IL-29: 107.06/ 159.16%, MxA: 153.25/ 175.51%, Viperin: 179.40/ 141.90% compared to vehicle treatment 100% $n = 8-10$).

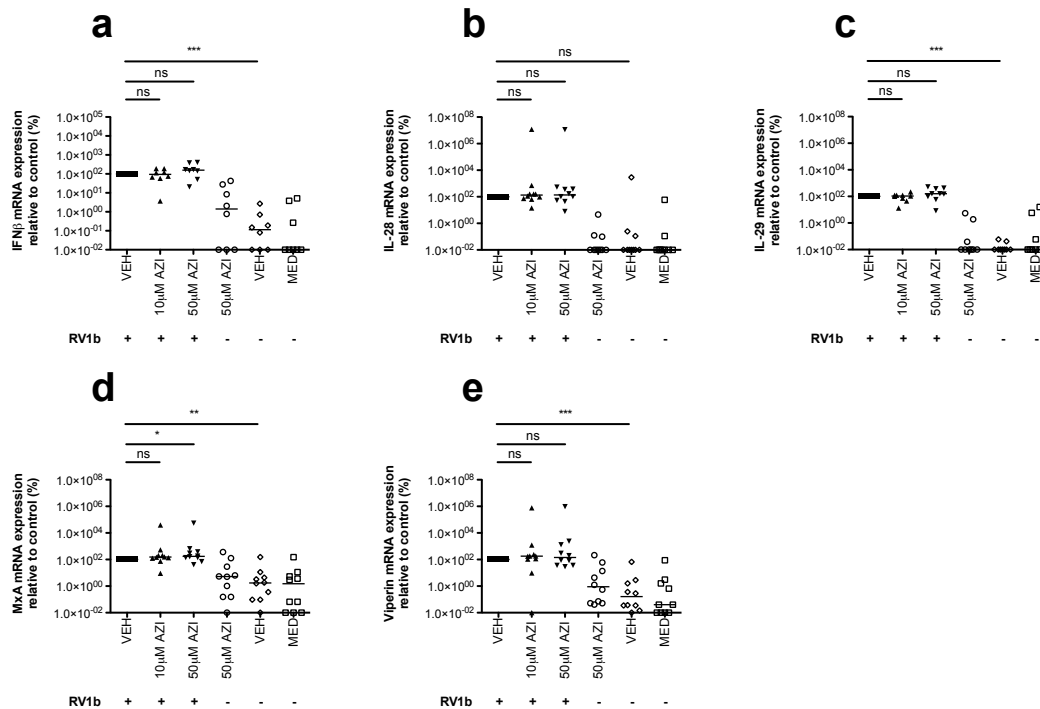


Figure 3.7 Azithromycin augmented ISG mRNA expression in RV1b-infected HBECs from AA.

Azithromycin (AZI) augmented levels of RV1b-induced MxA mRNA (d). AZI treatment showed trends for the augmentation of RV1b-induced (a) IFNβ, (b) IL-28, (c) IL-29 and (e) Viperin mRNA in HBECs cultured *ex vivo* from mild/moderate asthmatic adults. Data expressed as % of vehicle treated cells (VEH 100%) *p<0.05. **p<0.01. ***p<0.001. n=8-10.

3.4.6 Azithromycin showed a trend for augmenting levels of RV-induced IFN protein expression in HBECs cultured *ex vivo* from asthmatic adults

Azithromycin pre-treatment of HBECs from mild/moderate asthmatic adults showed a non-significant increase of secreted IFNλ protein expression in cultures infected with RV16 and a decrease in the median production of IFNλ protein expression in cultures infected with RV1b (Figure 3.8 median levels of 10μM/ 50μM AZI (RV16) IFNλ: 106.07/ 141.78%, (RV1b) IFNλ: 56.75/ 80.54%, compared to vehicle treatment 100% n=5-6). No IFNβ protein was detectable at this time point.

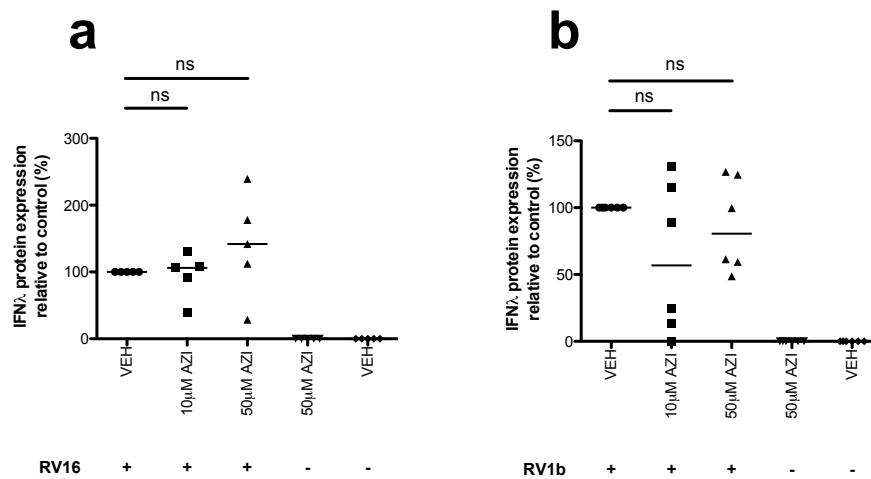


Figure 3.8 Azithromycin showed trends for the augmentation of secreted IFN λ protein (by MSD) in RV-infected HBECs cultured from AA patients.

In HBECs cultured from AA patients, Azithromycin showed trends for augmenting the median levels of (a) RV16-induced IFN λ protein expression and decreased levels of (b) RV1b-induced IFN λ protein. Data expressed as % change of RV1b + vehicle (VEH 100%) induction. n=5-6.

3.4.7 Azithromycin significantly reduced RV replication in HBECs cultured ex vivo from asthmatic adults

Azithromycin pre-treatment of HBECs from mild/moderate asthmatic adults significantly reduced RV1b replication and showed trends for the reduction of RV16 48 hours post infection (Figure 3.9 10 μ M/ 50 μ M AZI: 117.70/ 67.45% (RV16), 60.61/ 34.02% (RV1b) TCID₅₀ compared to vehicle 100% n=12).

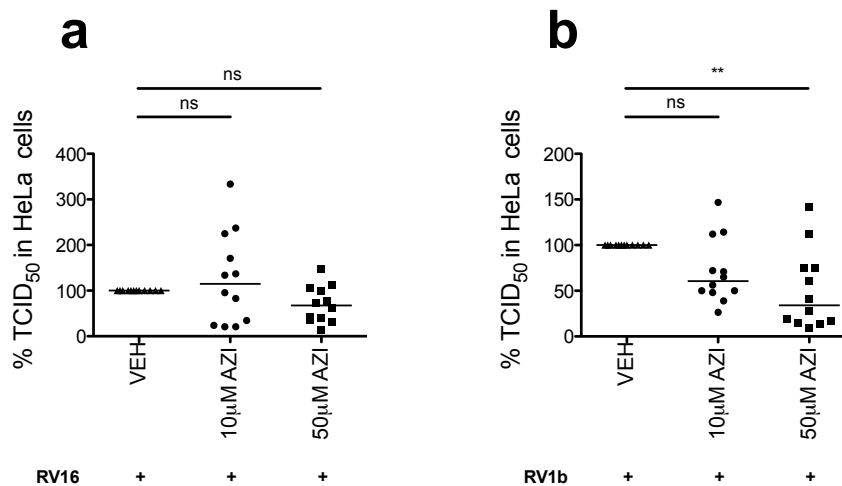


Figure 3.9 Azithromycin reduced RV replication in HBECs from asthmatic adults.

Azithromycin (AZI) inhibited (a) RV16 and (b) RV1b replication in HBECs cultured from AA after 48 hours. Supernatants from virally infected HBEC cultures were titrated onto HeLa cell cultures and TCID₅₀ was determined by observing CPE. Data expressed as % change of RV1b + vehicle (VEH 100%) induction. **p<0.01. n=12.

3.4.8 Azithromycin showed trends for augmenting anti-viral gene mRNA expression in RV16-infected HBECs from STRA children

Azithromycin pre-treatment of HBECs cultured *ex vivo* from STRA children showed a trend for augmenting levels of RV16-induced IFN β , IL-28, IL-29, MxA and Viperin mRNA (Figure 3.10 median levels of 10µM/ 50µM AZI IFN β : 160.60/270.68%, IL-28: 112.37/ 135.65%, IL-29: 183.81/ 189.37%, MxA: 163.47/ 401.69%, Viperin: 193.86/ 262.45% compared to vehicle treatment 100% n=2-4).

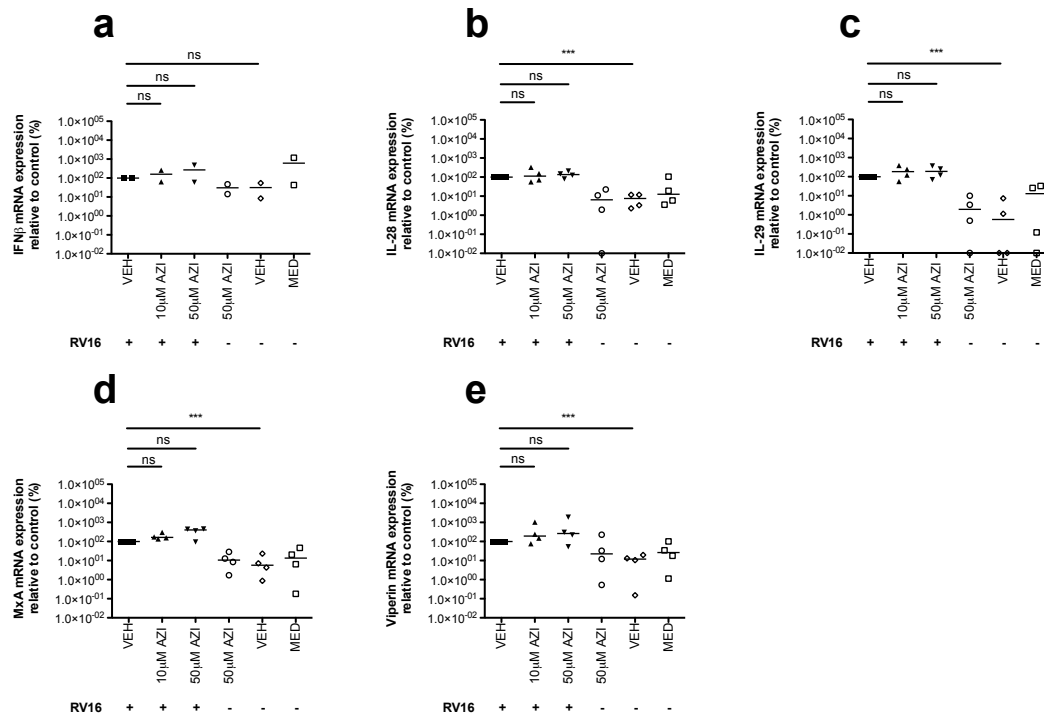


Figure 3.10 Azithromycin showed trends for augmenting IFN and ISG mRNA expression in RV16-infected HBECs from STRA children.

Azithromycin (AZI) treatment showed trends for the augmentation of RV16-induced (a) IFN β , (b) IL-28, (c) IL-29, (d) MxA, and (e) Viperin mRNA in HBECs cultured *ex vivo* from STRA children. Data expressed as % of vehicle treated cells (VEH 100%)* $p < 0.05$. *** $p < 0.001$. $n = 2-4$.

3.4.9 Azithromycin augmented anti-viral gene mRNA expression in RV1b-infected HBECs from STRA children

Azithromycin pre-treatment of HBECs cultured *ex vivo* from STRA children (with the 50 μ M dose) significantly augmented expression of RV1b-induced Viperin and showed a trend for augmenting levels of RV1b-induced IFN β , IL-28, IL-29 and MxA mRNA (Figure 3.11 median levels of 10 μ M/ 50 μ M AZI IFN β : 86.06/ 149.58%, IL-28: 98.29/ 190.24%, IL-29: 182.41/ 221.09%, MxA: 122.02/ 216.51%, Viperin: 160.21/ 420.24% compared to vehicle treatment 100% $n = 3-4$).

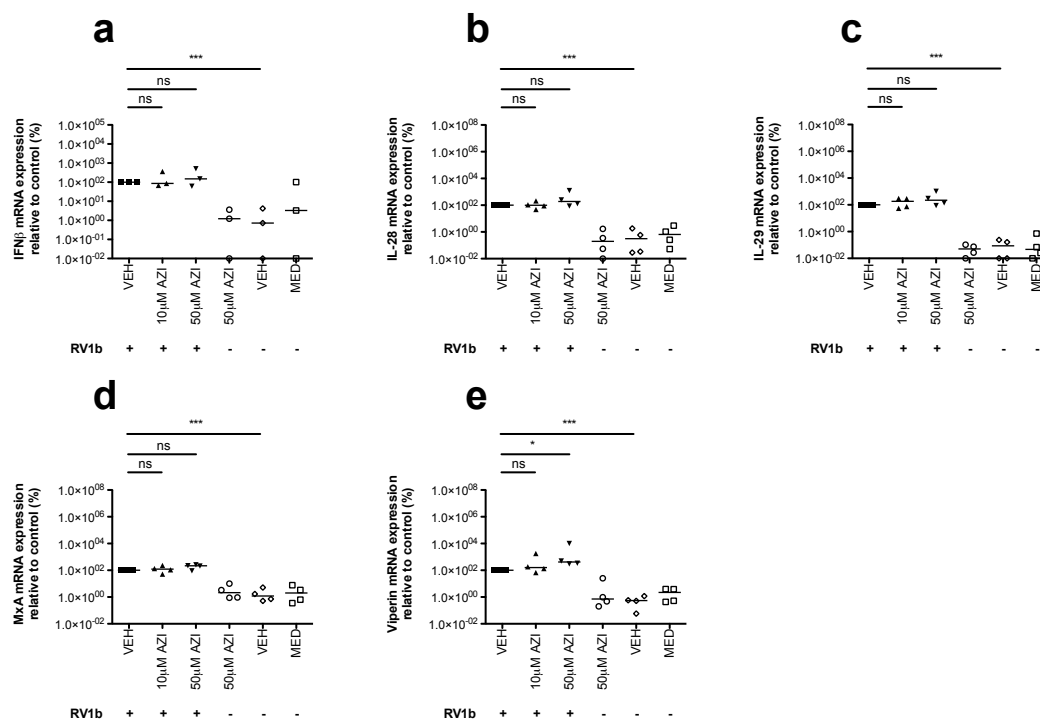


Figure 3.11 Azithromycin augmented IFN and ISG mRNA expression in RV1b-infected HBECs from STRA children.

Azithromycin (AZI) treatment showed trend for the augmentation of RV1b-induced (a) IFN β , (b) IL-28, (c) IL-29 and (d) MxA mRNA. AZI significantly augmented levels of RV1b-induced (e) Viperin mRNA in HBECs cultured *ex vivo* from STRA children. Data expressed as % of vehicle treated cells (VEH 100%) * $p < 0.05$. *** $p < 0.001$. $n = 3-4$.

3.4.10 Azithromycin augmented levels of RV-induced IFN protein in HBECs cultured from STRA children

Azithromycin pre-treatment of HBECs from STRA children showed a significant augmentation of secreted IFN β protein expression and a trend towards elevated concentrations of secreted IFN λ protein expression in cultures infected with RV1b (Figure 3.12 median levels of 10μM/ 50μM AZI IFN β : 125.63/ 173.61%, IFN λ : 82.61/ 667.50% compared to vehicle treatment 100% $n = 2-3$). No IFN β or IFN λ protein was detectable in cultured infected with RV16 at this time point.

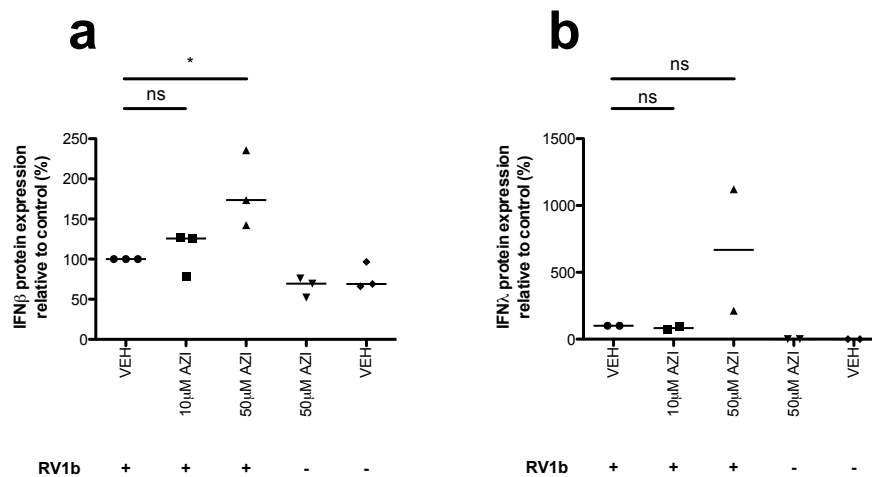


Figure 3.12 Azithromycin augmented IFN protein expression in RV1b-infected HBECs cultured from STRA children.

Azithromycin (AZI) pre-treatment of RV1b-infected HBECs cultured from STRA children augmented the expression levels of (a) secreted IFN β protein, AZI pre-treatment showed a non-significant increase in levels of (b) secreted IFN λ protein. Data expressed as % of vehicle treated cells (VEH 100%). n=2-3.

3.4.11 Azithromycin significantly reduced RV replication in HBECs cultured ex vivo from STRA children

Azithromycin pre-treatment of HBECs from STRA children significantly reduced RV16 and RV1b replication 48 hours post infection (Figure 3.13 10 μ M/ 50 μ M AZI: 89.23/ 56.69% (RV16), 60.61/ 44.12% (RV1b) TCID₅₀ compared to vehicle 100% n=4).

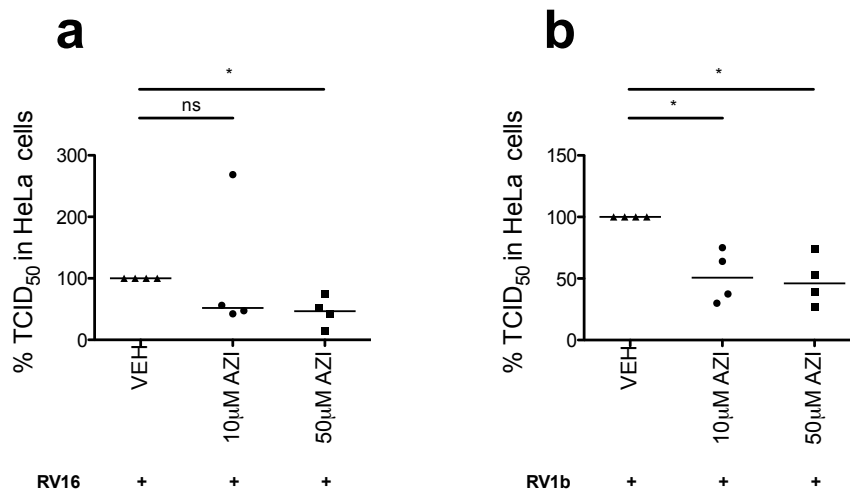


Figure 3.13 Azithromycin reduced RV replication in HBECs from STRA children.

Azithromycin (AZI) inhibited (a) RV16 and (b) RV1b replication in HBECs cultured from STRA children after 48 hours. Supernatants from virally infected HBEC cultures were titrated onto HeLa cell cultures and TCID₅₀ was determined by observing CPE. Data expressed as % change of RV1b + vehicle (VEH 100%) induction. *p<0.05. n=4.

3.4.12 Azithromycin augmented anti-viral gene mRNA expression in RV16-infected HBECs from non-atopic non-asthmatics

Azithromycin pre-treatment of HBECs from NANA (four adults, one child) showed trends for augmenting levels of RV16-induced IFN β , IL-28, IL-29, MxA and Viperin mRNA (Figure 3.14 median levels of 10µM/ 50µM AZI IFN β : 164.93/ 141.50%, IL-28: 104.94/ 79.11%, IL-29: 140.72/ 115.71%, MxA: 147.73/ 154.62%, Viperin: 141.03/ 70.07% compared to vehicle treatment 100% n=3-5).

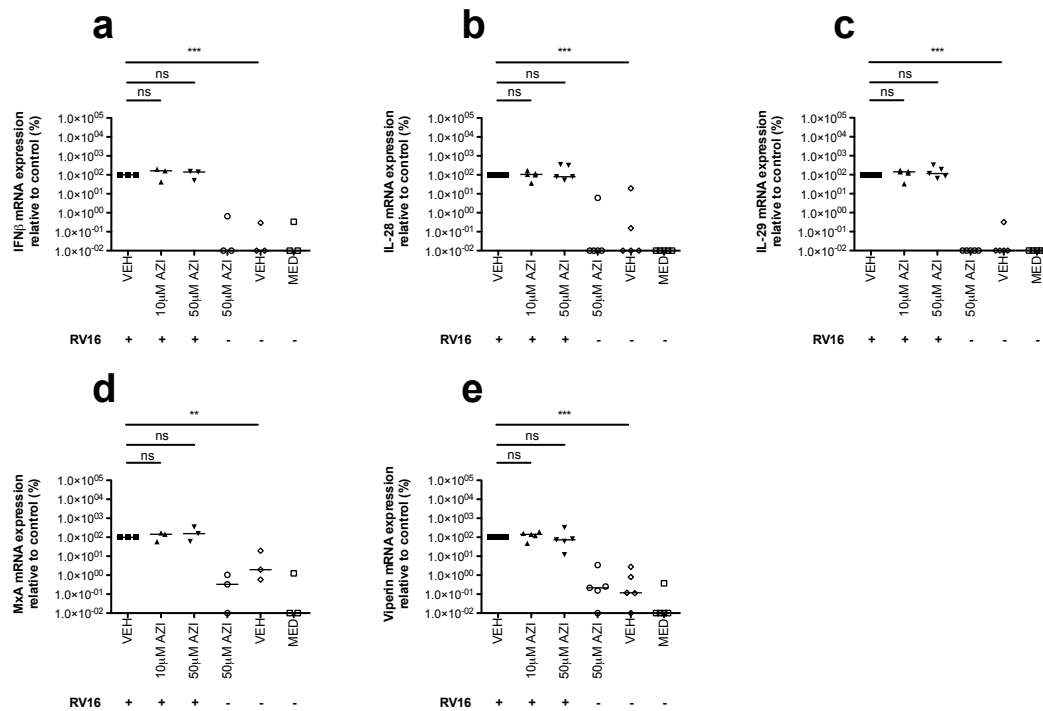


Figure 3.14 Azithromycin showed trends for augmenting IFN and ISG mRNA expression in RV16-infected HBECs from NANA patients.

Azithromycin (AZI) treatment showed trend for the augmentation of RV16-induced (a) IFN β , (b) IL-28, (c) IL-29, (d) MxA, and (e) Viperin mRNA in HBECs cultured *ex vivo* from NANA patients (4 adults, 1 child). Data expressed as % of vehicle treated cells (VEH 100%). **p<0.01. ***p<0.001. n=3-5.

3.4.13 Azithromycin augmented anti-viral gene mRNA expression in RV1b-infected HBECs from non-atopic non-asthmatics

Azithromycin pre-treatment of HBECs from NANA (four adults, one child) significantly augmented levels of RV1b-induced IL-28 and IL-29 mRNA expression (with the 50μM dose) and showed trends for augmenting levels of RV1b-induced IFN β , MxA and Viperin mRNA (Figure 3.15 median levels of 10μM/ 50μM AZI IFN β : 10.88/ 141.26%, IL-28: 109.07/ 214.81%, IL-29: 72.46/ 244.48%, MxA: 123.79/ 150.69%, Viperin: 65.42/ 196.03% compared to vehicle treatment 100% n=3-5).

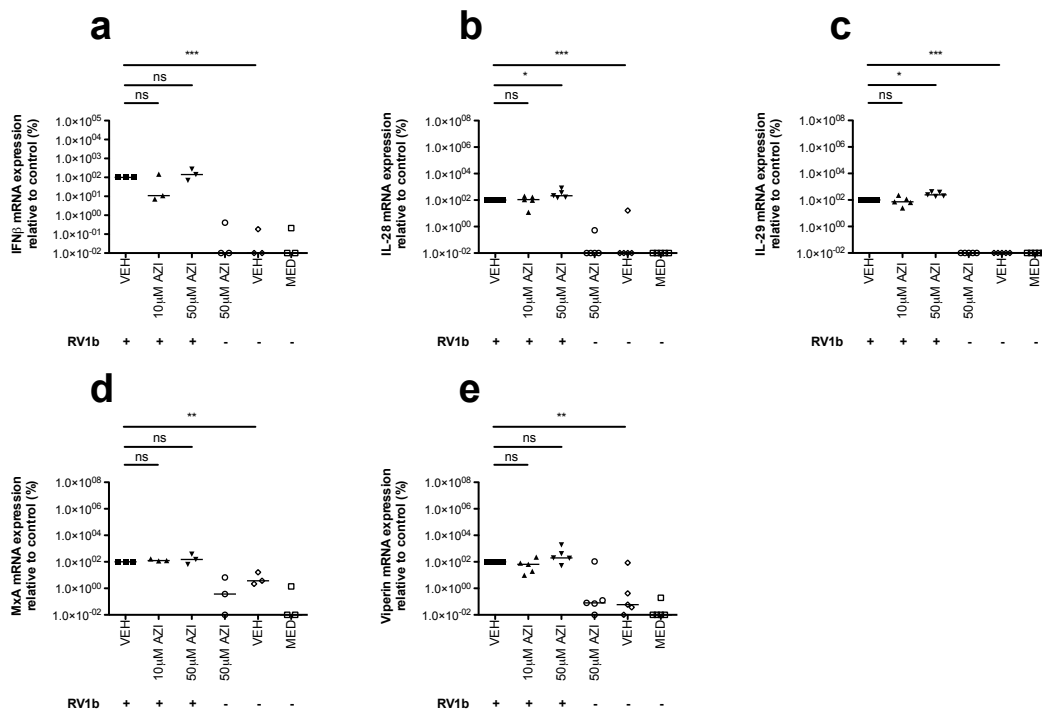


Figure 3.15 Azithromycin augmented IFN and ISG mRNA expression in RV1b-infected HBECs from NANA patients.

Azithromycin (AZI) treatment augmented levels of RV1b-induced (b) IL-28 and (c) IL-29 mRNA expression and showed a trend for the augmentation of RV1b-induced (a) IFNβ, (d) MxA, and (e) Viperin mRNA in HBECs cultured *ex vivo* from NANA patients (4 adults, 1 child). Data expressed as % of vehicle treated cells (VEH 100%)* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. $n = 3-5$.

3.4.14 Azithromycin augmented levels of RV-induced IFN protein in HBECs cultured non-atopic, non-asthmatic patients

50μM Azithromycin pre-treatment of HBECs from NANA patients showed a significant augmentation of secreted IFNλ protein expression in cultures infected with RV16 and a non-significant increase of secreted IFNλ protein expression in cultures infected with RV1b (Figure 3.16 median levels of 10μM/ 50μM AZI (RV16) IFNλ: 107.97/ 136.35%, (RV1b) IFNλ: 105.01/313.11% compared to vehicle treatment 100% $n = 2-4$). No IFNβ protein was detectable in cultured infected with RV16 or RV1b at this time point.

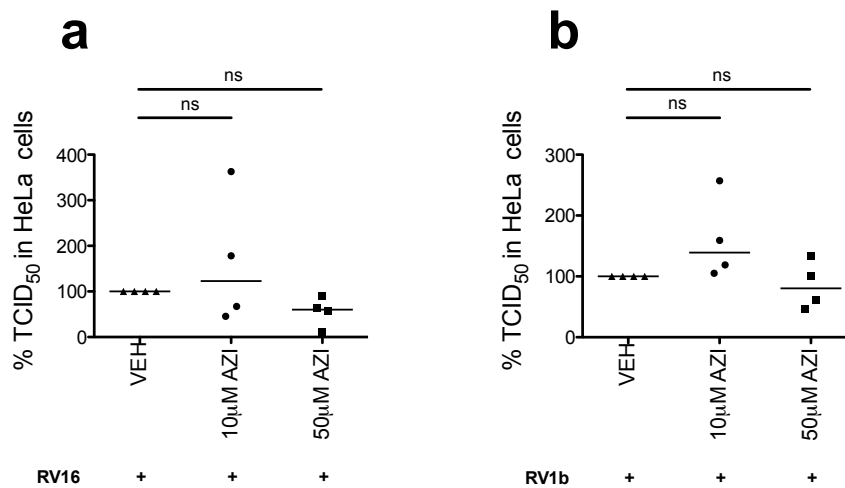


Figure 3.17 Azithromycin reduced RV replication in HBECs from NANA patients.

Azithromycin (AZI) showed a non-significant trend for the inhibition of (a) RV16 and (b) RV1b replication in HBECs cultured from NANA patients after 48 hours. Supernatants from virally infected HBEC cultures were titrated onto HeLa cell cultures and TCID₅₀ was determined by observing CPE. Data expressed as % change of RV1b + vehicle (VEH 100%) induction. n=4.

3.4.16 Azithromycin augmented antiviral gene mRNA expression and reduced RV replication in asthmatic HBECs from combined adults and children

As we only saw significant differences between azithromycin treated, infected and untreated, infected groups for some but not all readouts, we assessed the data from combined asthmatic children and adult groups. When the data was combined, RV-infected HBECs from AA adults and STRA children showed significant augmentation of MxA by Azithromycin pre-treatment (Figures 3.18-3.19 median levels of 10µM/ 50µM AZI RV16 IFNβ: 187.33/ 317.87%, IL-28: 150.87/ 120.39%, IL-29: 92.57/ 93.74%, MxA: 181.88/ 279.95%, Viperin: 167.82/ 207.48%. RV1b IFNβ: 86.06/ 149.58%, IL-28: 109.27/ 150.44%, IL-29: 104.06/ 155.58%, MxA: 142.96/ 197.05%, Viperin: 179.40/ 297.37% compared to vehicle treatment 100% n=10-14). Azithromycin was also able to significantly reduce viral replication in this pooled group of asthmatic HBECs (Figure 3.20 10µM/ 50µM AZI: 89.23/ 57.17% (RV16), 60.07/ 37.75% (RV1b) TCID₅₀ compared to vehicle 100% n=16).

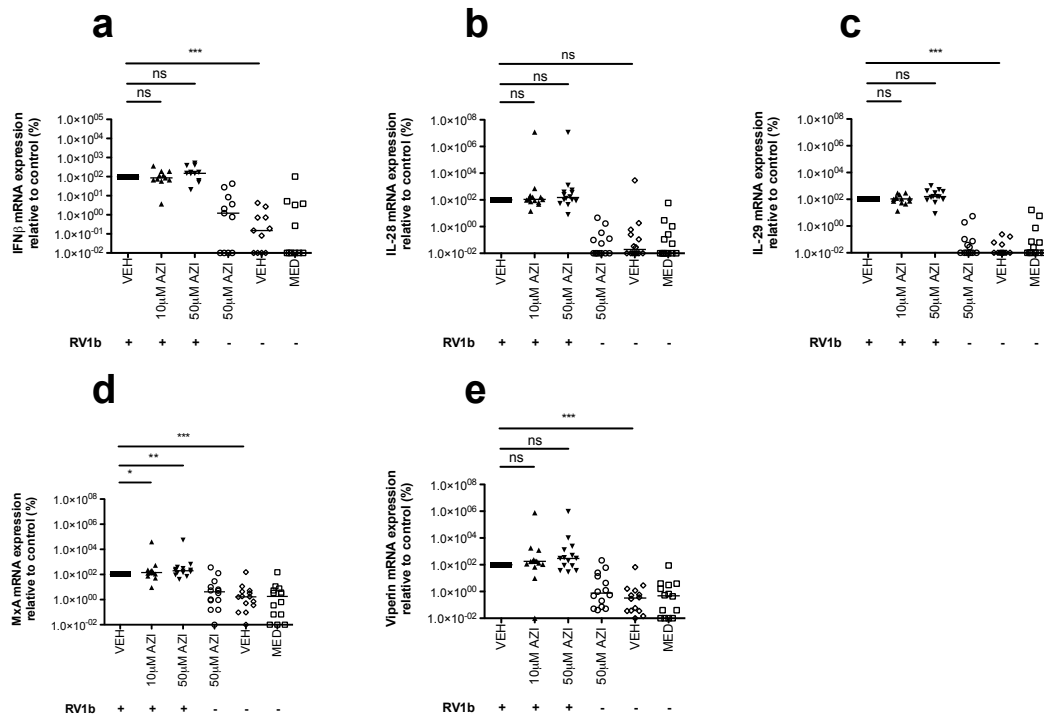


Figure 3.19 Azithromycin augmented ISG mRNA expression in RV1b-infected HBECs from asthmatic patients.

Azithromycin (AZI) treatment augmented levels of RV1b-induced (b) IL-28 and (d) MxA mRNA expression and showed a trend for the augmentation of RV1b-induced (a) IFNβ, (c) IL-29, and (e) Viperin mRNA in HBECs cultured *ex vivo* from asthmatic patients (8 adults, 4 children). Data expressed as % of vehicle treated cells (VEH 100%) * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. $n = 10-14$.

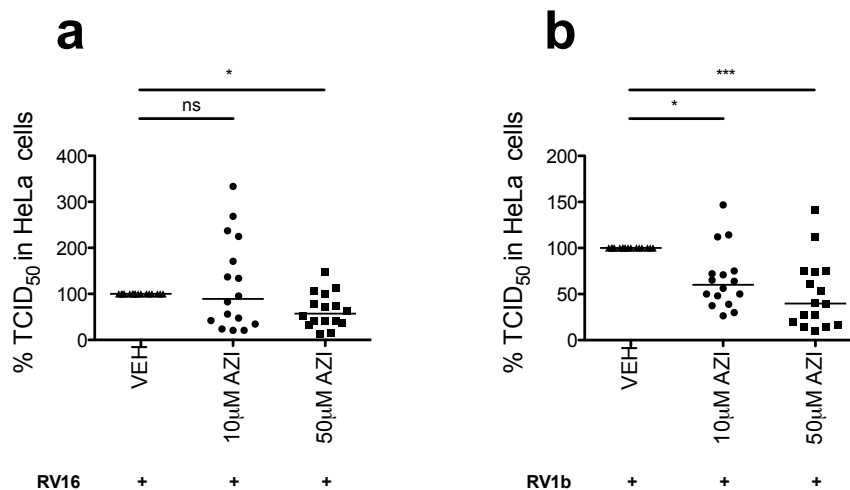


Figure 3.20 Azithromycin reduced RV replication in HBECs from asthmatic patients.

Azithromycin (AZI) inhibited (a) RV16 and (b) RV1b replication in HBECs cultured from AA Adults and STRA children (data pooled) after 48 hours. Supernatants from virally infected HBEC cultures were titrated onto HeLa cell cultures and TCID₅₀ was determined by observing CPE. Data expressed as % change of RV1b + vehicle (VEH 100%) induction. * $p < 0.05$ *** $p < 0.001$. $n = 16$.

