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The Roles of DUSPs in Respiratory Viral Infection

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Abstract

Exacerbations of airway disease are significant causes of morbidity and mortality. They are often associated with viral infections, most commonly rhinovirus. Rhinoviral infection of airway epithelial cells initiates several signalling pathways, including the MAPK pathways, leading to the production of inflammatory cytokines. It is of extreme importance that these pathways are regulated to prevent excessive inflammation. Dual specificity phosphatases (DUSPs) are known to negatively regulate the MAPKs in bacterial infection of macrophages. I hypothesised that DUSPs would play an important role in regulating the inflammatory response to rhinoviral infection.

The response of primary bronchial epithelial cells (PBECs) to rhinoviral infection, stimulation with the synthetic double-stranded RNA poly(I:C), or the inflammatory cytokine IL-1β, was characterised. Stimulation or infection of PBECs led to production of inflammatory cytokines, which was reduced by p38 or JNK inhibitors. Two DUSPs previously found to regulate these pathways, DUSPs 1 and 10, were expressed in PBECs. Interestingly, DUSP1 expression was not regulated in response to viral infection but was increased at the mRNA level by poly(I:C). All stimuli induced phosphorylation of DUSP1. In contrast, DUSP10 expression was found to be decreased rapidly and transiently in response to infection with the minor group virus RV1B, but not by the major group virus RV16, or poly(I:C), or IL-1β.

siRNA knock down of DUSP10 did not show a direct role in the regulation of cytokine production in response to rhinovirus or poly(I:C). However, DUSP10 knock down cells consistently produced higher CXCL8 in response to IL-1β stimulation, an important molecule in communication between macrophages and epithelial cells. Rhinovirus replicated well in monocytes, and transfer of supernatants from monocytes to PBECs induced CXCL8 production, which was increased when DUSP10 was knocked down.

These data suggest that the MAPK proteins p38 and JNK play important roles in the inflammatory response to rhinovirus. DUSPs 1 and 10 are both expressed in PBECs, and DUSP10 plays an important role in regulating the inflammatory response to IL-1β, and thus, airway inflammation. This study identified DUSP10 as a potential target for future therapeutics aimed at limiting the inflammation caused by rhinoviral infection.

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I'd like to dedicate this thesis to the memory of my grandparents.

Abbreviations

7-AAD	7-aminoactinomycin D
ANOVA	Analysis of variance
AP-1	Activator protein 1
ARE	AU-rich element
ASC	Apoptosis-associated Speck-like protein containing a CARD
ASK	Apoptosis signal regulating kinase
ATF	Activating transcription factor
ATP	Adenosine triphosphate
AUF1	AU-rich element ribonucleic acid binding protein 1
BAL	Bronchoalveolar lavage
BMDCs	Bone marrow derived dendritic cells
BMDM	Bone marrow derived monocytes
BSA	Bovine serum albumin
c/EBP	CCAAT/enhancer binding protein
C5a	Complement component 5a
CARD	Caspase recruitment domain
Cas9	CRISPR-associated protein 9
CCL	C-C motif chemokine ligand
CD40	Cluster of differentiation 40
CDHR3	Cadherin related family member 3
cDNA	Complementary deoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease
CREB	Cyclic adenosine monophosphate response element binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CXCL	C-X-C motif chemokine ligand
DAMP	Damage associated molecular pattern
Dex	Dexamethasone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
DTT	Dithiothreitol

DUSP	Dual-specificity phosphatase
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal regulated kinase
FCS	Foetal calf serum
G-CSF	Granulocyte colony stimulating factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDI	Guanosine nucleotide dissociation inhibitor
GM-CSF	Guanulocyte macrophage colony stimulating factor
GR	Glucocorticoid receptor
GRO-α	Growth related oncogene α
HBSS	Hanks balanced salt solution
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HTLV-1	Human T lymphotrophic virus 1
IκB	Inhibitor of nuclear factor of κ-light-chain-enhancer of activated B cells
IBV	Infectious bronchitis virus
ICAM-1	Intracellular adhesion molecule 1
IFIT	Interferon induced protein with tetratricopeptide repeats
IFN	Interferon
IFNAR	Interferon-α, β and ω receptor
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-1RI	Interleukin-1 receptor type 1
IP-10	Interferon-γ induced protein 10
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF	Interferon stimulated gene factor

JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
LDLR	Low density lipoprotein receptor
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRP1	Low density lipoprotein receptor-related protein 1
MAPK	Mitogen activated protein kinase
MAVS	Mitochondrial antiviral signalling protein
MDA-5	Melanoma differentiation-associated protein 5
MDMs	Monocyte derived macrophages
MEK	MAPK/ERK kinase
MEKK	MAPK/ERK kinase kinase
MIF	Macrophage migration inhibitory factor
MIP-1α	Macrophage inflammatory protein 1α
MK	MAPK-activated protein kinase
MKK	MAPK kinase
MKKK	MAPK kinase kinase
MKP	MAPK phosphatase
MLK	Mixed-lineage kinase
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MSK	Mitogen and stress activated protein kinase
mTORC	Mechanistic target of rapamycin complex
MyD88	Myeloid differentiation primary response gene
NF-κB	Nuclear factor of κ-light-chain-enhancer of activated B cells
NHS	National health service
NLR	Nucleotide binding oligomerisation domain-like receptor
NLRC5	NLR CARD domain containing 5
NLRP3	NLR family pyrin domain containing 3
NOD	Nucleotide binding oligomerisation
NS1	Non-structural protein 1
OA	Okadaic acid
PAC-1	Phosphatase of activated cells 1

PAMP	Pathogen associated molecular pattern
PBECs	Primary bronchial epithelial cells
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCAF	p300/CREB binding protein-associated factor
PI3K	Phosphoinositide 3-kinase
PMSF	Phenylmethane sulfonyl fluoride
Poly(I:C)	Polyinosinic:polycytidylic acid
PP	Protein phosphatase
PRR	Pattern recognition receptor
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RhoA	Ras homolog gene family member A
RIG-I	Retinoic acid inducible gene 1
RIP-1	Receptor interacting protein 1
RLR	Retinoic acid inducible gene-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcriptase polymerase chain reaction
RV	Rhinovirus
SCF-SKP2	Skp, cullin, F-box containing complex S-phase kinase associated protein 2
$SCF^{\beta\text{-}TrCP}$	Skp, cullin, F-box containing complex β-transducin repeat containing
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	Short hairpin ribonucleic acid
siRNA	Short interfering ribonucleic acid
SNP	Single nucleotide polymorphism
ssRNA	Single stranded ribonucleic acid
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase
TAB	Transforming growth factor β-activated kinase 1-binding protein
TAK1	Transforming growth factor β-activated kinase 1
TBK	TRAF family member-associated NF-kappa-B activator binding kinase 1

TBS	Tris buffered saline
$TCID_{50}$	Tissue culture infective dose 50
TEMED	Tetramethylethylenediamine
Th	T helper
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tpl2	Tumour progression locus 2
TRAF	Tumour necrosis factor receptor associated factor
TREM-1	Triggering receptor expressed on myeloid cells 1
TRIF	TIR-domain containing adaptor-inducing interferon-β
TRIM	Tripartite motif containing 25
TSLP	Thymic stromal lymphoprotein
TTP	Tristetraproline
Tyk	Tyrosine kinase
UK	United Kingdom
UV	Ultraviolet
VLDLR	Very low density lipoprotein receptor
VP	Viral protein

Contents

Figures

Tables

1 Chapter one: Introduction

1.1 Asthma and Chronic Obstructive Pulmonary Disease

Inflammatory airway diseases are major causes of morbidity and mortality. Asthma and chronic obstructive pulmonary disease (COPD) are becoming more and more prevalent, with COPD affecting over a million people in the UK, causing around 23,000 deaths per year, while asthma causes around 1,000 deaths per year (Darnton, 2014, Holton, 2012). There are also large costs associated with these diseases: asthma is estimated to cost the NHS one billion pounds per year (Holton, 2012). Furthermore, current therapies have significant side effects, and are poorly effective as they ease symptoms but do not address the underlying mechanisms of the disease (Mallia et al., 2011).

Both diseases are characterised by inflammation and airway obstruction; but while the inflammation in asthma is reversible and variable, in COPD it is irreversible and progressive (Barnes, 2008). COPD refers to a group of related progressive lung diseases, the main two being emphysema, characterised by abnormal dilation of the alveoli and the destruction of alveolar walls, and chronic bronchitis, characterised by inflammation of the bronchi (Reviewed in: Barnes et al., 2003). Each of these diseases leads to decreased air flow with common symptoms of breathlessness and persistent cough. COPD is thought to be most commonly caused by components of cigarette smoke activating the immune system, eventually leading to chronic inflammation, although it does not only affect smokers (Sabroe et al., 2007).

1.1.1 Exacerbations

The major cause of hospitalisations and mortality in asthma and COPD are acute exacerbations where inflammation causes obstruction of the small airways (Gern, 2010). This leads to cough, wheezing, shortness of breath, chest tightness and decreases in expiratory air flow (Fuhlbrigge et al., 2012).

Asthma exacerbations can be triggered by atmospheric and environmental factors as well as allergens (Johnston and Sears, 2006). One of the main triggers of exacerbations is respiratory viral infections. Several studies have examined the prevalence of respiratory viruses in acute exacerbations of asthma and COPD. In asthma, the percentage of cases of acute exacerbations where respiratory viruses were present

ranges from 62% to 85%; of these infections, 60% were rhinoviruses (Atmar et al., 1998, Heymann et al., 2004, Johnston et al., 2005, Johnston et al., 1995, Khetsuriani et al., 2007, Kling et al., 2005, Miller et al., 2016, Wark et al., 2002). In COPD, respiratory viruses were found in 37-56% of acute exacerbations, 64% of which were rhinoviruses (McManus et al., 2008, Rohde et al., 2003). In COPD, these exacerbations are more severe and frequent than non-virally induced exacerbations (Seemungal et al., 2001). Asthma morbidity correlates with the isolation of human rhinoviruses from the community, with peaks in September and spring (Johnston et al., 2006).

Asthma and COPD patients do not catch respiratory viral infections more frequently than healthy controls, but the infections are more likely to progress and spread to the lower respiratory tract, becoming much more severe. Rhinovirus is initially detected in nasal lavage 2 days post inoculation, then in bronchoalveolar lavage (BAL) and bronchial brushings by 4 days (Message et al., 2008, Mosser et al., 2005). The viral load detectable in BAL is higher in asthmatic patients (Message et al., 2008). This greater susceptibility to more severe illness could be due to damaged epithelium being more easily infected by virus (Gern, 2010, Jakiela et al., 2008), and the structural changes to the airways that occur in asthma have also been implicated. For example, a study in 2010 showed that goblet cells are particularly susceptible to rhinovirus infection (Lachowicz-Scroggins et al., 2010); one of the features of asthmatic airways is an increase in goblet cells, and thus mucus production, along with an increase in smooth muscle and airway remodelling with deposition of collagen and fibronectin under the epithelial basement membrane (Lambrecht and Hammad, 2012). Rhinovirus itself can also damage the airway epithelium, infection has been demonstrated to increase vascular leakage through the disruption of tight junctions (Sajjan et al., 2008).

It has been suggested that asthmatics have deficient immune responses, although this is controversial. Several studies have suggested that primary bronchial epithelial cells from asthmatics secrete reduced amounts of type I and type III interferons (IFNs) in response to infection with two serotypes of rhinovirus, RV16 and RV1B, in comparison to cells from healthy donors (Contoli et al., 2006, Edwards et al., 2013, Wark et al., 2005). This deficiency was also associated with an increase in viral replication, although the secretion of pro-inflammatory cytokines was no different between asthmatic and healthy cells (Wark et al., 2005). However, two studies utilising similar methods and cell

types found no significant difference between the levels of viral replication or of IFNs secreted in response to rhinoviral infection in asthmatics and non-asthmatics (Bochkov et al., 2010, Lopez-Souza et al., 2009). The reasons for these differences remain to be fully understood but could be dependent on the asthma phenotype, with more severe atopic asthma having a greater IFN deficiency (Sykes et al., 2012, Sykes et al., 2014). As well as IFN deficiency, many asthmatics have a skewed adaptive immune response, towards the T helper 2 (Th2) axis, leading to allergic eosinophilic inflammation (Message et al., 2008, Woodruff et al., 2009). This eosinophilic response has been correlated to lower lung function and greater lower-respiratory-tract symptoms in rhinoviral infection, and levels of Th2 cytokines correlate with the severity of asthma exacerbation (Message et al., 2008, Zambrano et al., 2003).

1.2 Rhinovirus

Rhinovirus (RV) causes the common cold; while the majority of people will recover from infections relatively quickly, RV can cause exacerbations in asthmatics and COPD patients which lead to hospitalisations and, in rare cases, death.

Human rhinoviruses belong to the family Picornaviridae and genus Enterovirus. They are non-enveloped, icosahedral, positive sense, single stranded RNA viruses enclosed within a protein capsid composed of four proteins, VP1 to VP4. Over 150 serotypes have been identified so far (Bochkov and Gern, 2012, Palmenberg et al., 2009). These serotypes are grouped into RV-A, RV-B and RV-C based on phylogenetic analysis. Group C was discovered in 2007 (Lau et al., 2007), but poor culture methods have limited investigation into this group (Hao et al., 2012). Rhinoviruses can also be classified based on the receptor they utilise to infect cells. The major group, which includes most of RV-A and all of RV-B, binds the receptor intracellular adhesion molecule 1 (ICAM-1) (Greve et al., 1989) while some serotypes in RV-A bind low density lipoprotein receptor (LDLR), very-LDLR (VLDLR) and LDLR-related protein 1 (LRP1) and are classified as the minor group (Hofer et al., 1994, Marlovits et al., 1998). RV-C has recently been found to bind cadherin related family member 3 (CDHR3) (Bochkov et al., 2015).

1.2.1 Rhinovirus Life Cycle

Rhinovirus replicates within the epithelial cells lining the respiratory tract. Rhinovirus replicates best at 33^{o}C and is therefore usually infects the nasal passages in the upper

respiratory tract. However, studies have shown the ability of rhinovirus to replicate well at 37^{o}C (Papadopoulos et al., 1999) and in experimental infection of human subjects rhinovirus has been recovered from the lower respiratory tract (Gern et al., 1997). Rhinovirus binds to receptors on the surface of epithelial cells, ICAM-1, LDLR or CDHR3, triggering receptor mediated internalisation. This internalisation can be via clathrin dependent or independent endocytosis, or micropinocytosis, depending on the serotype (Ganjian et al., 2017, Grassme et al., 2005, Khan et al., 2010, Snyers et al., 2003). Conformational changes of the rhinovirus capsid then occur, in the major group viruses these changes are induced by binding to ICAM-1 and in the minor group they are induced by the low pH environment within the endosome (Garriga et al., 2012, Xing et al., 2003). These changes release the VP4 protein from the capsid which forms pores in the endosomal membrane, allowing the viral RNA to move into the cytoplasm (Davis et al., 2008, Panjwani et al., 2014). Once within the cytoplasm, the RNA is translated into a polyprotein. This polyprotein is then cleaved by the viral protease 2A into P1 and P2/3. P1 is cleaved again into the structural proteins VP1, VP3 and VP0. Another viral protease, 3C, then cleaves P2/3 into the non-structural proteins (Dreschers et al., 2007). The virus then forms replication complexes within the cytoplasm where the genomic RNA is replicated by the viral RNA dependent RNA polymerase (Love et al., 2004). The virus then assembles, and VP0 is cleaved into VP2 and VP4 in the maturation step (Lee et al., 1993). The cell then lyses to release the nascent virus particles. This is summarised in figure 1.1.

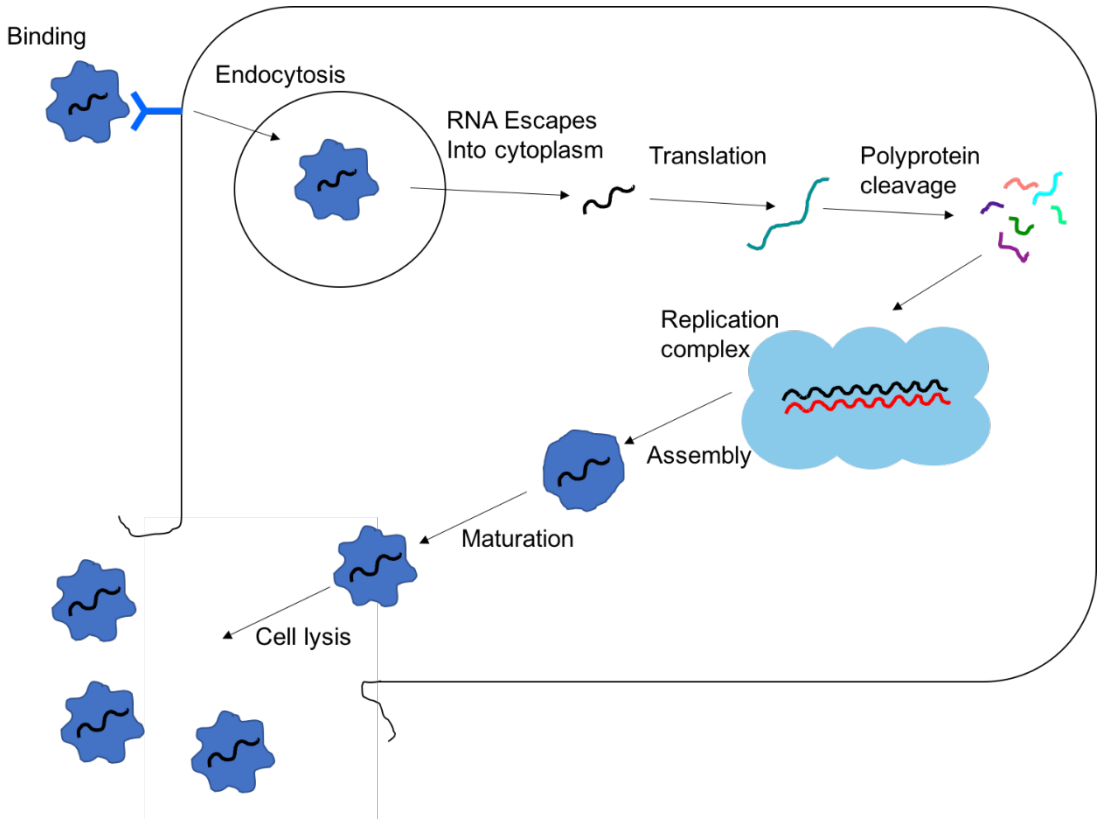

Figure 1.1 The life cycle of rhinovirus.

Rhinoviral particles bind to receptors on the cell surface triggering receptor mediated endocytosis. The viral RNA then escapes from the endosome and is translated into viral polyprotein. The polyprotein is then cleaved into viral proteins. The virus forms replication complexes where the RNA is replicated and the virus particles assemble. The VP0 protein is cleaved in the maturation step and the cell is lysed. (Adapted from: Dreschers et al., 2007, Whitton et al., 2005).

1.3 Innate Immune Response to Viruses in the Airway

The respiratory tract needs to be protected from the constant influx of inhaled pathogens. A layer of sticky mucus covers the epithelium, trapping pathogens before they are able to reach the cells below. This mucus is constantly moved upwards by beating cilia on the epithelial surface, removing any trapped pathogens. This mucus also contains immune factors, such as: secreted antibodies, and collectins, which bind and aggregate pathogens, enhancing their phagocytosis by resident immune cells (Bell et al., 1981, Vaniwaarden et al., 1990). These resident cells include macrophages, which are split into two populations: alveolar macrophages, on the alveolar epithelial surface, and interstitial macrophages within the alveolar walls (Franke-Ullmann et al., 1996). These macrophages are capable of phagocytosing and killing pathogens, and secreting inflammatory mediators. However, the epithelial cells lining the airways are often the first line of defence. These cells express pattern recognition receptors (PRRs), both on the plasma membrane and within the cell in the cytoplasm and endosomes, which recognise pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Section 1.3.1) (Sha et al., 2004). This gives these cells the ability to monitor both the external and internal environment. Binding of ligands to PRRs initiates signalling pathways (Section 1.3.2) which lead to the secretion of many different molecules: IFNs; cytokines, including chemotactic cytokines (chemokines); and adhesion molecules (Newton and Dixit, 2012). The secretion of these molecules initiates inflammation, attracting immune cells towards the site of infection (Figure 1.2).

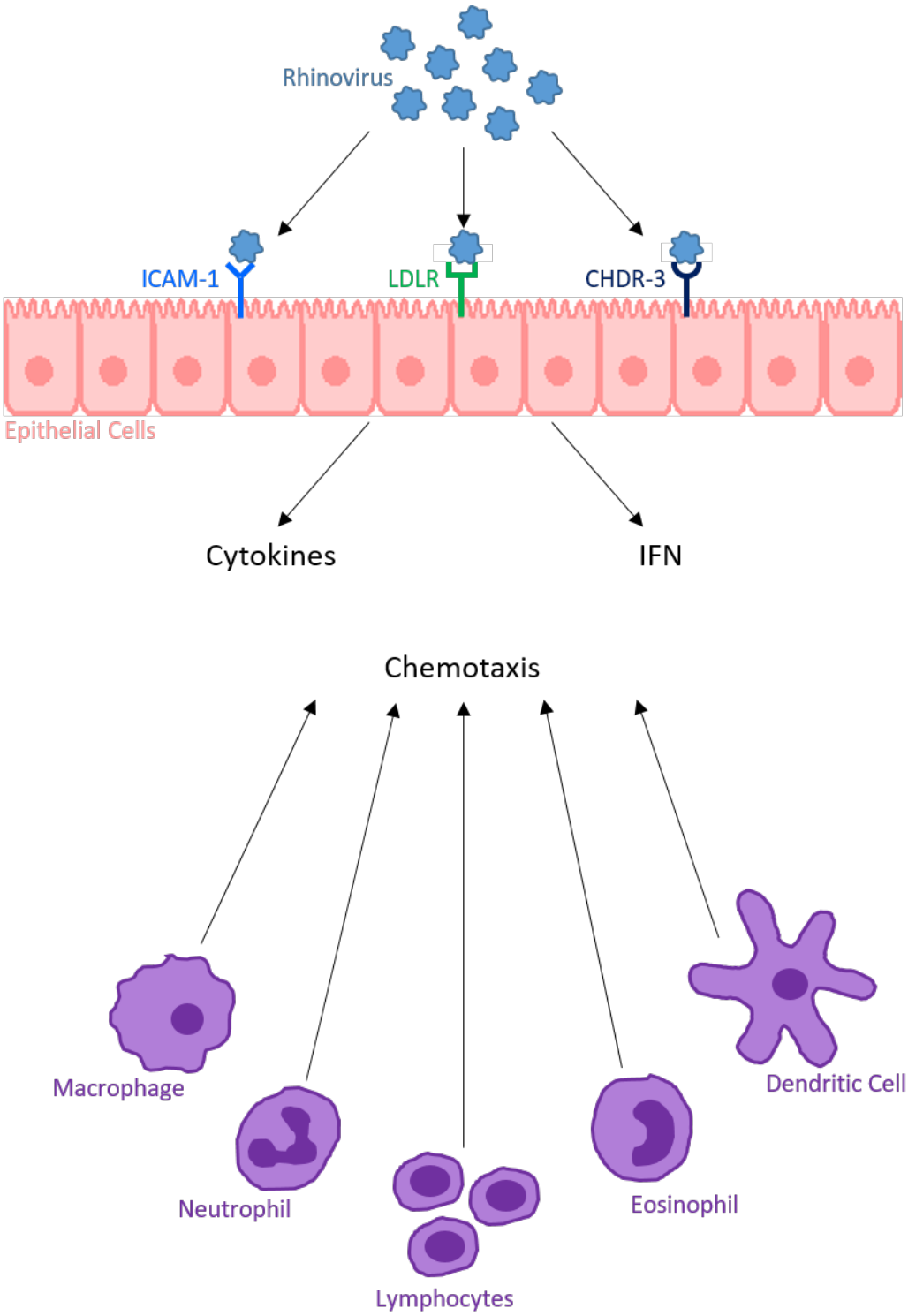

Figure 1.2 The initiation of an inflammatory response to rhinoviral infection.

Rhinovirus particles infect epithelial cells through binding to either ICAM-1, LDLR or CHDR-3. This induces epithelial cells to secrete cytokines including chemokines and IFN. Immune cells then move towards the site of infection through chemotaxis. (Adapted from: Ritchie et al., 2016, Saraya et al., 2014).

1.3.1 Pattern Recognition Receptors

The major family of PRRs is the Toll-like receptor (TLR) family. In humans, this consists of ten type I transmembrane proteins, each of which recognises a different PAMP (Table 1.1). When activated by a ligand, the TLRs oligomerise and form multi-subunit complexes which initiate signalling cascades leading to the activation and nuclear translocation of a variety of transcription factors, including NF-κB, AP-1, CREB, c/EBP and IRF3 (Section 1.3.2) (Newton and Dixit, 2012). A study in 2004 found that both primary cells and cell lines derived from airway epithelium express mRNA for TLRs 1-10 (Sha et al., 2004). Of relevance to viral recognition, three of these receptors, TLRs 3, 7 and 8, recognise RNA viruses and are present on endosomal membranes (Lund et al., 2004, Matsumoto et al., 2003). Here they can detect viruses entering the cell through endocytosis.

Table 1.1: Primary ligands recognised by toll-like receptors

TLR	Primary Ligand	Reference
TLR1	Triacyl lipopeptides	(Takeuchi et al., 2002)
TLR2	Peptidoglycan Lipoproteins Lipoteichoic acid	(Yoshimura et al., 1999) (Hirschfeld et al., 1999) (Schwandner et al., 1999)
TLR3	dsRNA	(Alexopoulou et al., 2001)
TLR4	Lipopolysaccharide (LPS)	(Hoshino et al., 1999)
TLR5	Flagellin	(Hayashi et al., 2001)
TLR6	Diacyl lipopeptides	(Takeuchi et al., 2001)
TLR7	ssRNA	(Heil et al., 2004)
TLR8	ssRNA	(Heil et al., 2004)
TLR9	CpG dinucleotides in DNA	(Hemmi et al., 2001)
TLR10	Ligand unknown, potential co-receptor for TLR2	(Guan et al., 2010)

RIG-I like receptors (RLRs), another virus-specific family of PRRs, are present in the cytosol where they can detect the double stranded RNA (dsRNA) produced by actively replicating viruses and 5'-triphosphorylated single stranded RNA (ssRNA) (Kato et al., 2008, Hornung et al., 2006). This family consists of three proteins: RIG-I, MDA5 and LGP2. Like TLRs 3, 7 and 8, RIG-I and MDA5 bind viral RNA and initiate signalling pathways, leading to the activation of the transcription factors NF-κB and IRF3 (Section 1.3.2) (Yoneyama et al., 2004). They are present in most cell types at low levels, and their expression is induced by type I IFNs in response to viral infection (Kang et al., 2004).

LGP2 lacks the caspase activation and recruitment domain (CARD)-like domain which is essential for RIG-I and MDA5 signalling, and is thought to act as a positive regulator of these two proteins (Satoh et al., 2010).

Both TLRs and RLRs are thought to detect rhinovirus infection but there is debate over which receptors are involved. Two studies have used small interfering RNA (siRNA) to silence different PRRs in rhinovirus infection and determined the effect on IFN and cytokine production (Wang et al., 2009, Slater et al., 2010). Both groups found that TLR3 and MDA5 play important roles in the detection of rhinovirus but they disagreed on the role of RIG-I. Wang et al found no difference in the cell response when RIG-I was silenced, whereas Slater et al. found that knockdown of RIG-I decreased the amount of IFN secreted and also led to a higher viral load (Wang et al., 2009, Slater et al., 2010). These differences may be due to the cell type used in each study, Wang et al. used a cell line derived from human bronchial epithelium (BEAS-2B) and Slater et al. used primary human bronchial epithelial cells. In addition, the study by Slater et al. found that expression of RIG-I and MDA5 was low at baseline and increased by four days post infection (Slater et al., 2010). Furthermore, when the TLR3 pathway was inhibited, RIG-I and MDA5 expression decreased (Slater et al., 2010). This suggests that TLR3 is the initiator of the response to rhinovirus and induces expression of RIG-I and MDA5 which then contribute to the antiviral signalling pathways.

A later study by Triantafilou et al. had very different findings (Triantafilou et al., 2011). They suggest that TLR2, TLR7 and TLR8 play a major role in rhinoviral recognition in primary human bronchial epithelial cells. However, several other studies have found that airway epithelial cells are unresponsive to the synthetic TLR7/8 ligands R848 and Gardiquimod (Parker et al., 2008, Slater et al., 2010, Morris et al., 2006, Sadik et al., 2009). These differences could be partly due to the fact that the Triantafilou study only included primary cells from one donor and one serotype of RV, RV6 (Triantafilou et al., 2011).

Macrophages are capable of recognising rhinovirus through TLR2 binding the rhinoviral capsid. The inflammatory response of bone marrow derived macrophages (BMDMs) to rhinovirus and UV-inactivated rhinovirus is comparable, suggesting that replication, and therefore dsRNA, is unnecessary for recognition of the virus (Saba et al., 2014). TLR2 knock out mice have much lower inflammatory cell influx in response to RV1B infection

compared to wild-type, and BMDMs taken from these mice have decreased mRNA levels of inflammatory cytokines, such as TNFα (Han et al., 2016, Saba et al., 2014). Recent evidence has suggested that epithelial cells are also capable of recognising RV through TLR2. BEAS-2B cells infected with RV16 or RV39 release comparable levels of CXCL8 to cells infected with UV-inactivated virus. This secreted CXCL8 is reduced when treated with TLR2 siRNA, although the reduction is not as dramatic as that seen in macrophages (Ganesan et al., 2016, Unger et al., 2012).

1.3.2 Signalling Pathways Induced by Viral Infection

Binding of a ligand to TLRs causes the receptor to hetero- or homodimerise (Liu et al., 2008b). The Toll/IL-1 receptor (TIR) domain then binds to TIR domains in adaptor proteins. All TLRs use the adaptor MyD88 with the exception of TLR3 which utilises the adaptor TRIF (Yamamoto et al., 2003). Binding of TRIF to TLR3 leads to the induction of two main signalling pathways: figure 1.3. The first pathway is initiated by TRIF forming a complex with TRAF3, TBK1 and IKKi. TBK1 and IKKi phosphorylate and activate IRF3 (Fitzgerald et al., 2003) which dimerises and translocates to the nucleus (Yoneyama et al., 1998). TBK1 and IKKi are also capable of activating IRF7 in the same way, however this is cell type dependent (Sharma et al., 2003). Transcription of IRF7 is also induced by interferon stimulation through the JAK-STAT pathway in a positive feedback mechanism. IRF3 and IRF7 induce the transcription of interferons, proteins extremely important in inducing an antiviral state in surrounding cells and limiting viral spread (Section 1.4.1).

The second signalling pathway induced by TRIF is mediated by TRIF binding TRAF6 (Sato et al., 2003). TRAF6 ubiquitinates and activates TAK1 (Sato et al., 2005), which activates IKKα and IKKβ by phosphorylation (Wang et al., 2001). IKKβ subsequently phosphorylates p105 and IκBα, targeting them for polyubiquitination by $SCF^{\beta\text{-}TrCP}$ and proteosomal degradation (Yaron et al., 1998). p105 is degraded leaving a smaller protein, p50, which after release from inhibition by the IκBα degradation, binds p65 forming the transcription factor NF-κB (Fan and Maniatis, 1991). Degradation of p105 also releases Tpl2 from inhibition leading to activation of the mitogen-activated protein kinase (MAPK) pathways (Section 1.3.3).

As mentioned above, all TLRs other than TLR3, utilise a different adaptor, MyD88. TLR4 is capable of signalling through both adaptors MyD88 and TRIF. Binding of MyD88 to TLR

leads to the recruitment of IRAK4, which autophosphorylates, allowing for the binding of IRAK1 and IRAK2 (Lin et al., 2010). The formation of this complex leads to recruitment of TRAF6, which then induces activation of NF-κB and the MAPKs through the same pathway as described above for TLR3 signalling (Gohda et al., 2004). However, unlike TLR3 signalling, MyD88 recruitment does not lead to activation of the IRF3 pathway and induction of IFNs.

The RLRs initiate signalling pathways through the adaptor protein mitochondrial antiviral signalling protein (MAVS) (Meylan et al., 2005). Binding of RIG-I to RNA causes a conformational change which exposes the CARD domain (Kowalinski et al., 2011). The CARD domain binds polyubiquitin chains generated by TRIM25 which enables MAVS to bind (Zeng et al., 2010). Activated MAVS forms large aggregates (Hou et al., 2011) which are able to bind TRAF3 and the IKK proteins (Meylan et al., 2005, Saha et al., 2006). This leads to the induction of the same signalling pathways as described above for TRIF-mediated signalling (Figure 1.3).

1.3.3 Mitogen-Activated Protein Kinases

TLRs also activate the mitogen-activated protein kinase (MAPK) pathways (Figure 1.3). These pathways consist of a three-tier kinase cascade of a MAPK kinase kinase (MKKK) phosphorylating a MAPK kinase (MKK) which then phosphorylates a MAPK on two residues, threonine and tyrosine. The MAPK then phosphorylates and activates a range of substrates, including transcription factors and other kinases. There are three families of MAPKs involved in innate immune signalling: p38, JNK and ERK.

Both the JNK and p38 pathways are activated by the MKKK TAK1. TAK1 phosphorylates MKK3 or 6 and MKK4 or 7, which in turn phosphorylate p38 and JNK, respectively (Wang et al., 2001). p38 and JNK then translocate to the nucleus and activate transcription of a variety of genes. Other MKKKs can also lead to p38 and JNK activation; for example, ASK1, MLK3, MEKK1 and MEKK3 have all been shown to induce JNK activation in response to inflammatory stimuli, but TAK1 is thought to be the most widely utilised in the immune system (Tobiume et al., 2001, Brancho et al., 2005, Xia et al., 2000, Huang et al., 2004).

The JNK pathway is the main activator of the AP-1 transcription factors. AP-1 refers to a group of transcription factors that bind to the AP-1 binding site on DNA. They are hetero-

or homodimers composed of Jun, Fos or ATF subunits (Curran and Franza, 1988). c-Jun is the central component of all AP-1 complexes and is phosphorylated by JNK1 and JNK2 (Hibi et al., 1993). This phosphorylation increases its stability and transcriptional activity (Musti et al., 1997, Smeal et al., 1994). JNK can also phosphorylate other AP-1 subunits, including JunD and ATF2, but less efficiently (Gupta et al., 1995, Kallunki et al., 1996). The genes of many inflammatory proteins contain AP-1 binding sites, including: IL-2, IL-3, IL-4, IL-5 and TNFα (Duncliffe et al., 1997, Jain et al., 1993, Rooney et al., 1995, Stranick et al., 1997, Tsai et al., 1996).

There are four proteins in the p38 family: p38α, p38β, p38γ and p38δ. Not much is known about the role of p38γ and p38δ in innate immune signalling, as most p38 chemical inhibitors do not affect them, but it has recently been suggested they may have a role in maintaining Tpl2 stability (Risco et al., 2012). p38α is thought to have the largest role in innate immune signalling pathways as knockout of p38α in mice protects them from lipopolysaccharide (LPS) induced endotoxic shock and decreases the amount of inflammatory cytokines produced by peritoneal macrophages from these mice in vitro, including TNFα, IL-12 and IL-18 (Kang et al., 2008). p38α also has a role in inducing anti-inflammatory molecules, such as IL-10 and dual specificity phosphatase 1 (DUSP1) (Section 1.5.1), in negative feedback mechanisms (Kim et al., 2008). p38β is thought to have less of a role, as knocking out the gene in mice has no effect on LPS stimulated cytokine production (Beardmore et al., 2005).

As well as directly phosphorylating transcription factors, p38α mediates some of its functions through activating other kinases, such as MAPK-activated protein kinase 2 and 3 (MK2 and 3) and mitogen and stress activated protein kinase 1 and 2 (MSK1 and 2). MK2 and 3 are produced by immune cells, such as macrophages, and are thought to have a role in maintaining the stability of TNFα (Kotlyarov et al., 1999). MSK1 and 2 can be activated by both p38α and the ERK proteins ERK1 and 2. They are nuclear proteins which phosphorylate and activate the transcription factors ATF1 and CREB (Ananieva et al., 2008). These transcription factors bind to promoters in the DNA and induce transcription of the anti-inflammatory proteins IL-10 and DUSP1. Dual knockout of MSK1 and 2 in mice made them highly susceptible to LPS-induced endotoxic shock (Ananieva et al., 2008) suggesting they have important roles in regulating inflammatory responses.