3.4.17 Associations of Azithromycin-augmented IFN and ISG mRNA with clinical outcomes

Post-hoc correlation analysis was used to determine whether clinical measurements (for atopic-asthmatic adults; Asthma Control Questionnaire- ACQ and sensitivity to histamine challenge- PC20, for all asthmatics; serum IgE levels and the total number of positive skin prick tests- SPTs) correlated with any observed Azithromycin-induced anti-viral gene augmentation.

3.4.17.1 Azithromycin-induced antiviral gene augmentation showed non-significant correlation with PC20 values in HBECs from atopic-asthmatic adults

In AA adults, ACQ and PC20 (histamine) values showed a non-significant trend for a negative correlation with the average gene augmentation (mean % augmentation of IFN β , IL-28, IL-29, MxA and Viperin) in HBECs pre-treated with 50 μ M AZI and infected with RV1b. No correlation was observed between ACQ or PC20 (histamine) with the average gene augmentation in HBECs pre-treated with 50 μ M AZI and infected with RV16. (Figure 3.21 ACQ value vs. mean antiviral gene augmentation with AZI + RV16: $p=0.98$, $R=0.0087$. AZI + RV1b: $p=0.99$, $R=0.0403$. PC20 histamine value vs. mean antiviral gene augmentation with AZI + RV16: $p=0.70$, $R=0.0074$. AZI + RV1b: $p=0.06$, $R=0.2215$ $n=11$).

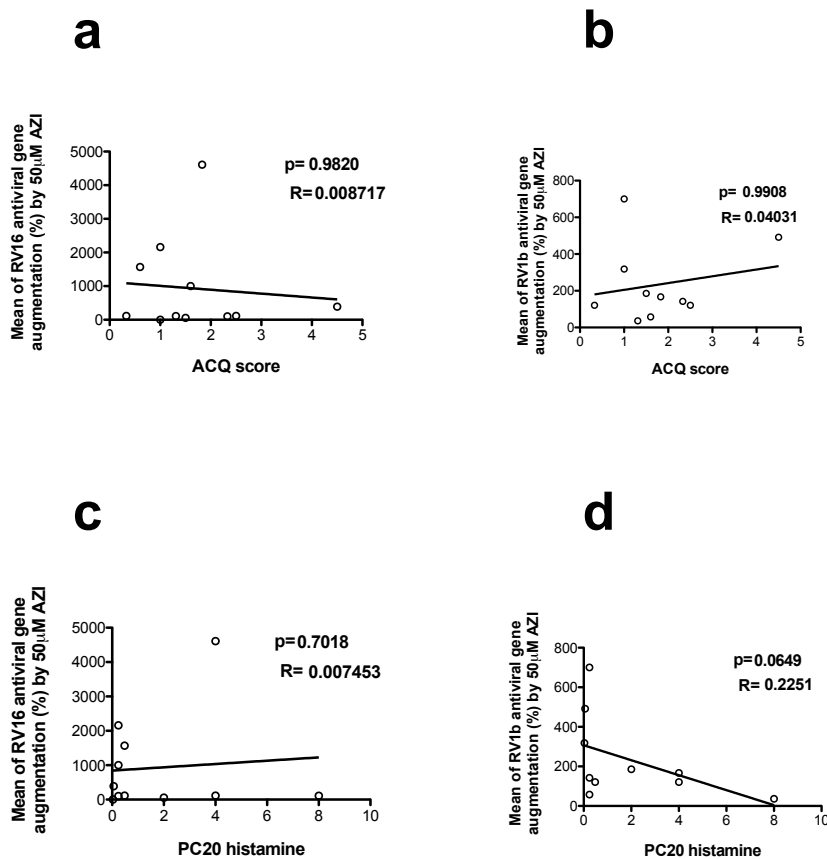


Figure 3.21 PC20 (histamine) values showed a non-significant negative correlation with the average gene augmentation observed with Azithromycin pre-treatment in RV-infected HBECs from AA adults.

PC20 (histamine) values negatively correlated (non-significantly) with the average gene augmentation caused by 50µM AZI treatment prior to infection with (d) RV1b but not with (c) RV16. No correlations were observed between the average AZI-induced gene augmentation and ACQ values (a and b). No data was statistically significant. $n=11$.

3.4.17.2 Azithromycin-induced $TCID_{50}$ showed a negative correlation with PC20 values in HBECs from atopic-asthmatic adults

$TCID_{50}$ values from Azithromycin-treated, RV16-infected HBECs from asthmatic adults showed a significant inverse correlation with patient PC20 histamine values, conversely RV1b infected HBECs showed a non-significant positive correlative trend between $TCID_{50}$ values and PC20 histamine values. No correlation was observed between $TCID_{50}$ values from Azithromycin-treated, RV16 or RV1b-infected HBECs from asthmatic adults and ACQ values (Figure 3.22 ACQ values vs. $TCID_{50}$ with AZI + RV16: $p=0.42$, $R=0.2968$. AZI + RV1b: $p=0.86$, $R=0.0412$. PC20 histamine values vs. $TCID_{50}$ with AZI + RV16: $p=0.03$, $R=0.2999$. AZI + RV1b: $p=0.52$, $R=0.2500$ $n=12$).

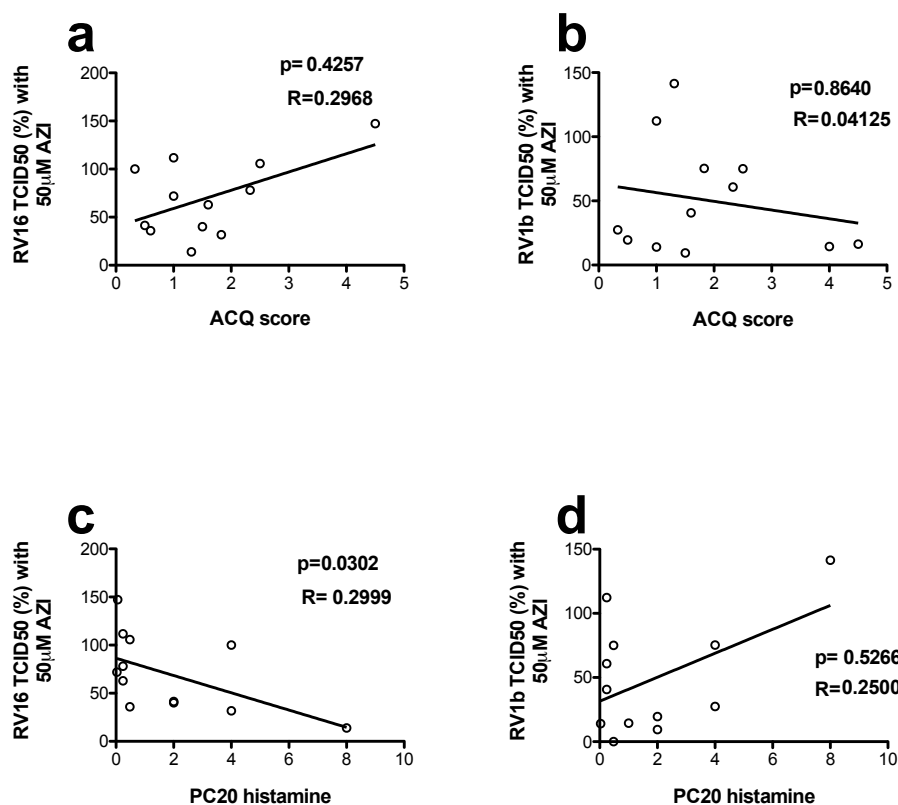


Figure 3.22 Azithromycin-mediated viral replication inversely correlated with PC20 histamine measurements in HBECs from asthmatic adults.

TCID₅₀ values of RV16 or RV1b-infected HBECs from asthmatic adults showed no correlation with ACQ values (a and b). TCID₅₀ values of RV16-infected HBECs showed a significant inverse correlation with PC20 histamine values (c) however showed a non-significant positive correlation with PC20 histamine values (d). n=12.

3.4.17.3 Azithromycin-induced antiviral gene augmentation showed no correlation with serum IgE levels and SPT positivity in asthmatic patients

In data pooled from asthmatic patients (mild/moderate asthmatic adults and STRA children) serum IgE showed no correlation with the average anti-viral gene augmentation (mean % augmentation of IFN β , IL-28, IL-29, MxA and Viperin) in HBECs pre-treated with 50µM AZI and infected with RV16 or RV1b. Again with pooled data from AA adults and STRA children the total number of positive skin prick tests values showed no correlation with the average anti-viral gene augmentation in Azithromycin-treated HBEC cultures infected with RV1b or RV1b (Figure 3.23 Serum IgE vs. mean antiviral gene augmentation with AZI + RV16: p=0.98, R= 0.0528. AZI + RV1b: p=0.47, R= 0.0003. Number of positive SPT vs. mean antiviral gene augmentation with AZI + RV16: p=0.72, R=0.0116. AZI + RV1b: p=0.75, R= 0.1327 n=15).

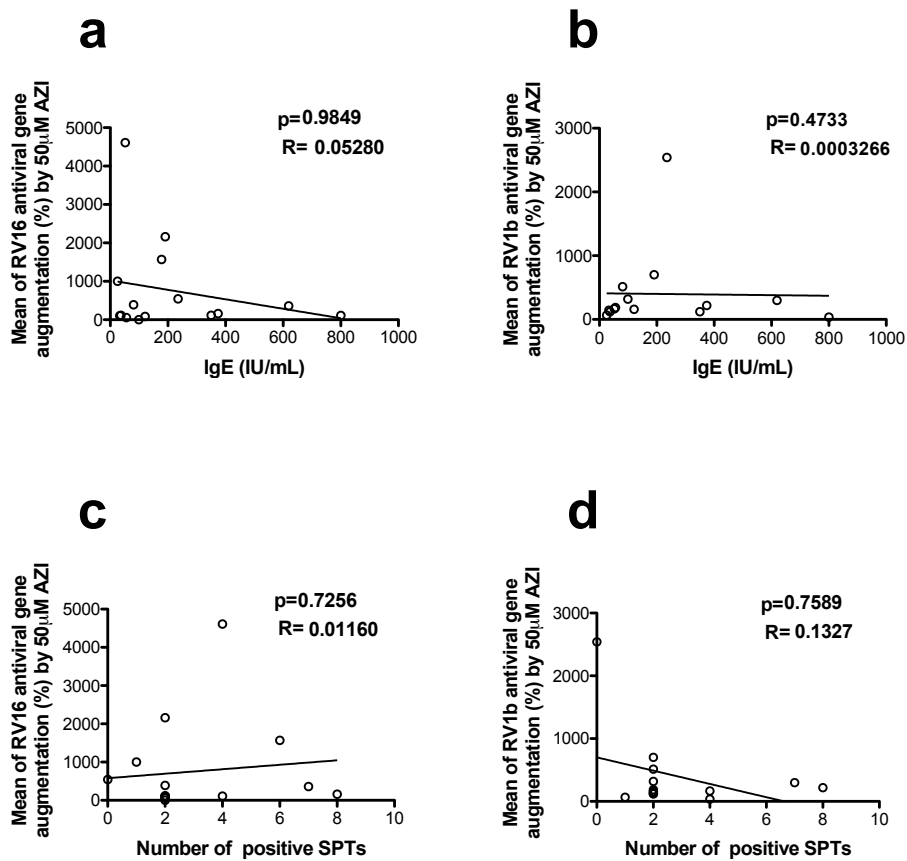


Figure 3.23 Serum IgE levels and the number of positive SPTs showed no correlation with the average gene augmentation observed with Azithromycin pre-treatment in RV-infected HBECs from asthmatics.

Serum IgE levels from pooled AA adults and STRA children showed no correlation with the average gene augmentation caused by 50 μ M AZI treatment prior to infection with (a) RV16 and showed no correlation with (b) RV1b. The number of positive SPTs also showed no correlation with the average gene augmentation caused by 50 μ M AZI treatment prior to infection with (d) RV1b or (c) RV16. No data was statistically significant. $n=15$.

3.4.17.4 Azithromycin-induced TCID₅₀ showed a significant correlation with the number of positive SPTs in HBECs from atopic-asthmatic adults and children

TCID₅₀ values from Azithromycin-treated, RV16-infected HBECs from pooled asthmatic adults and children showed an inverse correlation with the number of positive SPTs. No correlation was observed with TCID₅₀ values from Azithromycin-treated, RV1b-infected HBECs and the number of positive SPTs. TCID₅₀ values from Azithromycin-treated, RV16-infected or RV1b-infected HBECs from asthmatic adults showed no correlation with patient serum levels of IgE (Figure 3.24 Serum IgE vs. TCID₅₀ with AZI + RV16: $p=0.46$, $R= 0.1599$. AZI + RV1b: $p=0.45$, $R= 0.2420$. Number of positive SPTs vs. TCID₅₀ with AZI + RV16: $p=0.02$, $R= 0.2570$. AZI + RV1b: $p=0.88$, $R= 0.0066$ $n=15$).

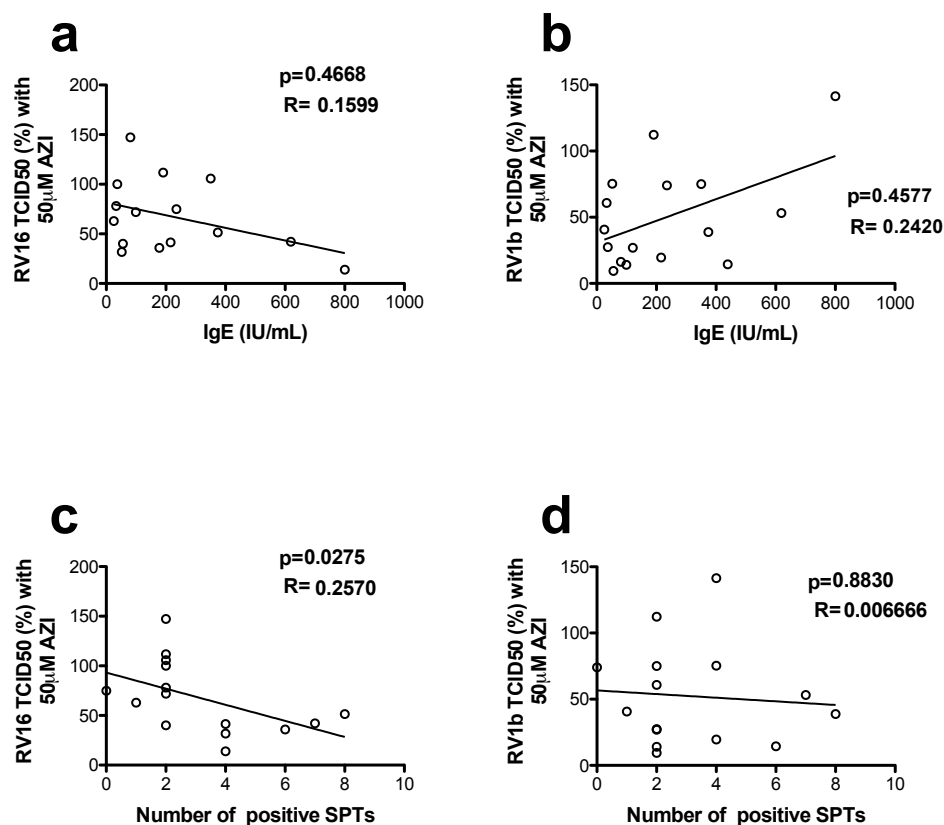


Figure 3.24 Azithromycin-mediated viral replication in asthmatic HBECs inversely correlated with the number of patients SPT positive tests.

RV16 TCID₅₀ values in HBECs from asthmatic adults and children (pooled data) showed no correlation with levels of serum IgE (a) or RV1b infection (b). A statistically significant inverse correlation was observed between RV16 TCID₅₀ values and the number of positive SPTs (c), no correlation was observed between RV1b TCID₅₀ and positive SPTs $n=15$.

3.5 Discussion

In this chapter we have replicated the published findings by Gielen *et al* and showed that the pre-treatment of normal HBECs with Azithromycin prior to infection with RV is able to augment the levels of anti-viral IFN and ISG mRNA expression. We have also confirmed that in addition to the augmentation of gene expression, Azithromycin pre-treatment can decrease viral replication by approximately 50%. The study by Gielen *et al* and the initial findings presented here were performed using commercially available HBECs grown from healthy donors, and no studies to date have explored the effects of Azithromycin on HBECs derived from asthmatic patients (which may be deficient in IFN production and therefore may be responsive to Azithromycin). We therefore next endeavoured to show that when Azithromycin was added to HBEC cultures grown *ex vivo* from a group of AA adults and STRA children, a similar Azithromycin- dependent gene augmentation occurred. We demonstrated that Azithromycin-treated HBECs from AA adults showed trends for augmentation of type I and type III IFN gene and protein expression and significantly augmented levels of ISG mRNA with RV infection and (in the case of RV1b infection) showed a significant decrease in viral replication. In the limited number of HBEC cultures from STRA children we have also shown that Azithromycin treatment can significantly augment RV-induced ISG mRNA and IFN λ protein levels, whilst significantly decreasing RV replication. In a small group of HBECs from NANA patients we have demonstrated that Azithromycin treatment can augment RV-induced type III IFN gene expression, IFN λ protein production and show a trend for decreasing RV replication. In these studies we also have shown that the Azithromycin dependent augmentation of the anti-viral response may negatively correlate with patient's PC20 histamine values and the number of positive SPTs. No association was seen between anti-viral gene augmentation and levels of serum IgE or ACQ values.

While numerous studies have identified asthmatics to be deficient in IFN responses to RV infection [46, 84-87], here we describe the augmenting effect of Azithromycin in cells from asthmatics although we cannot state definitively that Azithromycin is able to restore any IFN deficiency. The STRA samples used in this study had impaired levels of IFN β , IL-28 and IL-29 compared to age and sex matched controls (data published by Edwards *et al* [87]) however the HBECs from AA adults were from a study that demonstrated no impaired of IFNs versus age and sex matched controls (Dr. Jonathan MacIntyre, PhD thesis). While it was encouraging to see increased IFN and ISG responses in both groups, we cannot be certain the impaired IFN in STRA children was completely restored. This is partly due to the

fact that there currently exists no real definition of “impaired” IFN expression, and as the effects of Azithromycin on IFN production from healthy individuals was not compared side by side with the asthmatics it would be impossible to state definitively if Azithromycin treatment restored the IFN levels to those of age matched controls. Although the asthmatic status (well/poorly controlled) of the AA adults in this study was unknown, we would hypothesise that those patients whose asthma was poorly controlled would also be deficient in IFN production and (based on this study) Azithromycin may be able to restore this to some degree.

The major aim of this study was to determine whether the effects of Azithromycin augmenting antiviral responses could be observed in HBECs from asthmatic patients, we feel that we have addressed this aim however we were unable to directly compare responses between HBECs from healthy and asthmatic donors. This was due to different numbers of AA adults and STRA children compared to NANA patients, in order to better evaluate the differences between asthmatics and non-asthmatics ideally an equal number of AA adults, NANA adults, STRA children and NANA children. Overall we only saw significant augmentation of IFN and ISGs in a small number of gene measures. The data from HBECs grown *ex vivo* was also variable, with many non-significant trends being observed compared to the significant finding observed with commercially available HBECs, this is unsurprising as the genotype, diversity and disease status of donors will undoubtedly result in a lower degree of consistency of gene and protein expression.

The clinical presentation of asthma can be associated with a verity of differing clinical test scores. Here, patients whose HBECs were cultured displayed a range of clinical values for serum IgE levels and number of positive SPTs (markers of allergy and atopy) and ACQ values and PC20 histamine values (markers of asthma severity). We sought to identify if any of these scores correlated with the effectiveness of Azithromycin in augmenting anti-viral gene expression or decreasing RV replication, thus providing a possible marker for targeting patients for potential Azithromycin treatment. The data presented here is suggestive that Azithromycin treatment may be more effective in decreasing viral replication in patients with a higher PC20 and a higher number of positive SPT and thus may be suited more towards patients more atopic asthma, interestingly the gene data did not mimic this correlation. Larger numbers of patients would be required to further assess the validity of this correlation.

To further understand the impact of Azithromycin treatment in asthmatics addition studies should be performed, ideally one would need to evaluate the effects of Azithromycin on an equal number of asthmatic and non-asthmatic patients (as mentioned above). Following

further *in vitro* analysis, the effects of Azithromycin in an experimental RV infection study in asthmatic patients could determine Azithromycin's effectiveness. For potential long-term administration of Azithromycin to asthmatic patients, one would need to take the antibacterial nature of Azithromycin into account as perturbation of the lung microbiome may illicit changes in anti-viral immunity and inflammation [230, 231]. One potential way to circumvent this problem would be the use of a similar compound with no antibiotic activity. This study is suggestive that a short course of Azithromycin prior to infection may be effective; it is unlikely that major changes in the lung microbiome would occur after a 24-hour treatment (as this study describes) however this remains a possibility with a longer treatment regime.

Chapter 4 Azithromycin augments IFN responses via increased MAVS signalling

4.1 Introduction

The effective recognition of viral species within host cells is critical for the adequate expression of anti-viral host defences (such as IFNs and ISGs), the task of detecting viruses falls on a number of PRRs such as the TLRs and the RLHs. TLR-3 is able to bind viral RNA in the endosomal compartment, activated TLR-3 recruits a number of signalling intermediates such as TRIF, TBK1 and IKK ϵ , activating a signal cascade resulting in the activation of transcription factors including IRF-3 and NF κ B. These activated transcription factors are translocated to the nucleus and initialise the transcription IFNs and also pro-inflammatory genes [101, 232, 233]. Within the host cells cytoplasm the RLHs: RIG-I and MDA-5 bind viral RNA [110] and activate a signalling cascade through the adaptor protein MAVS [112]. Initiation of this signalling pathway also leads to the activation of IRF-3 and production of IFNs. In the context of RV infection, HBECs are known to produce IFN β , IFN λ 1 (IL-29) and IFN λ 2/3 (IL-28) mRNA at detectable levels by 4 hours and peaking within 24 hours of RV infection [81, 82, 142]. Initially, RV is detected by the host cell via TLR-3, RIG-I and MDA-5 in a coordinated manner to produce IFNs [50, 124, 234].

Key to the RLH signalling cascade is the adaptor protein MAVS, common to MDA-5 and RIG-I signalling however not known to be involved in TLR-3 activation. MAVS is a 75kDa, mitochondria-associated protein known to bind directly to RIG-I and MDA-5 via an N-terminal CARD-like domain and facilitate the activation of downstream signalling molecules such as TBK1 and FADD, ultimately leading to the activation of IRF3 and NF κ B [112, 235]. MAVS has been shown to be degraded in response to viral infection or cell stimulation with dsRNA or ssRNA, it is currently thought that this degradation serves as a form of regulation of IFN expression during infection [130]. This degradation of MAVS protein has been the subject of numerous studies, the common consensus is that after recruitment of TBK1 and FADD, MAVS is ubiquitinated and targeted for proteosomal degradation by a number of different ubiquitin E3 ligases and adaptors [132-134, 236-238].

After the initial recognition of viral species and subsequent expression of IFNs, these IFNs are able to bind the IFNAR1 (in the case of IFN β) or IFN λ R1 (in the case of IFN λ s) signalling

complexes on the host (or neighbouring) cells and initiate an auto-feedback loop, resulting in the amplified expression of IFN and the expression of ISGs [239, 240], the latter of which are known to be directly anti-viral [147, 241]. The importance of this in the context of RV infections has been highlighted by Bartlett *et al*, who showed that IFNAR1 signalling was required for robust RV-induced IFN and ISG expression *in vivo* [242]. It is a logical hypothesis that Azithromycin is able to interact directly or indirectly within the virus recognition or IFN signalling pathways when augmenting IFN and ISG expression in response to RV infection.

The immunomodulatory effects of Azithromycin have been studied in some detail. Azithromycin was shown to inhibit mucin production in epithelial cells through the inhibition of ERK1/2 in response to bacterial infection [243]. Azithromycin also decreased macrophage expression of IL-1 β through inhibition of AP-1 in mice treated with LPS [200]. Azithromycin has been shown to decrease levels of IL-6 and IL-8 through the inhibition of NF κ B in tracheal aspirate cells from premature infants [196]. Although, these studies demonstrate the effects of Azithromycin on transcription factor activity, no study confirmed how Azithromycin interacts with, and causes inhibition of transcription factor activation. Azithromycin is known to orchestrate its antibacterial activity through direct interaction with the 50S subunit of the bacterial ribosome [244], however direct interactions between Azithromycin and eukaryotic proteins (that might facilitate its immunomodulatory/ IFN augmenting effects) are less clear. Nujic *et al* showed that Azithromycin (and other macrolides) are able to bind valosin-containing protein (VCP) but this binding did not seem to mediate anti-inflammatory activity [245]. VCP is highly abundant within the cytoplasm and is known to be involved in protein turnover and autophagy [246]. It has been suggested that VCP is required for poliovirus replication [247], however no studies to date have identified VCP as a host factor in RV infection. Understanding any Azithromycin-protein interactions could provide useful drug-targets for augmenting IFN production in respiratory viral infections. An understanding of the mechanism of action of Azithromycin would further allow the design of macrolides with improved potency or efficacy.

In this chapter we used a variety of techniques to further investigate the effects of Azithromycin on IFN production in normal HBECs infected with RV. We demonstrated that although Azithromycin can augment RV-induced anti-viral gene expression when given at the point of infection, it is far more effective when administered 24 hours prior to infection. Through the use of synthetic agonists we showed that Azithromycin is capable of augmenting IFN responses to RIG-I and MDA-5 stimulation but not TLR-3 or recombinant

IFN β stimulation. Through a proteomic screen and Western blot analysis we showed that treatment of normal HBECs with Azithromycin (in the absence of RV infection) increases protein levels of the RIG-I/MDA-5 adaptor MAVS. Using small interfering RNAs (siRNA) we identified SMURF1, a negative regulator of MAVS, as a protein that is required for successful Azithromycin-mediated augmentation of anti-viral genes. We identified a range of possible Azithromycin binding proteins using a novel pull down assay with an Azithromycin analogue probe (AZI-Azide). Finally, we showed no anti-viral effects of Azithromycin in models of Influenza and RSV infection, two viruses which actively block MAVS signalling. Overall we propose a mechanism whereby Azithromycin binds MAVS or possibly a MAVS binding protein, preventing MAVs turnover through the proteosome. Increased abundance of MAVs generates pro-longed or greater IFN and ISG transcription, potentially explaining the IFN augmenting activity of Azithromycin.

4.2 Hypothesis

- The observed anti-viral activity of Azithromycin in HBECs is due wholly or partially through an interaction with the RLH or TLR virus sensing pathways.

4.3 Aims

- To determine whether Azithromycin can augment IFN/ISG expression in response to different synthetic agonists of the PRR pathways in normal HBECs.
- To determine whether Azithromycin can augment IFN/ISG expression in response to recombinant IFN in normal HBECs.
- To identify a target protein through which Azithromycin can bind to and possibly orchestrate its IFN augmenting ability.

4.4 Results

4.4.1 Azithromycin is more effective at augmenting IFN when administered prior to an RV infection

Studies by Gielen *et al* [47] and observations in previous chapters of this thesis have assessed the effects of Azithromycin pre-treatment of HBECs prior to infection with RV. In order to establish the effects of Azithromycin when administered with an RV infection normal HBECs were either pre-treated with 50 μ M Azithromycin (or vehicle control) for 24 hours prior to infection with RV1b or treated with 50 μ M Azithromycin post-treatment with an RV1b infection (MOI:1) for 24 hours. Post-treatment with Azithromycin augmented mRNA expression of IL-28 and showed trends for augmenting IFN β , IL-29, MxA and Viperin mRNA expression (Figure 4.1 IFN β augmentation pre/post treatment: 530.6/465.3, IL-28: 550.7/225.7, IL-29: 484.6/358.2, MxA: 264.7/145.6, Viperin: 353.9/216.1 data expressed as % compared to pre-treated Vehicle + RV1b 100%. n=6).

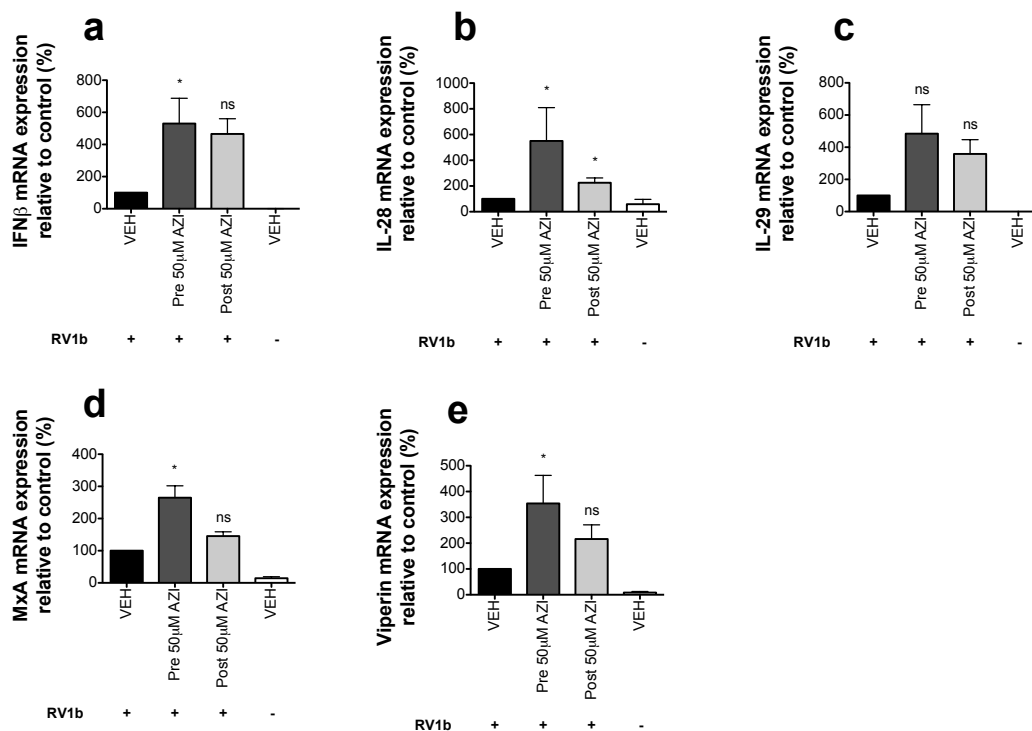


Figure 4.1 Azithromycin post-treatment augments responses to RV1b infection in normal HBECs.

Normal HBECs were either pre-treated with 50 μ M Azithromycin (AZI) or vehicle (VEH) or post-treated with 50 μ M AZI prior to infection with RV1b (MOI:1) for 24 hours, post treatment showed a trend for the augmentation of (a) IFN β , (c) IL29, (d) MxA and (e) Viperin. Post-treatment augmented expression of (b) IL-28. Data expressed as % change over RV1b + vehicle *p<0.05. n=6.

4.4.2 Azithromycin augmentation of IFN and ISG expression is restricted to RLH pathways

IFNs and ISGs are produced in response to RV infection in HBECs by distinct mechanisms, these can be divided into two groups; the virus recognition pathways (which can be subdivided into the TLR pathways and the RLH pathways) and the IFN signalling pathway. By using synthetic agonists specific for these different mechanistic pathways we assessed the limitations of Azithromycin's augmenting ability in normal HBECs.

4.4.2.1 Azithromycin was unable to augment IFN β responses to TLR agonists in normal HBECs

Normal HBECs were treated with 50 μ M Azithromycin or vehicle for 24 hours prior to stimulation with topical Poly(I:C) (a TLR-3 agonist) or R848 (a TLR7/8 agonist) for 8 hours. Azithromycin showed no augmentation of IFN β mRNA expression in Poly(I:C) treated cells (Figure 4.2 IFN β : 72.05% compared to vehicle treated cells 100%. n=7). R848 treatment failed to induce an IFN response in normal HBECs after 8 hours (data not shown).

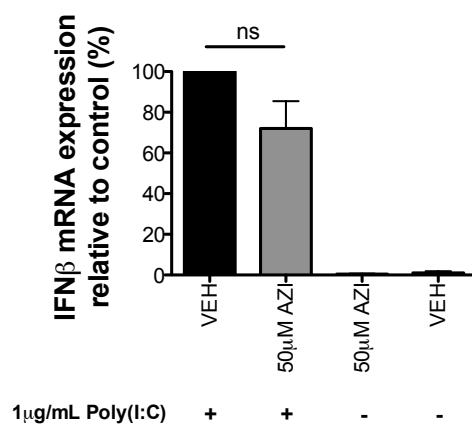


Figure 4.2 Azithromycin failed to augment IFN β expression in normal HBECs stimulated with TLR-3 agonist.

Normal HBECs were treated with 50 μ M Azithromycin (AZI) or vehicle (VEH) for 24 hours prior to stimulation with 1 μ g/mL of the TLR-3 agonists topical Poly(I:C) for 8 hours. AZI failed to augment IFN β mRNA expression. Data expressed as % change over RV1b + VEH. n=7.

4.4.2.2 Azithromycin showed trends for augmenting IFN and ISG expression in normal HBECs treated with RLH agonists

Normal HBECs were transfected with Poly(I:C) (Poly(I:C)/LyoVec) an MDA-5 specific agonist) or 5'ppp_{DS}RNA (a RIG-I specific agonist) in the presence or absence of Azithromycin pre-treatment. Cells were treated with 50 μ M Azithromycin or vehicle for 24 hours prior to transfection with 0.25 μ g/mL Poly(I:C)/LyoVec or 5'ppp_{DS}RNA for a further 24 hours. Azithromycin treatment showed a modest trend for augmenting IFN β and Viperin mRNA expression with the MDA-5 agonist and a clearer trend for augmenting IFN β and Viperin mRNA expression with the RIG-I agonist. (Figure 4.3 with MDA-5 agonist- IFN β : 129.7%, Viperin: 159.3%, with RIG-I agonist-IFN β 294.6%, Viperin 325.8% augmentation compared to vehicle treated cells 100%. n=6).