ERK1 and 2 also have roles in inducing pro-inflammatory cytokines. ERK1 and 2 are activated by phosphorylation by the MKK MEK1, which is activated by Tpl2 (Beinke et al., 2004). Peritoneal macrophages isolated from Tpl2 knockout mice produce significantly less TNFα in response to LPS stimulation. The same result is seen when MEK1 is inhibited in macrophages from wild type mice pharmacologically (Dumitru et al., 2000).

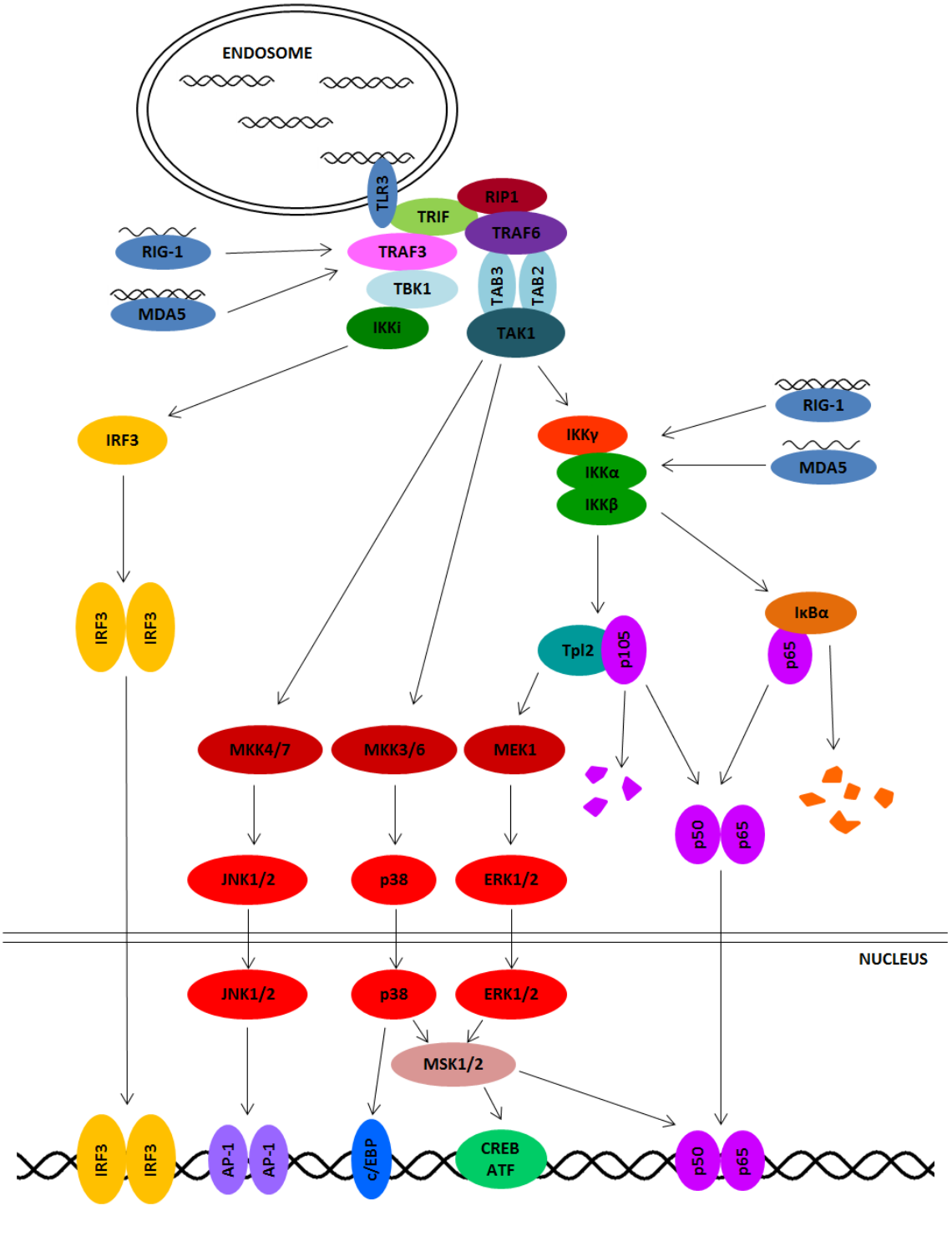

Figure 1.3 Innate immune signalling pathways initiated by viral infection of an epithelial cell.

TLR3 binds dsRNA within the endosome. TLR3 recruits TRIF which mediates two pathways. TRIF interacts with TRAF3, TBK1 and IKKi. TBK1 and IKKi phosphorylate the transcription factor IRF3 which dimerises and translocates to the nucleus. TRIF also interacts with TRAF6 and RIP-1. TRAF6 activates the TAK1/TAB2/TAB3 complex which

activates the IKK complex. This complex phosphorylates IκBα and p105 leading to their breakdown by the proteasome. p105 is broken down leaving p50 which binds p65, after release from inhibition by IκBα, to form NF-κB which translocates to the nucleus, binds DNA and activates transcription of many genes. Breakdown of p105 also releases Tpl2 from its inhibition, which phosphorylates MEK1, a MKK. Other MKKs, MKK3/6 and MKK4/7, are phosphorylated by TAK1. The MKKs phosphorylate the MAPKs which translocate to the nucleus and phosphorylate and activate a variety of transcription factors. RIG-I and MDA5 recognise both double and single stranded RNA in the cytoplasm and activate the IRF3 pathway and the IKK complex (Adapted from: Kawai and Akira, 2007, Newton and Dixit, 2012).

1.3.4 MAPKs in Rhinoviral Infection

Many viruses have been shown to induce phosphorylation and activation of the MAPK proteins upon infection, including influenza virus, simian immunodeficiency virus, vaccinia virus, human cytomegalovirus and murine coronavirus (Mizumura et al., 2003, Popik and Pitha, 1998, Andrade et al., 2004, Johnson et al., 2000, Banerjee et al., 2002). Infection with RV causes a time and dose dependent increase in the phosphorylation of the MAPKs (Griego et al., 2000, Schuler et al., 2014). Gern et al. studied the effect of TLR3 activation on the MAPKs using a synthetic double stranded RNA molecule: polyinosinic:polycytidylic acid (poly(I:C)). Poly(I:C) is composed of one strand of polyinosine base paired to a strand of polycytidine forming a synthetic dsRNA molecule which activates TLR3 and the RLRs. Stimulation of primary bronchial epithelial cells with poly(I:C) led to a gradual increase of phospho-p38 which peaked at 70 minutes (Gern et al., 2003).

Rhinovirus has also been found to activate p38 through signalling pathways other than TLR3, triggered by the viral internalisation pathway. In 2006, p38 was found to be activated by RV14 infection of HeLa cells through the G-protein RhoA (Dumitru et al., 2006). However, this study has limitations, for example the use of HeLa cells, which are not representative of the airway epithelium, and only one strain of rhinovirus. Other studies have demonstrated early, TLR3 independent, p38 activation induced by rhinoviral infection; with RV16 binding to ICAM-1 leading to activation of p38 through the protein kinase Syk (Wang et al., 2006). Again, this research was done in a cell line, BEAS-2B, and only used one rhinovirus serotype, from the major group. In addition, Syk activation was demonstrated to induce PI3K signalling in response to RV16 infection of BEAS-2B cells (Lau et al., 2008), and treatment of BEAS-2B cells with PI3K inhibitors decreased the release of inflammatory cytokines in response to RV16 infection (Ismail et al., 2014). PI3K was also shown to be activated by infection of the airway epithelial cell line 16HBE14o- with another major group rhinovirus, RV39, and this activation was reported to be responsible for a significant proportion of the CXCL8 released (Bentley et al., 2007). The PI3K pathway has previously been associated with the MAPK pathways, for example, the PI3K/Akt pathway can directly activate Tpl2, the MKKK associated with ERK activation in TLR3 signalling (Kane et al., 2002). Furthermore, PI3K signalling activates the JNK pathway in response to growth factor receptor activation (Kraus et al.,

2003). Of note, minor group rhinoviruses, such as RV1B, do not bind ICAM-1 and do not activate this pathway, although they may activate similar kinases through different means. For example, Newcomb et al demonstrated that infection of a human airway epithelial cell line with RV1B, a minor group rhinovirus, induced activation of Akt, a downstream target of PI3K, and mice treated with a PI3K inhibitor had decreased inflammatory cytokine release in response to RV1B (Newcomb et al., 2008). The various possible pathways leading to MAPK activation in RV infection are summarised in figure 1.4.

Pyridinyl imidazole compounds, such as SB203580, inhibit p38 by blocking its catalytic activity, possibly by competing with ATP for its binding site (Young et al., 1997). Griego et al. used two pyridinyl imidazole p38 inhibitors, SB203580 and SB239053, to examine the role of p38 in cytokine and chemokine production by the BEAS-2B human bronchial epithelial cell line in response to infection with the major group virus RV39 (Griego et al., 2000). They found that infection with RV39 caused a time and dose dependant increase in p38 phosphorylation. Treatment with either inhibitor prior to infection led to a significant decrease in the secretion of all cytokines and chemokines examined, including CXCL8, growth-related oncogene-α (GRO-α), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), all of which have important roles in neutrophilia (Griego et al., 2000).

The role of the ERK pathway in rhinoviral infection was examined by Liu et al. in 2008. The BEAS-2B cell line was treated with the ERK1/2 inhibitor U0126 before infection with RV16. U0126 inhibits the ERK1/2 pathway by blocking the activation of the MKKs MEK1 and MEK2 (Davies et al., 2000). Treatment with this drug reduced the secretion of CXCL8 by the cells in response to RV16, returning it to baseline levels (Liu et al., 2008a). Together, these studies demonstrate that the MAPK proteins play an important role in the production of cytokines in response to RV infection of airway epithelial cells.

As MAPKs have roles in many different cellular processes, they may also affect stages of the viral life cycle. For example, Ilnytska et al. have shown that enteroviruses utilise the host cell endocytosis machinery, mainly the protein Rab11, to traffic cholesterol to replication organelles (Ilnytska et al., 2013). p38 has been shown to have a role in the endocytosis pathway: it phosphorylates and activates guanyl-nucleotide dissociation inhibitor (GDI), a protein which facilitates cycling of Rab proteins between the

membrane and the cytosol (Cavalli et al., 2001). This suggests MAPKs may affect more aspects of viral infection than just innate immune signalling and affect viral replication.

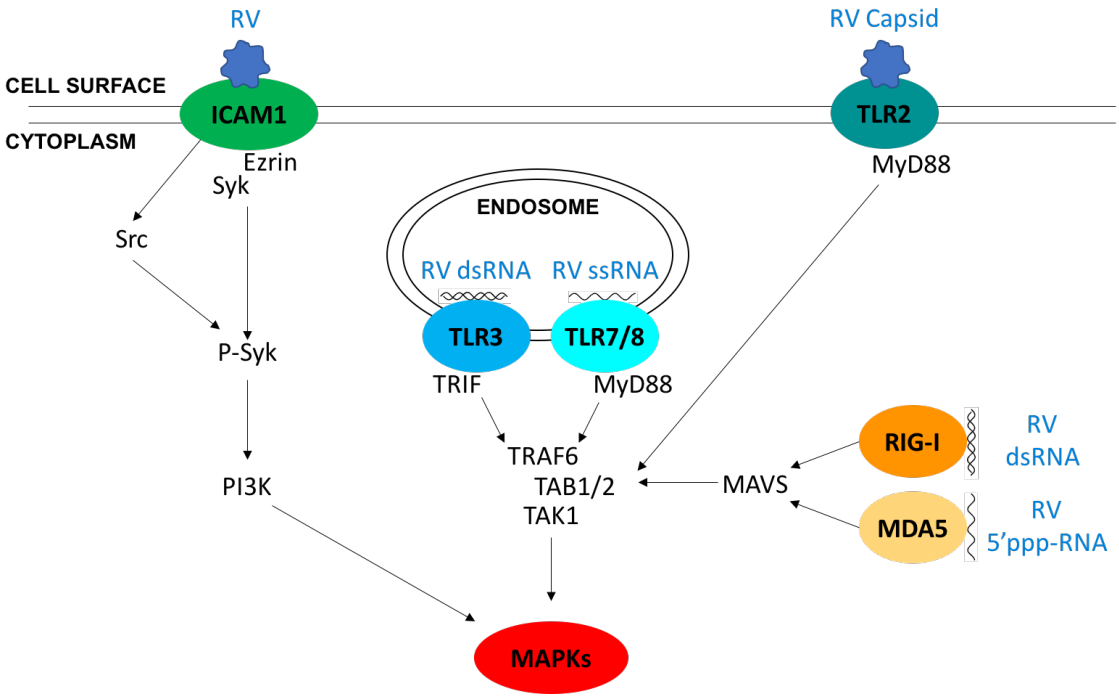

Figure 1.4 Activation of the MAPK pathways by RV infection.

Major group RV bind to receptor ICAM-1 which initiates phosphorylation of Syk and the PI3K pathway. RV can be recognised by various PRRs: TLR2 recognises RV capsid, TLR7/8 recognise ssRNA, TLR3 and RLRs recognise dsRNA and the RLRs also recognise 5' tri-phosphorylated RNA. All of these pathways converge on the TRAF6 pathway which leads to MAPK activation.

1.4 Cytokine Signalling

Epithelial cells have been demonstrated to secrete many cytokines in response to rhinoviral infection (Schroth et al., 1999, Spurrell et al., 2005, Zhu et al., 1996, Zhu et al., 1997). Each has a different role in the immune response to RV. In this thesis, I will be focusing on the following cytokines: type I IFN, IL-1β, CCL5 and CXCL8.

1.4.1 Interferon

Interferons play an important role in controlling viral infections. As discussed in section 1.3.2, activation of the IRF pathways in response to viral infection leads to induction and secretion of interferon. Secreted interferon acts in an autocrine manner to signal danger to the surrounding cells by binding to IFN receptors on the surface. This triggers activation of the JAK-STAT pathway, where the protein tyrosine kinases JAK1 and Tyk2 phosphorylate STAT1 and STAT2 which heterodimerise and translocate to the nucleus (Darnell et al., 1994, Muller et al., 1993). Within the nucleus, they bind IRF9 forming the complex IFN-stimulated gene factor 3 (ISGF3) (Fu et al., 1990). ISGF3 binds to promotors for interferon-stimulated genes (ISGs) inducing transcription (Sato et al., 1998). These ISGs prime the cell for enhanced virus detection and inhibit productive viral replication. Examples of ISGs include the RLRs, enabling enhanced detection of viral RNA, and PKR which phosphorylates and inhibits eIF-2α, blocking protein synthesis (Der and Lau, 1995, Kang et al., 2004).

Type I and III IFNs have important roles in the response to RV. The predominant type I IFNs are IFN-α and IFN-β. IFN-α and β have similar structures and bind the same receptor: a complex of two subunits IFN-α, β and ω receptor (IFNAR)-1 and IFNAR-2. Infection of primary bronchial epithelial cells with RV1B or RV16 was shown to induce production of IFN-α and β (Khaitov et al., 2009), and exogenous IFN-β suppressed replication of RV1B (Cakebread et al., 2011). Clinical trials have been conducted, with some success, using inhaled IFN-β to prevent exacerbations in asthmatics after the onset of cold symptoms (Djukanovic et al., 2014).

Type III IFN, IFN-λ, have similar properties to type I and their production is induced through the IRF pathways, although the promotor also contains binding sites for NF-κB (Onoguchi et al., 2007). Most cell types produce IFN-λ in response to TLR stimulation but only certain cells respond due to limited expression of the receptor, IL-28Rα. The

cell types known to express IL-28Rα include epithelial cells and plasmacytoid dendritic cells (Ank et al., 2008). Binding of IFN-λ to IL-28Rα activates the same signalling pathway as type I IFN (Kotenko et al., 2003). In 2007, Zhou et al. used a gene expression array to compare the response of the B lymphocyte cell line Raji to type I and type III IFNs. Type I and III IFN induced a similar subset of genes, with no genes upregulated only by type III (Zhou et al., 2007). IFN-λ mRNA production has been shown to be upregulated in PBECs in response to infection with RV16 or RV1B (Khaitov et al., 2009). Exogenous treatment of nasal epithelial cells with IFN-λ suppressed replication of RSV (Okabayashi et al., 2011).

1.4.2 Inflammatory Cytokines

Some of the cytokines secreted in response to infection signal to surrounding epithelial cells to induce defences against infection, including IL-1β. IL-1β binds to the receptor IL-1RI on the cell surface which dimerises with IL-1RAcP. This recruits the adaptor protein MyD88 and induces activation of the TRAF6 signalling pathway (Section 1.3.2) (Medzhitov et al., 1998). IL-1β has been shown to have an important role in inducing cytokine release in rhinoviral infection, as treatment of BEAS-2B cells with the IL-1 receptor antagonist (IL-1Ra) significantly reduces CXCL8 expression in response to infection (Stokes et al., 2011).

One ISG was examined in this study, CCL5. CCL5 is transcriptionally induced by IRF3 and secreted by epithelial cells in response to rhinoviral infection (Lin et al., 1999). CCL5 then acts as a chemokine, attracting many cell types to the site of infection, in particular eosinophils (Alam et al., 1993).

CXCL8 is an inflammatory cytokine, transcriptionally induced by NF-κB. It is secreted by cells in response to rhinoviral infection and acts as a neutrophil chemoattractant (Zhu et al., 1997). Asthma is associated with increased neutrophilic inflammation in response to respiratory viral infection (Jarjour et al., 2000, Ordonez et al., 2000, Wark et al., 2002) and this neutrophilia can induce epithelial necrosis and damage the airway (Wang et al., 1998). Indeed, higher levels of CXCL8 have been found to correlate with symptom severity in rhinoviral infection (Turner et al., 1998, Ordonez et al., 2000) and nasal challenge with CXCL8 alone can induce cold symptoms (Douglass et al., 1994). As rhinovirus itself only infects a small percentage of the epithelial cells within the airway

(Arruda et al., 1995, Mosser et al., 2002, Mosser et al., 2005), the damage seen in RV infections is likely due to these infiltrating immune cells.