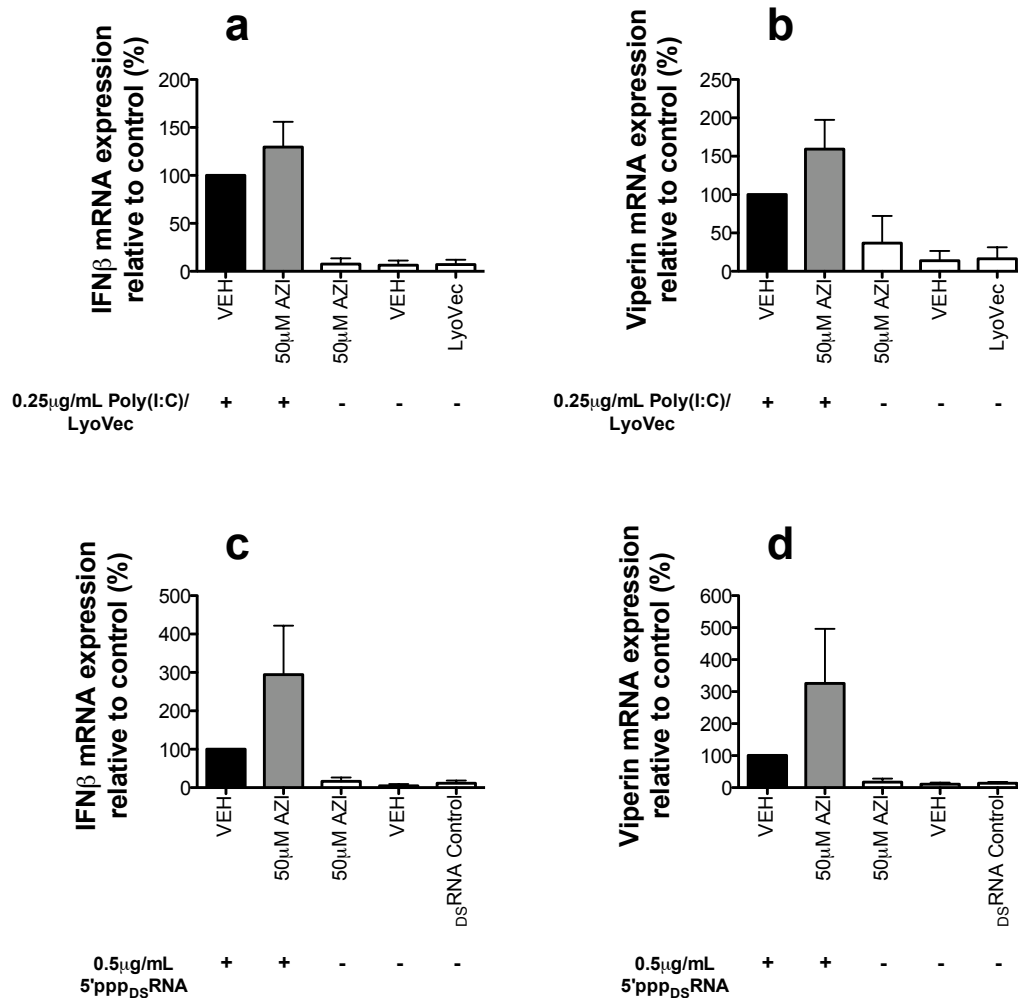


Figure 4.3 Azithromycin showed trends for augmenting IFN and ISG expression in normal HBECs stimulated with MAD-5 and RIG-I agonists.

Normal HBECs treated with Azithromycin (AZI) or vehicles (VEH) were stimulated with MDA-5 agonist (a-b) or RIG-I agonist (c-d) for 24 hours. AZI showed trends for augmenting expression of IFN β and Viperin in both MDA-5 and RIG-I stimulated cultures. Data expressed as % change over RV1b + VEH 100%. n=6.

4.4.2.3 Azithromycin fails to augment ISG expression in normal HBECs treated with recombinant IFN β

Normal HBECs were treated with 2.5ng/mL of recombinant IFN β for 8 hours following pre-treatment with 50 μ M Azithromycin or vehicle. Azithromycin treatment failed to augment levels of Viperin mRNA compared to vehicle control (Figure 4.4 Viperin: 100.9% augmentation compared to vehicle treated cells 100%. n=7).

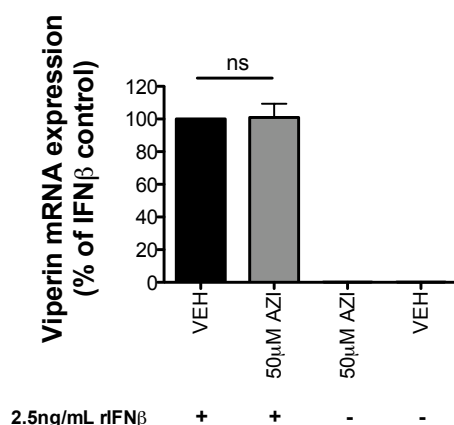


Figure 4.4 Azithromycin failed to augment Viperin expression in normal HBECs treated with recombinant IFN β .

Normal HBECs were treated with 50 μ M Azithromycin (AZI) or vehicle (VEH) for 24 hours prior to stimulated with recombinant IFN β (rIFN β) for 8 hours. AZI failed to augment mRNA expression of Viperin. Data expressed as % change over RV1b + VEH 100%. No data were statistically significant. n=7.

4.4.3 Azithromycin treatment increases levels of MAVS protein

Azithromycin is able to augment IFN responses to RV and synthetic agonists and in all instances it has been observed that Azithromycin treatment in the absence of infection/stimulation does not affect basal expression of IFNs or ISGs. It would therefore be logical to assume that Azithromycin is able to interact with components of the virus-sensing pathways (specifically the RLHs) in essence “priming” the cell for increased IFN production upon infection. In order to assess the effects of Azithromycin on uninfected normal HBECs (prior to infection) a proteomic screen was used. Normal HBECs were treated with 50 μ M Azithromycin for 24 hours, total protein lysates were isolated and assessed by mass spectrometry (mass spectrometry was performed by Dr. M. Khaitov of NRC Institute of Immunology, FMBA Russia). 149 proteins were detected to have a differential expression pattern between Vehicle and Azithromycin treated samples, from that 81 proteins were found to have a decreased expression profile and 68 proteins were found to have an increased expression profile in Azithromycin treated groups compared to vehicle treated groups. Of the 149 proteins, MAVS was the only protein with known anti-viral activity with an observed change in expression. (Figure 4.5 MAVS was increased by 2.00 fold in Azithromycin treated cells compared to Vehicle treated cells. n=1). This finding was confirmed in protein lysates from HBECs treated with 50 μ M Azithromycin for 24 hours by Western Blot and by densitometry analysis (Figure 4.6 MAVS protein density: 379% with 50 μ M AZI compared to VEH treatment control (100%). n=4). Interestingly, an increase in MAVS gene expression

was not observed (Figure 4.7 MAVS mRNA expression: 5.71×10^4 copies/ μ L compared to VEH control: 6.12×10^4 copies/ μ L. n=5).

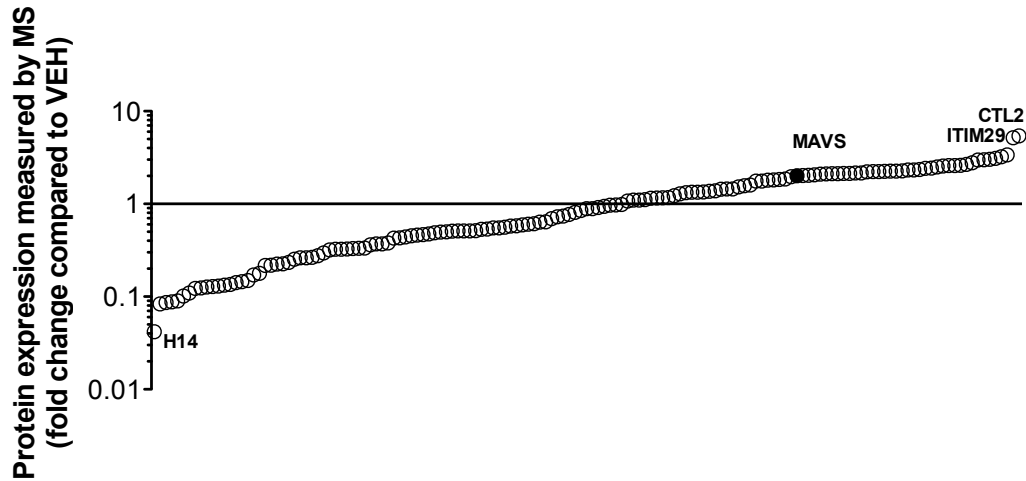


Figure 4.5 Azithromycin treatment in normal HBECs increases protein levels of MAVS .

Normal HBECs treated with 50 μ M Azithromycin or Vehicle (VEH) for 24 hours showed fluctuations in 149 different proteins (open circles ranging from CTL2 and ITIM29 to H14) with differing expressions between Veh and AZI treated samples were assessed by mass spectrometry. MAVS (closed circle) was increased by 2.00 fold compared to VEH. Data are expressed as fold change over VEH treatment, n=1.

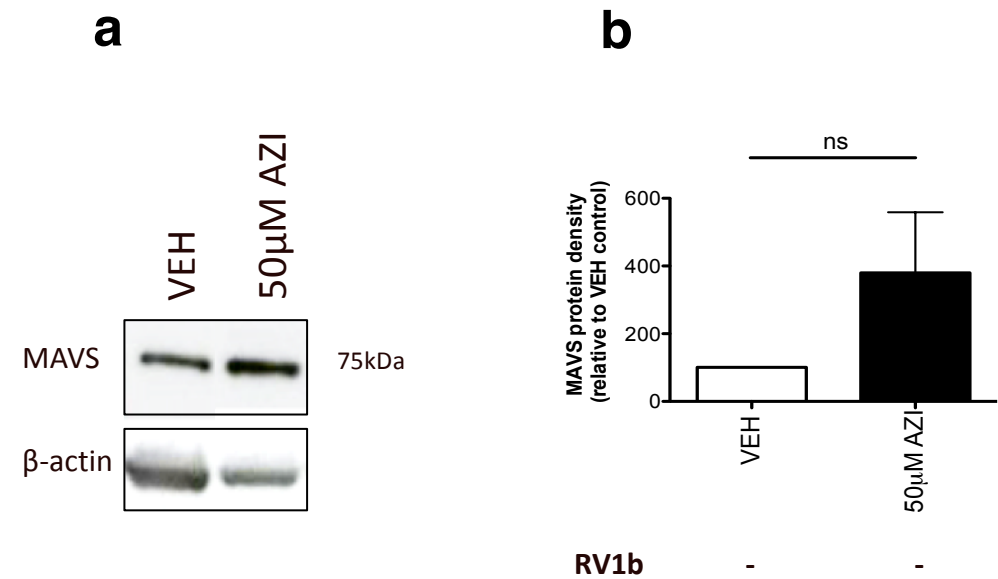


Figure 4.6 Azithromycin caused a non-significant increase in MAVS protein in HBECs.

Prior to infection, in HBECs treated with 50 μ M Azithromycin, MAVS protein was increased as detected by Western Blot (a). This was confirmed by densitometry analysis (b). Western Blot representative of 4 replicate experiments shown in (b). Data expressed as % change in MAVS protein density relative to vehicle (VEH) treated cells (100%) normalised to β -actin. n=4.

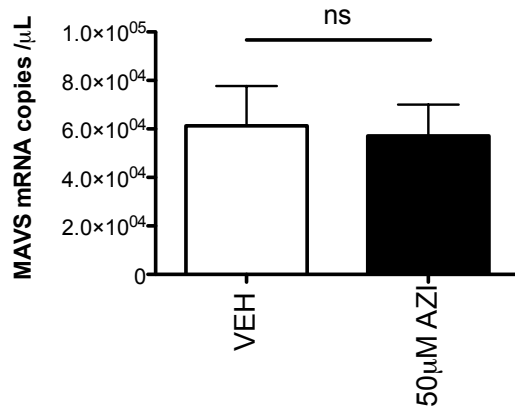


Figure 4.7 Azithromycin fails to increase levels of MAVS mRNA in HBECs.

Prior to infection, MAVS gene expression remains constant in HBECs treated with 50μM Azithromycin (AZI) 24 hours compared to Vehicle (VEH) treatment. n=5.

MAVS protein has previously been shown to be degraded post infection [130], we confirmed this degradation in normal HBECs infected with RV1b (Figure 4.8 RV infection caused the degradation of MAVS by 84.2% after 24 hours. n=4). 24 hours post infection with RV1b, normal HBECs pre-treated with Azithromycin showed increased levels of MAVS protein consistent with levels observed prior to infection (Figure 4.9 MAVS protein density: 1.65x10⁵% with 50μM AZI compared to VEH treatment control (100%). n=4). The MAVS band at 57kDa was inconsistently detected and was not included in any data analysis.

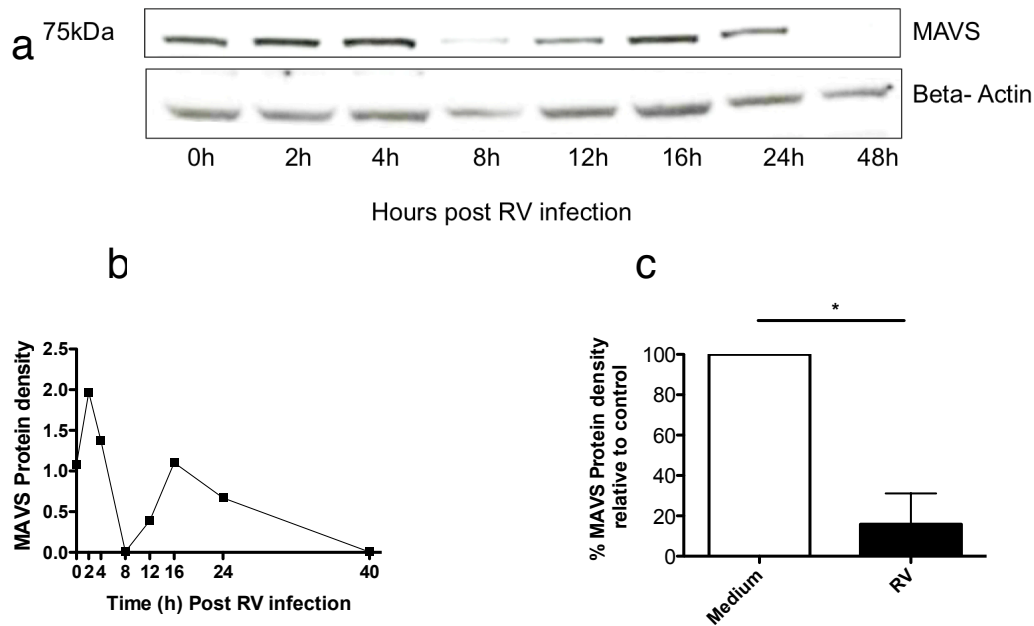


Figure 4.8 MAVS protein is degraded during infection with RV in normal HBECs

MAVS protein as detected by Western blot (a) is degraded over the course of a 48 hour RV infection in normal HBECs, this degradation appears biphasic in nature (b). At 24 hours post-infection, MAVS protein has degraded by 84.2% (c). Data is expressed as % change over un-infected (Medium) controls- 100% * $p < 0.05$. $n = 4$.

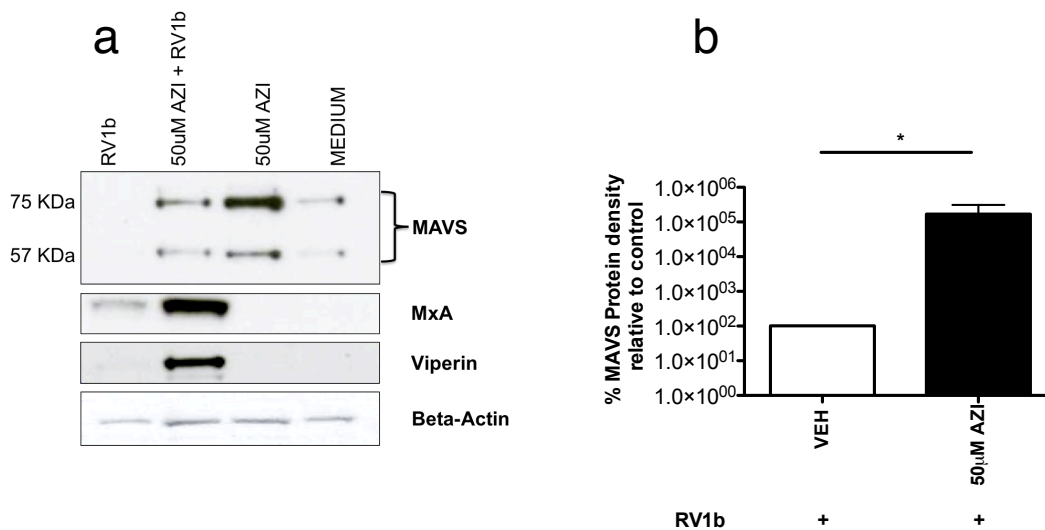


Figure 4.9 Azithromycin increases levels of MAVS protein 24 hours post RV1b infection.

In normal HBECs, MAVS was up regulated during RV infection by Azithromycin (AZI) pre-treatment as measured by Western blot. MxA and Viperin were also augmented by AZI treatment during RV infection (a) MAVS augmentation was confirmed by densitometry analysis (b). Data expressed as % change in MAVS protein density relative to vehicle (VEH) treated cells normalised to β -actin. * $p < 0.05$. $n = 3$.

4.4.4 The augmenting effects of Azithromycin may be dependent on MAVS protein regulators

MAVS is known to have a number of protein regulators that are associated with protein turnover, these include the chaperone protein NDFIP1 [132], Poly(C)-binding proteins PCBP1 [134] and PCBP2 [133], the ubiquitin ligases SMURF1 [132], RNF125 [236], ITCH (a.k.a AIP4) [133], the mitochondrial protein NLRX1 [237] and the proteasome subunit PSMA7 [238]. siRNA targeted towards MAVS and each of these regulators were transfected into HBEC cultures prior to 24 hours treatment with 50 μ M Azithromycin and infection with RV1b (MOI:1) for a further 24 hours. siRNA treatment reduced the expression of SMURF1, PCBP1, PCBP2, RNF125, ITCH, NLRX1 and PSMA7 by 75%-100% compared to non-targeting control siRNA over the course of 48 hours (Figures 4.9-4.15). siRNA was unable to reduce mRNA levels of MAVS and NDFIP1.

siRNA-mediated suppression of SMURF1 had little effect on the expression levels of RV-induced IFN β , IL-28, IL-29, or Viperin and caused a small decrease in basal levels of MxA expression. siRNA treatment suppressed Azithromycin-mediated augmentation of these genes (either completely or substantially compared to non-targeting control siRNA treatment) (Figure 4.10 percentage RV-induced mRNA expression with non-targeting control siRNA and 50 μ M AZI IFN β : 296.6%, IL-28: 135.1%, IL-29: 62.32%, MxA: 114.9% and Viperin: 44.0%. With SMURF1-targeted siRNA and VEH control IFN β : 269.6%, IL-28: 179.9%, IL-29: 94.4%, MxA: 126.8% and Viperin: 111.9%. Percentages relative to Vehicle treated RV-infected, non-targeting siRNA control treated normal HBECs (100%). n=4). Apart from confirmation of the knock down, no data were significant.

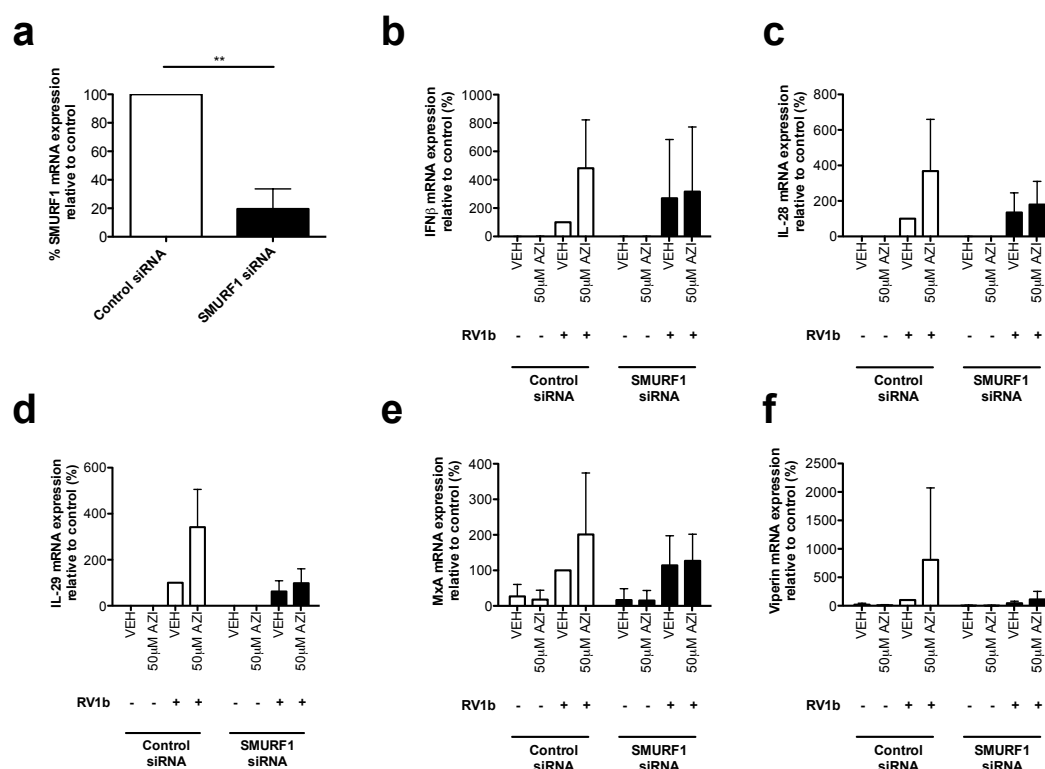


Figure 4.10 siRNA knock-down of SMURF1 inhibited Azithromycin-mediated augmentation of RV-induced anti-viral genes in normal HBECs.

siRNA treatment of normal HBECs targeted against SMURF1 demonstrated a reduction in mRNA expression in HBECs treated with 50 μ M AZI or VEH control over the course of 48 hours (a). In normal HBECs treated with siRNA, AZI was unable to augment levels of RV-induced (b) IFN β , (c) IL-28, (d) IL-29, (e) MxA and (f) Viperin. Data are expressed as % mRNA expression relative to non-targeting siRNA and VEH treated RV-infected HBECs, n=4.

siRNA-mediated suppression of PCBP1 suppressed RV-induced IFN β completely and reduced the expression of IL-28, IL-29, MxA and Viperin mRNA levels compared to non-targeting siRNA control. 50 μ M Azithromycin pre-treatment was able to augment levels of RV-induced IFN β , IL-28, IL-29, MxA and Viperin mRNA (Figure 4.11 percentage RV-induced mRNA expression with non-targeting control siRNA and 50 μ M AZI IFN β : 583.3%, IL-28: 432.6%, IL-29: 405.2%, MxA: 212.7% and Viperin: 991.3%. With PCBP1-targeted siRNA and VEH control IFN β : 0%, IL-28: 56.8%, IL-29: 77.7%, MxA: 85.5% and Viperin: 52.6%. With PCBP1-targeted siRNA and 50 μ M AZI IFN β : 251.5%, IL-28: 264.9%, IL-29: 178.5%, MxA: 140.2% and Viperin: 800.8%. Percentages relative to RV-infected, non-targeting siRNA control treated normal HBECs (100%). n=3). Apart from confirmation of the knock down, no data were significant.

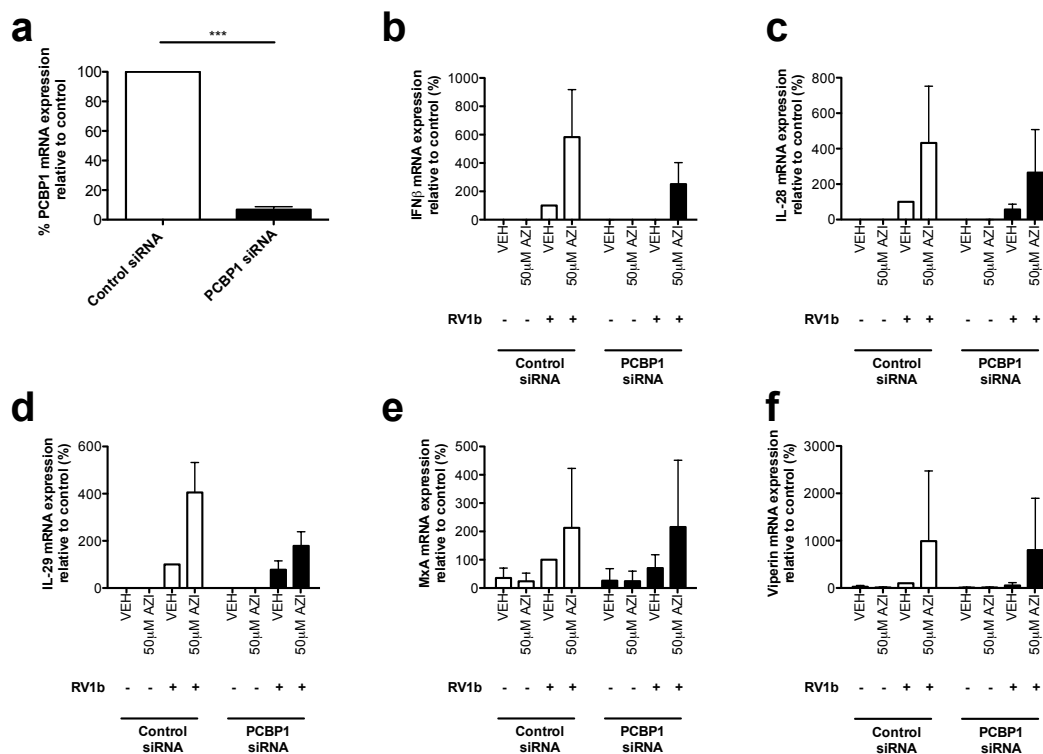


Figure 4.11 siRNA knock-down of PCBP1 did not inhibit Azithromycin-mediated augmentation of RV-induced anti-viral genes in normal HBECs.

siRNA treatment of normal HBECs with siRNA targeted against PCBP1 demonstrated a reduction in mRNA expression in HBECs treated with 50 μ M AZI or VEH control over the course of 48 hours (a). In normal HBECs treated with siRNA, AZI was able to augment levels of RV-induced (b) IFN β , (c) IL-28, (d) IL-29, (e) MxA and (f) Viperin. Data are expressed as % mRNA expression relative to non-targeting siRNA and VEH treated RV-infected HBECs, n=3.

siRNA-mediated suppression of PCBP2 slightly increased RV-induced IFN β , IL-28, IL-29, MxA and Viperin mRNA levels compared to non-targeting siRNA control. 50 μ M Azithromycin pre-treatment was able to augment levels of RV-induced IFN β , IL-28, IL-29 and MxA, however Viperin augmentation was reduced (Figure 4.12 percentage RV-induced mRNA expression with non-targeting control siRNA and 50 μ M AZI IFN β : 583.3%, IL-28: 432.6%, IL-29: 405.2%, MxA: 212.7% and Viperin: 991.3%. With PCBP2-targeted siRNA and VEH control IFN β : 156.5%, IL-28: 196.8%, IL-29: 117.0%, MxA: 128.8% and Viperin: 122.4%. With PCBP2-targeted siRNA and 50 μ M AZI IFN β : 346.1%, IL-28: 530.8%, IL-29: 293.4%, MxA: 200.3% and Viperin: 246.0%. Percentages relative to RV-infected, non-targeting siRNA control treated normal HBECs (100%). n=3). Apart from confirmation of the knock down, no data were significant.

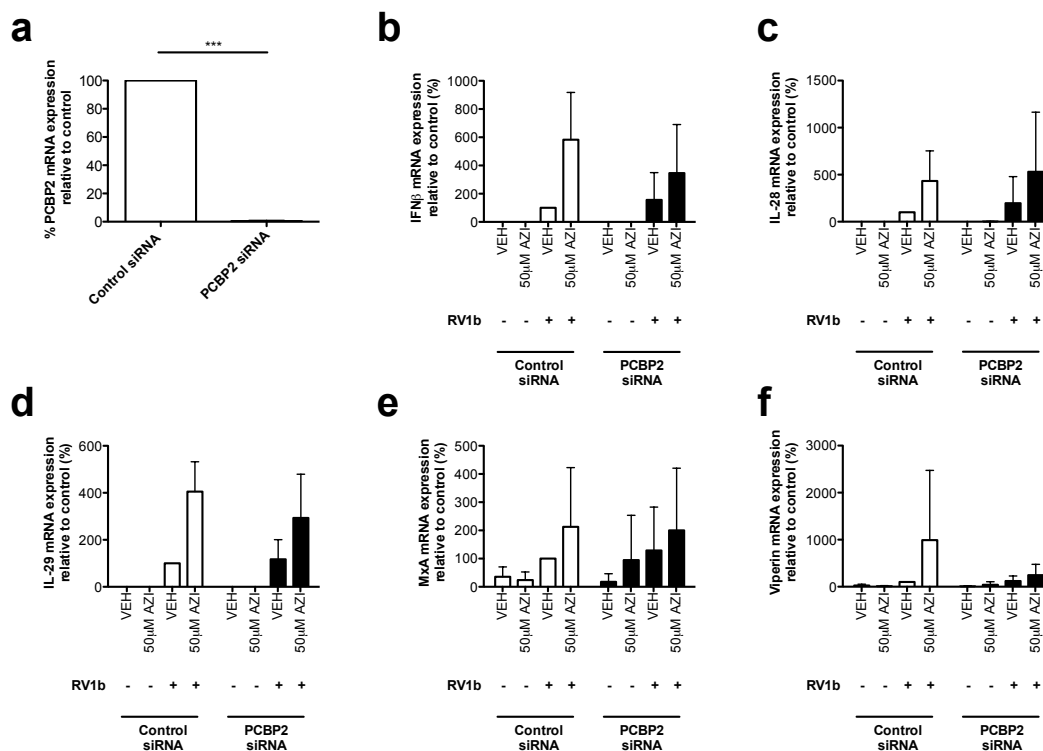


Figure 4.12 siRNA knock-down of PCBP2 did not inhibit Azithromycin-mediated augmentation of RV-induced anti-viral genes in normal HBECs. siRNA treatment of normal HBECs with siRNA targeted against PCBP2 demonstrated a reduction in mRNA expression in HBECs treated with 50 μ M AZI or VEH control over the course of 48 hours (a). In normal HBECs treated with siRNA, AZI was able to augment levels of RV-induced (b) IFN β , (c) IL-28, (d) IL-29, (e) MxA and (f) Viperin. Data are expressed as % mRNA expression relative to non-targeting siRNA and VEH treated RV-infected HBECs, n=3.

siRNA-mediated suppression of RNF125 reduced levels of RV-induced IL-28, IL-29, MxA and Viperin whilst increasing levels of IFN β mRNA compared to non-targeting siRNA control. 50 μ M Azithromycin pre-treatment was able to augment levels of RV-induced IFN β , IL-28, IL-29 and MxA however Viperin augmentation was reduced (Figure 4.13 percentage RV-induced mRNA expression with non-targeting control siRNA and 50 μ M AZI IFN β : 583.3% IL-28: 432.6%, IL-29: 405.2%, MxA: 212.7% and Viperin: 991.2%. With RNF125 -targeted siRNA and VEH control IFN β : 149.2%, IL-28: 65.7%, IL-29: 38.0%, MxA: 50.3% and Viperin: 48.5%. With RNF125-targeted siRNA and 50 μ M AZI IFN β : 734.4%, IL-28: 535.7%, IL-29: 380.0%, MxA: 183.7% and Viperin: 216.5%. Percentages relative to RV-infected, non-targeting siRNA control treated normal HBECs (100%). n=3). Apart from confirmation of the knock down, no data were significant.

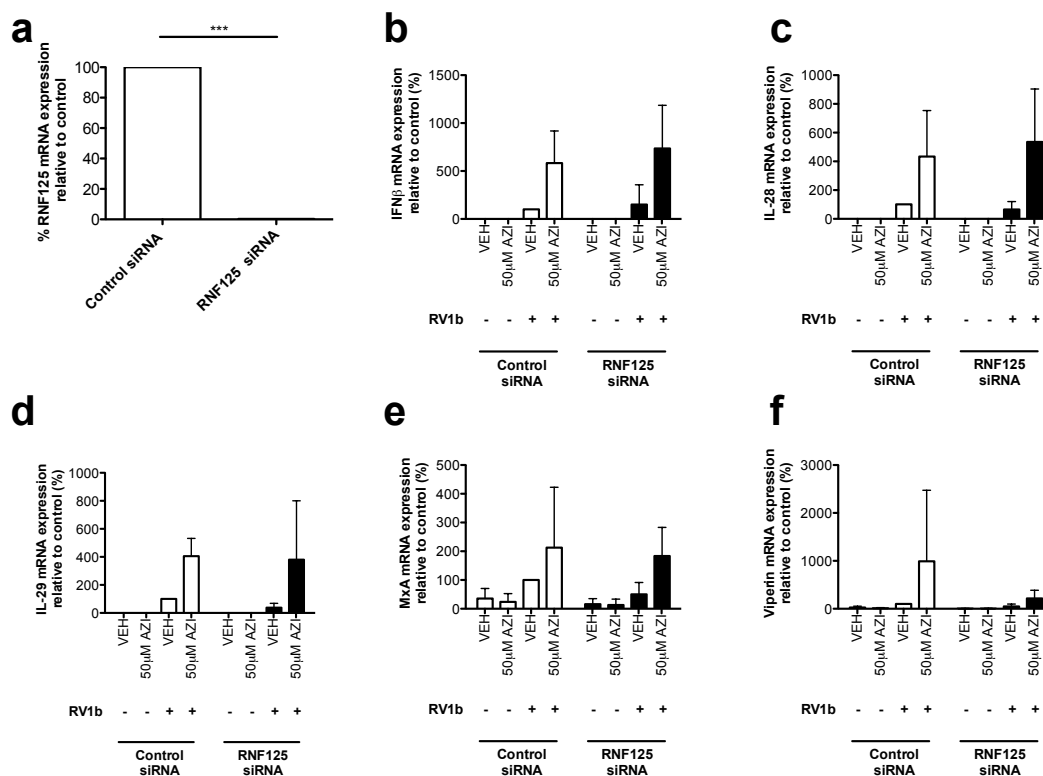


Figure 4.13 siRNA knock-down of RNF125 did not inhibit Azithromycin-mediated augmentation of RV-induced anti-viral genes in normal HBECs.

siRNA treatment of normal HBECs with siRNA targeted against RNF125 demonstrated a reduction in mRNA expression in HBECs treated with 50 μ M AZI or VEH control over the course of 48 hours (a). In normal HBECs treated with siRNA, AZI was able to augment levels of RV-induced (b) IFN β , (c) IL-28, (d) IL-29, (e) MxA and (f) Viperin. Data are expressed as % mRNA expression relative to non-targeting siRNA and VEH treated RV-infected HBECs, n=3.

siRNA-mediated suppression of ITCH showed a modest increase in RV-induced IFN β , a decrease in IL-29 and no change in IL-28, MxA or Viperin mRNA levels compared to non-targeting siRNA control. 50 μ M Azithromycin pre-treatment was able to augment levels of RV-induced IFN β , IL-28, IL-29 and MxA however Viperin augmentation was reduced (Figure 4.14 percentage RV-induced mRNA expression with non-targeting control siRNA and 50 μ M AZI IFN β : 583.5%, IL-28: 432.6%, IL-29: 405.2%, MxA: 212.7% and Viperin: 991.2%. With ITCH-targeted siRNA and VEH control IFN β : 182.9%, IL-28: 72.6%, IL-29: 35.2%, MxA: 84.6% and Viperin: 88.7%. With ITCH-targeted siRNA and 50 μ M AZI IFN β : 291.7%, IL-28: 231.7%, IL-29: 87.0%, MxA: 124.2% and Viperin: 95.5%. Percentages relative to RV-infected, non-targeting siRNA control treated normal HBECs (100%). n=3). Apart from confirmation of the knock down, no data were significant.