1.5 Regulation of Inflammation by DUSPs

In order to stop overproduction of inflammatory markers and immune mediated damage, pro-inflammatory signalling pathways are heavily regulated. A family of proteins called dual-specificity phosphatases (DUSPs) have roles in regulating signalling pathways by dephosphorylating both the threonine and tyrosine residues within one substrate simultaneously (Denu and Dixon, 1995). Sixty-one DUSP proteins have been identified so far with roles in many different processes, including the regulation of: actin polymerisation, cell cycle progression and DNA damage checkpoints (Reviewed in: Patterson et al., 2009).

A subset of the DUSP family, called MAPK phosphatases (MKPs), regulates the MAPK pathways triggered by the innate immune response (see figure 1.5). So far ten MKPs have been found, four of which have been shown to have roles in innate immune signalling. In this chapter I will be focussing on these four MKPs: DUSP1, DUSP2, DUSP4 and DUSP10 (Figure 1.5 and Table 2).

Table 1.2: Properties of DUSPs with roles in innate immune signalling.

Name	Alternative Names	Molecular Mass (kDa)	Substrate Specificity	Subcellular Localisation	Reference
DUSP1	MKP-1	39.30	p38 and JNK	Nuclear	(Zhao et al., 2005)
DUSP2	PAC-1	34.40	JNK	Nuclear	(Jeffrey et al., 2006)
DUSP4	MKP-2	42.95	ERK	Nuclear	(Cornell et al., 2010)
DUSP10	MKP-5	52.64	p38 and JNK	Nuclear and Cytoplasmic	(Zhang et al., 2004)

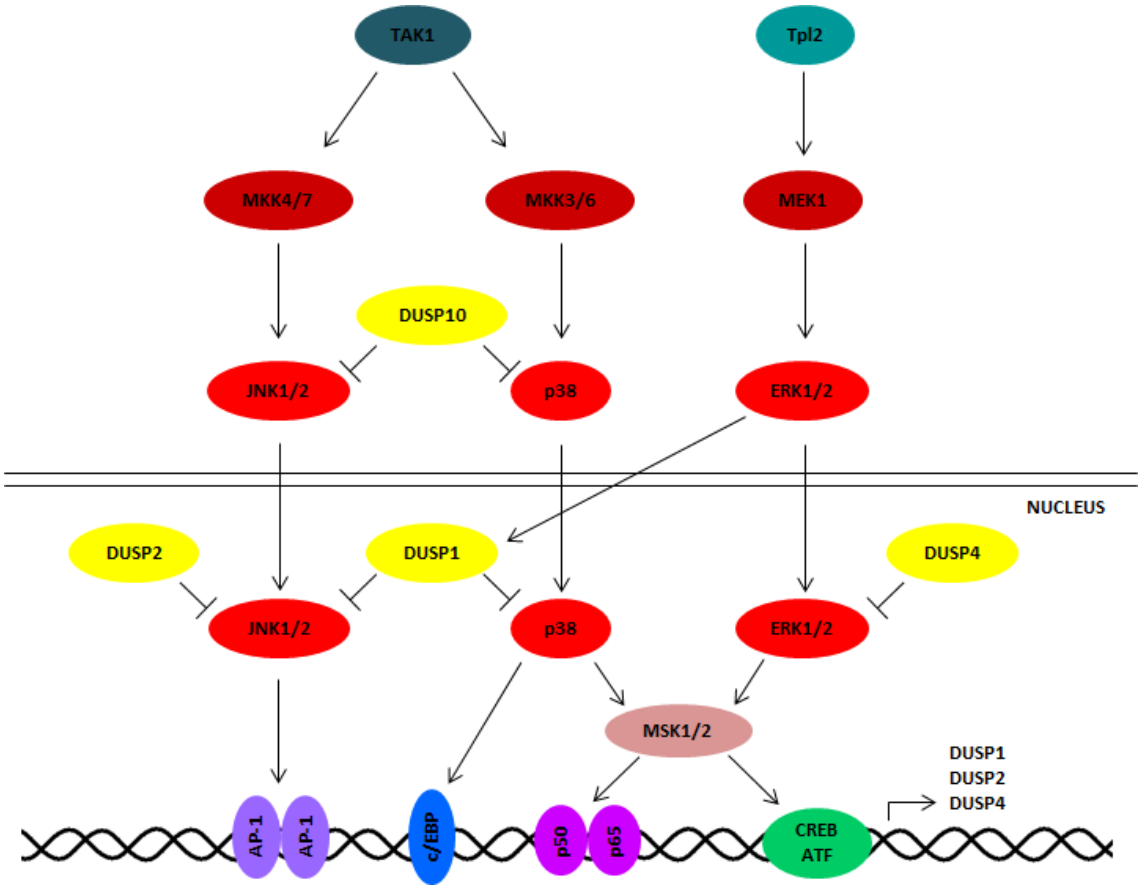

Figure 1.5 Regulation of the MAPK pathways by DUSP proteins in innate immune signalling.

The MAPKs are activated by a phosphorylation cascade: the MKKKs TAK1 and Tpl2 phosphorylate the MKKs MKK4/7, MKK3/6 and MEK1 which phosphorylate the MAPKs which then translocate to the nucleus and activate transcription factors and other kinases. Two kinases activated by p38 and ERK1/2 are MSK1 and MSK2. These activate transcription factors NF-κB and CREB/ATF. CREB/ATF then induces transcription of DUSPs 1, 2 and 4. The DUSP proteins inhibit these pathways by dephosphorylating the MAPKs: DUSP1 and DUSP10 dephosphorylate p38 and JNK1/2, DUSP2 dephosphorylates JNK1/2 and DUSP4 dephosphorylates ERK1/2. DUSP1 is also activated by phosphorylation by ERK (Adapted from: Arthur and Ley, 2013, Newton and Dixit, 2012).

1.5.1 DUSP1

The most well-studied of the DUSP proteins is the nuclear protein DUSP1. DUSP1 preferentially dephosphorylates p38 and JNK, but is capable of dephosphorylating ERK (Chi et al., 2006, Franklin and Kraft, 1997). Studies from knockout mice have shown the importance of DUSP1 in the immune system (Chi et al., 2006, Hammer et al., 2006, Frazier et al., 2009). For example, mice lacking DUSP1 are very susceptible to endotoxic shock when their immune systems are activated by stimuli such as LPS, and DUSP1$^{-/-}$ BMDMs overexpress many inflammatory mediators, such as TNFα, IL-1, IL-6, CCL3, CCL4 and CXCL2, when challenged with LPS, or live bacteria, in vitro (Frazier et al., 2009, Hammer et al., 2006, Talwar et al., 2017). In each study this excessive inflammatory response was associated with increased and prolonged activation of p38 and JNK MAPKs. Loss of DUSP1 also correlated with an increased release of the anti-inflammatory protein IL-10, BMDMs from knock out mice producing 5 to 10 times more than wild type in response to LPS stimulation (Chi et al., 2006, Hammer et al., 2006). This effect was negated when treated with p38 inhibitors (Chi et al., 2006). Furthermore, overexpression of DUSP1 in murine alveolar macrophages has been shown to significantly decrease the release of TNFα in response to LPS (Zhao et al., 2005). DUSP1 has also been implicated in the regulation of interferon production; DUSP1 knock out BMDMs produce much higher levels of IFN-β in response to LPS than wild-type (McGuire et al., 2017). This indicates that regulation of p38 and JNK signalling in response to bacteria by DUSP1 is extremely important in minimising the amount of damage caused by inflammation.

DUSP1 may also be able to positively regulate expression of some genes. During the course of this thesis, Shah et al. performed a gene expression array, comparing the response of A549 cells, with and without DUSP1 overexpression, to IL-1β stimulation. Several gene transcripts were found to be upregulated in DUSP1 overexpressing cells, including STAT5A, the interferon-stimulated genes IFIT1 and IFIT3, and the transcription factor IRF1. Treatment of the lung epithelial cell line A549 with DUSP1 siRNA was found to decrease IRF1 expression in response to IL-1β (Shah et al., 2016a). This study suggests that DUSP1 may have a positive regulatory role in the interferon pathways, however, overexpression studies must be interpreted carefully, as they create a synthetic environment.

DUSP1 may regulate cytokine expression both pre- and post-transcriptionally; a study in 2011 found that DUSP1 can also induce cytokine mRNA decay (Yu et al., 2011). The half-lives of several cytokine mRNAs were increased in BMDMs from DUSP1 knockout mice compared to wild type, and this was associated with a decrease in translocation of ARE/Poly(U) binding degradation factor 1 (AUF1), a protein which binds mRNA and recruits degradation machinery, from the nucleus to the cytoplasm (Yu et al., 2011). This may be an additional result of p38 dephosphorylation, as it has been found that treatment with another imidazole p38 inhibitor, SK&F 86002, promotes AUF1-dependant mRNA decay in primary human monocytes (Sirenko et al., 1997). DUSP1 also activates tristetraprolin (TTP), another mRNA destabilising factor, which binds to and induces the decay of mRNA for several inflammatory cytokines. TTP is inactivated by phosphorylation by p38; therefore, DUSP1 dephosphorylation of p38 prevents TTP inhibition (Prabhala et al., 2016). TTP has also been shown to destabilise DUSP1 mRNA in a negative feedback mechanism (Emmons et al., 2008).

1.5.2 DUSP1 and Viruses

Much of the literature discussed above has examined the role of DUSP1 in response to LPS, a component of bacterial cell membranes, not present in viruses. Recently, its role in the response to viral infection has begun to be investigated. DUSP1 has been shown to be induced by the synthetic viral mimic, poly(I:C), in a human airway epithelial cell line, NCI-H292 (Golebski et al., 2014). Knockdown of the DUSP1 protein in NCI-H292 cells using siRNA significantly increased both induction and secretion of two pro-inflammatory cytokines, CXCL8 and IL-6, in response to poly(I:C) (Golebski et al., 2015). DUSP1 is also induced by infection with viruses: Ebola infection of the human hepatoma cell line Huh7, human immunodeficiency virus (HIV) infection of human alveolar macrophages, and infectious bronchitis virus (IBV) infection of NCI-H1299 cells (a lung epithelial cell line), increased DUSP1 expression (Holzer et al., 2016, Liao et al., 2011, Tachado et al., 2005). This increase in response to HIV was diminished when infected with virus lacking the NS1 protein, suggesting that the virus may be targeting DUSP1 as a mechanism to dampen down the immune response (Tachado et al., 2005). Vaccinia virus has also been shown to upregulate DUSP1 mRNA and protein levels in HeLa cells, and knockout mice infected with this virus had increased TNFα release, and, interestingly, increased viral titres (Caceres et al., 2013).

Surprisingly, silencing DUSP1 in Huh7 cells inhibited the replication of hepatitis C virus (HCV). This was found to be due to increased STAT1 activation and induction of interferon stimulated genes, suggesting DUSP1 dephosphorylates STAT1 (Choi et al., 2015).

1.5.3 Regulation of DUSP1

DUSP1 is transcriptionally induced by a negative feedback mechanism: p38 and ERK activate the kinases MSK1 or MSK2 which phosphorylate the transcription factors CREB and ATF1, which then bind to the promoter of the gene for DUSP1 and initiate its transcription (Ananieva et al., 2008). DUSP1 has been shown to be transcriptionally induced in response to inflammatory cytokines, such as TNFα, and by ligands for TLRs 2, 3, 4 and 9: Pam_3CSK_4, poly(I:C), LPS and CpG (Chi et al., 2006, Manetsch et al., 2012). It is also upregulated by IL-1β in primary airway smooth muscle cells (Issa et al., 2007). This induction is through either the MyD88 or TRIF mediated pathways, with knock out mice for either unable to upregulate DUSP1 (Chi et al., 2006). However, it has been suggested that pretreatment of macrophages with IFN-γ can decrease the induction of DUSP1 by LPS (Zhao et al., 2005). DUSP1 is also regulated by the stability of its mRNA, inhibition of the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) has been found to increase DUSP1 mRNA stability in the RAW 264.7 macrophage cell line (Gao et al., 2017). DUSP1 protein stability can also be regulated: phosphorylation at two serine residues, Ser359 and Ser364, stabilises the protein, prolonging its half-life. The MAPK ERK has been shown to do this in a negative feedback mechanism (Brondello et al., 1999). However, sustained ERK activation has been shown, in cancer cells, to phosphorylate DUSP1 at two alternative serine residues, Ser296 and Ser323, which induces ubiquitination of DUSP1 by SCF-SKP2 leading to proteosomal degradation (Lin et al., 2003). Similarly to other phosphatases, oxidation of the catalytic site by reactive oxygen species (ROS) can inhibit DUSP1 activity (Kamata et al., 2005, Kim et al., 2012, Tephly and Carter, 2007). The catalytic activity of DUSP1 is increased when bound to substrates (Hutter et al., 2000), and the affinity of DUSP1 for its substrates is increased by acetylation of a lysine residue in the substrate binding domain (Cao et al., 2008). This is summarised in figure 1.6.

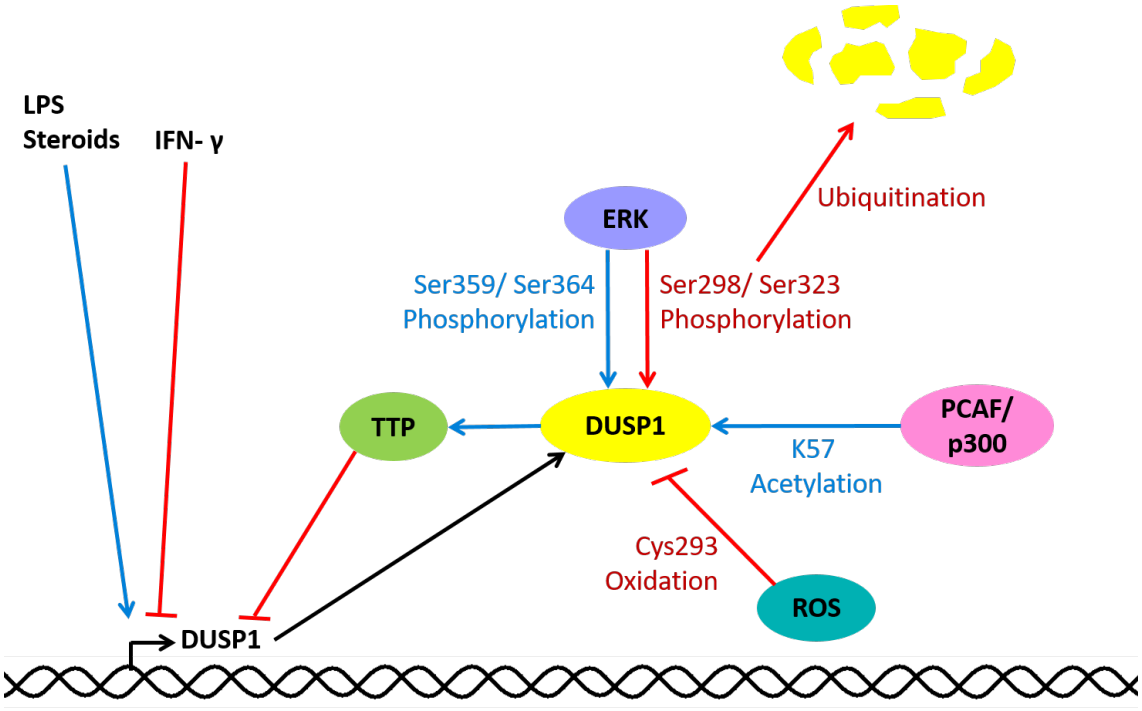

Figure 1.6 Regulation of DUSP1.

The various factors impacting DUSP1 mRNA or protein are depicted. Blue arrows indicate a positive regulation and red arrows a negative regulation. DUSP1 mRNA can be transcriptionally induced by LPS or steroids, or inhibited by IFN-γ. Once translated into protein, DUSP1 can be phosphorylated by ERK, which is either stabilising or leads to degradation, depending on the serine residue. ROS can oxidise DUSP1, inhibiting the catalytic activity. PCAF/p300 can acetylate DUSP1 which increases its affinity for substrates. DUSP1 can activate TTP, which destabilises DUSP1 mRNA. (Adapted from: Lloberas et al., 2016).

1.5.4 DUSP10

DUSP10 has a very similar role to DUSP1, as it has also been shown to attenuate the inflammatory response by dephosphorylating p38 and JNK (Tanoue et al., 1999). It is thought that DUSP10 may have more affinity for JNK, but activity towards p38 has also been demonstrated (Qian et al., 2009, Zhang et al., 2004). It is expressed ubiquitously in both the nucleus and cytoplasm (Tanoue et al., 1999), but is upregulated in response to innate immune signalling (Zhang et al., 2004). DUSP10 is stabilised by phosphorylation by the kinase mTORC2 in glioblastoma cells (Benavides-Serrato et al., 2014). mTORC2 is a regulator of cell growth but there may be other kinases involved in the innate immune signalling pathways that are also able to phosphorylate DUSP10. Moreover, DUSP10$^{-/-}$ mice suffer from lung tissue damage when challenged with LPS, and BMDMs isolated from these mice produced higher amounts of cytokines in response to LPS than wild type, which was associated with increased p38 and JNK activation (Qian et al., 2009, Qian et al., 2012, Zhang et al., 2004). DUSP10 knock out peritoneal macrophages also produce higher levels of inflammatory cytokines, including IL-6 and TNFα, to peptidoglycan, *Listeria monocytogenes,* and poly(I:C) (Zhang et al., 2004). Adoptive transfer of knock out BMDMs caused much more severe lung inflammation in response to LPS stimulation than transfer of BMDMs taken from wild-type mice (Qian et al., 2012). BMDMs taken from DUSP10 knock out mice have also been found to be skewed towards a pro-inflammatory M1 phenotype (Zhang et al., 2015). This indicates that, like DUSP1, DUSP10 plays an extremely important role in regulating the inflammatory response to bacterial infection, through inactivating p38 and JNK MAPKs.

DUSP10 expression can also be upregulated by viral infection: ebola infection of Huh7 cells and influenza infection of murine BMDMs upregulate DUSP10 expression (Holzer et al., 2016, James et al., 2015). When DUSP10 knockout mice were infected with influenza virus, production of the inflammatory cytokine IL-6 was increased in comparison to wild-type mice. The knock out mice were also found to have less severe disease and lower viral titres; this correlated with increased IFN-α and IFN-β release in BMDMs isolated from these mice. As they were able to co-immunoprecipitate DUSP10 and IRF3, the authors suggest this is due to the ability of DUSP10 to dephosphorylate and inactivate IRF3 (James et al., 2015).