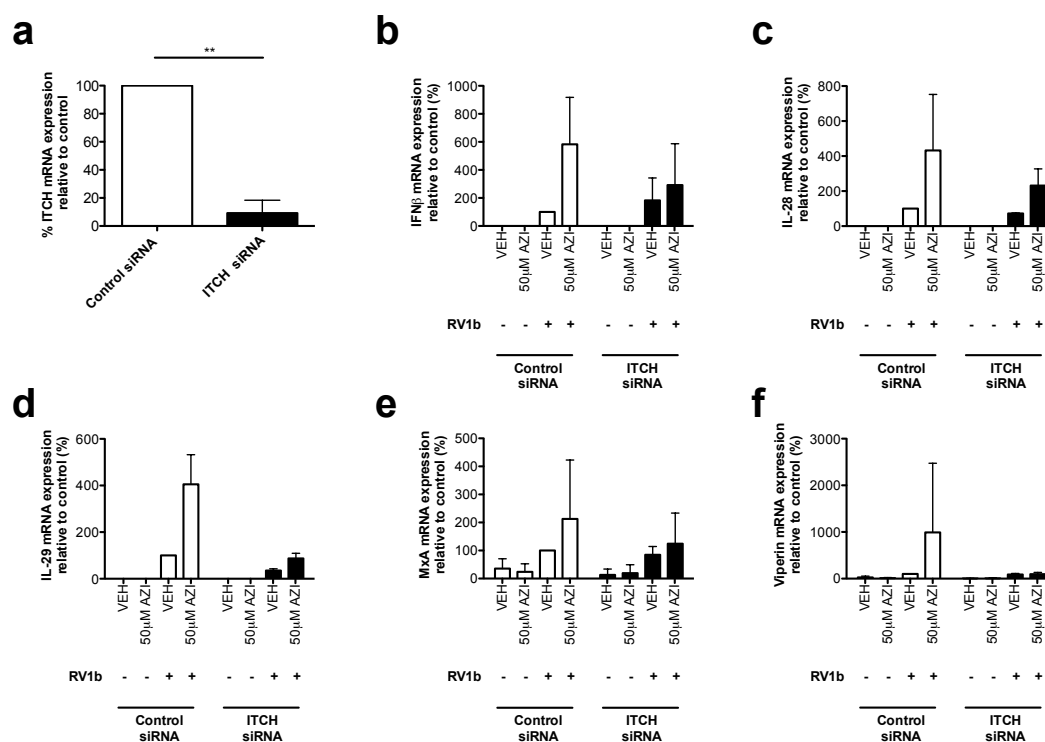


Figure 4.14 siRNA knock-down of ITCH did not inhibit Azithromycin-mediated augmentation of RV-induced anti-viral genes in normal HBECs.

siRNA treatment of normal HBECs with siRNA targeted against ITCH demonstrated a reduction in mRNA expression in HBECs treated with 50 μ M AZI or VEH control over the course of 48 hours (a). In normal HBECs treated with siRNA, AZI was able to augment levels of RV-induced (b) IFN β , (c) IL-28, (d) IL-29, (e) MxA and (f) Viperin. Data are expressed as % mRNA expression relative to non-targeting siRNA and VEH treated RV-infected HBECs, n=3.

siRNA-mediated suppression of NLRX1 showed an increase in RV-induced IFN β , IL-28 and MxA and a small reduction in IL-29 and Viperin mRNA levels compared to non-targeting siRNA control. 50 μ M Azithromycin pre-treatment was able to augment levels of RV-induced IFN β , IL-29, MxA and Viperin however IL-29 augmentation was reduced (Figure 4.14 percentage RV-induced mRNA expression with non-targeting control siRNA and 50 μ M AZI IFN β : 583.3%, IL-28: 432.6%, IL-29: 405.2%, MxA: 212.7% and Viperin: 991.2%. With NLRX1-targeted siRNA and VEH control IFN β : 280.8%, IL-28: 170.5%, IL-29: 53.49%, MxA: 130.6% and Viperin: 59.9%. With NLRX1-targeted siRNA and 50 μ M AZI IFN β : 815.4%, IL-28: 642.6%, IL-29: 89.7%, MxA: 332.1% and Viperin: 197.6%. Percentages relative to RV-infected, non-targeting siRNA control treated normal HBECs (100%). n=3). Apart from confirmation of the knock down, no data were significant.

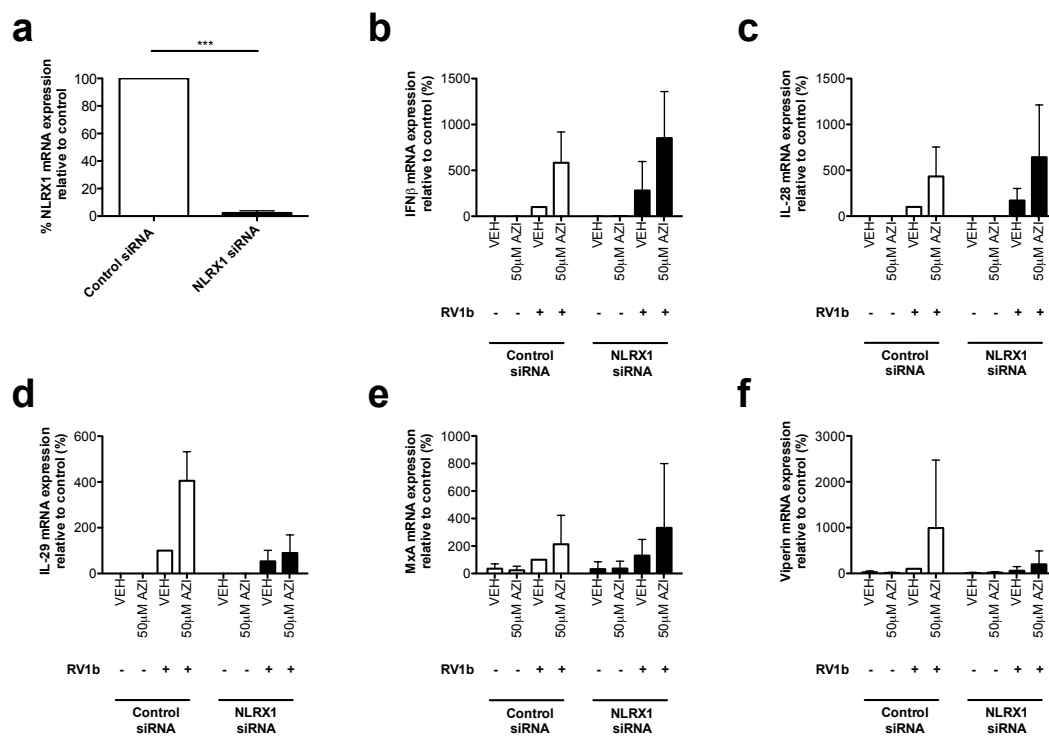


Figure 4.15 siRNA knock-down of NLRX1 did not inhibit Azithromycin-mediated augmentation of RV-induced anti-viral genes in normal HBECs.

siRNA treatment of normal HBECs with siRNA targeted against NLRX1 demonstrated a reduction in mRNA expression in HBECs treated with 50 μ M AZI or VEH control over the course of 48 hours (a). In normal HBECs treated with siRNA,, AZI was able to augment levels of RV-induced (b) IFN β , (c) IL-28, (d) IL-29, (e) MxA and (f) Viperin. Data are expressed as % mRNA expression relative to non-targeting siRNA and VEH treated RV-infected HBECs, n=3.

siRNA-mediated suppression of PSMA7 showed an increase in mRNA expression of RV-induced IFN β , IL-28, and MxA, with a reduction in IL-29 and Viperin compared to non-targeting siRNA control. 50 μ M Azithromycin pre-treatment was able to augment levels of RV-induced IFN β , IL-29, MxA and Viperin however IL-29 augmentation was reduced (Figure 4.14 percentage RV-induced mRNA expression with non-targeting control siRNA and 50 μ M AZI IFN β : 583.3%, IL-28: 432.6%, IL-29: 405.2%, MxA: 212.7% and Viperin: 991.2%. With PSMA7-targeted siRNA and VEH control IFN β : 231.6%, IL-28: 138.5%, IL-29: 55.8%, MxA: 124.87% and Viperin: 76.2%. With PSMA7-targeted siRNA and 50 μ M AZI IFN β : 848.9%, IL-28: 808.2%, IL-29: 121.7%, MxA: 512.0% and Viperin: 399.3%. Percentages relative to RV-infected, non-targeting siRNA control treated normal HBECS (100%). n=3). Apart from confirmation of the knock down, no data were significant.

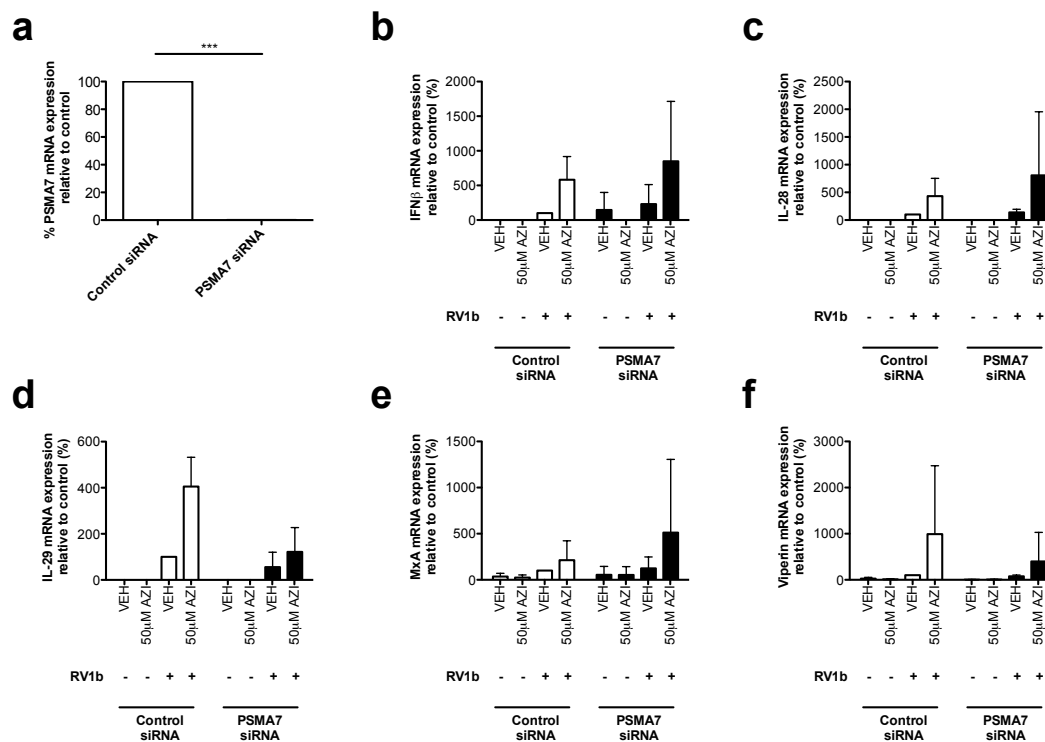


Figure 4.16 siRNA knock-down of PSMA7 did not inhibit Azithromycin-mediated augmentation of RV-induced anti-viral genes in normal HBECS.

siRNA treatment of normal HBECS with siRNA targeted against PSMA7 demonstrated a reduction in mRNA expression in HBECS treated with 50 μ M AZI or VEH control over the course of 48 hours (a). In normal HBECS treated with siRNA,, AZI was able to augment levels of RV-induced (b) IFN β , (c) IL-28, (d) IL-29, (e) MxA and (f) Viperin. Data are expressed as % mRNA expression relative to non-targeting siRNA and VEH treated RV-infected HBECS, n=3.

4.4.5 Azithromycin has multiple potential protein binding targets

To investigate a possible binding partner of Azithromycin, a novel Azithromycin analogue was synthesized by Pfizer Inc. containing an Azide handle (AZI-Azide Figure 4.17a and b). In a pull-down assay, AZI-Azide was bound to alkene-agarose beads before being incubated with total protein lysates from normal HBECs. Post incubation, AZI-Azide (bound to alkene-agarose beads and any potential binding proteins) were separated by centrifugation and analysed by mass-spectrometry. In parallel, total protein lysates were incubated with 50 μ M Azithromycin prior to incubation with AZI-Azide to act as a competition assay. Initially the AZI-Azide compound was tested for its ability to augment IFN responses in HBECs during RV infection. As per the previous studies, 50 μ M AZI-Azide was added to normal HBECs cultures for 24 hours prior to infection with RV1b for a further 24 hours, AZI-Azide augmented IFN β and Viperin mRNA levels to a similar level observed with Azithromycin (Figure 4.17 IFN β mRNA expression with AZI-Azide: 301.9%, Viperin mRNA: 254.9% compared to vehicle treated cells 100%. n=3).

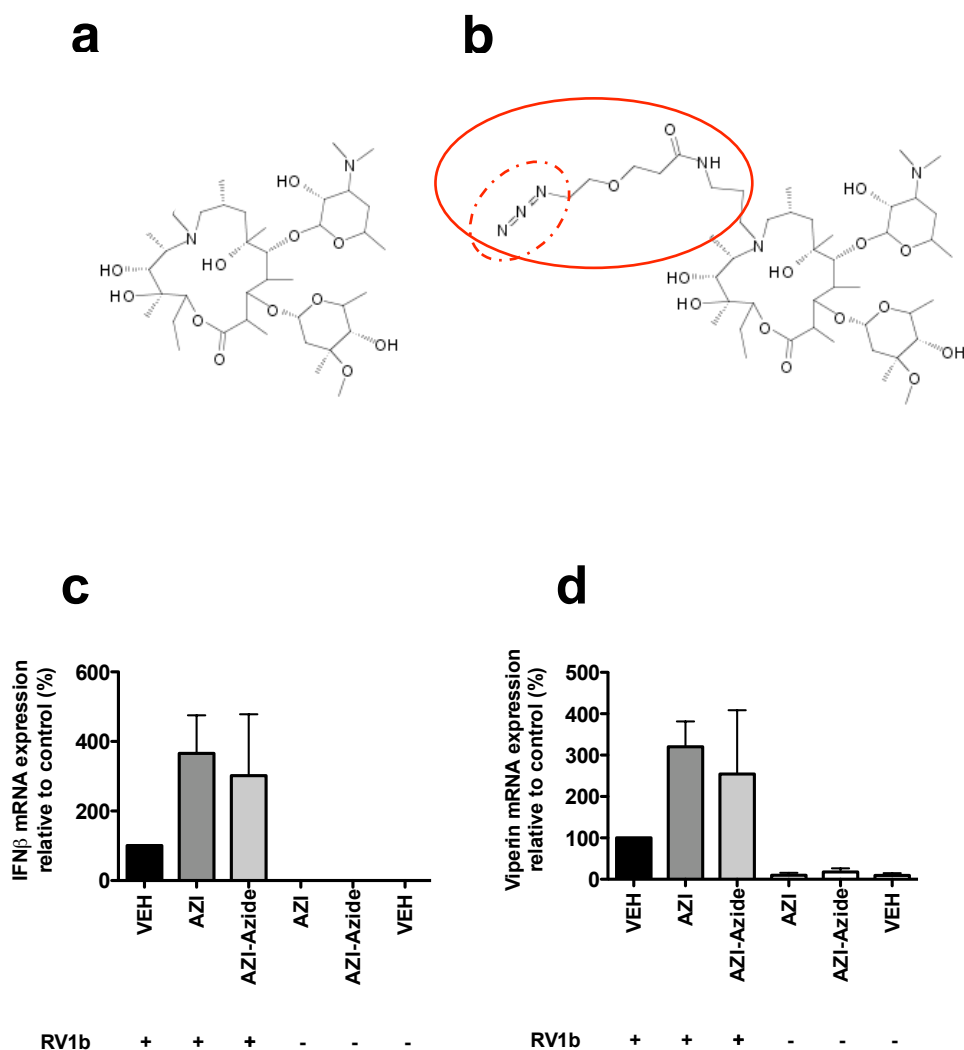


Figure 4.17 AZI-Azide showed trends for augmenting IFN and ISG mRNA to similar levels of Azithromycin.

The molecular structure of Azithromycin (a) and AZI-Azide probe, the synthesised 'arm' (solid ring) containing the Azide residue (dashed ring) is highlighted. (Normal HBECs treated with 50 μ M AZI-Azide show augmented levels of RV1b-induced IFN β (c) and Viperin (d) to similar levels to 50 μ M Azithromycin (AZI). No data was statistically significant. n=3.

From two independent experiments, a large number of proteins were identified as possible Azithromycin binding partners. Proteins pulled down by AZI-Azide can be divided into those that failed to bind AZI-Azide in the presence of Azithromycin competition (and thus are the strongest candidates for Azithromycin binders), those that bound AZI-Azide less in the presence of Azithromycin competition (that are potential Azithromycin binders) and proteins that bound AZI-Azide more in the presence of Azithromycin competition (that are not likely to be potential Azithromycin binders). MAVS was identified as a strong potential binding partner in the initial experiment, however it was not identified in the subsequent experiment. VCP

and PSMA7 (the proteosomal subunit known to be involved in MAVS degradation) were identified as potential Azithromycin binding partners in both experiments (Figure 4.18 details the range of identified proteins). In addition to VCP, a number of proteins were identified as potential binding partners in both experiments (Table 4.1 details the top 10 binding proteins n=2, see appendix for the full list).

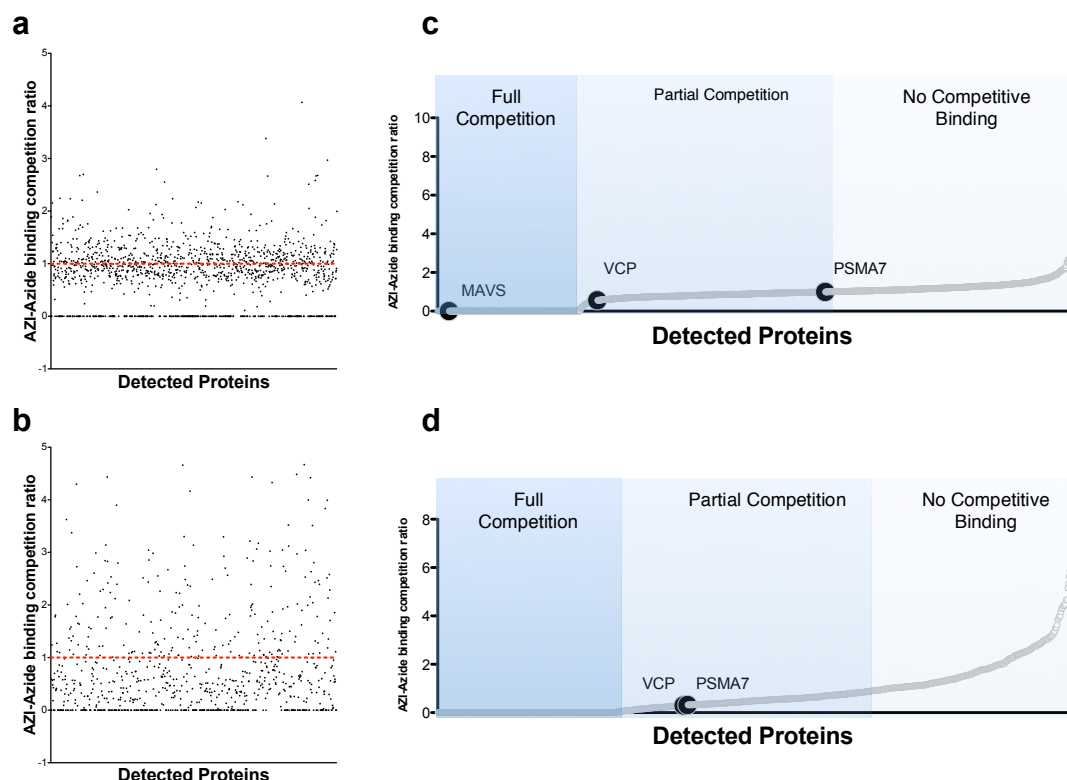


Figure 4.18 AZI-Azide binding experiments identified numerous possible Azithromycin binding partners.

Mass spectrometry analysis from two independent experiments (a/c and b/d) identified a large number of AZI-Azide binding partners. a-b: proteins with a competitive binding ratio of ≤ 1 (dashed line) are potential binding partners, proteins with a competitive binding ratio of ≥ 1 are non-binders. c-d: Proteins can be sub-grouped into ones failed to bind AZI-Azide with Azithromycin Competition (Full Competition, competition ratio of 0), bound AZI-Azide partially with Azithromycin Competition (Partial Competition, competition ratio of 0.001-1) or bound AZI-Azide greater with Azithromycin competition (No Competitive Binding competition ratio of ≥ 1). MAVS was detected in one experiment, PSMA7 and VCP were detected as potential binding partners in both experiments (closed circles).

Table 4.1 Top 10 AZI-Azide protein-binding partners identified on both experiments.

Protein ID	Known Function	Notes
Reticulocalbin 2	Calcium Binding Protein	
Biliverdin Reductase B	Converts biliverdin to bilirubin	
Ku70	DNA repair	
Elongation Factor 1-B	Ribosomal Translation	
Peroxiredoxin 1	Anti-oxidant	
Valosin-Containing Protein	Involved in autophagy	Known binding partner of Azithromycin. Known to suppress poliovirus replication.
ATP-Citrate Lyase	Critical enzyme for the synthesis of cytosolic acetyl-CoA	
Plastin 3	Actin Binding protein	
Pyruvate dehydrogenase (lipoamide) beta	Component of the PDH complex	
Adenylate Cyclase-Associated Protein 1	Multi-functional Actin binding protein	

4.4.6 Azithromycin-augmentation of IFN may be virus specific

To determine whether Azithromycin augmenting responses to RV in normal HBECs was an RV specific effect, 3 other respiratory viruses were tested; influenza H1N1, influenza H3N2 and RSV-2A.

4.4.6.1 Azithromycin showed trends for augmenting IFN and ISG responses in Normal HBECs infection with influenza H1N1

Normal HBECs were treated with 10 μ M or 50 μ M of Azithromycin or vehicle control for 24 hours followed by infection with influenza H1N1 (MOI:1) for a further 24 hours. mRNA expression of type I IFNs and ISG showed a slight trend to augmentation with 50 μ M Azithromycin treatment compared to vehicle control (Figure 4.19 10 μ M/50 μ M of Azithromycin IFN β : 99.6/117.4%, IL-28: 111.8/152.3%, IL-29: 119.0/118.6%, MxA: 100.6/110.4% and Viperin: 125.3/153.3% compared to vehicle treatment 100%).

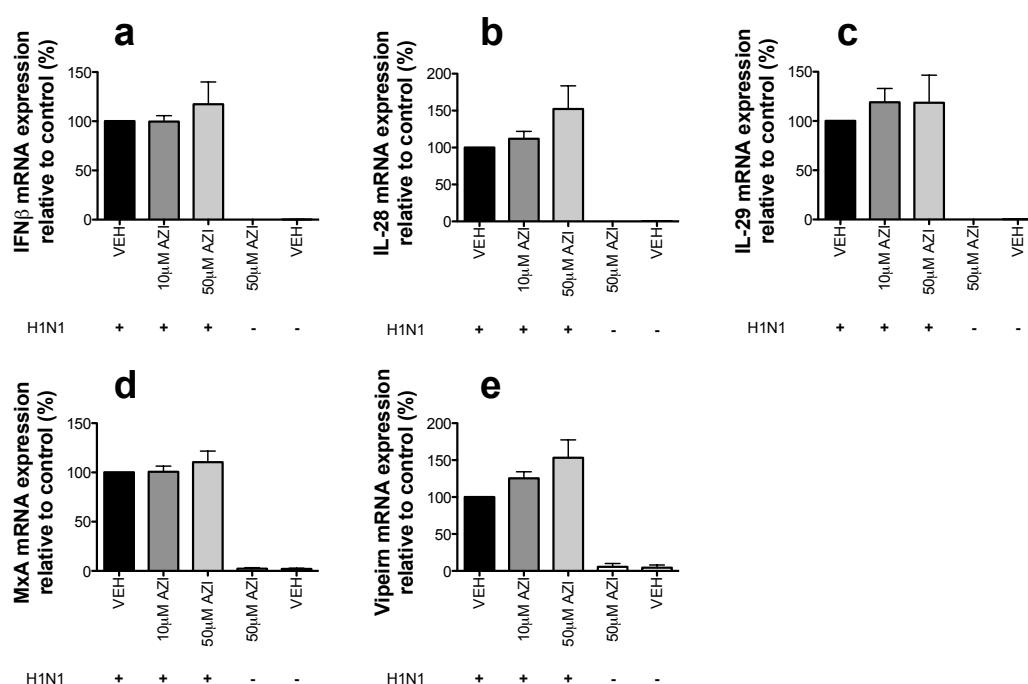


Figure 4.19 Azithromycin showed trends for augmenting IFN and ISG expression in normal HBECs infected with influenza H1N1.

Normal HBECs were treated with 50 μ M or 10 μ M Azithromycin (AZI) or vehicle (VEH) for 24 hours prior to infection with influenza H1N1 (MOI:1) for 24 hours. 50 μ M AZI showed trends for augmenting IFN β (a), IL-28 (b), IL-29 (c), MxA (d) and Viperin (e). Data expressed as % change over RV1b + VEH 100%. No data were statistically significant. n=3.

4.4.6.2 Azithromycin showed trends for augmenting IFN and ISG responses in normal HBECs infection with influenza H3N2

Normal HBECs were treated with 10 μ M or 50 μ M of Azithromycin or vehicle control for 24 hours followed by infection with influenza H3N2 (MOI:1) for a further 24 hours. mRNA expression of IFN β , IL-29 and MxA showed no augmentation with 50 μ M Azithromycin, IL-28 and Viperin showed a trend for augmentation with azithromycin treatment compared to vehicle control (Figure 4.20 mRNA expression with 10 μ M/50 μ M of Azithromycin, IFN β : 102.9/97.5%, IL-28: 99.7/125.0%, IL-29: 94.3/97.9%, MxA: 94.1/95.9% and Viperin: 99.7/125.0% compared to vehicle treatment 100%).

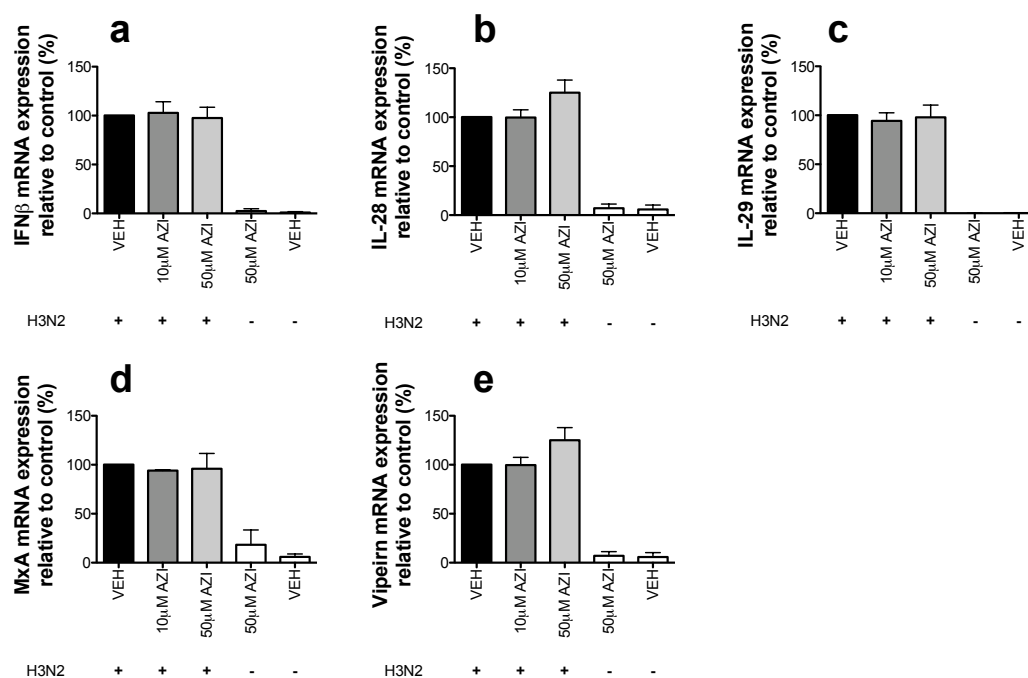


Figure 4.20 Azithromycin showed trends for augmentation of IFN and ISG expression in normal HBECS infected with influenza H3N2.

Normal HBECS were treated with 50μM or 10μM Azithromycin (AZI) or vehicle (VEH) for 24 hours prior to infection with influenza H3N2 (MOI:1) for 24 hours. 50μM AZI showed trends for augmenting IL-28 (b) and Viperin (e) but failed to augment expression of IFNβ (a), IL-29 (c) or MxA (d). Data expressed as % change over RV1b + VEH 100%. No data were statistically significant n=3.

4.4.6.3 Azithromycin failed to augment IFN and ISG responses to RSV infection in normal HBECS

Normal HBECS were treated with 10μM or 50μM of Azithromycin or vehicle control for 24 hours followed by infection with RSV-2A (MOI:1) for a further 24 hours. Azithromycin failed to augment mRNA expression of type I or type III IFNs or ISGs and conversely showed a trend towards a dose dependent decrease in mRNA expression (Figure 4.21 mRNA expression with 10μM/50μM of Azithromycin, IFNβ: 62.7/57.6%, IL-28: 65.9/40.8%, IL-29: 70.1/62.6%, MxA: 69.9/59.8% and Viperin: 74.1/64.5% compared to vehicle treatment 100%).

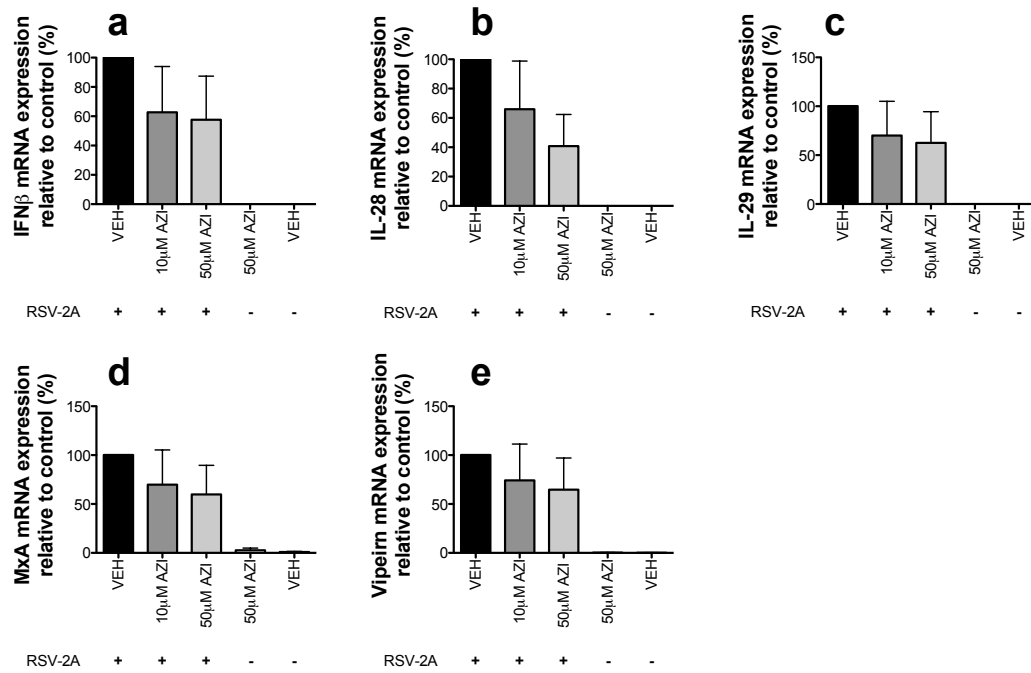


Figure 4.21 Azithromycin failed to augment IFN or ISG mRNA expression in normal HBECs infected with RSV-2A.

Normal HBECs were treated with 50 μ M or 10 μ M Azithromycin (AZI) or vehicle (VEH) for 24 hours prior to infection with RSV-2A (MOI:1) for 24 hours. 50 μ M AZI decreased mRNA expression of IFN β (a), IL-28 (b), IL-29 (c), MxA (d) and Viperin (e). Data expressed as % change over RV1b + VEH 100%. No data were statistically significant. n=3.

4.5 Discussion

In this chapter we described the effects of Azithromycin on a number of different stimuli known to induce IFN expression in normal HBECs. By substituting RV infection for specific PRR agonists we have shown that RIG-I and MDA-5 signalling pathways are responsive to augmentation by Azithromycin treatment. Conversely, we have shown that Azithromycin has no effects on anti-viral responses initiated by the stimulation of TLR-3 nor by recombinant IFN β and whilst we cannot comment on the effects of Azithromycin on TLR7/8-driven IFN production, it would seem apparent that Azithromycin's effects are limited to the RLH pathway. Through proteomic screening we have shown that MAVS, a protein limited to the RLH pathway is increased in normal HBECs treated with Azithromycin and that this increase is observed after 24 hours of infection with RV. The increase of MAVS protein does not originate by increased transcription, suggesting azithromycin affects MAVS at the protein level.

We next assessed the effects of Azithromycin whilst knocking down seven different MAVS-regulatory proteins by siRNA. Of the seven proteins assessed; SMURF1 was the only one to inhibit the augmenting effects of Azithromycin when knocked down by siRNA (although this was not statistically significant). Other MAVS regulatory proteins appeared not to be essential for the Azithromycin-mediated augmentation of all 5 RV-induced anti-viral genes. ITCH, another E3 ligase, appeared to be required for the Azithromycin-mediated augmentation of three anti-viral genes out of the five examined. We have identified a number of possible Azithromycin-binding proteins including MAVS and one of its regulators; PSMA7. Finally we have shown that the effects of Azithromycin are not readily observed in other models of respiratory viral infections compared to RV infection, suggesting that Azithromycin's augmentative ability may be limited to certain viral infections. Overall the data point towards Azithromycin's ability to modulate MAVS degradation, we hypothesise that this is responsible for the observed augmentation of RV-induced IFN and ISG expression by Azithromycin.

The effect of Azithromycin on the protein levels of MAVS were confirmed directly by two independent methods (mass spectrometry and WB analysis). MAVS is a key adapter in the RIG-I and MDA-5 signalling pathways however it is not utilised by TLR-3 [112], thus the involvement of MAVS was indirectly confirmed by the augmentation of IFN/ISG expression during RIG-I and MDA-5 stimulation but not TLR-3 stimulation [112, 248]. Our data is suggestive that Azithromycin is able to increase levels of MAVS prior to infection and prolong

the presence of MAVS during infection within the host cell, theoretically resulting in prolonged signalling of the RLH pathways and augmented IFN expression.