1.5.5 DUSP2

DUSP2 is a nuclear protein, which has so far only been detected in human leukocytes (Jeffrey et al., 2006). It is upregulated in response to LPS via the ERK pathway in a range of B cell lines and in response to human T-cell leukaemia virus type 1 (HTLV-1) in T cells (Grumont et al., 1996, Pise-Masison et al., 2002). When knocked out in mice the inflammatory signalling in response to LPS is decreased, suggesting, in contrast with DUSP1 and DUSP10, a positive rather than negative regulatory role for DUSP2 in the immune response. In mast cells and macrophages taken from these mice, the decreased release of inflammatory markers was associated with an increase in phosphorylation and activity of JNK, but a decrease in p38 and ERK, which the authors proposed was due to cross-talk between the JNK and ERK pathways (Jeffrey et al., 2006). This model is supported by an earlier study showing that JNK activity blocked the phosphorylation of ERK by MEK1, and that this inhibition was dependant on c-Jun mediated gene transcription. This implies JNK activation leads to induction of an unknown protein, through activation of AP-1, which blocks ERK phosphorylation (Shen et al., 2003). In this model DUSP2 would inactivate JNK, stopping activation of AP-1, the unknown protein which blocks ERK phosphorylation is therefore not expressed, and thus the pro-inflammatory activity of ERK is increased.

Contrastingly, DUSP2 has been suggested to negatively regulate production of the inflammatory cytokine IL-6 in endometriotic stromal cells, as overexpression of DUSP2 in this setting led to a significant decrease in IL-6 mRNA expression (Hsiao et al., 2017).

1.5.6 DUSP4

DUSP4 is a nuclear protein also induced by the ERK pathway (Brondello et al., 1997). Like DUSP1 it can be phosphorylated by ERK at Ser386 and Ser391, which stabilises the protein (Crowell et al., 2014). Two groups have performed studies using DUSP4 knockout mice and produced conflicting results. Cornell et al. showed that mice lacking DUSP4 have decreased pro-inflammatory cytokines present in BAL fluid after challenge with LPS in comparison to wild type mice (Cornell et al., 2012). BMDMs isolated from these mice showed this decrease was associated with an increase in ERK activity, decreases in p38 and JNK activity, and increased induction of DUSP1 protein. When DUSP1 was knocked down, using siRNA, in these DUSP4 knock out BMDMs, the release of pro-inflammatory cytokines increased (Cornell et al., 2010). The authors suggest

these results indicate that DUSP4 inactivates ERK, which leads to a decrease in the expression and stabilisation/activation of DUSP1, thereby increasing the activity of p38 and JNK, and the associated release of inflammatory mediators.

In contrast, a study by Al-Mutairi and colleagues used similar methods but found the opposite; BMDMs isolated from DUSP4 knockout mice released increased amounts of pro-inflammatory cytokines in response to LPS (Al-Mutairi et al., 2010). Furthermore, they found increased levels of JNK and p38 phosphorylation, and no change in the level of ERK phosphorylation (Al-Mutairi et al., 2010). It is currently not clear why the results of these studies should differ as they both studied the same cell types: BMDMs taken from DUSP4 knockout mice. One possible explanation could be the use of different strains of mice.

1.5.7 DUSPs and Asthma

It is possible that several of the DUSPs may be dysregulated in people with asthma. This could partially account for the excessive inflammatory response to respiratory infections. A study in 2008 isolated nasal epithelial cells from healthy individuals and patients with house dust mite allergy, a common allergy associated with asthma. They performed a microarray to determine any gene expression changes in response to stimulation with house dust mite. In non-allergic controls, DUSP1 mRNA expression was upregulated in response to house dust mite challenge, however, in allergic patients DUSP1 expression did not alter (Vroling et al., 2008). DUSP10 may also have differential expression in asthmatic patients. A transcriptional profile of Th2 cells taken from asthmatic and healthy subjects showed lower baseline mRNA expression of DUSP10 in the asthmatic cells than the healthy cells (Seumois et al., 2016).

Interestingly, a study in 2012 looking for genetic determinants for severe asthma suggested there may be a single nucleotide polymorphism (SNP) in the DUSP4 gene linked to asthma, however the result was not statistically significant, possibly due to the limited number of patients in the study (Wan et al., 2012).

As of yet, the expression of DUSPs in COPD patients and any differences with healthy controls, has not been explored.

1.6 Corticosteroids

Exacerbations of asthma and COPD are treated with corticosteroids (Cutrera et al., 2017, Rosenberg and Kalhan, 2017). Steroids act to limit inflammation by interacting with glucocorticoid receptors (GRs) in the cytosol, this causes a conformational change in the GR which allows it to translocate to the nucleus, where it interacts with and inhibits transcription factors, such as AP-1 and NF-κB (Heck et al., 1994, Ray and Prefontaine, 1994). More recent evidence suggests that glucocorticoids mediate many of their anti-inflammatory actions through DUSP1. Treatment of cells with the glucocorticoid dexamethasone causes an increase in DUSP1 expression in many different cell types, including: primary peripheral blood neutrophils; primary airway smooth muscle cells; murine BMDMs; the epithelial cell lines A549, BEAS-2B, and HeLa cells; the RBL-2H3 basophil line and the NIH-3T3 fibroblast cell line (Kassel et al., 2001, Keranen et al., 2017, King et al., 2009, Lasa et al., 2002, Prabhala et al., 2016, Rahman et al., 2016, Wang et al., 2016). Knock out mice have shown the importance of DUSP1 in dexamethasone treatment. Bone-marrow macrophages taken from these mice show that the inhibitory effect of dexamethasone treatment on p38 and JNK activation, in response to LPS, is impaired when DUSP1 is not present (Abraham et al., 2006). The suppressive effect of dexamethasone on inflammatory cytokine expression in response to LPS was also negated in the DUSP1 knock out cells (Abraham et al., 2006). Bone-marrow derived mast cells taken from DUSP1 knock out mice had higher levels of MAPK activation in dexamethasone treated cells. However, the expression of inflammatory cytokines in response to IgE crosslinking did not differ significantly between wild-type and knock out cells (Maier et al., 2007). The authors suggest this may be due to compensatory mechanisms, a theory supported by the fact that dexamethasone treatment also increased expression of other phosphatases, including DUSPs 2 and 4 (Maier et al., 2007). This suggests that the actions of DUSPs may differ depending on the cell type. DUSP1 has also been knocked down in the airway epithelial cell line A549 using siRNA. Knock down of DUSP1 blocked the anti-inflammatory action of dexamethasone on IL-1β induced MAPK activation and inflammatory cytokine expression (Newton et al., 2010, Shah et al., 2014).

Exacerbations of asthma and COPD caused by infection with respiratory viruses are more resistant to glucocorticoid treatment (Xia et al., 2017). Rhinoviral infections have

been found to impair their anti-inflammatory actions, partly through reducing nuclear translocation of the GR (Papi et al., 2013). RV16 infection of A549 cells impaired the dexamethasone-mediated suppression of inflammatory cytokines induced by IL-1β, and also the upregulation of DUSP1 mRNA (Papi et al., 2013). Bacterial infection has also been demonstrated to cause steroid resistance (Goleva et al., 2009). Treatment of A549 cells with the synthetic TLR2 ligand Pam_3CSK_4 induced steroid resistance, but did not affect the upregulation of DUSP1. However, treatment with Pam_3CSK_4 did induce oxidative stress, and a proportion of the DUSP1 present in these cells was oxidised (Rahman et al., 2016). As discussed in section 1.5.3, oxidation of the catalytic cysteine residue of DUSP1 inactivates its phosphatase activity. This suggests that the resistance of exacerbations caused by infection to steroid treatment may be in part due to a suppression of DUSP1 expression or inactivation of the DUSP1 protein. A study from 2010 has also suggested that polymorphisms within the DUSP1 gene are associated with the clinical response to steroids (Jin et al., 2010). The roles of other DUSPs in steroid treatment remains to be investigated.

The fact that a well-established therapy acts, in part, through DUSPs suggests that they are potential therapeutic targets. Corticosteroid treatment causes many harmful side effects (Fernandes et al., 2014). Therefore, a more targeted therapy, aimed at upregulating DUSP1, may be preferable.

1.7 Hypothesis and Aims

The literature supports a role for DUSPs in regulating the release of inflammatory cytokines in response to both bacteria and viruses. These studies also suggest roles for DUSPs in other pathways involved in antiviral control. However, the ability of DUSPs to regulate the response of bronchial epithelial cells to rhinoviral infection has not yet been studied. I hypothesised that DUSPs have important roles in regulating inflammatory cytokine release in epithelial cells in response to rhinoviral infection. If this hypothesis is correct, DUSPs could be a potential target for anti-inflammatory treatments for chronic lung disease.

Therefore, in the present study I aimed to characterise the expression of the four DUSPs discussed above in primary bronchial epithelial cells (PBECs) and determine their role in rhinoviral infection. This work aimed to:

- Characterise the response of PBECs to rhinoviral infection, including:
  - Describing the activation and role of the MAPK pathways
  - Determining the key DUSPs expressed in PBECs.
  - Assessing whether the expression and activity of the key DUSPs are regulated by infection with rhinovirus
- Knock down specific DUSPs in PBECs, using siRNA, and examine the effects upon rhinovirus infection, including:
  - Rhinoviral replication
  - The secretion of inflammatory and anti-viral cytokines in response to rhinovirus
  - The activation of the MAPK pathways

2 Chapter Two: Materials and Methods

2.1 Materials

Table 2.1: Cell Culture Materials

Name	Composition	Application
BEAS-2B complete media	RPMI (Gibco), supplemented with 10% FCS (Gibco), 1% penicillin and 1% streptomycin (Sigma-Aldrich)	BEAS-2B cell maintenance
BEAS-2B basal media	RPMI (Gibco), supplemented with 1% penicillin and 1% streptomycin (Sigma-Aldrich)	Removing serum prior to infection
BEAS-2B 2% media	RPMI (Gibco), supplemented with 2% FCS (Gibco), 1% penicillin and 1% streptomycin (Sigma-Aldrich)	Transfection of BEAS-2B cells
Airway epithelial cell complete media	Airway epithelial cell basal medium (Promocell) supplemented with 1% penicillin, 1% streptomycin (Sigma-Aldrich), 0.004 mg/ml bovine pituitary extract, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.5 μg/ml epinephrine, 6.7 ng/ml triiodo-L-thyronine, 10 μg/ml transferrin and 0.1 ng/ml retinoic acid (Promocell)	PBEC maintenance
Airway epithelial cell basal media	Airway epithelial cell basal medium (Promocell) supplemented with 1% penicillin, 1% streptomycin (Sigma-Aldrich)	Removing serum prior to infection
Airway epithelial cell recovery media	Airway epithelial cell basal medium (Promocell) supplemented with 1% penicillin, 1% streptomycin (Sigma-Aldrich), 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.5 μg/ml epinephrine, 6.7 ng/ml triiodo-L-thyronine, 10 μg/ml transferrin and 0.1 ng/ml retinoic acid (Promocell)	Post-PBEC infection
Detach Kit	30mM Hepes, 0.04% trypsin/EDTA, trypsin neutralising solution (Promocell)	PBEC maintenance
HeLa Ohio complete media	DMEM (Gibco) supplemented with 10% FCS (Gibco), 1% penicillin, 1% streptomycin (Sigma-Aldrich), 1% non-essential amino acids (Gibco), 1% L-Glutamine (Gibco)	HeLa Ohio maintenance
HeLa Ohio infection media	DMEM (Gibco) supplemented with 2% Hepes (Gibco), 1% Bicarbonate (Gibco), 2% FCS (Gibco), 1 % penicillin, 1% streptomycin (Sigma-Aldrich)	HeLa Ohio infection
PBMC Basal Media	RPMI (Gibco), supplemented with 1% penicillin and 1% streptomycin (Sigma-Aldrich)	Attachment of monocytes

Table 2.1 Continued: Cell Culture Materials

Name	Composition	Application
MDM Complete Media	RPMI (Gibco), supplemented with 10% FCS (Gibco), 1% penicillin and 1% streptomycin (Sigma-Aldrich)	MDM differentiation
Monocyte Infection Media	RPMI (Gibco), supplemented with 2% FCS (Gibco), 1% penicillin and 1% streptomycin (Sigma-Aldrich)	Monocyte infection

Table 2.2: Buffers and Reagents

Reagent	Composition	Application
MACS buffer	PBS 0.5% BSA 2 mM EDTA	Monocyte isolation
Phosphate lysis buffer	50 mM Tris base (pH7.5) 1% Triton 50 mM NaF 1 mM PMSF 10 mM Na_3VO_4 50 mM β-glycerophosphate Protease inhibitor (Calbiochem)	Protein extraction
4X Sample buffer	8% SDS 0.2 M DTT 20% Glycerol 0.125 M Tris-Cl (pH6.8) 0.008% Bromophenol blue	Protein storage
Agarose	Agarose molecular grade powder (Melford)	Agarose gel electrophoresis
50X TAE	242 g Tris base 37.2 g EDTA 57.1 ml Acetic acid	Agarose gel electrophoresis
ELISA coating buffer	0.14 M NaCl 2.7 mM KCl 1.5 mM KH_2PO_4 8.1 mM Na_2HPO_4	ELISA
ELISA wash buffer	0.5 M NaCl 2.5 mM NaH_2PO_4 7.5 mM Na_2HPO_4 0.1% Tween pH to 7.2 with NaOH	ELISA
10X running buffer	0.26 M Tris base 1.92 M Glycine 1% SDS	Running SDS-PAGE gels
10X transfer buffer	0.25 M Tris base 1.93 M Glycine	Western blot
1X PBS/Tween	PBS tablets (Oxoid) 0.2% Tween	Western blot
1X TBS/Tween	6.05 g Tris base 8.76 g NaCl 0.1% Tween	Western blot
Blocking buffer	5% Non-fat milk 1X PBS/Tween or 1X TBS/Tween	Western blot

Table 2.3: Commercially Available Kits

Name	Components	Application	Supplier
MAPK Inhibitor Tocriset	U0126 SB203580 PD98059 SB202190 SP600125	MAPK inhibitor	Tocris (1879)
DNA-free	10X DNase I buffer rDNase I DNase inactivation reagent	Genomic DNA removal during RNA extraction	Ambion (AM1906M)
High-capacity cDNA reverse transcription kit	10X RT buffer 10X random primers 25X dNTP mix MultiScribe Reverse Transcriptase RNase inhibitor	cDNA synthesis	Applied Biosystems (4368814)
GoTaq Hot Start Polymerase	GoTaq Hot Start Polymerase 5X Green GoTaq Flexi Buffer 5X Colourless GoTaq Flexi Buffer $MgCl_2$	RT-PCR	Promega (M5001)
q-PCR mastermix	2X Mastermix $MgCl_2$	qRT-PCR	Eurogentec (RT- QP2X-03-075+)
GoTaq Probe q-PCR Master Mix	GoTaq Probe q-PCR Master Mix CXR Reference Dye	qRT-PCR	Promega (A6101/2)
GeneJET Plasmid Mini-Prep Kit	Resuspension solution Lysis solution Neutralisation solution Wash solution RNase A Elution buffer GeneJET spin columns Collection tubes	qRT-PCR	Thermo Fisher (K0502)
MACS Human Monocyte Isolation Kit II	FcR Blocking Reagent Monocyte-Biotin Antibody Cocktail Anti-Biotin MicroBeads	Monocyte isolation	MACS (130-091-153)

Table 2.3 Continued: Commercially Available Kits

Name	Components	Application	Supplier
Human Cytokine Array	Human Cytokine Array Membranes Array Buffer 4 Array Buffer 5 Wash Buffer Concentrate Detection Antibody Cocktail Streptavidin-HRP Chemi Reagent 1 Chemi Reagent 2 4-Well Multi-dish	Detection of cytokines in cell culture supernatants	R&D (ARY005B)
Human IL-1β ELISA DuoSet	IL-1β Capture Antibody IL-1β Standard IL-1β Detection Antibody Streptavidin-HRP	IL-1β ELISA	R&D (DY201-05)

Table 2.4: Antibodies

Antibody	Isotype	Application	Concentration Used	Supplier
Capture antibody, anti-human CXCL8	Mouse IgG	ELISA	0.3 μg/ml	R&D (MAB208)
Detection antibody, anti-human CXCL8	Biotinylated goat IgG	ELISA	0.32 μg/ml	R&D (BAF208)
Capture antibody, anti-human CCL5	Mouse IgG	ELISA	2 μg/ml	R&D (MAB678)
Detection antibody, anti-human CCL5	Biotinylated goat IgG	ELISA	0.08 μg/ml	R&D (BAF273)
Anti-human actin	Rabbit IgG	Western blot	0.04-0.08 μg/ml	Sigma-Aldrich (A2066)
Anti-human DUSP1	Rabbit IgG	Western blot	1 μg/ml	Merck Millipore (07-535)
Anti-human phosphorylated DUSP1	Rabbit IgG	Western blot	1:500	Cell-Signalling (2857)
Anti-human DUSP10	Rabbit IgG	Western blot	0.35 μg/ml	Abcam (ab140123)
Anti-human DUSP4	Rabbit IgG	Western blot	2.67 μg/ml	Abcam (ab72593)
Anti-human phosphorylated ERK 1/2	Rabbit IgG	Western blot	1:1000	Cell-Signalling (9101)
Anti-human activated p38	Rabbit IgG	Western blot	1:2000	Promega (V1211)
Anti-human phosphorylated JNK	Rabbit IgG	Western blot	1:1000	Cell-Signalling (4668)
Anti-rabbit secondary antibody	Polyclonal goat – HRP conjugated	Western blot	0.125 μg/ml	Dako (P0448)
Anti-rabbit secondary antibody	Goat IgG – HRP conjugated	Western blot	1:2000	Cell-Signalling (7074)
Anti-mouse secondary antibody	Polyclonal goat – HRP conjugated	Western blot	0.5 μg/ml	Dako (P0447)