A large number of studies have highlighted a number of post-translational regulators of MAVS, including NDFIP1, PCBP1, PCBP2, RNF125, ITCH, SMURF1, NRLX1 and PSMA7 [132-134, 236-238]. We were able to demonstrate that knocking down the expression of SMURF1 appeared to be able to disrupt the augmenting effects of Azithromycin on RV-induced IFN and ISG mRNA expression although no data was significant. Interestingly, knock-down of SMURF1 (or any of the other regulators that were successfully suppressed) did not increase the overall IFN and ISG expression pattern as one might expect from the suppression of a negative regulator of RLH signalling. This may be due to the redundancy observed in the negative regulation of MAVS, compensating for the loss of SMURF1. The fact that no change in Azithromycin-mediated augmentation was observed with siRNA targeted against PCBP1, PCBP2, RNF125, ITCH, NLRX1 or PSMA7 may explain the modest and variable effects of Azithromycin, if it can only augment IFN production by acting through one of the many MAVS regulatory proteins.

Previously, the only known binding proteins of Azithromycin were the prokaryotic 50S ribosomal subunit (mediating the molecule's antibiotic effects) [244] and (in eukaryotes) valosin-containing protein (VCP) [245]. Using an Azithromycin analogue probe we have detected a vast number of possible protein binding partners within total protein lysates from normal HBECs, including MAVs and one of its known regulators. Over the course of two independent experiments VCP was in the top-ten likely binding proteins. Interestingly, a large number of other possible binding proteins were known to be mitochondria-associated and involved in cell metabolism, MAVS protein is also mitochondria-associated and effective mitochondrial function has previously been associated with efficient anti-viral activity [235, 249]. Azithromycin is also known to favourably associate with phospholipid membrane structures within cells [250] suggesting that Azithromycin may interact with a number of mitochondria-associated proteins that may indirectly affect MAVS function. At this point we can not discriminate between the possible processes that Azithromycin may affect mitochondrial function as a whole and thus also affect MAVs signalling; or primarily binds to and affects MAVs, and thus indirectly affects the mitochondria. This novel technique of using an AZI-Azide probe to detect Azithromycin binding partners has yielded a vast number of possible protein interactions, (a total of 153 possible proteins were observed in both experiments- see appendix for the list), in order to verify the validity of these interactions

numerous repeats of the assay should be performed. Due to time and economical restraints only two duplicate experiments could be performed here.

The effects observed with Azithromycin in models of RSV and influenza infection support Azithromycin's mechanism of action during RV infection. Our data suggested that Azithromycin had little effect on the levels of RSV or influenza-induced IFN/ISG expression, this may be due to the presence of immune subversion mechanisms known to be present during these viral infections. Firstly, RSV is known to produce NS1/2 proteins capable of interacting with MAVS, it is thought that this interaction acts as a viral mechanism of inhibiting IFN production [129]. Similarly, during influenza infection the inhibition of MAVS by the non-structural protein PB1 was demonstrated in human and murine dendritic cells [127]. If Azithromycin mediated its effects through MAVS, then the inhibition of MAVS signalling (present within RSV and Influenza but critically not during RV infection) could prevent or lessen the effects of Azithromycin. Further studies making use of RSV/influenza variants that lack functional NS1 or PB1 could be used to confirm this effect, one would expect that Azithromycin would be able to augment IFN production during infection with these defective mutants.

Although the stimulation of cells with foreign RNA (or in our case, RV infection) is known to cause the degradation of MAVS [130], the current literature suggests that MAVS is not degraded during influenza and RSV infection. It may be that NS1 and PB1 (respectfully) are able to perturb MAVS turnover and thus Azithromycin may not show signs of augmenting anti-viral genes during infection with these viruses. Unfortunately due to time constraints we were unable to determine MAVS turnover in our model of Azithromycin-induced gene augmentation, future experiments should investigate this as it may confirm Azithromycin acting through MAVS.

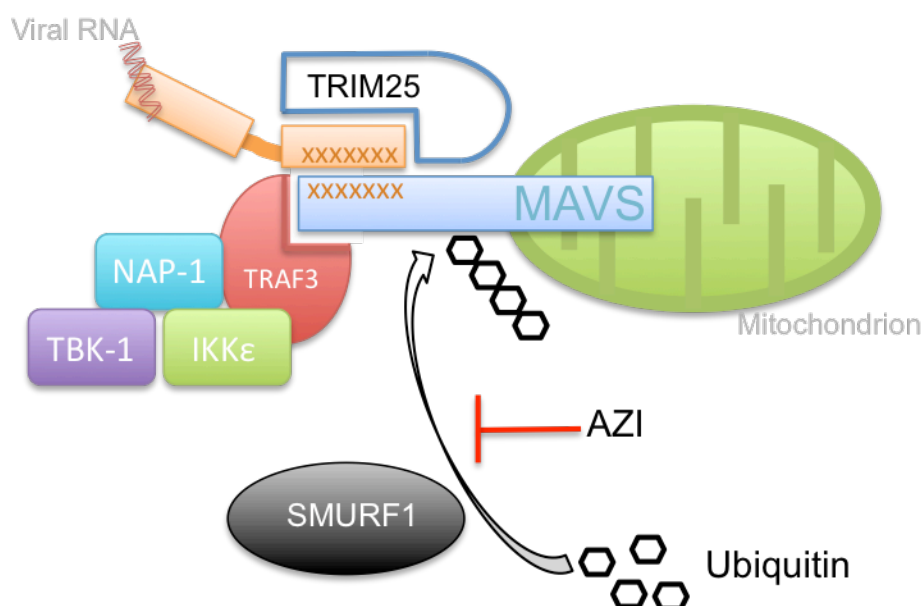


Figure 4.22 Proposed mechanism of Azithromycin-mediated anti-viral activity.

Azithromycin is able to prevent the degradation of MAVS via direct or indirect interaction with the E3-ubiquitin ligase SMURF1, possibly inhibiting ubiquitin-dependent proteasomal degradation..

In this chapter we described a possible mechanism of action of Azithromycin-mediated RV-induced IFN and ISG augmentation. Due to the multitude of immuno-modulatory effects associated with the molecule, it's variable levels of gene augmentation observed here and in other studies, it's affinity for membranes and high cellular retention, it is likely that Azithromycin is capable of a vast number of cellular interactions (be them transient or robust). A number of studies would be required to identify all of these interactions and to fully understand the nature of the molecule. Our data suggested that small molecules such as Azithromycin or novel analogues that are capable of binding MAVS and preventing it's degradation (as proposed in Fig 4.22) may prove effective in the treatment of RV-associated asthma exacerbations.

Chapter 5 Azithromycin augments IFN responses *in vivo*

5.1 Introduction

We have previously shown that Azithromycin is able to augment anti-viral activity to RV infection and responses to synthetic agonists that stimulate the RLH pathways *in vitro*. However to date few studies have assessed the effects of Azithromycin on an *in vivo* model of viral infection. As previous studies have shown that infection of mice with high-titre RV1b is able to induce a robust IFN response [51, 52], we therefore hypothesised that the administration of Azithromycin prior to infection may be able to augment the IFN response similarly to that observed *in vitro*. To date very few studies have assessed the role of macrolides in the context of innate responses to respiratory viral infection *in vivo*. Azithromycin has been shown to attenuate Sendai virus-induced weight loss, reduce the levels of BAL neutrophils and decrease the production of inflammatory mediators in a mouse model of viral infection [251]. Sato *et al* showed that Erythromycin significantly improved the survival rate of mice infected with a lethal dose of influenza [252]. No studies so far have evaluated the affect of Azithromycin on anti-viral IFN production in an animal model of disease.

Unlike previous studies reported here and by Gielen *et al* when Azithromycin was applied as a topical treatment to cells in culture, the treatment of rodents with Azithromycin required an understanding of the pharmacokinetics of the macrolide. Azithromycin is known to have a relatively high tissue concentration (compared to it's serum concentration), Vallée *et al* demonstrated that in mice, oral administration of 50mg/kg Azithromycin yielded a lung tissue concentration of approximately 13mg/kg compared to a serum concentration of approximately 4mg/kg after 24 hours [216]. In humans, Azithromycin accumulates to approximately 8mg/kg in the lung compared to serum levels of approximately 0.8mg/kg 24 hours post inoculation with a single 500mg oral dose [215]. Both studies suggest that the phenomenon of high tissue retention of Azithromycin is consistent in both humans and mice. Azithromycin is also known to be poorly metabolised, with few metabolites produced and much of the compound is excreted unchanged in faeces [211, 253]. Interestingly, Azithromycin has been shown to accumulate in phagocytes, Bosnar *et al* show that this accumulation can be up to 200-fold higher than in serum levels [222]. This retention of Azithromycin may act as an innate delivery mechanism to sites of infection and inflammation.

Phagocytes, such as neutrophils and macrophages are recruited via chemotaxis to the site of infection where they engulf bacteria, exposing the pathogens to high levels of Azithromycin retained within the phagocytic vesicles. It has been proposed that this is at least in part how Azithromycin acts as such an effective anti-bacterial agent.

Azithromycin is a potent anti-bacterial agent with broad-spectrum activity and is commonly prescribed against atypical bacterial infection in the lungs. Until relatively recently it was thought that the lower respiratory tract was a sterile environment however this position has changed and it is now reported that the lungs house a plethora of commensal bacteria [17]. Multiple studies on the respiratory microbiome suggest that alterations of the bacterial flora may have implications in other areas of respiratory diseases including inflammation and anti-viral immunity [230, 231]. Whilst this antibiotic activity must be taken into account when assessing anti-viral effects, it is likely that significant changes in the microbiome would only be observed after days, weeks or months of antibiotic treatment, for our purposes it is unlikely that a short course of Azithromycin (24-48 hours) would perturb the microbiome significantly. However, potential effects on commensal bacteria and how this may impact on respiratory immunology in the wider sense is an important point worthy of consideration.

In this chapter we describe the effects of oral Azithromycin on RV1b infection in BALB/c mice. Azithromycin was administered orally at two doses (25mg/kg and 50mg/kg following the studies performed by Vallée *et al*) prior to infection with high titre RV1b. We observed that although Azithromycin was able to augment IFN/ISG gene expression, it caused small increases in RV load (possibly driving this augmentation) within the lungs. We found that Azithromycin had no impact on the composition of BAL leukocytes including levels of neutrophils, lymphocytes and macrophages.

5.2 Hypothesis

- Azithromycin treatment is able to augment IFN and ISG expression in response to RV1b in a mouse model of infection.

5.3 Aims

- To establish whether oral Azithromycin is able to augment IFN/ISG gene expression in lung tissue.
- To establish the effects of Azithromycin treatment on BAL leukocyte numbers in mice infected with RV1b.

5.4 Results

5.4.1 Azithromycin in a mouse model of RV infection

7-8 week old female BALB/c mice were gavaged orally with 50mg/kg, 25mg/kg Azithromycin or EtOH vehicle in 200 μ L CMC gavage fluid 48 hours and 24 hours prior to intra-nasal infection with high-titre RV1b (5×10^6 TCID₅₀) for 8 or 24 hours. Bronchoalveolar lavage (BAL) was performed and BAL leukocytes numbers were assessed by cytospin and differential staining. IFN protein levels from BAL fluid were quantified by ELISA and gene expression levels from lung tissue were assessed by quantitative PCR. Data was pooled from two independent experiments (with five mice per group per experiment). Prior to each of the two experiments, virus stocks were tested by inoculating BALB/c mice intra-nasally with 5×10^6 TCID₅₀ RV1b or UV-inactivated RV1b, after 24 hours neutrophils within the BAL were only present within the RV1b treated mice and not within the UV-RV1b infected, indicating the lack of pro-inflammatory mediators within the virus preparation (Figure 5.1 percentage of BAL neutrophils from RV1b/ UV-RV1b inoculated mice: 79.44/ 0.56% n=4. Data is pooled from 2 independent experiments with n=2 per group).

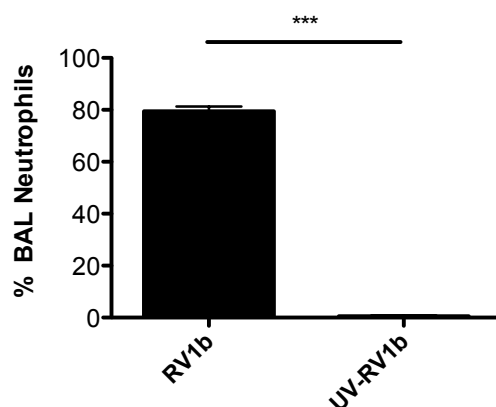


Figure 5.1 UV-inactivated RV1b did not induce neutrophilia in the BAL of BALB/c mice.

RV1b were tested in BALB/c mice, after 24 hours infection, mice that received UV-inactivated RV1b showed no neutrophilia in the BAL fluid compared to RV1b inoculated mice. *** $p < 0.001$. n=4. Data is pooled from 2 independent experiments with 2 mice per group.

5.4.2 Azithromycin had no significant effect on BAL leukocyte composition

At 24 hours post infection total leukocytes within the BAL fluid showed a non-significant increase in mice infected with RV1b. Azithromycin treatment prior to infection had no effect on the total number of leukocytes present within the BAL fluid of BALB/c mice (Figure 5.2 8 hours post infection total BAL leukocytes with Vehicle, 25mg/kg or 50mg/kg AZI treatment: 2.53×10^5 , 2.17×10^5 , 1.79×10^5 respectively and 24 hours post infection: 3.48×10^5 , 2.65×10^5 , 3.08×10^5 respectively. $n=10$ (2 pooled independent experiments each $n=5$ mice per group). RV1b infection showed a significant influx of neutrophils at 8 hours and 24 hours post infection compared to mock infection. Azithromycin treatment alone resulted in no significant changes in neutrophil influx; however the lower dose of 25mg/kg with infection demonstrated a slight trend for decreasing neutrophil percentages in the BAL. At 24 hours post infection, macrophages made up a significantly lower proportion of BAL leukocytes however again Azithromycin treatment failed to perturb this effect. Finally, at 24 hours post infection the percentage of BAL lymphocytes was significantly increased, Azithromycin treatment showed a non-significant dose-dependent decrease in the percentage of lymphocytes in the BAL fluid (Figure 5.3 8 hours post infection total BAL leukocytes with Vehicle, 25mg/kg or 50mg/kg AZI treatment neutrophils: 1.50×10^5 , 1.30×10^5 and 1.00×10^5 respectively. Macrophages: 1.00×10^5 , 8.29×10^4 and 7.63×10^4 . Lymphocytes: 1.75×10^3 , 3.16×10^3 and 1.94×10^3 . 24 hours post infection neutrophils: 2.80×10^5 , 1.84×10^5 and 2.32×10^5 . Macrophages: 5.69×10^4 , 7.26×10^4 and 6.97×10^4 . Lymphocytes: 1.03×10^4 , 8.11×10^3 and 6.07×10^3 . $n=10$ (2 pooled independent experiments each $n=5$ mice per group).

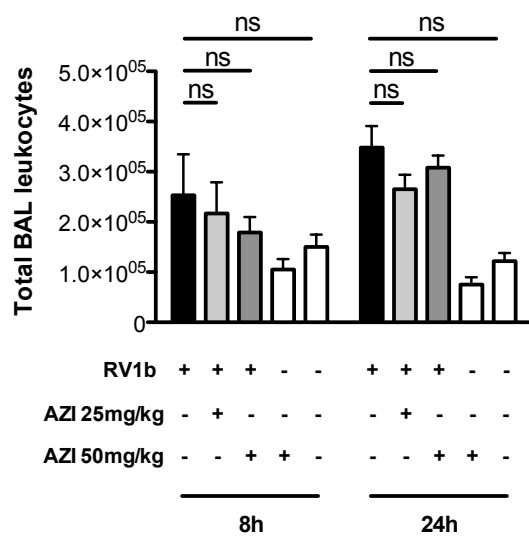


Figure 5.2 Azithromycin treatment had no effect on the total number of leukocytes in BAL fluid.

Infection with RV1b showed a trend for increasing total number of leukocytes within the BAL fluid of BALB/c mice after 8 and 24 hours. Azithromycin had no effect on the total number of leukocytes. $n=10$ (pooled from 2 independent experiments each $n=5$ mice per group).

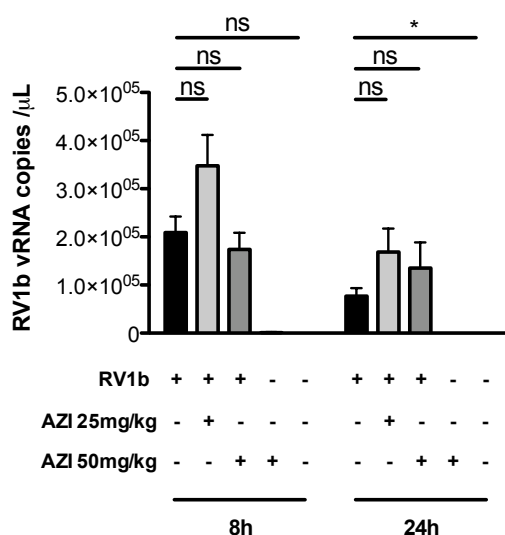


Figure 5.4 Azithromycin treatment showed a non-significant increase in viral load in BALB/c mice.

Azithromycin increased levels of RV1b in lung tissue compared to vehicle treatment. * $p < 0.05$. $n = 10$ (pooled from 2 independent experiments each $n = 5$ mice per group).

5.4.4 Azithromycin augmented levels of RV1b-induced IFN mRNA in lung tissue

RV1b failed to induce significant levels of IFN β mRNA expression in whole-lung tissue compared to mock infected control, Azithromycin did however (non-significantly) augment levels of IFN β mRNA compared to vehicle treatment with infection after 24 hours. RV1b infection caused a clear (albeit non-significant) increase in IFN λ 2/3 mRNA expression in whole lung tissue compared to mock infected control. 50mg/kg Azithromycin treatment significantly augmented levels of RV1b-induced IFN λ mRNA expression whilst 25 mg/kg Azithromycin treatment showed a non-significant trend for augmenting IFN λ mRNA (Figure 5.5 24 hours post infection IFN β mRNA copy number with Vehicle, 25mg/kg or 50mg/kg AZI treatment: 3.02×10^4 , 1.01×10^4 and 1.05×10^5 respectively. IFN λ 2/3 mRNA copy number: 9.43×10^4 , 2.50×10^5 and 2.17×10^5 . $n = 10$ (2 pooled independent experiments each $n = 5$ mice per group). Neither IFN β nor IFN λ mRNA was detected at 8 hours post infection in whole lung tissue.

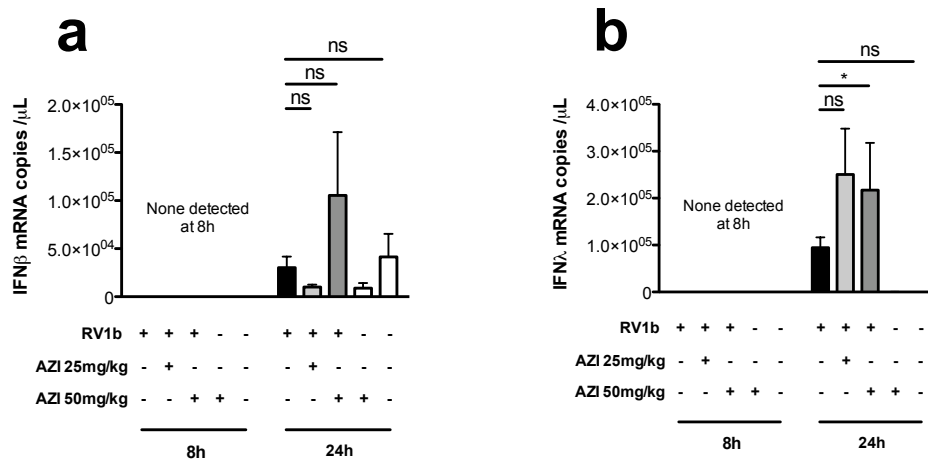


Figure 5.5 Azithromycin augmented levels of RV1b-induced IFN mRNA *in vivo*.

At 24 hours post infection, Azithromycin showed a non-significant trend for augmenting levels of (a) IFN β and significantly augmented levels of (b) IFN λ mRNA expression in lung tissue compared to vehicle treatment. * $p < 0.05$. $n = 10$ (pooled from 2 independent experiments each $n = 5$ mice per group).

5.4.5 Azithromycin augmented levels of RV1b-induced ISG mRNA *in vivo*

Levels of Viperin and PKR mRNA expression were induced by RV1b infection at both 8 hours and 24 hours post infection. Azithromycin showed a trend for augmenting levels of RV1b-induced Viperin and significantly augmented levels of RV1b-induced PKR at 24 hours (Figure 5.6 8 hours post infection Viperin mRNA copy number with Vehicle, 25mg/kg or 50mg/kg AZI treatment: 7.70×10^6 , 1.44×10^7 and 3.88×10^6 respectively. PKR mRNA copy number: 2.23×10^6 , 2.86×10^6 and 1.25×10^6 . 24 hours post infection Viperin mRNA copy number with Vehicle, 25mg/kg or 50mg/kg AZI treatment: 3.06×10^6 , 6.17×10^6 and 3.18×10^6 respectively. PKR mRNA copy number: 1.14×10^6 , 1.41×10^6 and 2.56×10^6 . $n = 10$ (2 pooled independent experiments each $n = 5$ mice per group).

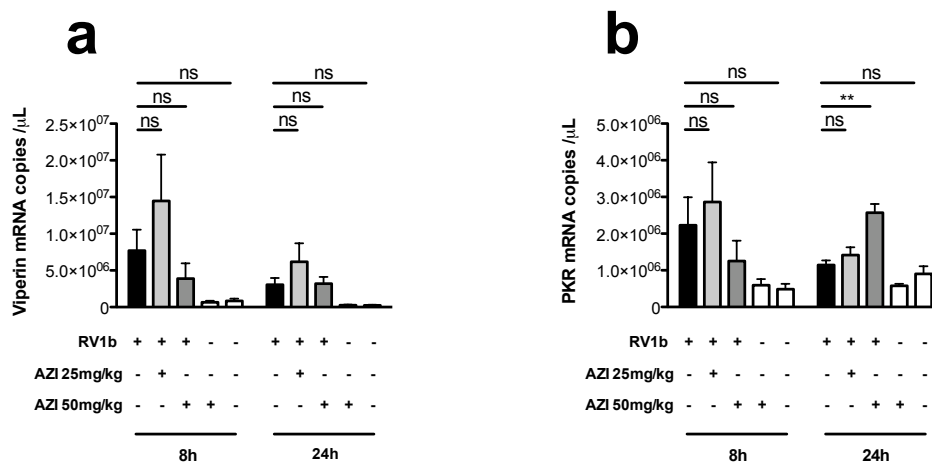


Figure 5.6 Azithromycin augmented levels of RV1b-induced ISG mRNA *in vivo*.

Azithromycin showed a non-significant trend for augmenting levels of (a) Viperin and significantly augmented levels of (b) PKR mRNA expression in lung tissue compared to vehicle treatment. ** $p < 0.01$. $n = 10$ (pooled from 2 independent experiments each $n = 5$ mice per group).

5.4.6 Azithromycin failed to augment levels of RV1b-induced IFN protein expression *in vivo*

RV1b infection significantly induced levels of IFN λ protein within the BAL fluid at 24 hours post-infection. 25mg/kg Azithromycin treatment had no effect on levels of RV1b-induced IFN λ protein expression, 50mg/kg Azithromycin treatment showed a slight increase in RV1b-induced IFN λ protein expression (Figure 5.7 24 hours post infection IFN λ BAL protein with Vehicle/ 25mg/kg/ 50mg/kg AZI treatment: 59.3pg/mL/ 60.6pg/mL/ 75.49pg/mL $n = 10$ (2 pooled independent experiments each $n = 5$ mice per group). IFN λ protein was not detected at 8 hours post infection. IFN β was not detected at either 8 hours or 24 hours post-infection.

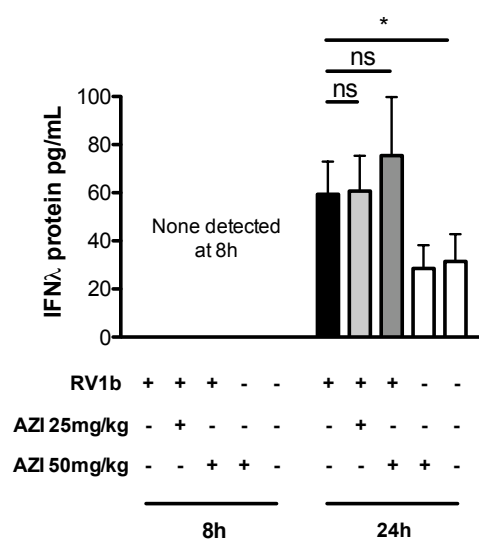


Figure 5.7 Azithromycin failed to augment levels of RV1b-induced IFN λ protein expression in BAL fluid.

At 24 hours post infection, 25mg/kg Azithromycin treatment had no effect on RV1b-induced IFN λ protein in BAL fluid. * $p < 0.05$. $n = 10$ (pooled from 2 independent experiments each $n = 5$ mice per group).

5.5 Discussion

In this chapter we have demonstrated that in BALB/c mice, pre-treatment with oral Azithromycin is able to augment levels of IFN λ and ISG mRNA expression in response to RV1b infection. We have also shown that Azithromycin treatment is able to increase the levels of RV1b viral RNA, however Azithromycin had no significant effects on the composition of leukocytes within the BAL fluid. Although the increase in viral RNA seen with Azithromycin was not statistically significant, we cannot rule out that the observed augmentation of IFN gene and ISG expression is entirely independent of the observed increase in vRNA. We would expect however that an increase in viral load would correspond to an increase in RV1b-induced neutrophilia, this was not observed. Due to the likelihood of increased viral load causing the observed augmentation of IFN/ISG expression, we cannot definitively report that oral Azithromycin enhances lung IFN responses *in vivo* to RV with any certainty and further experiments are required to do so.

No studies to date have investigated the innate anti-viral effects of Azithromycin *in vivo*, with a limited number of studies assessing the effects of antibiotics on anti-viral adaptive immunity [231]. The data presented here are suggestive that Azithromycin is able to increase the susceptibility of lung tissue to infection with RV1b (presumably resulting in augmented expression of IFN and ISGs, however we cannot be certain that Azithromycin is not having any augmenting effects). The lack of an observed increase in neutrophilia within the BAL may be indirectly suggestive that Azithromycin is augmenting IFN responses, rather than increasing susceptibility to infection. The lack of enhanced neutrophilia may be evidence that Azithromycin is also decreasing levels of neutrophil chemokines, this has previously been reported in human cells [195, 196], although this observation would need to be confirmed.

Previous studies here and by Gielen *et al* have demonstrated that Azithromycin augments anti-viral responses in epithelial cells. In the context of *in vivo* infection, IFN/ISG levels within the lung tissue may not originate solely from the epithelium. It has been well documented that Azithromycin can accumulate at high levels within macrophages [222], where it is naturally carried to the sites of infection. One might suggest that due to this preferential uptake by macrophages, Azithromycin has limited effects on the epithelium. Additionally, BAL macrophages may only have limited exposure to RV1b, therefore the limited augmentation observed with Azithromycin may originate from both RV and Azithromycin having different target cell preferences within the lung. Intra-nasal administration of

Azithromycin could be one way to increase the likelihood of interaction of the compound with the epithelial layer, which is where the majority of RV infection occurs *in vivo*. Azithromycin levels from whole serum, whole-lung tissue, BAL fluid and/or BAL leukocytes (separated populations) could be assessed by mass spectrometry to further investigate the role of cellular uptake and Azithromycin distribution.

In previous chapters we have demonstrated a potential mechanism of action of Azithromycin, if the partial augmentation observed *in vivo* is due to the effects of Azithromycin on the mouse epithelium this would suggest a similar mechanism of action to that proposed in HBECs *in vitro*. To investigate the effects of Azithromycin on PRR signalling and MAVS degradation one could assess the effects of intra-nasal Poly(I:C) compared to RV1b infection (one would expect to see no Azithromycin-mediated augmentation with Poly(I:C) treatment as per previous experiments performed in Chapter 4). Additionally, measuring levels of MAVS protein within the lung tissue would reveal if the effects of Azithromycin were consistent from HBECs to the mouse lung environment, if so one would expect Azithromycin to prevent MAVS degradation in response to RV1b infection (as per *in vitro* studies). Unfortunately due to time constraints we were unable to perform these tests.

Current knowledge of the impact of the lung microbiome on viral and inflammatory diseases is developing quickly with the use of non-culture based detection techniques [254]. It is now understood that the respiratory tract is not sterile and that differing commensal flora are able to impact on disease status [17]. Another potential application of studying Azithromycin *in vivo* is to assess the longer term anti-bacterial effects of Azithromycin on the immune system including immunity to RV1b infection. Several studies have questioned the use of long term Azithromycin use in chronic airway diseases due to effects on natural flora and bacterial resistance [182, 255, 256]. A potential solution to the use of Azithromycin in the longer term could be the use of a non-antibiotic analogue of Azithromycin that exhibited the same anti-viral effects and crucially acted via the same mechanism. This hypothetical molecule could then be tested in a model as outlined in this chapter without the confounding effects caused by any anti-bacterial activity.

This investigation displays the combined results from 2 independent experiments, ideally this study would need to be repeated again to confirm the findings displayed here. Additionally RV1b replication should be assessed by titration of virus in whole lung-lobe lysates onto HeLa cells and TCID₅₀ values for RV1b within the lung quantified to determine the effects of Azithromycin on viral replication (unfortunately, due to time constraints these assays were not performed). Different methods of administration of Azithromycin may yield differing

results as discussed above and should be considered, if intra-nasal administration of Azithromycin does yield significant effects on IFN/ISG expression in rodents this may lend confidence to the use of inhaled Azithromycin for the treatment of asthma exacerbations in humans.

Chapter 6 Identification of novel macrolides with anti-viral activity

6.1 Introduction

IFN production is one of the first initiators of the host's innate immunity against virus infection [88], and are typically the inducers of the 'anti-viral' state. IFNs are known to initiate the expression of ISGs via signalling through the type I and type III interferon receptor signalling complexes [137, 257, 258] and the protein products of these ISGs have been shown to be directly anti-viral. The proteins of ISGs can be extracellular, such as chemokines or cytokines that attract other cells of the immune system, or IFNs themselves with a classical example being IFN β inducing IFN α and IFN λ [52]. Most ISGs however are intracellular proteins that act to prevent virus protein trafficking, virus RNA synthesis, or virion assembly and release. Viperin and MxA are two examples of intracellular anti-viral ISGs [147, 259]. Viperin is thought to act upon viral protein trafficking within the host cell [260], whilst MxA is a small GTPase that localises to the ER and inhibits viral replication [146].

Studies by Wark in 2005 and later by Contoli in 2006 initially described deficiencies in the ability of cells from asthmatic patients to produce type I and type III IFNs in response to RV infection [84, 85]. Subsequent studies by Baraldo, Sykes and Edwards have confirmed the role for deficient IFN production in cells cultured from both children and adults with asthma [46, 86, 87]. This growing body of evidence suggests that augmenting IFN production in response to RV infection could be a novel target for therapeutic intervention in the context of RV associated asthma exacerbations.

Currently there are no therapies targeted at supplementing IFN levels in the treatment of asthma however recently, inhaled recombinant IFN β therapy has been used in phase II clinical trials examining naturally occurring colds in a cohort of mild- moderate asthmatics [173]. The data showed that IFN β therapy reduced symptoms and improved lung function recovery time post infection compared to placebo. The use of IFN as a prophylactic or therapeutic is not a novel idea however, as many clinical trials have used recombinant IFNs in an attempt to treat respiratory viral infections. In two studies in the 1980s, Hayden *et al* demonstrated that whilst intranasal recombinant IFN α therapy may reduce replication of RV in experimental and naturally occurring colds, it had no effects on patient symptoms or the duration of infection [171, 172]. Other studies have shown that treating RSV-infected children

with IFN may improve illness severity [261]. Away from respiratory diseases, IFN therapy is currently a mainstay in the treatment of viral hepatitis, used in combination with the anti-viral agent Ribavirin [262]. IFN inducers have been shown to directly translate to antiviral activity both in a clinical context and an animal model of disease [263, 264].

A limited number of studies have also identified small molecules that are capable of augmenting host anti-viral responses to viruses. In theory a small molecule capable of boosting the host's anti-viral responses to viral infection could prove to be an effective alternative to recombinant IFN therapy in the prophylactic treatment of virally associated asthma exacerbations. IFN production in response to viral infection is typically initiated by the activation of host cell PRRs such as the TLRs [82]. R-837 (Imiquimod) is a TLR7/8 agonist and has been shown to be anti-viral against HPV infection and is currently approved for use as a topical anti-viral therapy [174, 175] Rintatolimod, an analogue of Poly(I:C) and TLR3 agonist has been tested in phase II clinical trials for the treatment of HIV . Gielen *et al* demonstrated that Azithromycin augmented RV-induced IFNs and ISGs whilst decreasing viral replication *in vitro* [142]. Thomas *et al* has shown that Ribavirin-treated cells augment ISG responses in a model of Hepatitis C Virus infection [265]. Furthermore, Holtzman has described a novel high throughput screen that identified Idarubicin, a small molecule capable of augmenting the IFN signalling pathway *in vitro* leading to increased ISG induction, however this molecule has yet to be tested *in vivo* [266]. The ability to identify molecules with IFN augmenting characteristics in a high-throughput, biologically relevant screen would be a powerful tool for drug discovery and may provide novel therapies for the treatment of RV-associated asthma exacerbations as well as other viral infections.

One major challenge of screening assays is selecting a reproducible readout that is biologically relevant to the desired host response *in vivo*. The ability to detect changes in host cells' IFN responses to virus infection using a library of potential therapeutic molecules provides an extremely useful tool in the development of novel drugs for virus infections. Models of RV infection in bronchial epithelial cells show virus-dependent induction of IFN that correlate with increases in the ISGs MxA and Viperin [81, 124, 267], therefore detection of IFN production serves as a viable surrogate readout of antiviral protein production and antiviral activity. A promoter-linked bio-luminescent detection assay as a method of detecting promoter activity in a high throughput screen of small molecules has previously been described as a robust way of analysing a diverse library of compounds in a cost and time effective manner [266]. The use of immortalised cell lines to generate data as opposed to primary cells that can be expensive and time-consuming to culture is also favourable, as is

the ability to scale down experiments to increase throughput. It is however essential to verify effects seen in immortalised cell lines in a more clinically relevant model such as primary cell culture (in our case primary bronchial epithelial cells) as inherent differences may exist between the two models.

We were provided with a library of 226 novel macrolide compounds (which we identified as MAC1-MAC226) by Pfizer Inc. The aim in this chapter was to identify one or more compounds with anti-viral augmenting ability that had similar or greater potency or efficacy to that of Azithromycin. The antiviral effects of Azithromycin shown in previous chapters here and by Gielen *et al* were both modest and variable, producing a normal range of 150%-300% augmentation of type I and type III IFN induction following RV infection, with poor potency (50 μ M) [142]. It is possible that novel macrolide compounds may have stronger effects on innate host responses to RV in terms of both efficacy and potency. With new treatment options for asthma and COPD exacerbations being sought by the healthcare sector and the pharmaceutical industry, novel macrolide libraries provide a large repertoire of structurally similar compounds that may elicit anti-viral and/or anti-inflammatory effects. In order to screen the compound library in an economically viable and time efficient manner we implemented a multi-phase screening process using an *in vitro* luciferase reporter system based on the IFN β and IFN λ 1 (and later IL-6) promoters in the bronchial epithelial cell line BEAS-2B, where luciferase production is driven by infection with RV1b and any augmentation of IFN production by macrolide compounds can be assessed by changes in luciferase. This was followed by a more robust evaluation of the top performing macrolides (hits) in normal primary HBECs measuring intrinsic mRNA responses to RV1b infection by qPCR. The lead compound generated by the first three phases of the screen was assessed for effects on viral replication in normal and asthmatic HBECs and structure-activity-relationship (SAR) was assessed. We also assessed the antibiotic ability of the novel macrolides by measuring their ability to inhibit the growth of *E.coli* in liquid culture. We identified a novel macrolide, MAC5 as a compound that could augment normal HBEC gene expression of RV-induced type I and type III IFNs (IFN β and IL-28/IL-29 respectively) and the ISGs MxA and Viperin. MAC5 was also able to decrease RV replication in normal and asthmatic HBECs. This augmentation of antiviral activity was far superior to that seen with Azithromycin treatment. Additionally MAC5 showed the ability to inhibit HBEC expression of IL-8 however did not show any ability to inhibit *E.coli* growth. We then further assessed 30 novel analogues of MAC5 with similar structures for any structure-activity relationships pertaining to anti-viral, anti-inflammatory or anti-bacterial effects observed.

6.2 Hypothesis

- One or several novel macrolide compounds could augment RV-induced innate anti-viral immunity in normal HBECs in a manner superior to Azithromycin.

6.3 Aims

- To establish a medium throughput screening assay using IFN β and IFN λ 1 promoter activity as a readout. This assay involved the use of transiently-transfected BEAS-2B cells, using luminescence to measure promoter activity and would be able to easily measure the ability of 226 novel macrolide compounds for any potential IFN augmenting ability.
- To identify at least one novel macrolide compound from the promoter activity screen that was able to augment intrinsic anti-viral responses to RV1b in an *in vitro* model of infection using normal HBECs and that this augmentation was equal to or greater Azithromycin in terms of efficacy or potency.
- To compare a novel augmenting macrolide to 30 other compounds with related structure to investigate SAR.

6.4 Results

6.4.1 The development of a multi-phase screening program

A three-phase screening process was implemented in order to identify a lead compound from the initial library of 226 molecules, with only the top-augmenting compounds progressing to the next phase. The first two phases were promoter activity screens in RV-infected BEAS-2B cell cultures, the third phase served to confirm the findings of the first two phases in a model of normal HBEC RV infection. In the initial phase all 226 compounds were tested at 50 μ M concentration in a promoter activity based screen, after 24 hours incubation cells were examined for signs of toxicity and any compounds found to disrupt the monolayer were excluded and re-tested at a lower dosage (see appendix figures).

Table 6.1 Multi-phase screen design

Phase	No. Compounds	Cell type	Read out	Notes
1	226	BEAS-2B.	IFN β -Luc.	Compounds that alter cell morphology/ disrupt the monolayer were excluded. Compounds that augment IFN β -Luc activity 2-fold above AZI were progressed to Phase 2.
2	15	BEAS-2B.	IFN β -Luc, IFN λ 1-Luc.	Compounds that significantly augment IFN β -Luc or IFN λ -Luc activity were progressed to Phase 3.
3	5	Normal HBEC.	IFN β , IL-28, IL-29, MxA, Viperin, IL-6 and IL-8 mRNA expression.	One "lead" compound was chosen and assessed for SAR.

6.4.2 Luciferase reporter system as a screening model for macrolide compounds

BEAS-2B cell cultures were transiently transfected with plasmid DNA containing IFN β , IFN λ 1 or IL-6 promoter regions upstream of fluorescent Luciferase (IFN β -Luc, IFN λ 1-Luc or IL-6-Luc respectively) and treated for 24 hours with 50 μ M Azithromycin or each of the 226 novel macrolide compounds followed by infection with RV1b (MOI 1) for a further 24 hours before levels of promoter activity were measured by detection of luciferase. Relative Light Units (RLU) were attained by comparison to a constitutively active fluorescent control vector (Renilla) co-transfected with IFN β -Luc, IFN λ -Luc or IL-6-Luc (see Figure 6.1).

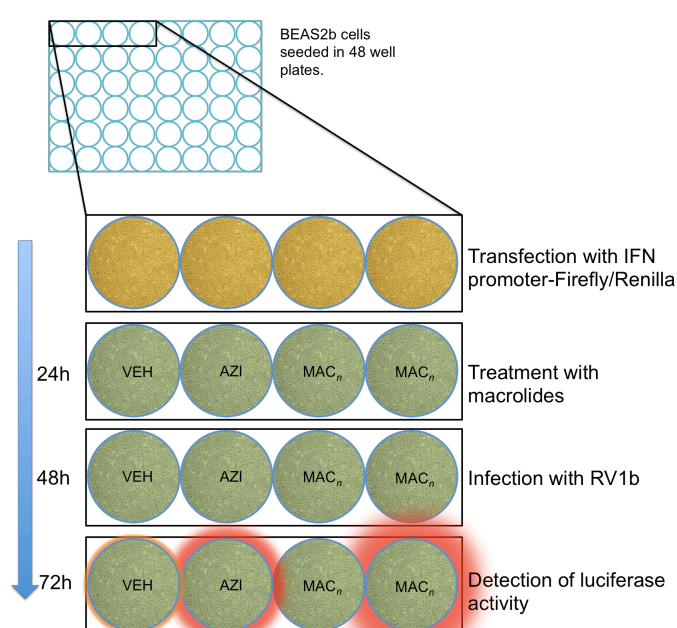


Figure 6.1 Luciferase based promoter activity assay

Beas-2B cells were transiently transfected with reporter constructs containing IFN β / λ 1 or IL-6 promoter linked to luciferase (Firefly) and the transfection control construct (Renilla). After 24 hours the cells were treated with novel macrolides for a further 24 hours. Cultures were then infected with RV1b for 24 hours before detection of luciferase was assessed to determine promoter activity.

6.4.3 Azithromycin augmented IFN β and IFN λ promoter activity in BEAS-2B cells

Treatment of BEAS-2B cells with Azithromycin prior to infection with RV1b demonstrated increased relative IFN β -Luc and IFN λ 1-Luc activity compared to cultures treated with vehicle (Figure 6.2 IFN β promoter activity in RV1b-infected BEAS-2Bs with 50 μ M Azithromycin: 216.0%, IFN λ promoter activity: 219.5% compared to vehicle treated cultures (100%) n=3). These data justified the use of this promoter based assay in BEAS-2B cells for the screening of a novel macrolide library for potential IFN augmenting ability.

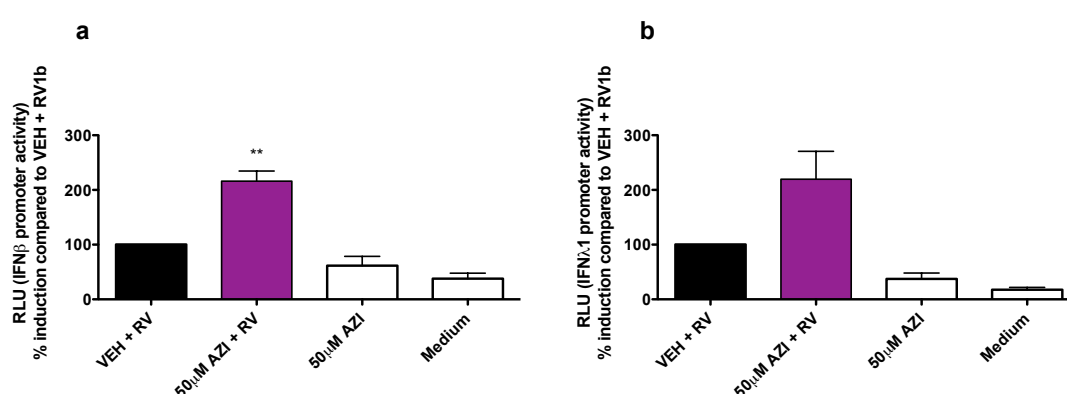


Figure 6.2 Azithromycin augmented IFN β and IFN λ 1 promoter activity in BEAS-2B cells

Beas-2B cell cultures transfected with (a) IFN β -Luc or (b) IFN λ -Luc promoter demonstrated that Azithromycin treatment prior to RV1b infection can augment promoter activity. Data expressed as % change of RV + vehicle (VEH) induction **p<0.01 AZI+RV versus VEH+ RV n=3.

6.4.4 Phase one- Novel macrolides differentially augmented IFN β promoter activity in BEAS-2Bs

The initial phase of the novel macrolide screen measured promoter activity for IFN β -Luc only as to maximise throughput and minimise time and labour. After transfection with IFN β -Luc (and Renilla control) cell cultures were incubated with 50 μ M Azithromycin or novel macrolide at 37°C for 24 hours, after this time cells were infected with RV1b (MOI:1) for a further 24 hours before being harvested and promoter activity was assessed by measuring RLU. After macrolide treatment (prior to infection), cell cultures were observed under light microscopy for signs of cellular toxicity, compounds that exhibited damage to the cell monolayer were excluded from this portion of the screen. An arbitrary threshold of 2-fold augmentation above the level of Azithromycin treatment was deemed sufficient to progress the compound into the second phase of screening. 15 compounds were able to augment IFN β promoter activity to a level greater than 2 fold above the level of Azithromycin (Figure 6.3 MAC2:

263.0%, MAC5: 317.6%, MAC12: 389.8%, MAC14: 343.7%, MAC26: 486.0%, MAC39: 280.1%, MAC51: 267.4%, MAC122: 431.5%, MAC132: 354.3%, MAC153: 269.7%, MAC183: 255.7%, MAC185: 278.5%, MAC202: 255.2%, MAC206: 310.6% increase over vehicle + RV1b 100% n=1) these compounds were progressed to phase two. A number of compounds showed an inhibition or decrease in IFN β reporter activity following virus infection, these compounds may be directly anti-viral or inhibitors of IFN production, for the interests of this study however these compounds were not pursued.

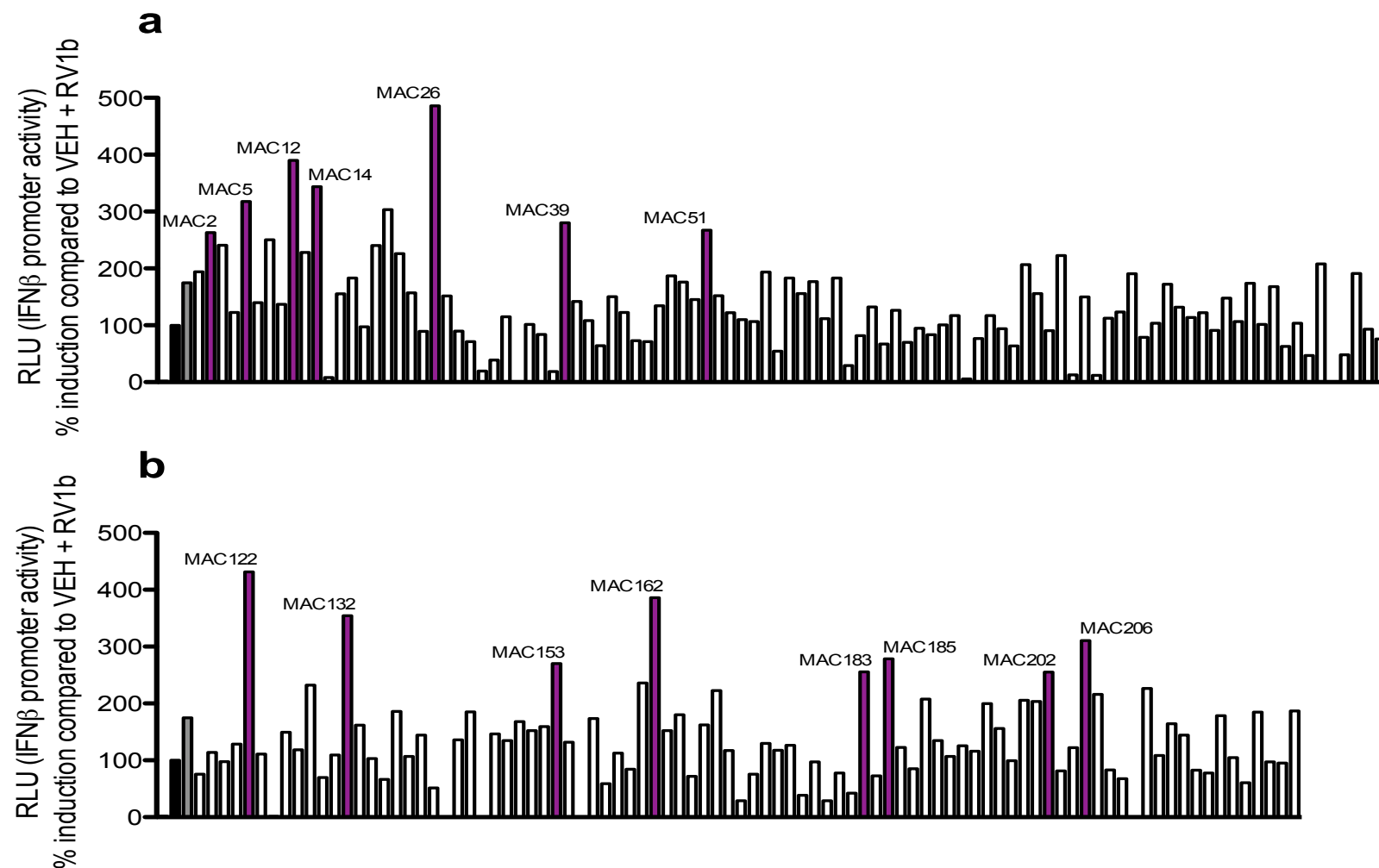


Figure 6.3 Novel macrolides differentially augmented RV1b-induced IFN β promoter activity in BEAS-2Bs.

BEAS-2B cell cultures were treated with 50 μ M 225 novel macrolide compounds (top: 1-115, bottom: 116-225) for 24 hours prior to RV1b infection for 24 hours. 15 compounds (purple bars) augmented IFN β promoter activity 2-fold above the level of Azithromycin (grey bar). Compounds that were toxic at 50 μ M were excluded. Data are expressed as a % of RV1b + Vehicle (Black bar 100%). n=1.

6.4.5 Phase two- 15 Novel macrolides augmented IFN β and IFN λ 1 promoter activity in BEAS-2Bs

Phase two of the screen utilised the IFN β promoter activity assay used in phase one however with the addition of measuring IFN λ 1 promoter activity in parallel. BEAS-2B cell cultures were transiently transfected with either IFN β -Luc or IFN λ 1-Luc and Renilla control for 24 hours after which 50 μ M of Azithromycin or novel macrolide (or vehicle control) diluted in serum-free medium was added to cells and incubated for 24 hours at 37°C, cultures were then infected with RV1b (MOI:1) for 24 hours and RLU was measured and promoter activity was determined. 5 novel macrolide compounds showed a statistically significant augmentation of RV1b-induced IFN β promoter activity when compared to vehicle control (Figure 6.4 MAC5 278.9%, MAC26 289.8%, MAC162 291.8%, MAC202 190.6% and MAC206 291.3% increase over vehicle + RV1b 100% n=3). These 5 novel macrolide compounds also augmented RV1b-induced IFN λ 1 promoter activity above levels of vehicle (Figure 6.5) however this was not statistically significant. Although only able to significantly augment RV1b-induced IFN β promoter activity, levels of IFN β and IFN λ 1 promoter augmentation correlated across the 15 tested compounds (Figure 6.6) and the five novel macrolides (MAC5, MAC26, MAC162, MAC202 and MAC206) were progressed into the third phase of the screening process.

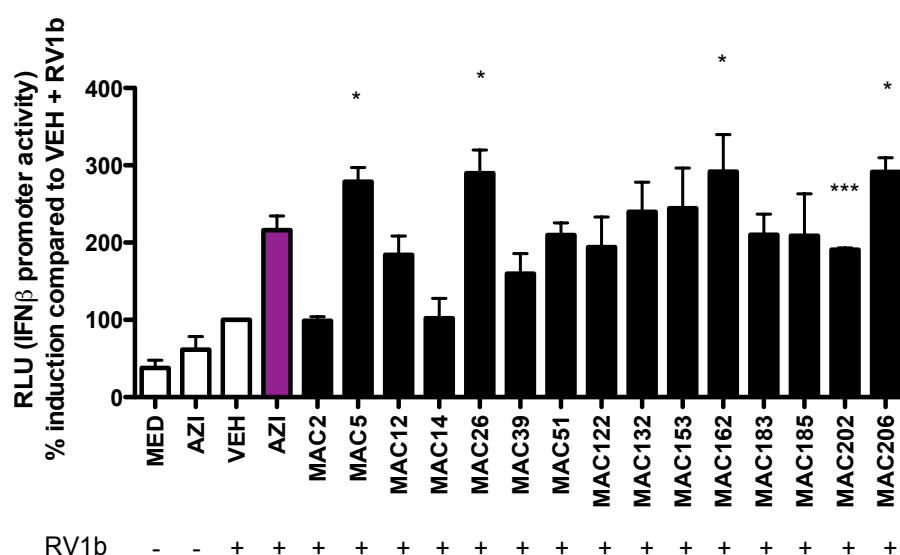


Figure 6.4 Novel macrolides augmented RV1b-induced IFN β promoter activity

5 novel macrolides significantly augmented RV1b-induced IFN β promoter activity in BEAS-2B cell cultures. Data expressed as % change of RV1b + vehicle (VEH) induction *p<0.05 ***p<0.001 versus RV1b + Vehicle (VEH) induction. n=3.

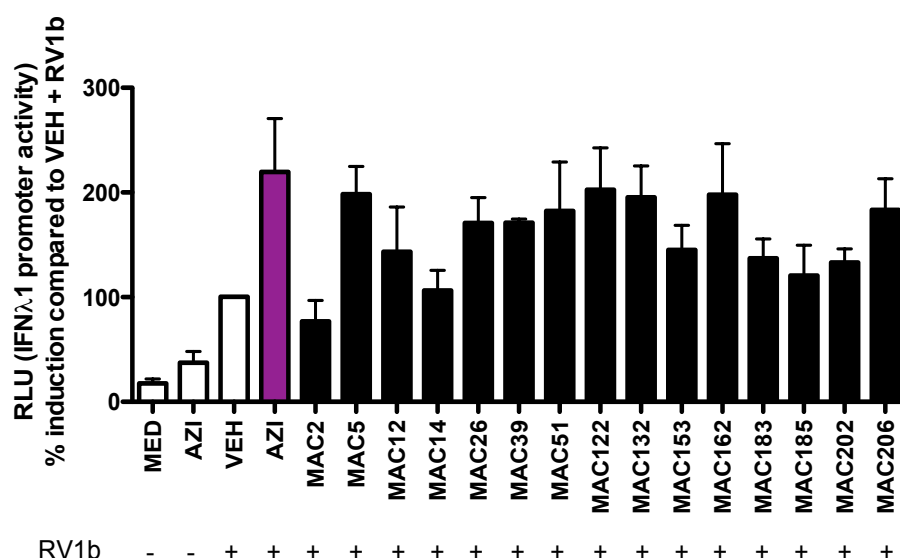


Figure 6.5 Novel macrolides showed trends for augmenting RV1b-induced IFNλ1 promoter activity

Novel macrolides showed trends for augmenting RV1b-induced IFNλ1 promoter activity in BEAS-2B cell cultures although none to a significant level. Data expressed as % change of RV1b + vehicle (VEH) induction n=3.

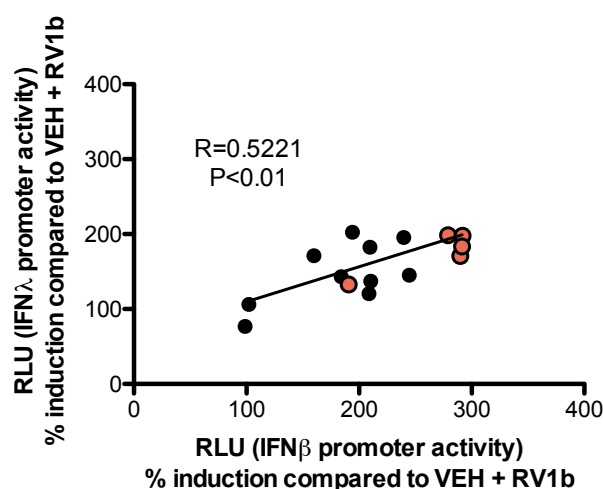


Figure 6.6 IFNβ promoter activity correlated with IFNλ1 promoter activity in RV1b-infected BEAS-2Bs treated with novel macrolides

BEAS-2B cell cultures treated with novel macrolides and subsequently infected with RV1b exhibited a strong correlation between levels of IFNβ promoter activity and IFNλ1 promoter activity. Data expressed as % change of RV1b + vehicle (100% assessed by Spearman correlation). Macrolides that were progressed to phase 3 are highlighted in red. n=3.

6.4.6 Phase three – Novel macrolides augmented IFN and ISG expression in primary cell culture

The third phase of the screening process measured the augmentation of antiviral effects in normal HBEC cultures infected with RV1b. Cultures of normal HBECs were treated with

50 μ M and 10 μ M solutions of each of the five novel macrolide compounds in supplement-free medium for 24 hours followed by infection with RV1b for a further 24 hours. Intrinsic gene expression of IFN, ISG and pro-inflammatory cytokines was measured by qPCR.

6.4.7 Novel Macrolides augmented virus-induced IFN mRNA expression in normal HBECs

MAC5 showed a significant augmentation of RV1b-induced mRNA expression of type I and type III IFNs (Figures 6.7-6.9 50 μ M/10 μ M IFN β : 1192.6%/199.3%, IL-28: 1882.4%/261.4%, IL-29: 1897.9%/291.7% increase over vehicle 100% n=8). MAC26 and MAC162 at 50 μ M and 10 μ M showed no significant increase in levels of virus-induced IFN β , IL-28 or IL-29. MAC202 at 50 μ M showed a relatively small increase in RV1b-induced IFN β , IL-28 and IL-29 (50 μ M/10 μ M IFN β : 167.2%/133.6% IL-28: 164.9%/107.8%, IL-29: 242.6%/135.1% increase over vehicle 100% n=8). MAC206 showed a statistically significant increase in levels of RV1b-induced IL-28, and increased levels of IL-29 and IFN β (50 μ M/10 μ M IFN β : 245.2%/133.3%, IL-28: 245.5%/137.8%, IL-29: 245.8%/135.3% increase over vehicle 100% n=8). None of the compounds tested induced type I or type III IFNs in the absence of RV1b infection (see appendix figures).

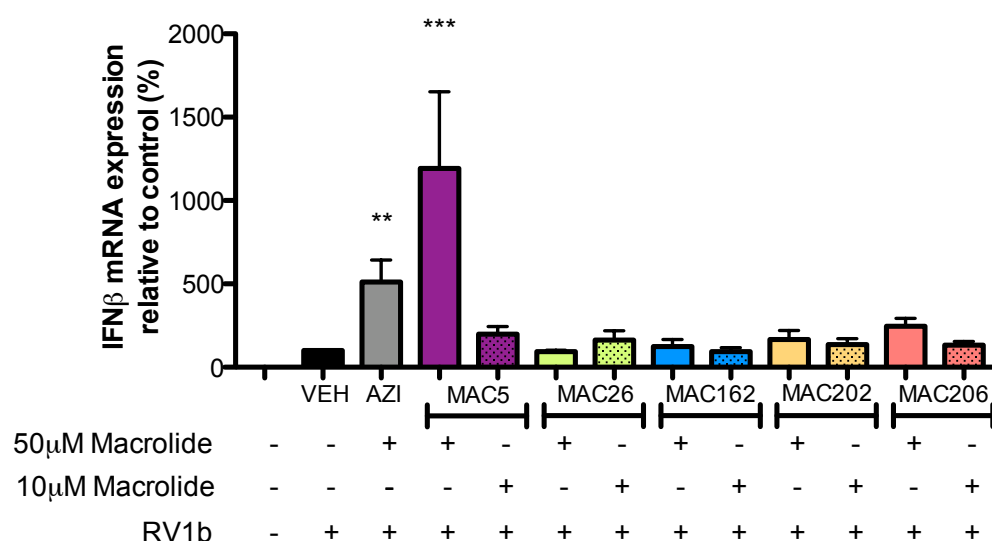


Figure 6.7 Novel macrolides augmented levels of RV1b-induced IFN β mRNA.

MAC5 augmented RV-induced IFN β in normal HBEC cultures. MAC26, MAC162, MAC202 and MAC206 failed to significantly augment mRNA levels of IFN β . Data expressed as % change of RV1b + vehicle (black bar 100%) induction *p<0.05 **p<0.01 versus RV1b + VEH n=8.

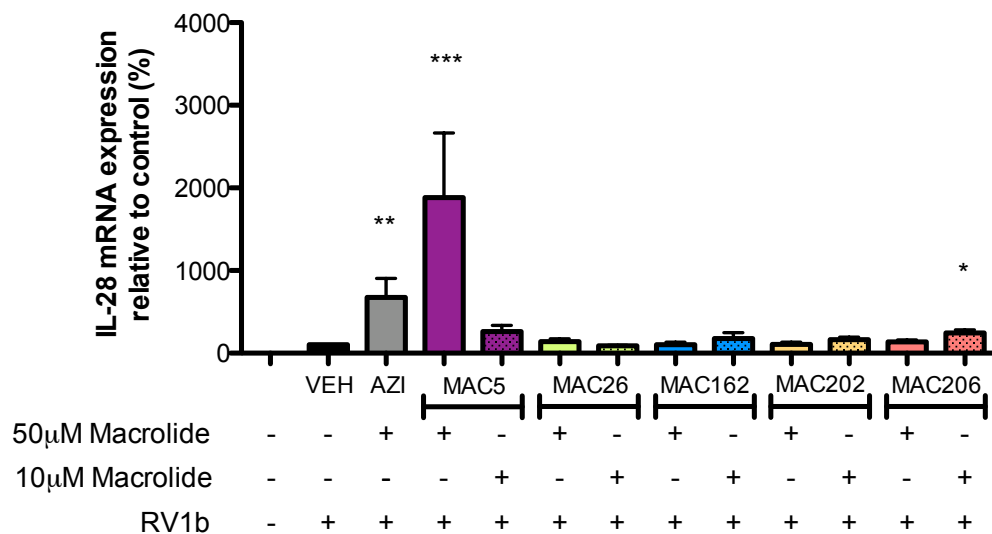


Figure 6.8 Novel macrolides augmented levels of RV1b-induced IL-28 mRNA.

MAC5 and MAC206 significantly augmented RV-induced IL-28 in normal HBEC cultures. MAC26, MAC162 and MAC202 failed to significantly augment mRNA levels of IL-28. Data expressed as % change of RV1b + vehicle (black bar 100%) * $p < 0.05$ ** $p < 0.01$ versus RV1b + VEH $n = 8$.

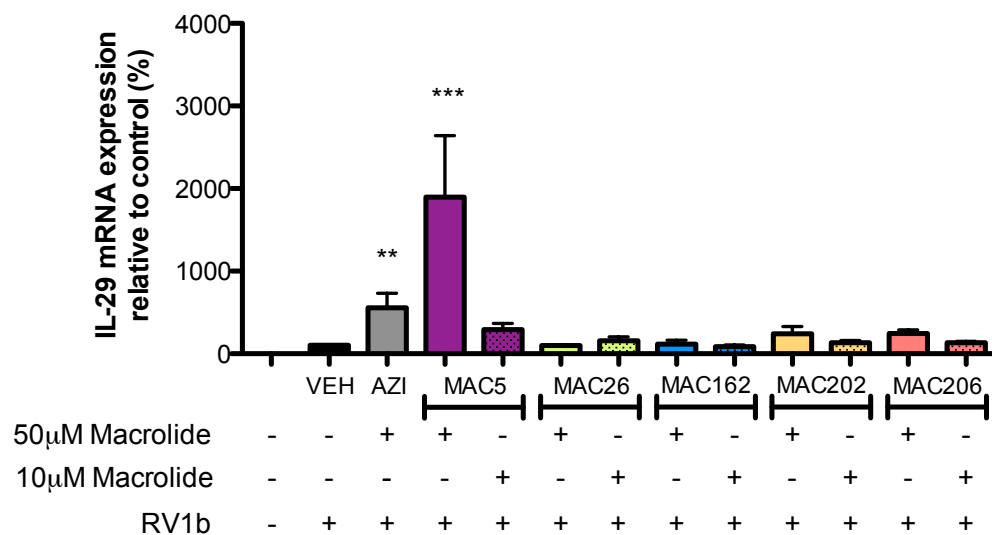


Figure 6.9 Novel macrolides augmented levels of RV1b-induced IL-29 mRNA.

MAC5 augmented IL-29 mRNA in normal HBEC cultures. MAC26, MAC162 failed to augment mRNA levels of IL-29. MAC202 and MAC206 showed trends to augment IL-29 levels above that of RV1b alone. Data expressed as % change of RV1b + vehicle (black bar 100%) induction no data were statistically significant. $n = 8$.

6.4.8 Novel Macrolides augmented virus-induced ISG mRNA in normal HBECs

MAC5 augmented mRNA expression of the intracellular ISG Viperin and showed a trend for augmentation of RV1b-induced MxA expression (Figures 6.10 and 6.11 50 μ M/10 μ M MxA: 539.3%/209.0%, Viperin: 2807.5%/312.6% increase over vehicle 100% n=8). MAC26 showed no augmentation of RV1b-induced mRNA expression of MxA however a slight increase of RV1b-induced Viperin was observed with the lower dose (50 μ M/10 μ M Viperin: 80.8%/171.5% n=8). MAC162 showed a trend for augmented virus-induced MxA mRNA however this effect was not seen in Viperin mRNA levels (50 μ M/10 μ M MxA: 216.6%/177.6%, Viperin: 73.4%/68.9% increase over vehicle 100% n=8). MAC202 showed a trend for augmented virus-induced MxA and Viperin mRNA expression (not to a statistically significant level (50 μ M/10 μ M MxA: 228.8%/132.9% Viperin: 250.9%/90.0% increase over vehicle 100% n=8). MAC206 augmented RV1b-induced MxA and Viperin mRNA levels (50 μ M/10 μ M MxA: 224.5%/131.6%, Viperin: 413.6%/164.2% increase over vehicle 100% n=8). None of the compounds tested induced MxA or Viperin mRNA above constitutive levels in the absence of RV1b infection (see appendix figures).

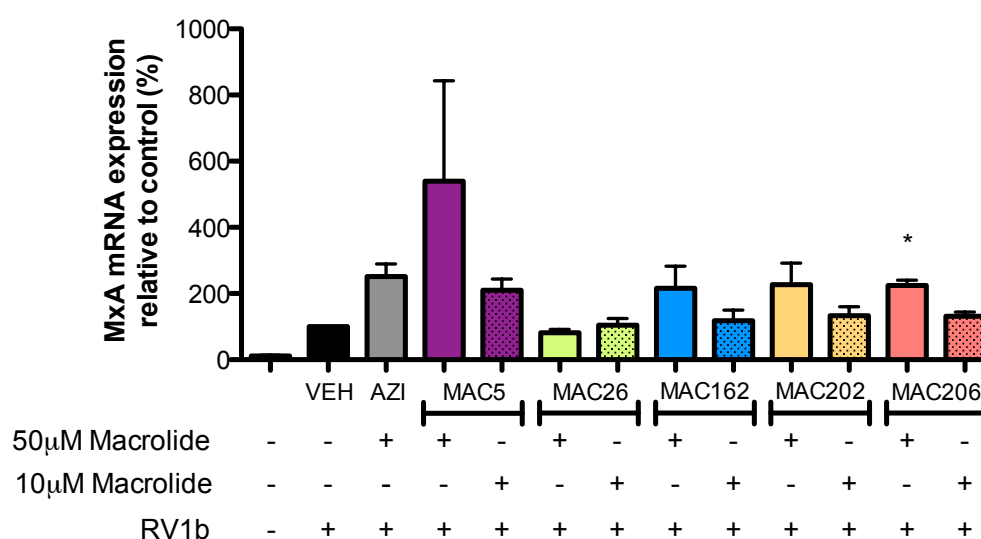


Figure 6.10 Novel macrolides augmented levels of RV1b-induced MxA mRNA.

MAC206 augmented RV-induced MxA in normal HBEC cultures. MAC5, MAC162 and MAC20 all showed a trend for augmenting RV-induced MxA. MAC26 failed to augment MxA levels. Data expressed as % change of RV1b + vehicle (black bar 100%). *p<0.05, **p<0.01 versus RV1b + VEH n=8.

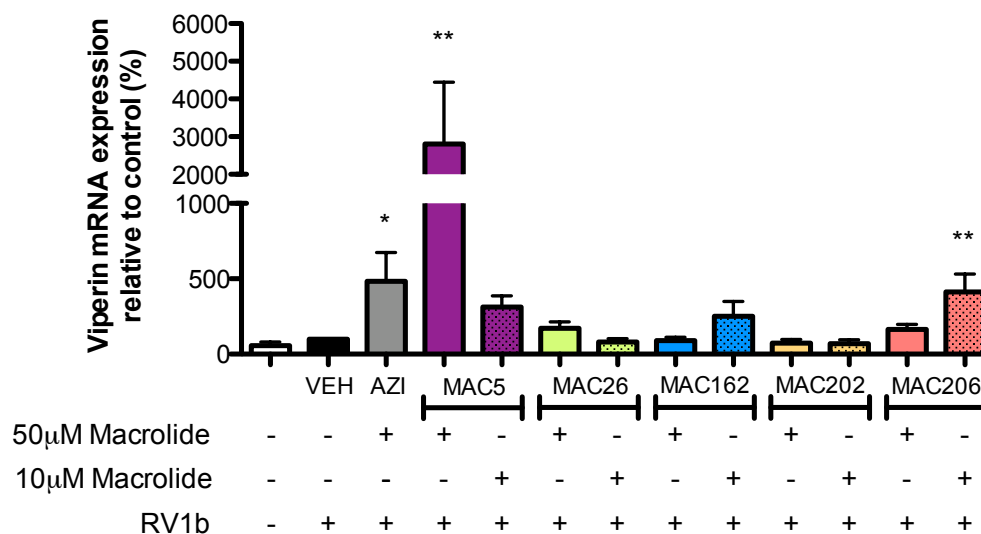


Figure 6.11 Novel macrolides augmented levels of RV1b-induced Viperin mRNA.

Azithromycin, MAC5 and MAC206 augmented RV1b-induced expression of Viperin. MAC162 showed trends for augmenting Viperin mRNA however this was statistically significant. MAC26 and MAC202 failed to augment. Data expressed as % change of RV1b + vehicle (black bar) induction no data were statistically significant. * $p < 0.05$, ** $p < 0.01$ versus RV1b + VEH $n = 8$.