Table 2.5: RT-PCR Primers

Target	Sequence	Annealing Temp (°C)	Product Size (bp)	Supplier	Reference
DUSP1 (Fwd)	GTCGTGCAGCAAACAGTCGA	67.9	432	Sigma-Aldrich	(Imasato et al., 2002)
DUSP1 (Rvs)	CGATTAGTCCTCATAAGGTA	53.4		Sigma-Aldrich	
DUSP2 (Fwd)	TTGCCCTACCTGTTCCTGGG	68.2	398	Sigma-Aldrich	(Givant-Horwitz et al., 2004)
DUSP2 (Rvs)	GTCTCAAACTGCAGCAGCTG	64		Sigma-Aldrich	
DUSP4 (Fwd)	TTCAACAGGCATCCATCCCT	66.7	297	Sigma-Aldrich	This study
DUSP4 (Rvs)	TGGCTTTGGGAGGGAATGAT	67.6		Sigma-Aldrich	
DUSP10 (Fwd)	ATGACCAAATGCAGCAAG	59.8	397	Sigma-Aldrich	(Givant-Horwitz et al., 2004)
DUSP10 (Rvs)	GGAGCTGGAGGGAGTTGTCAC	67.4		Sigma-Aldrich	
GAPDH (Fwd)	GGTGAAGGTCGGTGTGAAC	63.2	233	Sigma-Aldrich	Ismail, S., unpublished
GAPDH (Rvs)	CTCGCTCCTGGAAGATGGTG	67.2		Sigma-Aldrich	

Table 2.6: qRT-PCR Primer-Probes

Target	Concentration used at	Sequence	Supplier
DUSP1 (pre-mixed)			Applied Biosystems (Hs00610256_g1)
Forward + Reverse	900 nM	-	
Probe	250 nM	-	
DUSP10 (pre-mixed)			Applied Biosystems (Hs00200527_m1)
Forward + Reverse	900 nM	-	
Probe	250 nM	-	
DUSP4 (pre-mixed)			Applied Biosystems (Hs01027785_m1)
Forward + Reverse	900 nM	-	
Probe	250 nM	-	
CXCL8 (pre-mixed)			Applied Biosystems
Forward + Reverse	900 nM	-	(Hs00174103_m1)
Probe	250 nM	-	
GAPDH (pre-mixed)			Applied Biosystems (Hs02758991_g1)
Forward + Reverse	900 nM	-	
Probe	250 nM	-	
IFN-β			Sigma-Aldrich
Forward	300 nM	5'CGCCGCATTGACCATCTA	(SY150506722-061)
Reverse	900 nM	5'TTAGCCAGGAGGTGCTCTAACAATAGTCTCA	(SY150504450-060)
Probe	175 nM	5'[6FAM]TCAGACAAGATTCATCTAGCACTGGCTGGA[TAM]	(HA07784503-002)
RV			Sigma-Aldrich
Forward	900 nM	5'GTGAAGAGCCSRTGTGCT	(SY150600935-024)
Reverse	900 nM	5'GCTSCAGGGTTAAGGTTAGCC	(SY150600935-025)
Probe	175 nM	5'[6FAM]TGAGTCCTCCGGCCCCTGAATG[TAM]	(HA07878670-002)

Table 2.7: siRNA

Target	siRNA Sequence Sense	Supplier
Control	5'UCACACAACAUGUAAACCA	Dharmacon (D-001810-01-05)
DUSP1 #1	Pool of: 5'AAACGCUUCGUAUCCUCCU 5'UUCGCCUCUGCUUCACAAA 5'UAGUCCUCAUAAGGUAAGC 5'UGACCCUCAAAAUGGUUGG	Dharmacon (L-003484-02-0005)
DUSP1 #2	Pool of: 5'GGUUCAACGAGGCUAUUGA 5'CGAGGCUAUUGACUUCAUA 5'GCAAGACAUUUGCUGAACU	Santa Cruz (sc-35938)
DUSP1 #3	5'GAGGCGAAGCAUCAUCUC	Santa Cruz (sc-35937)
DUSP1 #4	5'CUGUACUAUCCUGUAAAUAUA	Qiagen (SI00374808)
DUSP10	Pool of: 5'CCAUCGUCAUCGCUUACUU 5'CGAGAAUCCUUACACCAAA 5'CAGCUUAAGUGGUCUAAGA	Santa Cruz (sc-61048)

Table 2.8: Solutions for making 10% SDS-PAGE gels

Solution	10% Resolving gel (ml)	5% Stacking gel (ml)	Supplier
Water	1.9	0.68	
30% acrylamide mix	1.7	0.17	Geneflow
1.5 M Tris (pH 8.8)	1.3	0	BioRad
1.0 M Tris (pH6.8)	0	0.13	BioRad
10% SDS	0.05	0.01	Panreac
10% ammonium persulfate	0.05	0.01	Fluka
TEMED	0.002	0.001	Sigma-Aldrich

2.2 Cell Culture

2.2.1 BEAS-2B Cell Culture

The BEAS-2B lung epithelial cell line was purchased from ATCC. Cells were maintained in BEAS-2B complete media (Table 2.1) in 75 cm^2 culture flasks (Thermo Fisher). Flasks were kept in a humidified 37^oC incubator with 5% CO_2, and passaged at 80-90% confluency using cell dissociation solution (Sigma-Aldrich). Briefly, media was removed from cells and replaced with 2 ml cell dissociation solution. The flask was then incubated at 37^oC, 5% CO_2 for 5 minutes to allow cells to detach. The cell dissociation solution was inactivated with complete media, and cells transferred to fresh 75 cm^2 culture flasks.

2.2.2 HeLa Ohio Cell Culture

HeLa Ohio cells were purchased from ATCC and grown in 75 cm^2 culture flasks in HeLa Ohio complete media (Table 2.1) and incubated at 37^oC, 5% CO_2 and passaged at 80-90% confluency using cell dissociation solution (Sigma-Aldrich).

2.2.3 Primary Bronchial Epithelial Cell Culture

Primary bronchial epithelial cells (PBECs) isolated from healthy human volunteers were purchased from Promocell. They were grown in 75 cm^2 culture flasks in airway epithelial cell complete media (Table 2.1) and incubated in a humidified 37^oC incubator with 5% CO_2. The media was removed and replaced with fresh media every two to three days. Cells were passaged at 80-90% confluency using the Promocell detach kit (Table 2.1) in accordance with manufacturer's instructions.

2.2.4 Mycoplasma Testing

All cells were tested monthly for the presence of mycoplasma by technicians in the department. The EZ-PCR mycoplasma test kit (Geneflow) was used according to the manufacturer's instructions.

2.2.5 Peripheral-Blood Mononuclear Cell Isolation

Peripheral venous blood was obtained from healthy human volunteers with fully informed consent, in accordance with a protocol approved by South Sheffield Local Research Ethics Committee.

Peripheral-blood mononuclear cells (PBMCs) were isolated from blood by other members of the department. Freshly drawn blood was added to 3.8% tri-sodium citrate (Martingdale Pharmaceuticals) and mixed gently before centrifuging at 350 *g*, 20 minutes, room temperature. The upper phase, containing platelet-rich-plasma, was transferred to a fresh 50 ml falcon tube and centrifuged at 800 *g*, 20 min, 20^{o}C, in order to remove platelets. The supernatant was transferred to a fresh tube and the pellet discarded. To the lower phase of cells, 6% dextran (Pharmacosmos) was added, and made up to 50 ml with saline (Gibco), before gently mixing. This was incubated at room temperature for 20 to 30 minutes, with the lid left loose, to allow the dextran to bind the red blood cells and sediment. The upper layer was transferred into a fresh tube and centrifuged at 320 *g*, 6 min, 20^{o}C. The supernatant was discarded and the pellet resuspended in 2 ml platelet-poor-plasma. A percoll gradient was made, containing a lower phase of 51% percoll (GE Healthcare) and 49% platelet-poor-plasma and an upper phase containing 42% percoll and 58% platelet-poor-plasma. The cells were overlaid onto the gradient and centrifuged at 320 *g*, 11 min, 20^{o}C. The solution separated into three layers, with the PBMCs in the interphase between the upper and middle layers. The PBMC layer was transferred into a fresh falcon tube, and made up to 40 ml with 1X HBSS (Gibco), before centrifuging 320 *g*, 6 min, 20^{o}C.

2.2.6 Monocyte-Derived Macrophage Culture

PBMCs isolated from percoll gradient centrifugation (Section 2.2.5) were resuspended in monocyte basal media (Table 2.1) at 4 x 10^{6} cells/ml and 500 µl added to each well of a 24-well culture plate (Costar). Cells were incubated at 37^{o}C, 5% CO_2 for one hour. Media was then replaced with MDM complete media (Table 2.1) to remove non-adherent cells. The remaining monocytes were cultured for 7 or 14 days (as indicated in figure legends) to allow differentiation into macrophages, replacing media with fresh MDM complete media every two days.

2.2.7 Monocyte Isolation and Culture

Monocytes were isolated from PBMCs (Section 2.2.5) using the MACS Human Monocyte Isolation Kit II (Table 2.3) according to the manufacturer's instructions. Any non-monocytes are magnetically labelled and removed by passing over a magnetic column. Briefly, PBMCs were resuspended in 1 ml MACS buffer (Table 2.2), transferred to a 1.5 ml tube, and centrifuged at 1000 *g*, 2 min, room temperature. The supernatant was

discarded and the pellet resuspended in 30 μl buffer per 10^7 cells. 10 μl of FCR blocking reagent, and 10 μl of biotin antibody cocktail were added per 10^7 cells before gently mixing and incubating at 4°C for 10 min. 30 μl of buffer per 10^7 cells was then added. The anti-biotin beads were vortexed and 20 μl added per 10^7 cells. The solution was mixed gently and incubated at 4°C for 15 min. The solution was then made up to 1 ml with MACS buffer and spun at 300 *g*, 10 min, room temperature. The supernatant was discarded and the pellet resuspended in 500 μl MACS buffer.

A MACS LS column was placed into the field of a MACS separator. The column was rinsed using 3 ml MACS buffer. The cell suspension was added to the column and the flow through, containing monocytes, collected in a 50 ml falcon tube. The column was washed three times using 3 ml MACS buffer and the effluent collected into the same falcon tube. The cell solution was centrifuged at 1000 *g*, 5 min, room temperature, the supernatant discarded and the pellet resuspended in monocyte basal media (Table 2.1) at 4 x 10^5 cells/ml. 500 μl was added to each well of a 24 well plate and incubated at 37°C, 5% CO_2.

2.3 Rhinovirus

2.3.1 Viral Propagation

Confluent 175 cm^2 culture flasks of HeLa Ohio cells were washed twice with HeLa Ohio infection media (Table 2.1), and 7.5 ml infection media and 5 ml viral stock, from the last batch, added to each before incubating at 37°C, 5% CO_2, for one hour with agitation. 12.5 ml infection media was added before overnight incubation. Once around 90% cytopathic effect was observed, three cycles of freeze-thawing lysed the cells, and cell debris was removed by centrifuging at 4000 *g* for 15 minutes. Supernatant was filtered through a 0.22 μm filter, and aliquoted for storage in liquid nitrogen.

2.3.2 Viral Titration

50 μl of infection media, undiluted RV or sample, or 10-fold serial dilutions, up to 10^{-9}, were added to each well of a 96 well plate (Costar), 8 repeats of each dilution. 1.5x10^4 HeLa Ohio cells were seeded into each well on top of the virus. The plate was incubated for 4 days before the number of wells with cytopathic effect at each viral dilution was

determined using light microscopy, and the $TCID_{50}$ calculated using the Spearman Karber formula (Hamilton et al., 1977).

2.4 Cell Stimulation

PBECs were seeded at $7x10^5$ or BEAS-2Bs were seeded at $1x10^6$ cells per plate onto 12 well culture plates. Once cells reached 80-95% confluency media was replaced with 1 ml basal media (Table 2.1) and incubated at 37°C, 5% CO_2 overnight. Media was replaced with basal media containing stimulant as required for each time point: 1 - 100 ng/ml IL-1β (Peprotech) or 25 μg/ml poly(I:C) (Invivogen). Supernatant, mRNA or protein was collected from each well (Section 2.9).

PBECs from at least three different donors were used for each experiment. Donors were chosen at random. Table 7.1 (Appendix 1) indicates which donors were used for each figure and their characteristics.

2.5 RV Infection

2.5.1 RV Infection of PBECs

PBECs were seeded at $7x10^5$ cells per plate onto 12 well culture plates. Once cells reached 80-95% confluency media was replaced with 1 ml basal media (Table 2.1) and incubated at 37°C, 5% CO_2 overnight. Media was removed, and 250 μl of basal media, RV1B or RV16 was added as required for each time point; titres used are recorded in figure legends. The plate was incubated at 37°C, 5% CO_2, for one hour, with agitation. The wells were washed twice with PBS before adding 1 ml PBEC recovery media (Table 2.1). Supernatant, mRNA or protein was collected from each well (Section 2.9), or cell viability assay performed (Section 2.10).

2.5.2 RV Infection of Monocytes and MDMs

Monocytes were isolated and plated as in section 2.2. After one hour incubation (monocytes), or after differentiation (MDMs), media was removed and replaced with 250 μl basal PBMC media (Table 2.1) or rhinovirus; serotypes and titres are recorded in figure legends. The plate was incubated at 37°C, 5% CO_2, for one hour, with agitation. The wells were washed twice with PBS before adding 500 μl of basal media or monocyte 2% media (Table 2.1), as indicated in figure legends. Cells were incubated at 37°C, 5%

CO_2 for the indicated time point. Supernatant or mRNA was collected from each well (Section 2.9).

2.5.3 RV Controls

To determine that the effects of the virus were due to viral infection, two controls were used, UV-inactivated virus and virus filtrate. UV-inactivated RV1B or RV16 was obtained by exposing the virus to 1000 mJoules/cm^2 UV light for 10 minutes. Filtrate was obtained by adding 500 μl of virus to a centrifugal filter with a membrane nominal molecular weight limit of 30 kDa (Amicon). Filters were centrifuged at 12000 *g*, 5 min, room temperature, and any liquid left in the upper chamber discarded. These controls were then used to treat cells in the same way as virus (Sections 2.5.1 and 2.5.2).

2.6 Inhibitor Treatment

Prior to cell stimulation (Section 2.4) or infection (Section 2.5) PBECs were treated with 20 μM MAPK inhibitor (Table 2.3), diluted in DMSO, or vehicle control for one hour, or with 100 nM okadaic acid (Abcam), diluted in DMSO, or vehicle control for 30 minutes.

2.7 Dexamethasone Treatment

At the same time as, or 4 h prior to, cell stimulation (Section 2.4) or infection (Section 2.5) (as indicated in figure legends) PBECs were treated with 1 - 100 nM dexamethasone (Sigma-Aldrich) diluted in basal media (Table 2.1).

2.8 siRNA Treatment

2.8.1 DUSP10 Knock Down

Short interfering RNA (siRNA) was used to knock down DUSP10 mRNA expression in PBECs. PBECs were seeded at $7x10^5$ cells per plate onto 12 well culture plates (Costar) and incubated at 37°C, 5% CO_2 until around 80% confluent. For each well 5 μl Lipofectamine 2000 (Invitrogen) was mixed with 95 μl Opti-MEM (Gibco) and incubated at room temperature for 5 minutes. 5 μl of 20 μM DUSP10 or control siRNA (Table 2.7) diluted in 95 μl Opti-MEM was added to this mixture, and complexes allowed to form at room temperature for 20 minutes. Media was removed from cells and replaced with 800 μl PBEC basal media (Table 2.1). 200 μl of the siRNA mixture was added dropwise to each well and plates were incubated at 37°C, 5% CO_2, for 4 h before media was

removed and replaced with 1 ml PBEC recovery media (Table 2.1). Plates were incubated at 37°C, 5% CO_2 for 48 h. Cells were then stimulated (Section 2.4) or infected (Section 2.5).

2.8.2 DUSP1 Knock Down

siRNA knock down of DUSP1 was attempted in PBECs, BEAS-2Bs and HeLa Ohio cells. Cells were seeded into 12 well culture plates and incubated at 37°C, 5% CO_2 until they reached the desired confluency, indicated in figure legends. The above protocol was used for each of four different DUSP1 siRNAs (Table 2.7), varying the final concentrations of siRNA and Lipofectamine 2000, and replacing Lipofectamine 2000 with Mission transfection reagent (Sigma-Aldrich). Knock down was attempted for 24 and 48 h. In BEAS-2B cells knock down was attempted in both basal and 2% media (Table 2.1).

Knock down in BEAS-2B cells was also attempted using Viromer Blue transfection reagent (Lipocalyx) according to the manufacturer's instructions. DUSP1 or control siRNA (Table 2.7) was diluted to 2.8 μM in 10 μl Buffer Blue per well. 1 μl of Viromer Blue was mixed with 99 μl Buffer Blue per well and added to the diluted siRNA, before incubating at room temperature for 15 min. Media was removed from cells and replaced with 900 μl BEAS-2B basal media (Table 2.1). 100 μl of the siRNA solution was added dropwise to each well and plates incubated at 37°C, 5% CO_2, for 4 h before media was removed and replaced with 2% media (Table 2.1). Plates were incubated at 37°C, 5% CO_2 for 48 h before cell lysates were collected.

2.9 Sample Collection

2.9.1 Supernatant Collection

The media from each well was collected in a 1.5 ml tube before being centrifuged 1000 *g*, 2 min, to remove cell debris; hereafter called supernatant. Cell-free supernatant was transferred into a clean tube, and stored at -80°C. Where indicated, supernatant collection was performed under sterile conditions.

2.9.2 mRNA Collection

Cells were scraped and lysed in 1 ml of Tri reagent (12 well plates) or 500 μl Tri reagent (24 well plates) (Sigma-Aldrich). The sample was collected in a 1.5 ml tube, and stored at -80^{o}C.

2.9.3 Protein Collection

Cells were scraped and lysed in 75 μl of phosphate lysis buffer (Table 2.2) for 15 minutes on ice. Lysates were collected in 1.5 ml tubes and centrifuged at 12000 *g* for one minute; the soluble fraction was transferred to fresh 1.5 ml tubes. Lysates were boiled for 5 min in 1X sample buffer (Table 2.2). Samples were stored at -80^{o}C.

2.10 Cell Viability Assay

Cell-titre glo viability assay (Promega) was performed in accordance with the manufacturer's instructions 24 or 48 h post infection of PBECs (Section 2.5.1). Briefly, media was removed, and 200 μl PBEC recovery media (Table 2.1) added to each well, and one empty well as a control. Cell titre glo reagent (Table 2.3) was made by mixing equal volumes of buffer and reagent. 200 μl of this mixture was added to each well. The plate was wrapped in foil and incubated at room temperature on a shaking platform for 2 min, and then incubated at room temperature for 10 min. 200 μl was then taken from each well and added to a well of a white 96 well plate (Thermo Fisher), in duplicate. The optical density at 480 nm was read by Thermo Scientific Varioskan Flash plate reader, and the reading for the control wells deducted from each reading.