6.4.9 Novel Macrolides showed trends for augmentation of virus-induced pro-inflammatory cytokine mRNA expression in normal HBECs

MAC5 showed a trend for augmenting mRNA expression levels of RV1b-induced IL-6 and IL-8 (Figures 6.12 and 6.13 50µM/10µM IL-6: 108.0%/108.2%, IL-8: 178.9%/125.4% increase over vehicle 100% $n = 8$). Neither MAC26, MAC162, MAC202 nor MAC206 showed any change in virus-induced pro-inflammatory cytokine mRNA expression. None of the compounds tested induced IL-6 or IL-8 mRNA above constitutive levels in the absence of RV1b infection (see appendix figures).

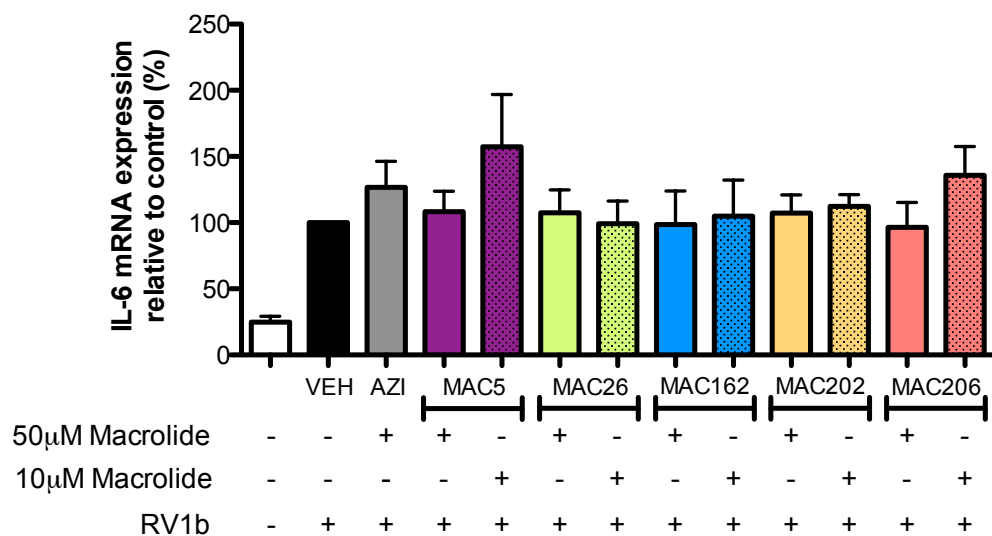


Figure 6.12 Novel macrolides failed to augment levels of RV1b-induced IL-6 mRNA.

None of the novel macrolides tested were able to augment levels of IL-8 mRNA above the level of RV1b. Data expressed as % change of RV1b + vehicle (black bar 100%) induction no data were statistically significant. n=8.

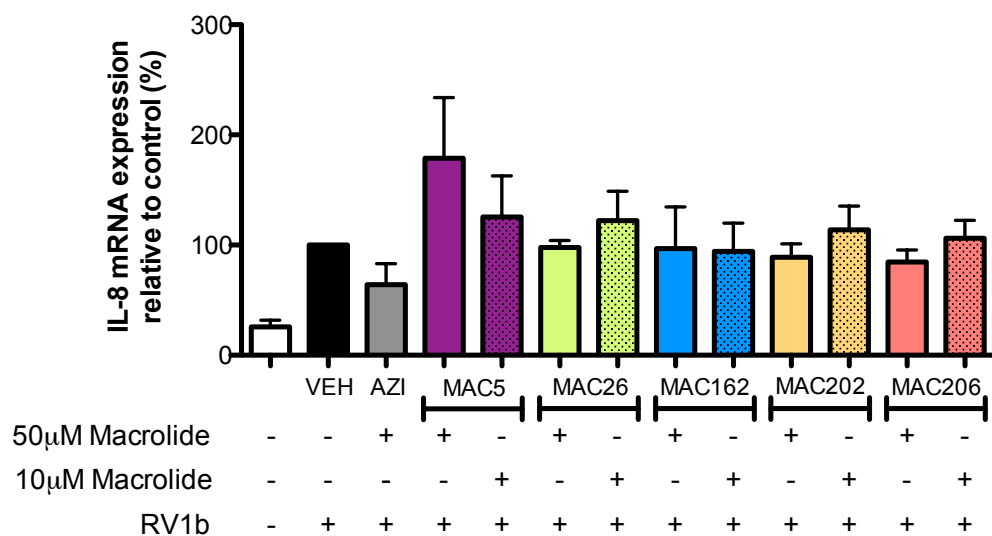


Figure 6.13 Novel macrolides showed trends for augmenting levels of RV1b-induced IL-8 mRNA.

MAC5 showed limited trends for augmenting levels of IL-8 mRNA above the level of RV1b. Data expressed as % change of RV1b + vehicle (black bar) induction no data were statistically significant. n=8.

6.4.10 Characterisation of MAC5

MAC5 was identified as a “lead” compound from the original 226 macrolides and was further assessed for anti-viral activity against RV, potential anti-inflammatory and anti-bacterial activity.

6.4.10.1 MAC5 showed a dose dependent IFN and ISG augmenting ability in normal HBECs

MAC5 was serially diluted 1:2 (a total of seven times) in supplement free BEBM medium (50-0.78 μ M) and added to normal HBEC cultures for 24 hours prior to infection with RV1b (MOI:1) for a further 24 hours. IFN β , IL-28, IL-29, MxA and Viperin mRNA all showed a sigmoidal dose response with EC₅₀ values of 9.804 μ M, 10.99 μ M, 9.914 μ M, 5.1 μ M and 7.5 μ M respectively (Figure 6.14). IL-6 and IL-8 did not show a dose dependent augmentation of mRNA expression (see appendix figures).

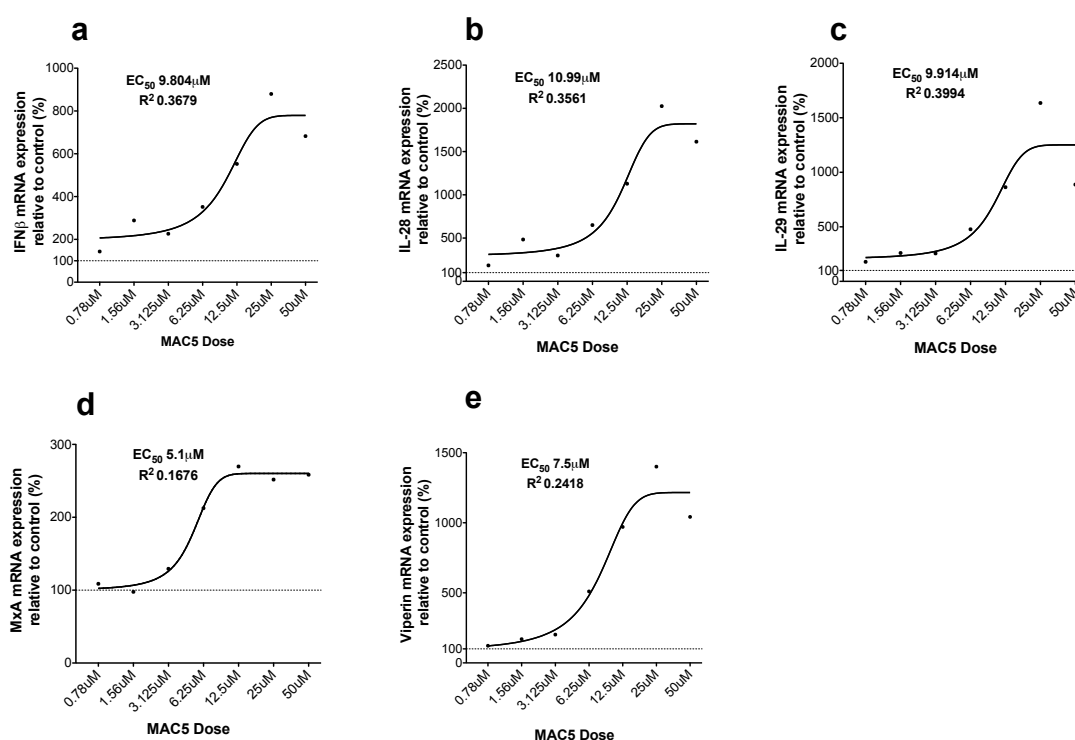


Figure 6.14 MAC5 showed a dose dependent augmentation type I and type III IFN and ISG mRNA in normal HBECs.

MAC5 was titrated (1:2 dilution) and shows a sigmoidal dose response to augmenting RV1b-induced (a) IFN β , (b) IL-28, (c) IL-29 mRNA, (d) MxA and (e) Viperin. Data expressed as % change of RV1b + vehicle (100% dashed line) induction. n=3.

6.4.10.2 MAC5 inhibited RV1b replication in normal HBECs

MAC5 treatment was shown to inhibit RV1b replication. Supernatants from normal HBECs treated with novel macrolides and infected for 48 hours with RV1b were incubated with HeLa cells for 96 hours and the HeLa cells were then assessed for CPE. HBEC cultures treated with 50 μ M MAC5 showed a significantly decreased TCID₅₀ than vehicle treated (Figure 6.15 50 μ M MAC5 reduced RV1b TCID₅₀ by 61.6% compared to vehicle 100% n=4).

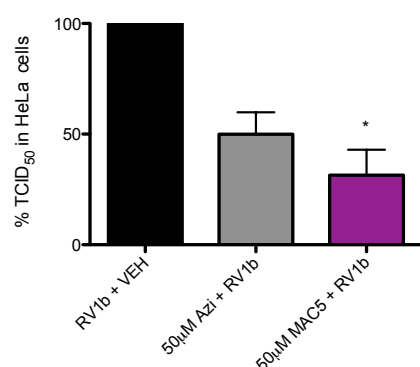


Figure 6.15 MAC5 inhibited RV1b replication *in vitro* in normal HBECs.

Azithromycin and MAC5 inhibited RV1b replication in HBEC cultures after 48 hours. Supernatants from virally infected HBEC cultures were titrated onto HeLa cells and TCID₅₀ was determined by observing CPE. Data expressed as % change of RV1b + vehicle (black bar 100%) induction. *p<0.05 versus RV1b + VEH. n=4.

6.4.10.3 MAC5 showed trends for inhibiting RV replication in asthmatic HBECs

Supernatants from 48 hour RV infected asthmatic HBECs pre-treated with 50 μ M AZI or MAC5 were titrated onto HeLa cells for 96 hours and the HeLa cells were then assessed for CPE. MAC5 treated asthmatic HBECs showed trends for inhibiting both RV16 and RV1b replication (Figure 6.16 50 μ M MAC5: 46.0% (RV16), 51.4% (RV1b) TCID₅₀ compared to vehicle 100% n=4).

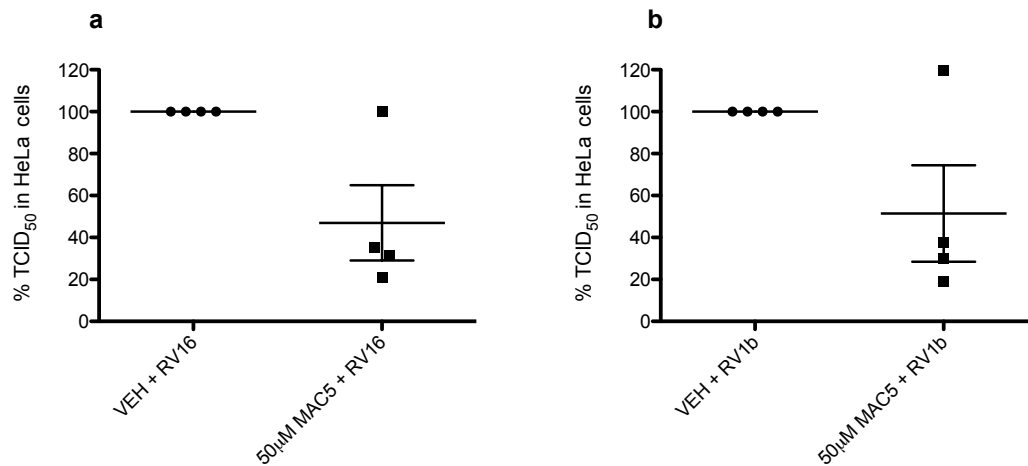


Figure 6.16 MAC5 showed trends for inhibiting RV replication in asthmatic HBECs.

Azithromycin and MAC5 inhibited (a) RV16 and (b) RV1b replication in asthmatic HBEC cultures after 48 hours. Supernatants from virally infected HBEC cultures were titrated onto HeLa cell cultures and TCID₅₀ was determined by observing CPE. Data expressed as % change of RV1b + vehicle (VEH 100%) induction. No data were significant. n=4

6.4.10.4 MAC5 showed a trend for decreasing IL-8 protein production in normal HBECs

HBECs treated with MAC5 showed a decrease in RV1b-associated IL-8 protein production measured by ELISA, this inhibition occurred regardless of infection with RV1b (Figure 6.17 Medium: 29.0%, 10µM MAC5 + RV1b: 84.2%, 50µM MAC5 + RV1b: 49.6%, 10µM MAC5: 19.2%, 50µM MAC5: 9.1% compared to Vehicle (VEH) + RV1b 100%. n=4).

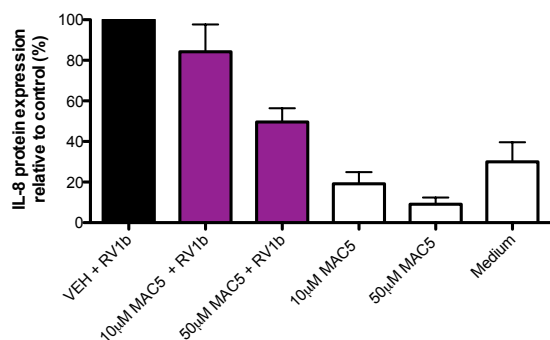


Figure 6.17 MAC5 shows a trend for decreasing IL-8 protein production in HBECs.

MAC5 shows a dose-dependent decrease in IL-8 proteins levels as measured by EILSA, this inhibition is not dependent on RV1b infection. Data expressed as % change of RV1b + VEH (black bar 100%) induction. No data were significant (n=4).

6.4.10.5 Novel macrolides did not exhibit anti-bacterial properties

Log-phase *E.coli* was grown in LB broth containing 50µM novel macrolide, Azithromycin or vehicle control, over an 8 hour time course. Only LB supplemented with Azithromycin was able to prevent the growth of *E.coli*. LB supplemented with novel macrolides had no effect on *E.coli* growth compared to Azithromycin supplemented broth (Figure 6.18 at 8 hours 50µM Azithromycin OD₆₀₀: 0.04, MAC5: 1.29, MAC26: 1.29, MAC162: 1.29, MAC202: 1.29, MAC206: 1.26, Vehicle: 1.22, no supplement control: 1.29. n=3).

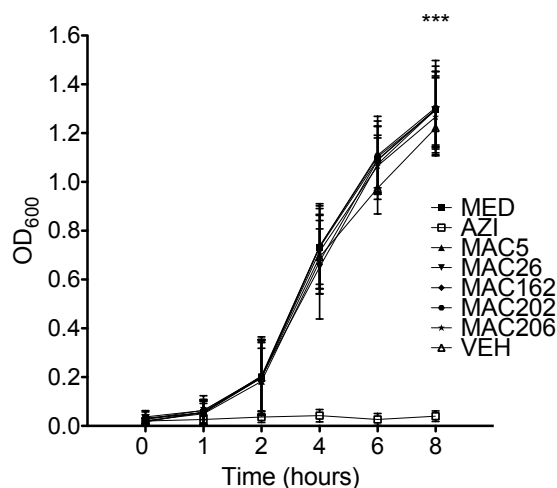


Figure 6.18 Novel macrolides did not exhibit anti-bacterial properties.

E. coli was grown in the presence or absence of 50 μ M macrolide compounds for 8 hours, only Azithromycin (AZI) showed inhibition of bacterial cell growth. *** $p < 0.001$ compared to AZI positive control calculated from area under curve. $n = 3$.

6.4.10.6 Structure activity (SAR) studies on MAC5 analogues

30 additional MAC5 analogues (designated MAC5A-MAC5AD) were investigated for their ability to augment RV associated IFN and pro-inflammatory cytokine induction. These novel macrolides were assessed for their ability to augment IFN β and IL-6 promoter activity in RV1b-infected BEAS-2B cell cultures and for their ability to perturb the growth of *E. coli*. MAC5 analogues were initially assessed for toxicity by an MTT assay (see appendix Figures), molecules that were toxic at 50 μ M were excluded (a total of 11), data was presented on the remaining molecules (19). Additionally, molecules that disrupted the cell monolayer or caused a change in cell morphology were also discarded at this point.

6.4.10.6.1 Analogues of MAC5 showed trends for augmentation of IFN β promoter activity

MAC5A-MAC5AD were assessed for their ability to augment RV-associated IFN β promoter activity in BEAS-2B cells. As per phase two of the novel macrolide screen (see section 6.3.4), BEAS-2B cell cultures were transiently transfected with IFN β -Luc (and Renilla control) for 24 hours followed by treatment with 50 μ M of MAC5 analogue solution for a further 24 hours, cultures were then infected with RV1b (MOI:1) for 24 hours before levels of promoter activity were assessed. Fig 6.19 shows that with the exception of MAC5M all novel analogues showed trends for augmenting IFN β promoter activity above the level of Vehicle,

MAC5Q and MAC5AC showed the greatest ability compared to MAC5 however this was not statistically significant (MAC5Q 236.35%, MAC5AC 267.06% above the level of Vehicle 100%. n=5).

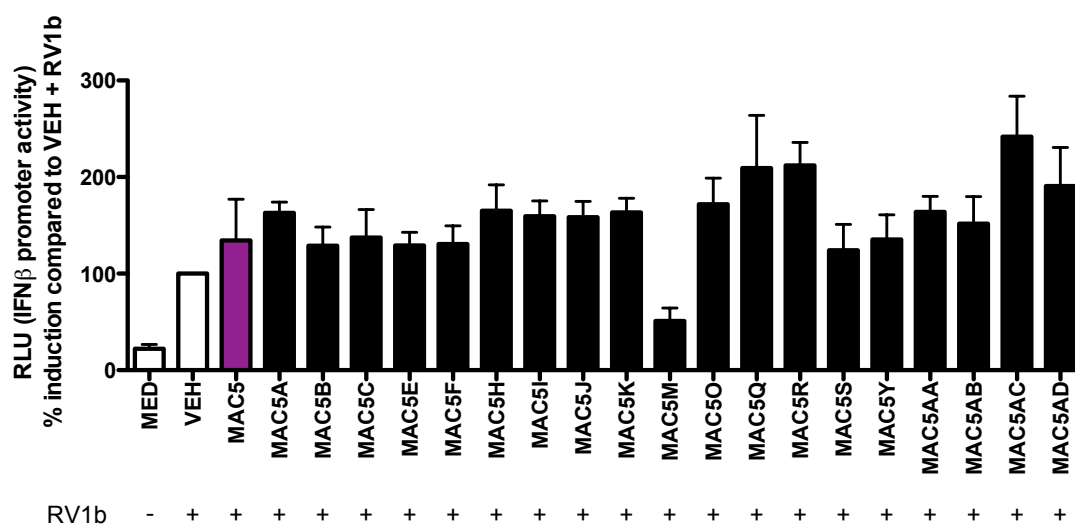


Figure 6.19 Novel analogues of MAC5 showed trends for augmentation of IFN β promoter activity.

Novel macrolide analogues of MAC5 showed trends for augmenting RV1b-induced IFN β promoter activity in BEAS-2B cell cultures although none to a significant level above MAC5. Data expressed as % change of RV1b + vehicle (VEH) induction 100%. n=5.

6.4.10.6.2 Analogies of MAC5 inhibited IL-6 promoter activity

MAC5A-MAC5AD were assessed for their ability to augment or suppress RV-associated IL-6 promoter activity in BEAS-2B cells. BEAS-2B cell cultures were transiently transfected with IL-6-Luc (and Renilla control) for 24 hours followed by treatment with 50 μ M of MAC5 analogue solution for a further 24 hours, cultures were then infected with RV1b (MOI:1) for 24 hours before levels of promoter activity were assessed. In Fig 6.20 MAC5, MAC5K, MAC5M, MAC5Y and MAC5AB significantly inhibited IL-6 promoter activity in RV1b infected BEAS-2B cell cultures (MAC5 32.50%, MAC5K 14.66%, MAC5M 28.67%, MAC5Y 38.42%, MAC5AB 37.73% over vehicle 100%. n=3).

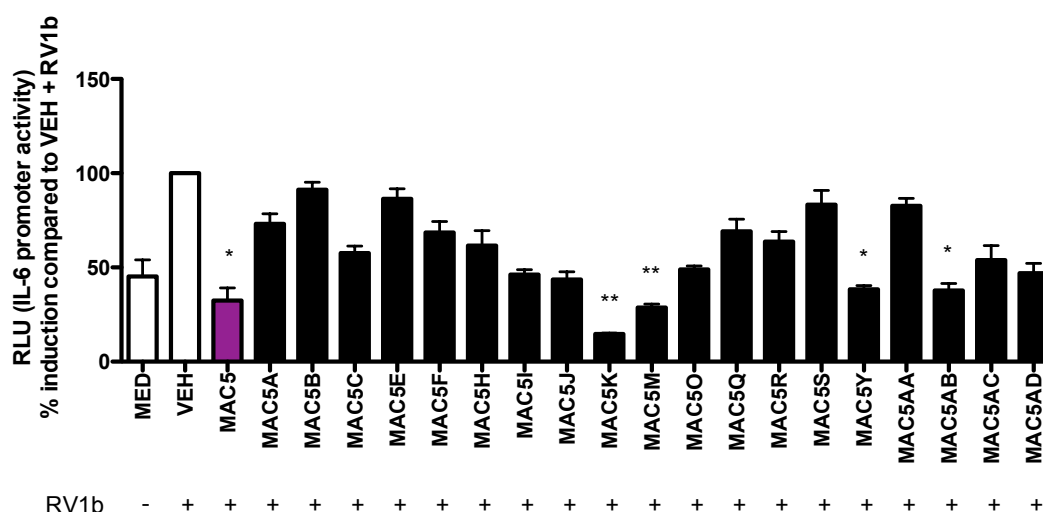


Figure 6.20 Novel analogues of MAC5 inhibited IL-6 promoter activity.

MAC5, MAC5K, MAC5M, MAC5Y and MAC5AB inhibited RV1b-induced IL-6 promoter activity in BEAS-2B cell cultures, many other MAC5 analogues also showed trends for augmentation of IL-6 promoter activity. Data expressed as % change of RV1b + vehicle (VEH) induction. * $p < 0.05$, ** $p < 0.01$ versus RV1b + VEH. $n = 3$.

6.4.10.6.3 Analogues of MAC5 exhibited anti-bacterial properties

Log-phase *E.coli* was grown in LB broth containing 50 μ M of MAC5, MAC5 analogues, Azithromycin or vehicle control over 12 hours (Figure 6.21a). Azithromycin, MAC5A, MAC5B, MAC5H, MAC5M, MAC5Y, MAC5AB and MAC5AC inhibited the growth of *E.coli* compared to Vehicle (Figure 6.21b at 12 hours MAC5A OD_{600} : 0.77, MAC5B: 0.97, MAC5H: 0.48, MAC5M: 0.67, MAC5Y: 0.46, MAC5AD: 0.47 and MAC5AC: 0.69, Vehicle: 0.95. $n = 3$).

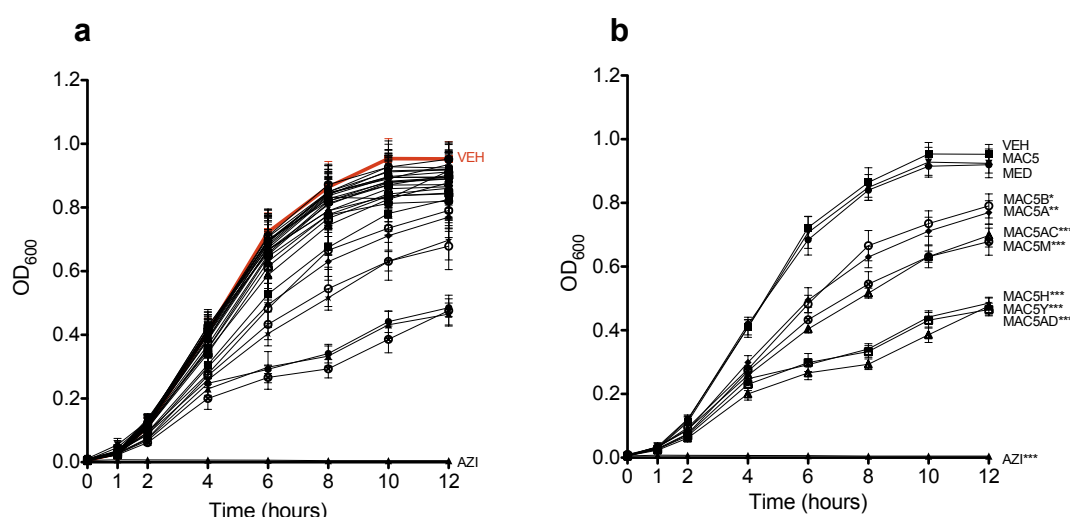


Figure 6.21 Novel analogues of MAC5 showed antibacterial activity against *E.coli*.

(a) *E. coli* was grown in the presence or absence of 50 μM of MAC5 analogues for 12 hours, (b) MAC5A, MAC5B, MAC5H, MAC5M, MAC5Y, MAC5AD and MAC5AC inhibited the growth of *E.coli* compared to Vehicle (VEH) treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for each macrolide compared to vehicle (VEH) treatment calculated from area under curve. $n = 3$.

6.4.10.6.4 Novel analogues of MAC5 did not show correlations between anti-viral, anti-inflammatory and anti-bacterial characteristics.

When plotted together, the level of IFN β promoter augmentation failed to correlate with the level of IL-6 promoter suppression in RV1b-infected BEAS-2B cell cultures (see Figure s6.10 $R = 0.0009$, $p = 0.51$). Additionally, neither anti-viral nor anti-inflammatory compounds correlated with those that were found to be anti-bacterial against *E.coli*, for example MAC5 and MAC5Y had similar anti-viral and anti-inflammatory activity however differed markedly in their antibacterial ability (Figure 6.22).

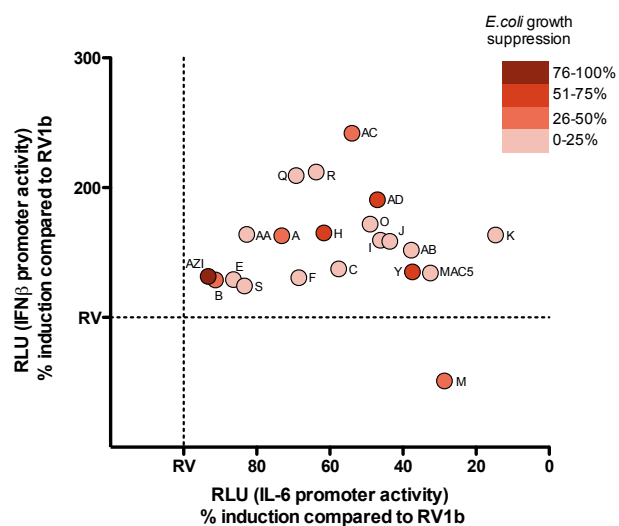


Figure 6.22 Novel analogues of MAC5 showed varying IFN-augmenting, anti-microbial and anti-inflammatory properties.

MAC5, Azithromycin (AZI) and all of the novel MAC5 analogues tested showed some degree of IL-6 promoter suppression in RV1b infected BEAS-2B cells, only MAC5M (M) failed to show augmentation of the IFN β promoter. IFN β -Luc induction did not correlate with IL-6-Luc induction. Several compounds inhibited the growth of *E. Coli*, which appeared to not be related to either IL-6 suppression or IFN β induction.

6.5 Discussion

In this study we described a screening assay that identified a novel macrolide compound (MAC5) that showed a striking ability to augment levels of IFN and ISG mRNA in a model of RV infection in normal HBECs. This screening assay was made feasible (both in terms of cost and workload) by dividing it into three phases, the initial macrolide library of 226 compounds was scaled down to 15, then further scaled to five compounds for in-depth analysis of effects on intrinsic gene expression in primary HBECs. MAC5 produced a dose dependent effect on RV induced IFN and ISG induction suggesting it may be more effective at augmenting IFN and ISGs at a lower dose than used here (the range of EC_{50} observed over the five genes tested was between 5.1 μ M-10.9 μ M). We have also shown that MAC5 is able to reduce RV replication in normal HBEC cultures and replicated this trend in HBECs cultured from asthmatics. Upon further investigation, MAC5 and its structural analogues showed some anti-inflammatory characteristics, MAC5 showed a trend for decreasing secreted IL-8 protein in RV infected normal HBECs and MAC5 along with its structural analogues showed the ability to reduce the promoter activity of IL-6 in RV infected BEAS-2B cells suggesting novel macrolides may have both antiviral and anti-inflammatory effects. MAC5 did not show any anti-bacterial activity against *E.coli*, however we have shown that analogues of MAC5 do indeed inhibit bacterial growth in liquid culture however not to the levels of Azithromycin. Taken together, this study has developed a useful medium-throughput screening assay capable of measuring the potential IFN augmenting ability of novel macrolides and identified a novel compound capable of augmenting RV-induced IFN gene expression in RV-infected HBECs, with more efficacy than that seen with Azithromycin.

Current treatment guidelines for asthma do not include anti-viral therapies (ginasthma.org). Given the high association between RV infection and asthma exacerbations [25, 59, 228] it would seem logical that an anti-viral agent or molecule capable of boosting the host's innate immunity to viruses would be a favourable option in asthma therapy. Conventional systemic IFN therapy is associated with a vast plethora of adverse side-effects [268]. MAC5 and Azithromycin were able to augment IFN and ISGs only in response to RV infection, and did not cause IFN induction in the absence of infection. [142]. Novel agents that are able to augment virally-induced IFN levels, whilst leaving basal levels unchanged (as is the case with Azithromycin) may be a preferable therapeutic option as IFN levels would only be increased in response to incoming infection, therefore potentially avoiding side-effects. Azithromycin or MAC5 could also be used prophylactically, for example given prior to a seasonal trend in disease, such as September in the case of RV-associated asthma

exacerbations in the Northern Hemisphere [228, 269]). It should be noted that the use of recombinant IFN therapy is relatively expensive [270] compared to the use of small molecules; and the inhaled IFN β therapy being developed by Synairgen requires nebulisation. Therefore treatment of a chronic disease such as asthma and COPD could favour the use of a small molecule augmenter of IFN that could be delivered by an easier route such as oral therapy.

Although not statistically significant, IL-8 protein levels were reduced in MAC5 treated cultures (either with or without infection), this combined with the observed decrease in IL-6 promoter activity seen with MAC5 and several derivatives thereof may translate into an anti-inflammatory effect that may be beneficial in the context of asthma [58, 271, 272]. MAC5 structural analogues showed some antibiotic activity in addition to their anti-viral and anti-inflammatory properties suggesting that a single novel macrolide (for example MAC5AD) may be anti-viral, anti-inflammatory and anti-bacterial. A therapeutic compound with anti-bacterial, anti-inflammatory and anti-viral activity may be a favourable option as many chronic inflammatory airway diseases (such as COPD) are associated with virally induced exacerbations that are compounded by secondary bacterial infections [273, 274]. However it must be noted that a growing body of evidence suggests that perturbation of the commensal flora at the mucosal surfaces (including the lungs) by antibiotics may alter host defences to viral infections [231]. A major concern with the use of antibiotics is the generation of resistant bacteria [275-277], it is therefore essential that careful consideration be made when assessing the use of novel (potentially antibiotic) therapeutic agents. This type of screening (specifically the examination of MAC5 analogues) may yield a number of structurally similar molecules with different combinations of anti-viral, anti-inflammatory and anti-bacterial properties, the ability to “pick-and-choose” a compound based upon the specific clinical need would no doubt prove to be a powerful tool against inflammatory disease with and without bacterial or viral infection.

In the limited examples of other attempts to identify small molecules capable of enhancing anti-viral activity in vitro, Holtzman *et al* identified the chemotherapeutic agent Idarubicin, as an augmenter of ISGs in a stably transfected immortalised cell line [266]. Although the study presented here was testing 226 compounds in a medium throughput, non-automated system (compared to 2240 compounds screened in an automated, high throughput assay by Holtzman *et al*), we have shown MAC5 to be antiviral in a clinically relevant cell type, augmenting IFN and reducing RV replication in primary HBECs. Separate studies by Zhang *et al* and Thomas *et al* show Ribavirin, a commonly used anti-viral agent is capable of

augmenting IFN and ISGs in models of RSV and hepatitis C virus infection (respectively) [265, 278]. Ribavirin clinically is used in combination with recombinant IFN α therapy for maximum effect and is modest in its ISG augmenting ability alone [265]. We observed that MAC5 fails to alter baseline IFN and ISG expression in the absence of infection, this may prove a favourable characteristic compared to IFN therapy as it may not induce IFN levels throughout the host organism but only within infected cells.

This approach to screening was successful in generating a smaller selection of compounds and eventually a lead compound, however there were certain limitations and factors that could have been improved. Although 15 compounds augmented RV1b-induced IFN β promoter activity to a threshold in the initial screen those 15 compounds when re-tested in the same model (phase two- this time measuring IFN β and IFN λ 1 promoter activity), showed variation in their ability to augment promoter activity. Phase one of the screening assay delivering false positives (for example MAC2) was most likely due to a lack of replicates in the initial screen and could be rectified in future screens by performing a number of replicates of the initial screen (although this may not be a cost or time effective pursuit). Phase two of the screen, where 15 top-performing compounds were screened for their ability to augment RV1b-associated IFN β and IFN λ 1-promoter activity showed non-significant augmentation of levels of IFN λ 1 promoter activation compared to IFN β (Figures 6.4 and 6.5). It should be noted however that (with the exception of MAC26) the pattern of promoter activity in the IFN λ 1 assay mimicked that of the IFN β assay albeit to a lower extent and IFN β -Luc correlated strongly with IFN λ 1-Luc.