2.11 Supernatant Transfer

Cell-free supernatant was collected under sterile conditions (Section 2.9.1) from monocyte cultures from three donors infected with RV1B or RV16 for 24 or 48 h (Section 2.5.2) and stored at -80^{o}C. All samples were thawed and all three donors pooled into fresh bijou tubes for each treatment. The pooled supernatant was filtered through centrifugal filters with a membrane nominal molecular weight limit of 30 kDa (Amicon), as in section 2.5.3, to remove viral particles. PBECs which had been treated with control or DUSP10 siRNA for 48 h (Section 2.8.1) were washed in PBS and 500 μl PBEC recovery media added to each well (Table 2.1). 500 μl of the filtered supernatant from each treatment was then added to one well treated with control siRNA and one well treated

with DUSP10 siRNA. Cells were incubated at 37°C, 5% CO_2 for 24 h, and supernatant and RNA collected (Section 2.9).

2.12 Reverse Transcription PCR (RT-PCR)

2.12.1 Primer Design

Specific primer sequences for DUSP1, DUSP2 and DUSP10 were found in the literature (Table 2.5). Primer sequences were designed for DUSP4 (gene ID 1846) with NCBI BLAST primer design (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were synthesised by Sigma-Aldrich. All primer sequences, and their associated conditions, are listed in Table 2.5.

2.12.2 RNA Extraction and Purification

RNA was extracted using Tri reagent in accordance with the manufacturer's instructions. Briefly, after sample collection (section 2.9.2), 200 μl of chloroform was added, mixed vigorously, and stood at room temperature for 10 minutes before centrifuging at 4°C for 15 minutes at 12000 *g*. The solution separates into the upper aqueous phase (containing RNA), interphase (containing DNA), and the lower organic phase (containing protein). The aqueous phase was transferred to a clean 1.5 ml tube. 500 μl isopropanol was added, and samples stood at room temperature for 10 minutes before centrifuging at 4°C for 10 minutes at 12000 *g*, and discarding the supernatant. The pellet was washed in 70% ethanol, centrifuged at 4°C for 5 minutes at 7500 *g*, and the supernatant discarded. Once the pellet was completely dry it was resuspended in 20 μl sterile water. Any contaminating genomic DNA was removed using the Ambion DNase treatment kit (Table 2.3). 2 μl of 10X DNase I buffer and 1 μl of rDNase I was added to each sample, and incubated at 37°C for 25 minutes. 2.3 μl of DNase Inactivation Reagent was added to each sample, before centrifuging at 10000 *g* for 2 minutes. Supernatant was transferred to fresh 1.5 ml tubes, and the quantity and quality of RNA assessed using a Nanodrop-1000 spectrophotometer (Thermo Fisher).

2.12.3 cDNA Synthesis

High capacity cDNA reverse transcriptase kit (Table 2.3) was used to convert 1 μg mRNA into cDNA. 1 μg mRNA was made up to 20 μl with sterile water. To each sample 4 μl 10X RT buffer, 1.6 μl 25X 100nM dNTPs, 4 μl 10X random primers, 2 μl multiscribe reverse

transcriptase, 2 μl RNase inhibitors, and 6.4 μl of sterile water was added. Two controls were included, one Taq polymerase negative, and one containing master mix only. The samples were reverse transcribed using a Peltier Thermal Cycler PCR machine (25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes).

2.12.4 RT-PCR

PCR was carried out using the Promega GoTaq Hot Start Polymerase kit (Table 2.3). 0.025 μg of each cDNA sample, or 1 μl control, was added to a master mix containing: 0.28 μM of each forward and reverse specific primers, 0.4 mM of each dNTP (Promega), 1 unit Taq polymerase, buffer to a final concentration of 1x, 1.5 mM $MgCl_2$, and sterile water to 25 μl total volume. The samples were run in a Bibby Scientific ^{3}Prime PCR machine (94°C for 2 minutes, then cycled 38 times through 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute, followed by 72°C for 5 minutes).

2.12.5 Agarose Gel Electrophoresis

Samples (10 μl) were electrophoresed through a 1.5% agarose gel containing 0.3 μg/ml ethidium bromide (Sigma-Aldrich) at 100 V, and then imaged using a Bio-Rad ChemiDoc XRS+.

2.13 Quantitative RT-PCR (qRT-PCR)

2.13.1 ΔΔCt qRT-PCR

Quantitative RT-PCR (qRT-PCR) was used to determine cellular mRNA levels of DUSP1, DUSP10 and DUSP4 in comparison to a control gene, GAPDH. 1 μl of primer probe (Table 2.6), 10 μl of q-PCR mastermix, 8 μl of water and 0.025 μg cDNA sample, or control (Section 2.12.3) was added to each well of a 384 well plate. Two different qRT-PCR mastermix were used: Promega, mixed with 0.2 μl CXR reference dye per well, or Eurogentec (Table 2.3). Each sample was run in duplicate. The plate was centrifuged for 2 minutes at 689 *g* before running on the ABI 7900HT Fast Real-Time PCR system using the ΔΔCT setting (50°C for 2 minutes, 95°C for 10 minutes, then cycled 40 times through 95°C for 15 seconds, and 60°C for 1 minute). Data was analysed using SDS 2.2.1 software (ABI systems) to find the fold change in DUSP mRNA expression normalised to GAPDH expression, and the untreated control (Livak and Schmittgen, 2001).

2.13.2 Absolute Quantification qRT-PCR

2.13.2.1 Plasmid Preparation

Standard curves for each gene of interest were made using plasmid DNA. Plasmids expressing the genes of interest and an antibiotic resistance gene were transformed into *Escherichia coli* by other members of the group. Plasmid DNA was purified using the GeneJET plasmid mini-prep kit (Table 2.3) according to the manufacturer's instructions. A single colony of *E. coli* was used to inoculate 5 ml LB media and incubated at 37^{o}C, shaking, over-night. The culture was then centrifuged 8000 *g*, 10 min, 4^{o}C, and the supernatant discarded. The pellet was resuspended in 250 μl resuspension solution and mixed by inversion. 250 μl of lysis solution and 350 μl neutralisation solution were added before centrifuging 12000 *g*, 5 min, room temperature to remove cell debris and chromosomal DNA. The supernatant was transferred to the supplied spin column and centrifuged 12000 *g*, 1 min, room temperature and flow through discarded. The column was washed twice with 500 μl wash solution, centrifuging 12000 *g*, 1 min, room temperature, and flow through discarded. 50 μl elution buffer was then added to the column and incubated for 2 min at room temperature, before centrifuging at 12000 *g*, 2 min, room temperature and collecting the flow through in a fresh 1.5 ml tube. This last step was repeated in order to collect the maximum amount of plasmid DNA. The quantity and quality of plasmid DNA was assessed using a Nanodrop-1000 spectrophotometer (Thermo Fisher) and the DNA stored at -20^{o}C.

2.13.2.2 qRT-PCR

Absolute quantification qRT-PCR was used to find cellular mRNA levels of CXCL8 and IFN-β mRNA, and to determine viral replication using primers specific to part of the rhinoviral genome. The RNA concentration of each sample was calculated from a standard curve made from a sample of known copy number per μl (plasmid standard). Plasmid standards were diluted to 10^{8} copies per μl and then a serial 1:10 dilution performed up to 10^{0} copies per μl.

For CXCL8 and GAPDH 1 μl of primer probe (Table 2.6), 10 μl of q-PCR mastermix (Promega), 0.2 μl CXR (Promega), 7.8 μl of water and 0.025 μg cDNA sample or 1 μl plasmid standard was added to each well of a 384 well plate. For RV and IFN-β, 1 μl of each of diluted forward and reverse primers and probe, 10 μl q-PCR mastermix

(Promega), 0.2 μl CXR (Promega), 5.8 μl water and 0.025 μg cDNA or 1 μl plasmid standard or controls added to each well of a 384 well plate. Samples, standards, and controls were run in duplicate. The plate was centrifuged for 2 minutes at 689 *g* before running on the ABI 7900HT Fast Real-Time PCR system, using the absolute quantification setting (50°C for 2 minutes, 95°C for 10 minutes, then cycled 40 times through 95°C for 15 seconds, and 60°C for 1 minute). Data was analysed using SDS 2.2.1 software (ABI systems) to find the number of copies per μg RNA, normalised to GAPDH.

2.14 Enzyme-Linked Immunosorbent Assay (ELISA)

2.14.1 CXCL8 and CCL5 ELISAs

Cell-free supernatants were collected (section 2.9.1) and CXCL8 or CCL5 generation were determined by ELISA. 100 μl of coating antibodies (Table 2.4) diluted in coating buffer (Table 2.2) was added to each well of a 96 well plate with high-binding surface (Costar), and incubated at room temperature overnight. The plate was washed four times with wash buffer (Table 2.2) using a plate washer (Labtech International), then blocked in 1% albumin (from chicken egg, Sigma-Aldrich) or 1% BSA (Sigma-Aldrich) in coating buffer for 1 h on a shaking platform (300 rotations/minute) at room temperature. Plates were washed four times, and 100 μl of standard (19.6 to 5000 pg/ml for CXCL8; 39 to 10000 pg/ml for CCL5) or sample was added in duplicate to each well. The plate was incubated at room temperature for 1.5 h on a shaking platform before washing four times. 100 μl of biotinylated detection antibody (Table 2.4), diluted in wash buffer, was added to each well, and incubated at room temperature on a shaking platform for 1.5 h. The plate was washed four times, and 100 μl of Streptavidin-HRP (R&D Systems), diluted 1:200 in wash buffer, was added to each well, and incubated in the dark at room temperature for 20 minutes on a shaking platform. After washing four times, 100 μl of substrate solution (R&D systems) was added to all wells, before incubating in the dark at room temperature for 15-25 minutes on a shaking platform. The reaction was stopped using 50 μl of 1M sulphuric acid (Fisher) per well. The optical density of each well at 450 nm was read by the Thermo Multiscan EX plate reader. The amount of CXCL8 or CCL5 present in each sample, above the minimum detection level of 78.125 pg/ml for CXCL8 and 156.25 pg/ml for CCL5, was determined using the standard curve from the same plate.

2.14.2 IL-1β ELISA

The R&D Systems IL-1β ELISA duo-set (Table 2.3) was used to determine IL-1β generation in cell-free supernatants by ELISA, according to the manufacturer's instructions. 96 well plates (Costar) were coated overnight using 100 μl per well of IL-1β coating antibody, diluted to 4 μg/ml in PBS. The plate was washed four times with wash buffer (Table 2.2) using a plate washer (Labtech International), then blocked in 1X reagent diluent (R&D Systems) for one hour on a shaking platform at room temperature. Plates were washed four times and 100 μl of standard (3.91 to 250 pg/ml) or sample added in duplicate to each well. The plate was incubated at room temperature for 1.5 h on a shaking platform before washing four times. 100 μl of biotinylated detection antibody, diluted to 200 ng/ml in 1X reagent diluent, was added to each well, and incubated at room temperature on a shaking platform for 1.5 h. The plate was washed four times, and 100 μl of Streptavidin-HRP (R&D Systems), diluted 1:40 in reagent diluent was added to each well, and incubated in the dark at room temperature for 20 minutes on a shaking platform. After washing four times, 100 μl of substrate solution (R&D systems) was added to all wells, before incubating in the dark at room temperature for 15-25 minutes on a shaking platform. The reaction was stopped using 50 μl of 1M sulphuric acid (Fisher) per well. The optical density of each well at 450 nm was read by the Thermo Multiscan EX plate reader. The amount of IL-1β present in each sample, above the minimum detection level of 7.81 pg/ml, was determined using the standard curve from the same plate.

2.15 Cytokine Array

Cell-free supernatants were collected (Section 2.9.1) and stored at -80^{o}C. Generation of 36 cytokines was determined using the R&D Systems cytokine array (Table 2.3). All reagents from the kit were brought to room temperature and supernatant samples defrosted on ice. Membranes were each blocked in 3 ml array buffer 4 in the supplied 4-well multi-dish on a rocking platform, at room temperature, for 1 h. 900 μl of each sample was transferred to a fresh 1.5 ml tube, and mixed with 500 μl array buffer 4 and 100 μl array buffer 5. The antibody cocktail was reconstituted in 100 μl water and 15 μl added to each sample mixture. Sample mixtures were inverted and incubated at room temperature for 1 h. Blocking buffer was removed from the dish and the sample

mixtures added to each well, before the membranes were incubated on a rocking platform, at 4°C, overnight. Membranes were then washed three times in 1X wash buffer for 10 minutes. Streptavidin-HRP was diluted 1:2000 in array buffer 5, and 3 ml added to each membrane. This was incubated at room temperature on a rocking platform for 30 min. Membranes were then washed 3 times in 1X wash buffer for 10 minutes. Each membrane was then incubated for 1 minute with Chemi Reagent before being imaged using a BioRad ChemiDoc XRS+.

2.16 Western Blot

2.16.1 SDS-PAGE Gel Electrophoresis

SDS-PAGE gel apparatus was set up according to the manufacturer's instructions. Gels were prepared as in table 2.8 with approximately 2.5 cm of 5% stacking gel, containing a 10 or 15 well comb, layered above the 10% resolving gel.

Gels were assembled in a running tank filled with 1X running buffer (Table 2.2). Cell lysates (10 μl) (section 2.9.3) and prestained protein ladder (GeneFlow) were electrophoresed through the stacking gel at 100 V, increased to 200 V through the resolving gel.

2.16.2 Transfer

Proteins were transferred onto 0.22 μm nitrocellulose membrane (Pall) using the Thermo Scientific Pierce G2 Fast Blotter. Filter paper and nitrocellulose membrane were soaked in 1X transfer buffer (Table 2.2) for 5 minutes, and the transfer sandwiches assembled on the Fast Blotter. Proteins were allowed to migrate for 15 minutes at 3 A and 25 V, and membranes stained with 1% Ponceau S (Sigma-Aldrich) to assess protein transfer. Ponceau S was washed off with 1X PBS/Tween or 1x TBS/Tween (for antibodies specific to phosphorylated proteins) (Table 2.2), and the membranes incubated in blocking buffer (Table 2.2) for 1 h at room temperature on a rotating platform.

2.16.3 Primary Antibody

Primary antibodies (Table 2.4) were diluted in blocking buffer (Table 2.2) or 5% BSA in PBS/Tween, and incubated with the membrane overnight at 4°C, rolling.

2.16.4 Secondary Antibody

Membranes were washed 4 times with 1X PBS/Tween or 1X TBS/Tween for 5 minutes. The HRP-conjugated secondary antibody (Table 2.4) was diluted in blocking buffer, and incubated with the membrane at room temperature for one hour, rolling.

2.16.5 Visualising the Membrane

Membranes were washed 4 times with 1X PBS/Tween or 1X TBS/Tween for 5 minutes. The membranes were incubated with ECL substrate (BioRad) for 5 minutes before being imaged using a BioRad ChemiDoc XRS+.

3 Chapter Three – Results: Characterising the Response of PBECs to Rhinoviral Infection

3.1 Aims

Rhinovirus infection of bronchial epithelial cells activates pattern recognition receptors, including TLR3, which initiate a range of different signalling pathways, such as the MAPK pathways: ERK, p38 and JNK (Slater et al., 2010, Wang et al., 2009). These pathways consist of a three-tier kinase cascade, culminating in the phosphorylation of the MAPK protein on two residues, threonine and tyrosine. The phosphorylated MAPK then translocates to the nucleus and activates transcription factors, such as NF-κB and AP-1; inducing cytokine transcription (Griego et al., 2000). Previous work has identified the MAPKs as having important roles in the response to rhinoviral infection (Griego et al., 2000, Liu et al., 2008a).

It is important to regulate these pathways to limit the inflammation produced in response to the virus. A family of proteins called DUSPs have previously been found to regulate the MAPK pathways by dephosphorylating both the threonine and tyrosine residues within the MAPK proteins simultaneously. However, much of this work has been investigated in bacterial infection of macrophages (Hammer et al., 2006, Qian et al., 2012, Cornell et al., 2012). The expression of DUSPs in epithelial cells has not been well studied, and the regulation of these proteins in response to rhinoviral infection is not known.

The aim of this chapter was to examine the role of the three MAPK proteins: ERK, p38 and JNK, in the response of primary bronchial epithelial cells to rhinoviral infection and to characterise the expression of DUSP proteins thought to regulate these pathways.

3.2 The Role of the MAPK Pathways in the Response of PBECs to RV Infection

3.2.1 Cytokine Release in Response to Viral Infection

It is well documented that epithelial cells respond to viral infection by secreting cytokines, such as CXCL8 and CCL5. CXCL8 is a neutrophil chemoattractant, its transcription being induced by NF-κB, whereas CCL5 production is mainly induced by the IRFs. Therefore, secretion of these two molecules indicates that an immune response has been induced and can give an indication of which transcription factors have been activated. PBECs were infected with rhinovirus to confirm that airway epithelial cells respond to rhinoviral infection by secreting these cytokines. Both major, RV16, and minor, RV1B, group rhinoviruses were utilised to further ascertain if the cells respond differently to the two serotypes. Poly(I:C) was also used to stimulate PBECs, to determine whether the response to rhinovirus was due to TLR3 signalling pathways. As a positive control, IL-1β was used, as it is known to activate the MAPK pathways.

Unstimulated PBECs released around 1 ng/ml CXCL8 at baseline and the CCL5 release was below the minimum level of detection (Figure 3.1). Stimulation with poly(I:C) for 24 h induced significant secretion of both CXCL8 and CCL5, as measured by ELISA (Figure 3.1). Simulation with RV1B consistently induced secretion of both cytokines, however this only reached statistical significance for CCL5 release. Infection with RV16 did not upregulate CXCL8 release, but caused a consistent, statistically significant, increase in CCL5 release. Stimulation of PBECs with the proinflammatory cytokine IL-1β caused release of CXCL8 but not CCL5, for which two out of six donors were below the minimum detection level. This was in keeping with the literature and confirms that IL-1β does not activate the IRF and IFN pathways. These data confirm that under these conditions, stimulation of PBECs with poly(I:C) or infection with rhinovirus leads to activation of both the NF-κB and IRF pathways, whereas IL-1β only induces the NF-κB pathway.