Perhaps the major limitation with this approach to screening is the ability of the first 2 promoter activity screens to identify compounds that could augment intrinsic gene expression in HBECs. Here we took 5 compounds that augmented RV-induced IFN promoter activity in BEAS-2B cell cultures and assessed their ability to augment intrinsic gene expression in normal HBECs infected with RV1b. Although we identified a compound with the ability to augment RV1b-induced type I and type III IFN as well as ISG expression, and to decrease pro-inflammatory cytokines, the other 4 novel macrolides tested did not augment levels of IFN mRNA above the level of Azithromycin (Figures 6.6.-6.10). This failure to augment cytokine mRNA expression in one assay vs. another is likely due to a combination of the different cell types being used and the assay system employed. BEAS-2B cells are an immortalised human bronchiolar cell line and are likely to behave differently from normal primary HBECs (primary cells from cadavers) in response to both macrolide compounds and virus infection. Secondly the first two phases of the screen involved the transfection of cells

with a reporter plasmid that has been shown to stimulate host cell inflammatory responses through the activation of pattern recognition receptors [279] that may have played a role in the differential observations between BEAS-2Bs and HBECs. Finally and perhaps most importantly, in this study we looked at mRNA expression at 24 hours in HBECs, due to the transient nature of mRNA within cells, examining mRNA expression at a single time point only provided a snapshot of the effects of the novel macrolides. When this is compared to the accumulation of stable luciferase produced from promoter activation over the course of 24 hours (as in phase one and phase two of the screen), it is no wonder why the study in HBECs yielded different results to that in the first two phases. Assessing the effects of macrolides on HBEC gene transcription over the course of 24 hours may confirm previous non-augmenters to indeed have IFN augmenting ability, but has a peak augmenting effect at a time other than 24 hours.

MAC5 has potentially useful biological properties as a therapeutic agent in the treatment of virally associated asthma exacerbations, however more experiments are required to realise this potential. Further investigations utilising HBECs from asthmatic patients and *in vivo* models need to be performed in order to confirm the effects of MAC5 and its analogues. Protein production of MAC5 treated HBECs infected with RV must be further assessed, here we failed to detect IFN protein production in the supernatant of HBEC cultures (24 hours post-infection) however this was most likely due to limitations in the detection assay as antiviral effects were seen at later time points. Although MAC5 did not show any anti-bacterial effects against *E.coli*, it should be fully assessed for anti-bacterial activity against a number of bacterial species (specifically the respiratory pathogens *H.influenzae* and *S. pneumoniae*). Unfortunately time and costs did not allow the pursuit of MAC5 *in vivo* however examination of its potential effects should be tested initially in an animal model of viral infection to allow translation from *in vitro* to *in vivo* effects prior to being tested in a human virus challenge model. Although MAC5 shows a striking improvement in efficacy compared to Azithromycin *in vitro* this does not allude to its mechanism of action and further studies including protein-drug interactions must be performed. Finally, for MAC5 or any of its structural analogues to be suitable as a therapeutic agent, *in vivo* pharmacokinetic and pharmacodynamic studies will have to be performed in order to establish drug absorption, distribution, metabolism and excretion in addition to determining the stability and optimal route of administration. Only until these studies are performed, can MAC5 or any of its derivatives be seriously considered as a potential experimental drug for virus induced asthma exacerbations.

Chapter 7 Summary, discussion and future directions

7.1 Summary of findings

Over the last four chapters we have built on the observations published by Gielen *et al* [142] that Azithromycin can augment anti-viral responses to RV infection. We have shown that Azithromycin is able to augment levels of RV-induced IFN and ISG mRNA in HBECs from asthmatic patients. Crucially we demonstrated that Azithromycin is able to suppress viral replication in these cells suggesting that Azithromycin may be able to restore deficient IFN in asthmatic HBECs [84-87]. We have shown that in a mouse model of RV infection, oral Azithromycin may be able to augment anti-viral responses, however more experiments are required to robustly confirm this hypothesis. We have shed light on the mechanism of Azithromycin-mediated augmentation of anti-viral activity identifying MAVS, a key RLH signalling intermediate as a potential target for Azithromycin. Finally, we have identified novel macrolide compounds that not only augment RV-induced anti-viral activity but may also have anti-bacterial and anti-inflammatory effects.

7.2 Azithromycin and the future of asthma therapy

Current asthma therapy is only partially effective at relieving the symptoms of acute asthma exacerbations and asthma morbidity and mortality rates have shown no decline, thus novel therapies are highly sought after. Macrolides, although currently not recommended for the treatment of asthma, have previously demonstrated immuno-modulatory activity that may prove effective in the context of asthma [1]. In particular, Azithromycin has been shown to reduce pro-inflammatory and T_H2 -associated cytokines whilst augmenting RV-induced IFN production in HBECs [142, 195-197] and could, based upon these characteristics, be a potential therapeutic agent in asthma. The observation that Azithromycin can augment host anti-viral activity in asthmatic cells as demonstrated here, adds credence to the idea of its use in the treatment of asthma exacerbations due to the high association of asthma exacerbations with RV infections [25, 27, 28]. Also, the observation that IFN production in asthmatics can be impaired compared to healthy controls [46, 84-87] further supports that IFN-augmenting therapies may be useful in the treatment of asthma exacerbations.

Asthmatics exhibit various clinical read-outs that may be indicative of disease severity. We showed that Azithromycin-mediated augmentation of anti-viral genes in asthmatic HBECs showed a non-significant correlation with PC₂₀ histamine values in asthmatic adults, whilst Azithromycin-associated TCID₅₀ values inversely correlated with PC₂₀ histamine values and the number of positive SPTs. These correlations suggested the more allergic donors may have better responses to Azithromycin regarding anti-viral immunity. Conversely, RV16 replication with Azithromycin negatively correlated with PC₂₀ histamine data, suggesting that Azithromycin has less of an effect on asthmatics with more hyper-reactive airways. It is possible that these correlations are spurious and more studies are required to confirm these findings. Interestingly, correlations were only observed with some gene and replication data and not all, suggesting that Azithromycin treatment could possibly have some beneficial effects on asthmatic patients regardless of asthma severity (however a larger number of HBEC donors would be required to confirm this). Future therapies for asthma may be decided based upon an individual's clinical scores in a drive towards personalised medicine. Azithromycin therapy (and other novel macrolides, such as those identified in chapter six) may be matched to a particular asthma phenotype. Whilst the data presented in chapter three are restricted to early-stage *in vitro* analysis, one might conclude that an agent capable of augmenting RV-induced IFN would prove effective in the treatment of RV-associated asthma as the use of inhaled IFN has also proved effective in clinical trials [173].

Although IFN has been a target of many studies within the context of asthma, most studies have relied on the use of recombinant IFN as a therapeutic agent. In this thesis (and in the original observations by Gielen *et al*), we used Azithromycin as an agent to boost intrinsic anti-viral activity and did not observe any effects on IFN or ISG expression when administered without viral infection. This characteristic of Azithromycin makes it and other molecules exhibiting the same effect ideal for potential prophylactic use. Potentially, Azithromycin could be administered to asthmatics during the peak of cold seasons and would act only if the patient was infected with RV [269, 280]. This would have an advantage over current recombinant IFN-based therapeutics, as they are expensive to produce and historically associated with side-effects [268]. Conversely, long-term antibiotic usage is associated with the development of antibiotic-resistant bacterial species and should be approached with caution [281-283].

Current asthma therapy is hampered by non-compliance and non-adherence and it is hypothesised that if all asthmatics complied with their therapy regime, rates of exacerbations may fall [284, 285]. Oral Azithromycin is known to have a relatively high tissue retention

(including the lungs) compared to other macrolides [224], if used as an oral therapy for asthma it could potentially be dosed at reduced intervals whilst achieving an adequate lung concentration potentially aiding therapy compliance concerns. Oral medication is often associated with better compliance rates, oral Azithromycin administered to mice in chapter five seemed to augment RV-induced IFN suggesting that systemically distributed Azithromycin is capable of acting on the host responses of lung cells. Unfortunately we did not have the time to check if oral Azithromycin did reach and was retained by lung cells; these studies could be the subject of future experiments. To validate our present data the systemic distribution of Azithromycin after oral dosing would need to be confirmed by tissue analysis (such as detecting Azithromycin via MS), however if it is confirmed that oral Azithromycin is present in lung epithelium, this could address potential compliance issues in asthmatics that may further support the use of Azithromycin in asthma.

The study of Azithromycin as a therapy in asthma is still in its infancy, although we have investigated the effect of Azithromycin in the context of RV in cells cultured from asthmatics and *in vivo* in mice, a large number of future investigations are required to confirm the efficacy of Azithromycin. The most comprehensive evaluation of Azithromycin's effects in RV-associated asthma would be a randomised, double blind placebo controlled experimental infection study with RV in asthmatics. This would evaluate the effects of Azithromycin on RV-induced cytokine environment within the lungs including IFNs and ISG expression (measured by taking BAL and sputum samples) changes in lung function (measured by FEV₁ and PEF), symptom scores (with particular emphasis on duration of lower airway symptoms) and RV replication. A study of this kind could also utilise microbiological sampling techniques to control for changes to the lung microbiome possibly confounding the anti-viral effects of Azithromycin. The findings of this type of study could be assessed for correlations with baseline clinical scores (such as the outcomes measures in chapter three; serum IgE, SPT positivity, FEV₁ and PC₂₀ histamine) to determine if Azithromycin-mediated anti-viral responses do indeed inversely correlate with SPT positivity (as seen in chapter three). These analyses would be helpful in confirming data from the present study.

Prior to experimental infection with Azithromycin in asthmatics, further experiments conducted *in vitro* and *in vivo* could be performed to assess Azithromycin's efficacy. Azithromycin was observed to augment antiviral responses in HBEC cultures; other cells of the airways should be assessed for similar effects. Macrophages and dendritic cells are important producers of IFNs and known to produce a robust response to RV infection [46]. The effects of Azithromycin treatment on these cells grown in culture either from cell lines or

purified from BAL samples (from asthmatics and healthy donors) could assess if the effects observed in the studies reported above are limited to the epithelium.

Azithromycin's effects in mouse models of infection were only briefly examined in this thesis and would require repeat experiments of the study outlined in chapter five to confirm the results. If repeat experiments were deemed conclusive of Azithromycin's ability to augment anti-viral responses *in vivo*, several different studies could be performed to build on this finding. The effects of intra-nasal administration of Azithromycin in the mouse should be performed, all previous experiments conducted here have assessed the effects of Azithromycin on the epithelium, oral Azithromycin would be dispersed systemically and the exact concentration of Azithromycin within the epithelium as a result is not known. Intra-nasal administration of Azithromycin is more likely to come into direct contact with the epithelial lining and this may prove more effective as eliciting augmented anti-viral responses. Quantification of Azithromycin levels within the lung tissue as a result of oral dosing vs. intra-nasal dosing in mice could easily be determined by MS and may promote the use of inhaled Azithromycin over oral dosing for any future human studies. Azithromycin administered in an allergic mouse model of RV infection would investigate the complex interplay between the known anti-inflammatory effects of Azithromycin combined with any augmentation of anti-viral immunity. Due to the high degree of complexity existing between signalling pathways leading to the activation of NF κ B, IRF-3/7 and IRF-9 and their potential effects on innate immunity to RV; a study in this kind of model is unlikely to untangle or isolate a single effect of Azithromycin (be it anti-inflammatory or anti-viral). It may be harder to deduce whether beneficial effects of Azithromycin on virus induced allergic airways inflammation are down to anti-inflammatory effects via NF κ B, or anti-viral effects mediated through IRF or a combination of IRF/NF κ B.

7.3 MAVS as a target for anti-viral therapies

Azithromycin is able to augment anti-viral activity in HBECs through interacting with the RLH pathway of viral recognition. Previous studies have demonstrated the importance of the RLHs during RV infection [82]. We have identified MAVS as a potential Azithromycin-interacting protein. Previously, only VCP has been identified as a direct Azithromycin-binding partner [245] and we were able confirm this interaction with the use of an AZI-Azide probe. To date, no associations between MAVS and Azithromycin-mediated anti-viral activity have been published. Our data suggests that small molecules (such as Azithromycin) are able to interact with MAVS and have an effect on anti-viral responses providing a possible target for

therapeutic agents of the future. The negative regulation of MAVS protein expression is mediated by a number of different proteins, theoretically providing a group of possible drug targets that may be able to augment virus-induced IFN.

In order to validate MAVS as a potential target for therapeutic intervention in RV-associated asthma further studies are required. The siRNA knock-down of MAVS and its negative regulators as described in chapter four was only partially conclusive. Our data was interesting in light of the hypothesis that Azithromycin acts through MAVS, as SMURF1 (an E3 ubiquitin ligase that in conjunction with the chaperone NDFIP1 is capable of targeting MAVS for proteasomal degradation [132]). Our data generally suggested that SMURF1, and possibly to a lesser extent, ITCH were required for Azithromycin-mediated IFN and ISG mRNA augmentation. In general, all data was not statistically significant so no definitive conclusion can be drawn. Our data supported the requirement for SMURF1 in Azithromycin-mediated anti-viral gene augmentation however the hypothesis that Azithromycin acts through MAVS cannot be deemed conclusive as we failed to knock-down MAVS itself (and NDFIP1). In the future, knock-down may be achieved with the use of alternative siRNA pools and if MAVS expression could be knocked down then we would expect to see no augmentation by Azithromycin, confirming our hypothesis that Azithromycin acts on MAVS. It is unclear what could be expected of NDFIP1 but until these experiments are performed we cannot conclude that SMURF1 is the only negative regulator that Azithromycin may influence.

MAVS knock-out mice strains have been used in respiratory virus models of infection (albeit not with RV), these strains have demonstrated the importance of MAVS for IFN and pro-inflammatory cytokine expression during RSV infection [286]. One would expect that in MAVS deficient mice, much like an siRNA-mediated *in vitro* model, Azithromycin treatment prior to RV infection would fail to augment innate anti-viral responses as complete ablation of MAVS expression at the genomic level would remove the means for Azithromycin to mediate its actions, thus further confirming our hypothesis. Our data suggests that Azithromycin is either able to bind to MAVS directly and prevent it being targeted for degradation (possibly by prevention of formation of a complex with E3 ligases), or Azithromycin is able to bind SMURF1 although we did not identify it as a potential binding protein in our experiments and prevent its specific binding and ubiquitination of MAVS (see fig 7.1).

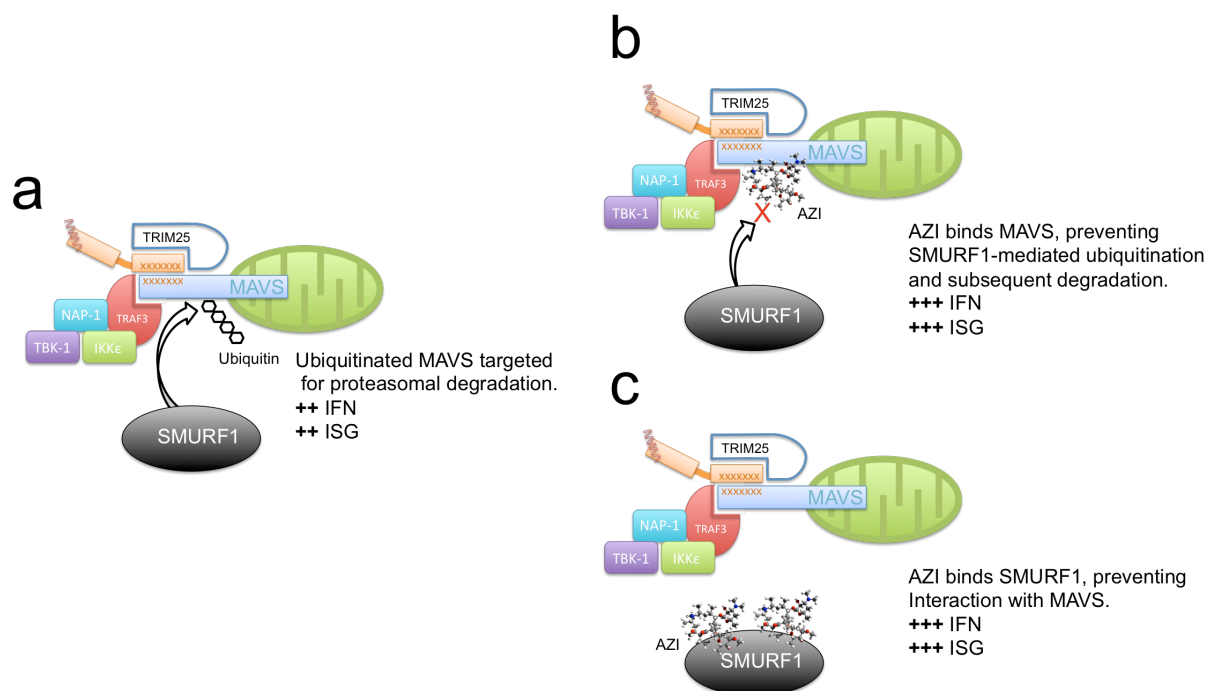


Figure 7.1 Proposed actions of Azithromycin.

During RV infection, SMURF1 mediated the ubiquitination of MAVS leading to negative regulation of IFN and ISG expression (a). Azithromycin may directly interact with MAVS and block the actions of SMURF1 (b) or inhibit the interaction between MAVS and a MAVS binder e.g. SMURF1 or PSMA7 (c) leading to increased IFN and ISG production in response to RV infection.

7.4 Novel macrolides and the future of drug discovery in asthma

Although Azithromycin has displayed anti-viral activity here and anti-inflammatory activity in previous studies the use of Azithromycin as a possible therapeutic agent in asthma is hampered by the potential development of bacterial resistance. Azithromycin is a powerful antibiotic; prophylactic use may result in the development of Azithromycin-resistant bacterial infections [281-283]. For this reason novel macrolides that possess the anti-viral activity seen with Azithromycin without the antibiotic characteristics may be highly sought after as potential therapeutic agents in asthma. In chapter six we identified a macrolide (MAC5) with not only a superior IFN augmenting ability to Azithromycin that did not display antibiotic activity against *E. coli*, but another macrolide (MAC5K) that displayed antibiotic, anti-inflammatory and anti-viral ability. MAC5K is a structural analogue of MAC5. The discovery of MAC5K is suggestive that changing the structure of a molecule (the exact molecular structure is unknown at this time) can add, remove or alter the anti-viral, anti-inflammatory or antibiotic characteristics. The role of bacteria in asthma exacerbation is not as important as viruses hence may not warrant any antibiotic activity, however inflammatory diseases such

as COPD that are known to be associated with viral and bacterial infections may benefit from an antibiotic, anti-viral and anti-inflammatory macrolide such as MAC5K.

The study of MAC5 and MAC5K is still at an early stage, ultimately proof of concept for both of these molecules may benefit from clinical trials in the context of asthma exacerbations however before studies are conducted in humans it is important to further understand their effects in cell culture and animal models and to perform additional SAR. Firstly, the effects of MAC5K should be assessed for effects on intrinsic gene expression (effects on both anti-viral and anti-inflammatory gene expression) in HBECs. Mechanistic analysis of both MAC5 and MAC5K should be performed, perhaps utilising synthetic PRR agonists/recombinant IFN, proteomic screening and use of a binding probe (as per the experiments performed in chapter four with Azithromycin) to identify the target. The identification of a potential binding partner of MAC5 and MAC5K could shed light on the mechanism of MAC5 and MAC5K, possibly aiding SAR analysis between MAC5, MAC5K and Azithromycin. The use of similar Azide-bound probes (like that of AZI-Azide used in chapter four) is a powerful tool for detecting protein-drug interactions and could be applied to MAC5 and MAC5K to determine their potential binding partners. Oral or intra-nasal dosing of MAC5 or MAC5K in a RV infection model similar to the model used in chapter five could be used to examine the effects of MAC5 and/or MAC5K *in vivo*. Such an experiment would aim to assess the effects of the novel macrolides on IFN/ISG augmentation and BAL cytokines in addition assessing toxicity of the compounds. MAC5 and MAC5K could also be tested in a virus-induced model of allergic airways inflammation to test efficacy of their anti-inflammatory and antibiotic properties.

The methods that were used in the discovery of MAC5 and MAC5K serve as an example of a biologically relevant assay that can be scaled down to a medium throughput screen. Typical compound screening assays that are designed to identify binding activity against a specific target protein do not fully assess the effects of compounds within cellular systems. The screening assay described in chapter six has the advantage over protein-binding assays of being able to identify novel compounds within a cell culture-based system that is directly related to a biologically relevant, therapeutic outcome (in this instance; RV-induced IFN augmentation). Assessing the effects of novel compounds on the outcome of a disease model rather than screening for a molecule with activity towards a particular protein target may yield novel therapies in a more time and cost-effective manner. Here, we were limited by resources and did not have access to the machinery required to perform high throughput screening (such as fluid robotics) and as a result we were limited to the screening of 225

compounds. With sufficient downscaling and optimisation the screen we performed in chapter six could be adapted to a high throughput screen, potentially allowing for the screening of thousands of compounds for IFN augmenting activity or anti-inflammatory activity that could prove invaluable in the future treatment of asthma.

Therapeutic approaches towards asthma have not yet embraced the use of anti-viral compounds. In this thesis we have suggested that Azithromycin may possess the ability to augment innate anti-viral responses to RV infection and thus may fill a gap in RV-associated asthma therapy. Azithromycin appears to act through the RLH pathway of viral recognition, specifically acting at the point of MAVS signalling and thus identifying a potential target for future therapeutic intervention. We have demonstrated that other small molecules are able to augment the innate host anti-viral defences to a greater extent than seen with Azithromycin and may prove to be a better asthma therapeutic option (especially if combined with anti-inflammatory and/or antibiotic activity). The ability to up regulate host type I and III IFN production may prove to be a novel therapeutic breakthrough in the treatment of RV-associated asthma exacerbations.

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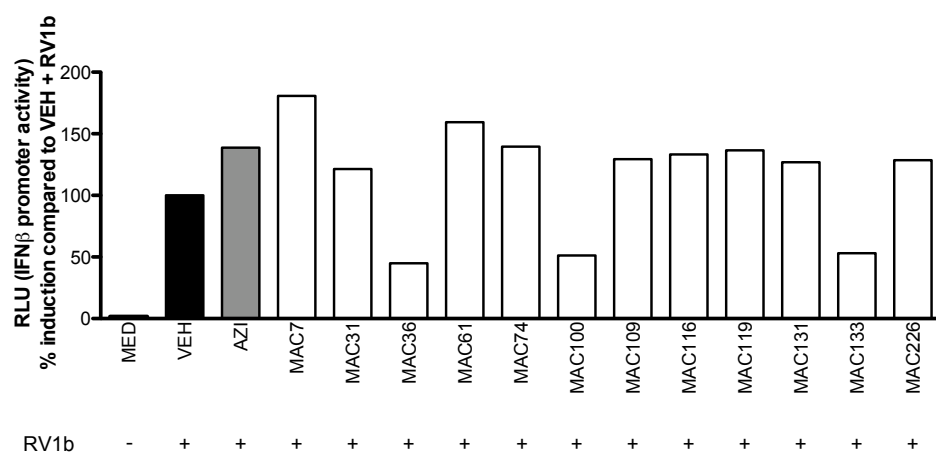
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Appendix

Azithromycin binding proteins ranked by confidence.

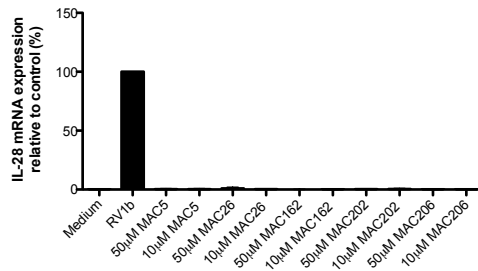
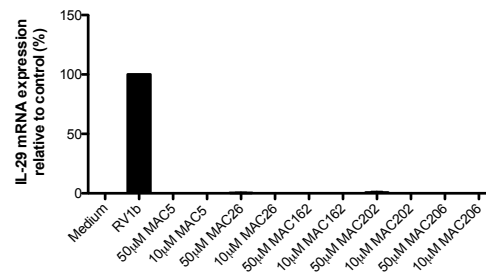
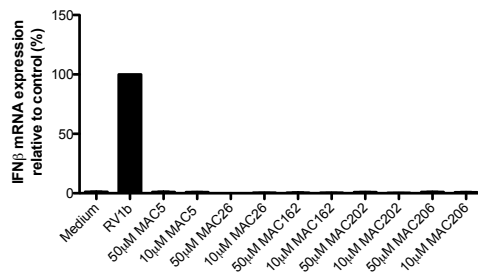
Confidence ranking	Gene name	Confidence ranking	Gene name
1	RCN2	76	CAPG
2	BLVRB	77	ARPC3
3	XRCC6	78	UPP1
4	EEF1B2	79	CKMT1A
5	PRDX1	80	NOP58
6	VCP	81	PSMD1
7	ACLY	82	PSMD3
8	PLS3	83	RPL21
9	PDHB	84	LDHB
10	CAP1	85	TKT
11	EEF1G	86	TUBB2A;TUBB2B
12	MVP	87	FARSB
13	THRAP3	88	PTBP1
14	SERPINB2	89	PFN1
15	ACO2	90	RPS25
16	MAP4	91	EVPL
17	KARS	92	ACTN4
18	NCKAP1	93	ARPC1B
19	UGP2	94	ACOT9
20	RARS	95	PSMC2
21	HNRNPA2B1	96	EPRS
22	UGGT1	97	RPL35
23	NPEPPS	98	MDH2
24	PRDX3	99	DARS
25	ACTN1	100	C14orf166
26	PDIA4	101	SND1
27	PRMT1	102	YWHAE
28	ERH	103	S100A2
29	P4HA1	104	SEP09
30	KHDRBS1	105	EEF1A1;EEF1A1P5
31	PYGL	106	IDH3A
32	LARS	107	CORO1C
33	ARHGAP1	108	HNRNPA1;HNRNPA1L2
34	ATP5B	109	RPL7
35	PDIA3	110	SPTAN1
36	SFPQ	111	FH
37	SDHA	112	AHNAK
38	EEF2	113	IARS2
39	ARCN1	114	PKM2
40	RPL23	115	GLUD1;GLUD2

41	KIF5B	116	CCT3
42	RPL23A	117	RPL3
43	GSTP1	118	FDXR
44	ILF3	119	PPP1CA
45	EIF2S1	120	TAGLN2
46	YWHAG	121	DECR1
47	RPL10;RPL10L	122	PA2G4
48	RPL30	123	FLNB
49	TNC	124	UBA1
50	HNRPDL	125	SERPINH1
51	RPL24	126	PTPN1
52	EIF3L	127	RPS3
53	RPSA	128	EIF3C
54	HNRNPL	129	RPS14
55	RPLP2	130	PSMA7
56	RPS18	131	PYGB
57	RPS10;RPS10P5	132	FBL
58	RPS24	133	ALDH2
59	PSMA3	134	RPL19
60	PGD	135	AHCY
61	HSD17B10	136	EIF4G1
62	PPL	137	FSCN1
63	ALDH18A1	138	ANXA1
64	WDR1	139	NAMPT
65	CAPZB	140	PFKP
66	RPL5	141	FASN
67	PRDX5	142	LDHA
68	EEF1D	143	MYL6
69	RPL13A	144	EIF4A1
70	ACOT7	145	RAB11B;RAB11A
71	P4HB	146	RPS2
72	SYNCRIP	147	RPS9
73	PSMC6	148	DHX9
74	PGK1	149	RPS6
75	TLN1	150	CS
		151	HNRNPD
		152	ATP5A1
		153	CLIC1



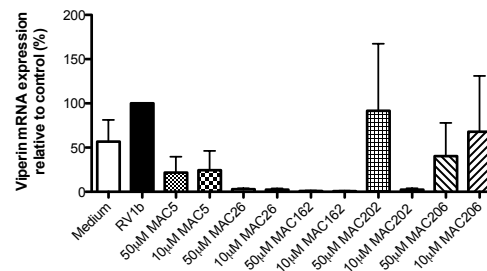
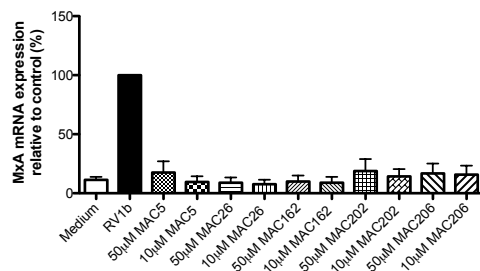
Toxic macrolides tested at a lower dosage showed trends for augmenting IFN β promoter activity.

BEAS-2B cell cultures were transfected with IFN β -Luc and treated with 25 μ M of toxic macrolides prior to infection with RV1b. These macrolides showed limited ability to augment IFN β promoter activity and were not carried through to the next phase of screening. Data expressed as a % change of RV1b + vehicle (VEH) induction. n=1.



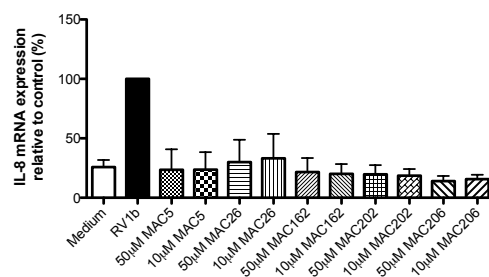
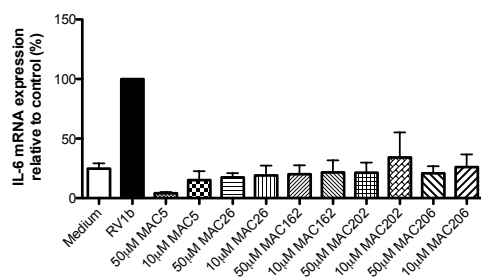
Novel macrolides failed to augment IFN mRNA in the absence of RV1b infection.

Normal HBECs were treated with either 10 μ M or 50 μ M novel macrolide for 24h prior to a mock RV1b infection (supplement-free medium) for 24 hours. Data expressed as % change of RV1b + vehicle (black bar 100%). n=5.



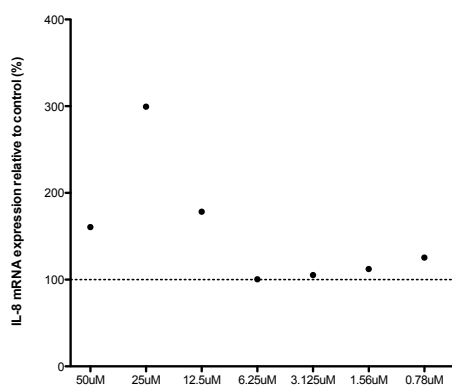
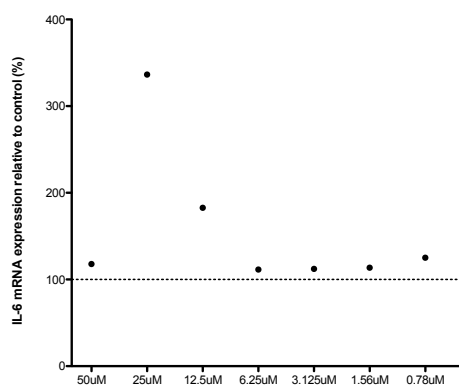
Novel macrolides failed to augment ISG mRNA in the absence of RV1b infection.

Normal HBECs were treated with either 10 μ M or 50 μ M novel macrolide for 24h prior to a mock RV1b infection (supplement-free medium) for 24 hours. Data expressed as % change of RV1b + vehicle (black bar 100%). n=5.



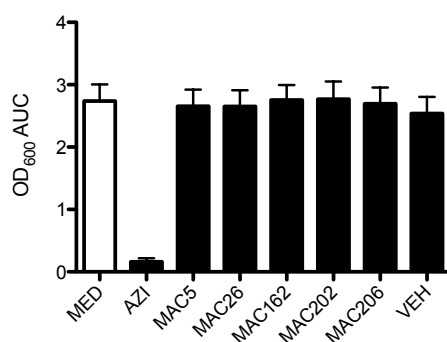
Novel macrolides failed to augment IL-6 or IL-8 mRNA in the absence of RV1b infection.

Normal HBECs were treated with either 10µM or 50µM novel macrolide for 24h prior to a mock RV1b infection (supplement-free medium) for 24 hours. Data expressed as % change of RV1b + vehicle (black bar 100%). n=5.



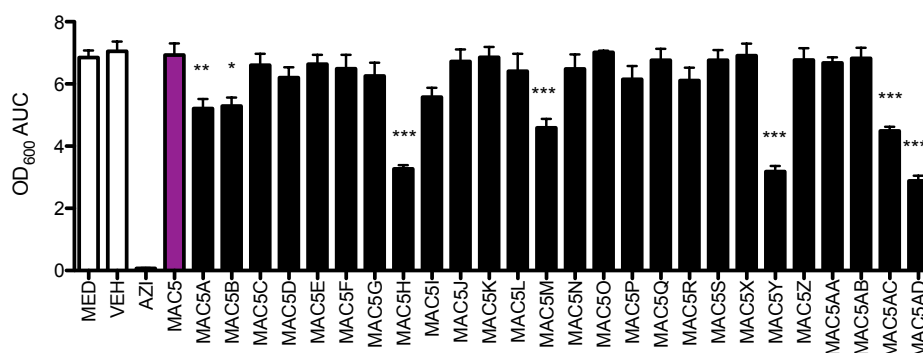
MAC5 does not appear to be dose dependent in augmenting RV1b-induced IL-6 and IL-8 mRNA in HBECs.

MAC5 was titrated (1:2 dilution) and shows a non-sigmoidal dose response to augmenting RV1b-induced Viperin and MxA mRNA. An EC₅₀ value could not be determined by non-linear regression analysis. Data expressed as % change of RV1b + vehicle (100% dashed line) induction. n=3



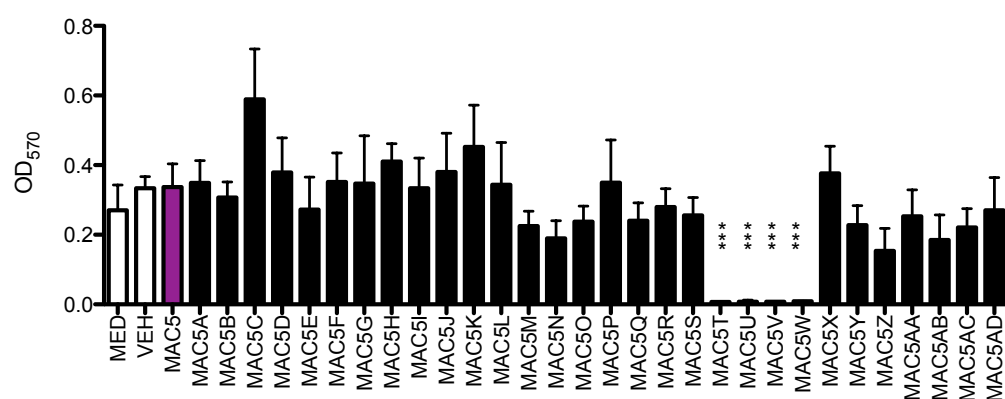
Novel macrolides do not show any antibacterial effects against *E.coli*.

E.coli were grown in LB broth supplemented with 50µM Azithromycin (AZI), Vehicle (VEH), novel macrolides or Vehicle control (VEH). Data expressed as OD₆₀₀ area under curve (AUC) over the course of 8 hours.



MAC5 analogues show antibacterial effects on the growth of *E.coli*.

E.coli were grown in LB broth supplemented with 50µM Azithromycin (AZI), Vehicle (VEH), novel macrolides or Vehicle control (VEH). Data expressed as OD₆₀₀ area under curve (AUC) over the course of 12 hours.



MAC5 analogues show cellular toxicity.

BEAS-2B cell cultures were treated with 50µM novel MAC5 analogues for 24 hours. An MTT assay was performed to assess the levels of cell viability. ***p<0.001 n=3.