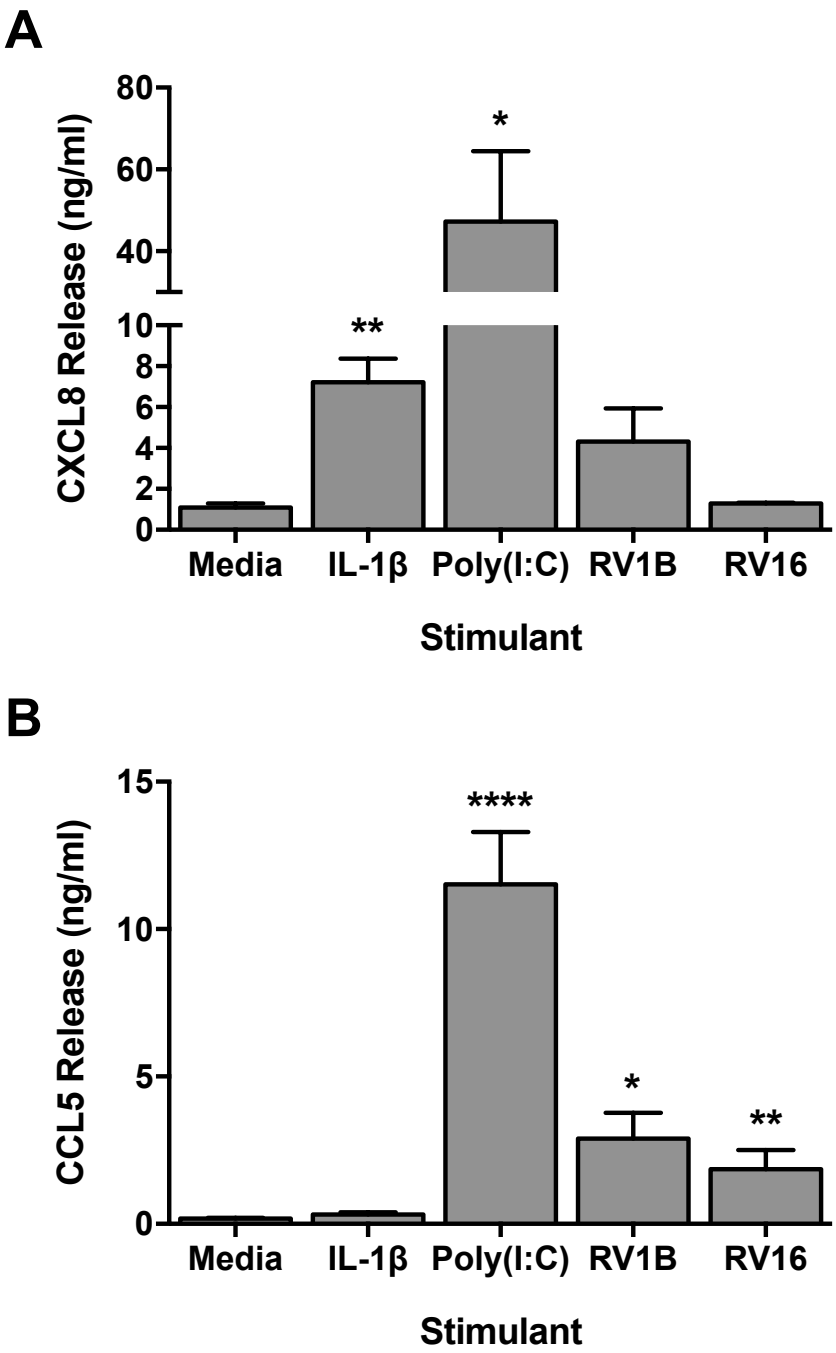

Figure 3.1 PBECs respond to major or minor rhinoviral infection or stimulation with poly(I:C) or IL-1β by secreting pro-inflammatory cytokines.

PBECs were stimulated with IL-1β (100 ng/ml), poly(I:C) (25 μg/ml), RV1B (MOI 3) or RV16 (MOI 4) for 24 hours. Supernatant was collected and ELISA used to measure the release of CXCL8 (A) and CCL5 (B). Data shown are mean ± SEM of n=4 for CXCL8, except RV16 n=3, and n=6 for CCL5, except poly(I:C) and RV16 n=3. Significance is indicated by * $p \leq 0.05$, ** $p \leq 0.01$, or **** $p \leq 0.0001$ as measured by t-test, compared to media control.

3.2.2 The Roles of the MAPK Pathways in Cytokine Release in the Response of PBECs to Viral Infection

It is well established that the MAPK pathways have roles in inducing cytokine release. To determine the specific role of each MAPK pathway in the release of cytokines from PBECs in response to viral infection (Figure 3.1), cells were treated with a panel of MAPK inhibitors before stimulation with poly(I:C), as the greatest cytokine release was observed in response to this stimulant.

Inhibition of either p38 or JNK dramatically decreased release of both CXCL8 and CCL5, almost to the level of media control (Figure 3.2). This was statistically significant for CXCL8 release for both p38 and JNK inhibitors; and there was a consistent decrease in CCL5 release following p38 or JNK inhibition in all three PBEC donors. Release of both cytokines was unaffected when ERK was inhibited (Figure 3.2).

The effect of MAPK inhibition on CXCL8 mRNA generation in response to both strains of rhinovirus was determined by qRT-PCR and ELISA. Similarly to results obtained when studying responses to poly(I:C), at 48 h inhibition of either p38 or JNK decreased mRNA and secreted protein levels of CXCL8 in response to RV1B, whereas ERK inhibition had no effect (Figure 3.3). Due to the large amount of donor variation in mRNA levels, this is only statistically significant for p38 inhibition, however, the effect is much clearer at the secreted protein level. At 24 h post infection, RV1B caused a small increase in CXCL8 levels compared to the media control, which was unaffected by JNK inhibition, and inhibition of p38 caused only a modest downregulation of CXCL8 (Figure 3.3).

The response of PBECs to RV16 infection was similar, with only a small effect of 24 h RV16 infection on CXCL8 production. These data are in keeping with those shown in figure 3.1, where no change was seen in CXCL8 protein levels in response to RV16. After 48 h, the response to RV16 was larger, with a trend towards lower CXCL8 levels in cells treated with p38 or JNK inhibitors, but again no change in response to ERK inhibition. However, this only reached statistical significance in the secreted protein level in response to SB202190 treatment (Figure 3.4).

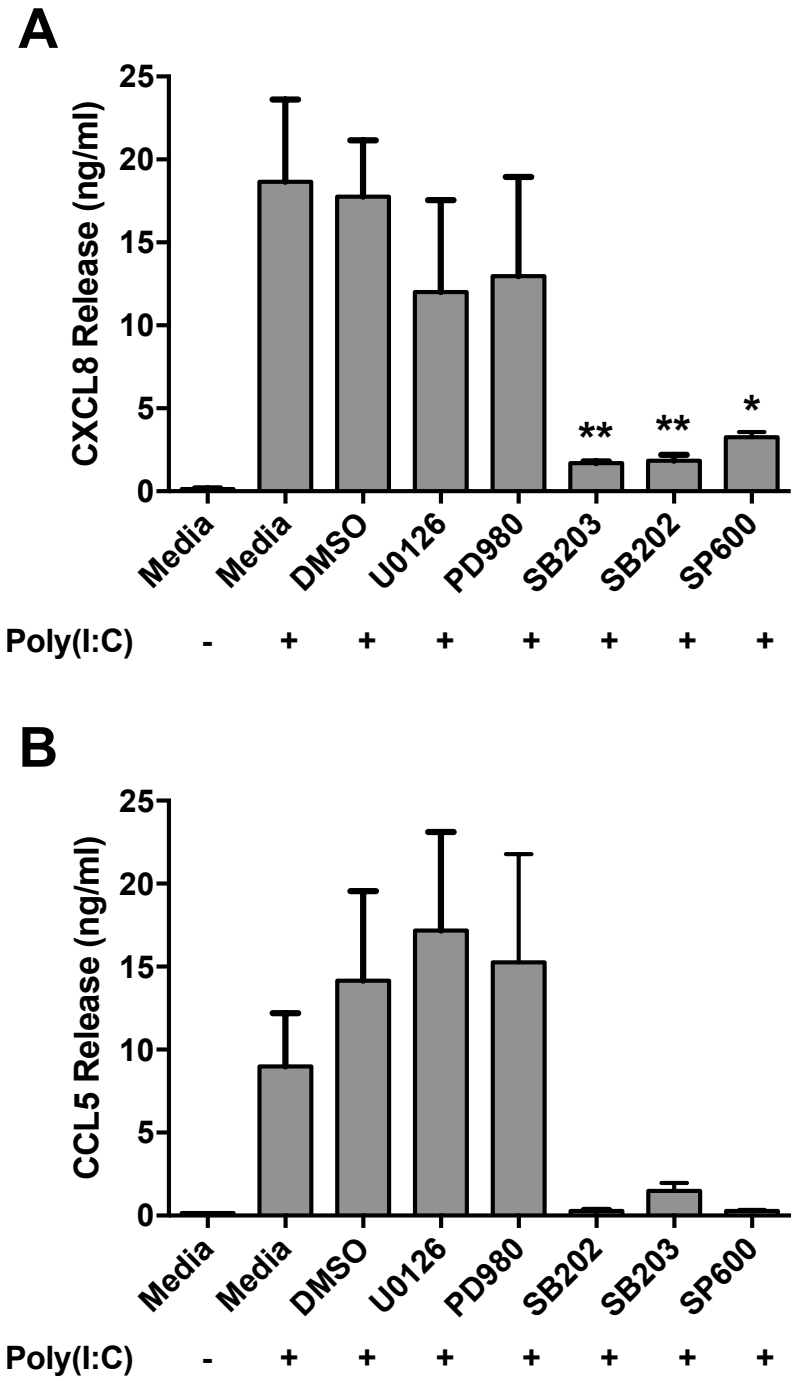

Figure 3.2 Treatment of PBECs with MAPK inhibitors decreases cytokine release in response to poly(I:C) stimulation.

PBECs were treated with MAPK inhibitor (20 μM); U0126 (ERK), PD98059 (ERK), SB203580 (p38), SB202190 (p38) or SP600125 (JNK); vehicle control; or untreated (media) for one hour prior to 24 h poly(I:C) stimulation (25 μg/ml). Supernatant was collected and ELISA used to measure the release of CXCL8 (A) and CCL5 (B). Data shown are mean ± SEM of n=3. Significance is indicated by * $p \leq 0.05$, ** $p \leq 0.01$ verses poly(I:C) + DMSO**,** as measured by paired t-test.

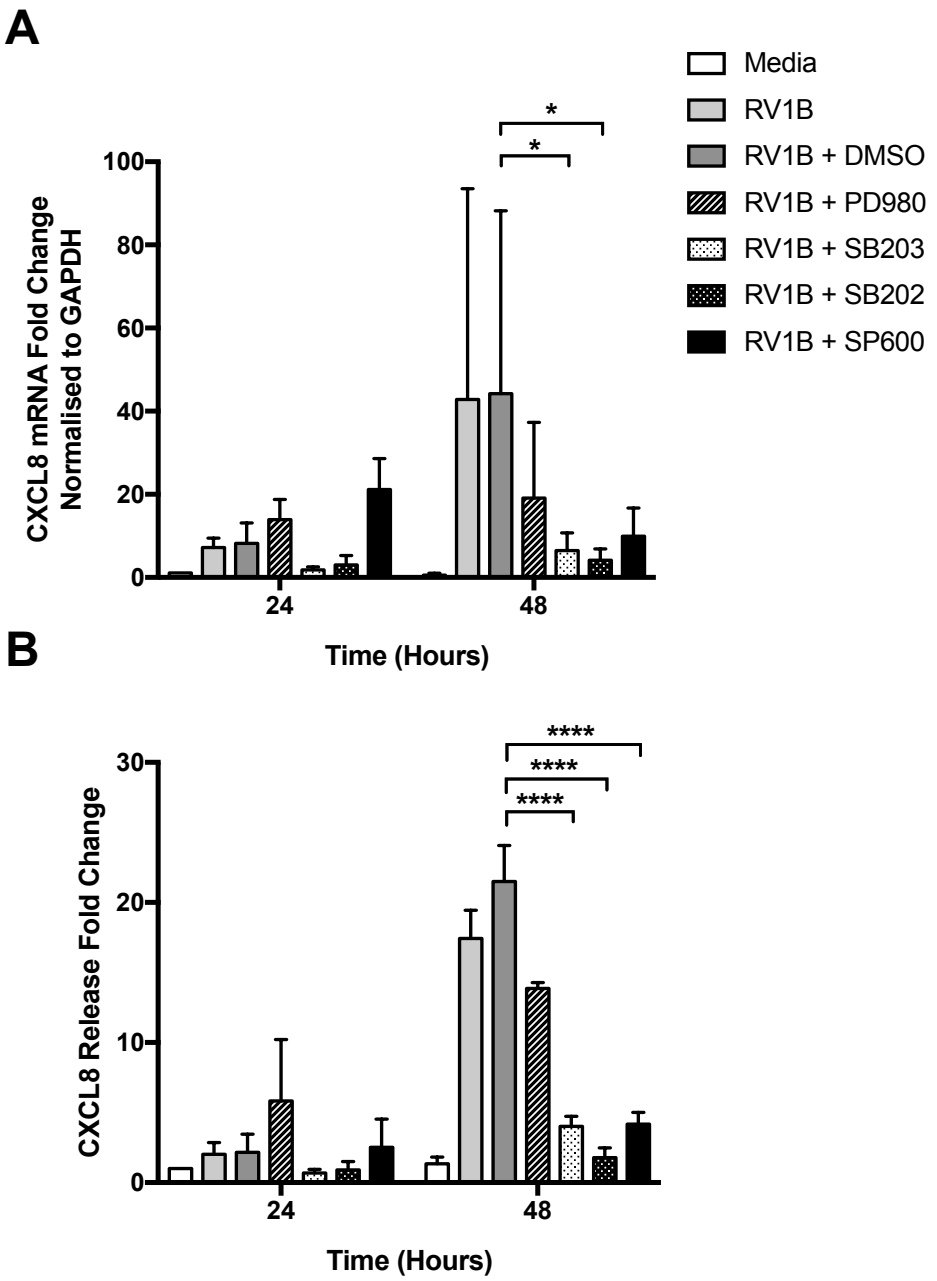

Figure 3.3 Treatment of PBECs with MAPK inhibitors decreases cytokine release in response to RV1B infection.

PBECs were treated with MAPK inhibitor (20 μM); PD98059 (ERK), SB203580 (p38), SB202190 (p38) or SP600125 (JNK); vehicle control; or untreated (media) for one hour prior to RV1B infection (MOI 3). RNA was collected and qRT-PCR performed for CXCL8 and a GAPDH control. Data shown mean ± SEM of n=3 normalised to the GAPDH control and media control (A). Supernatant was collected and ELISA used to measure the release of CXCL8. Data shown are mean ± SEM of n=3, normalised to media control. Significance is indicated by * $p \leq 0.05$, **** $p \leq 0.0001$ verses RV1B + DMSO, as measured by two-way ANOVA, Dunnett's post-test.

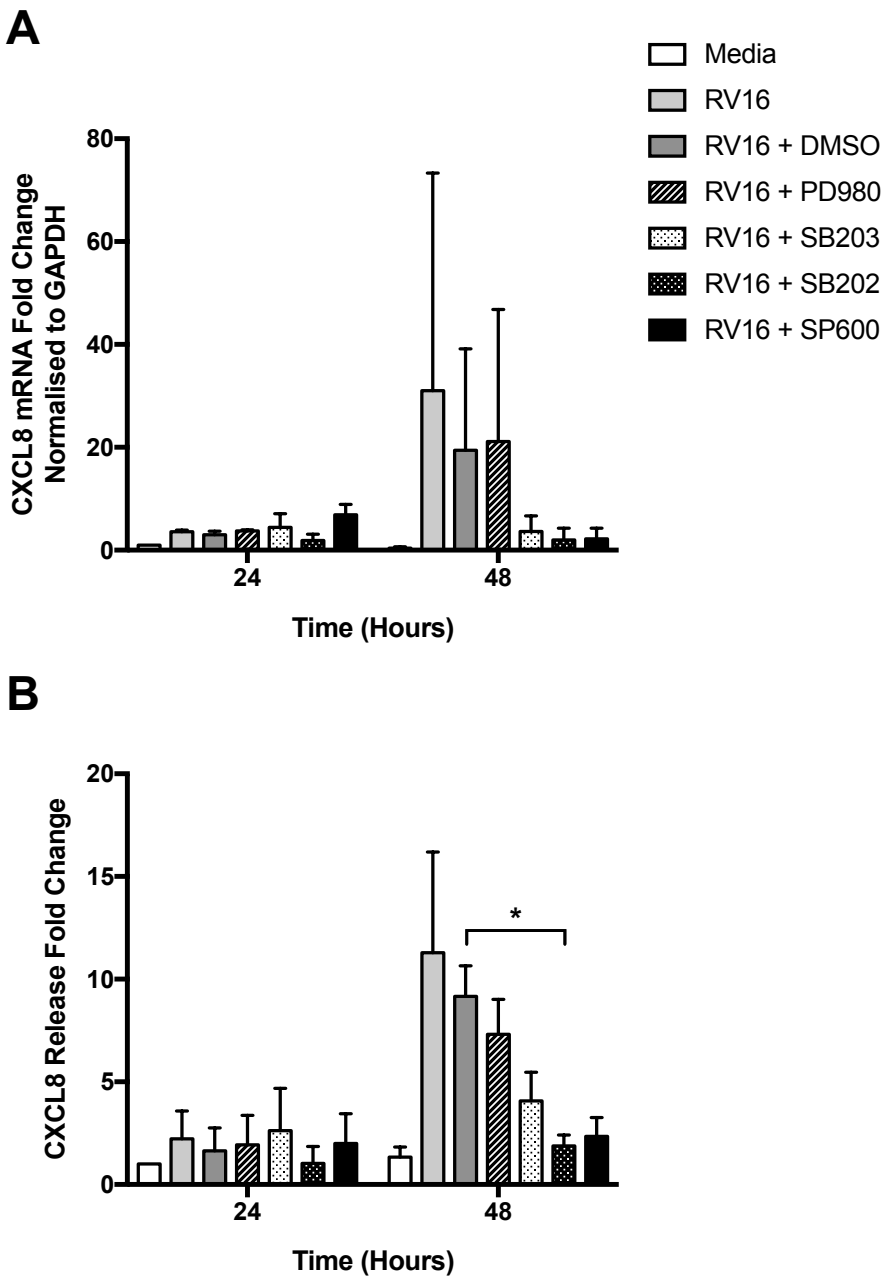

Figure 3.4 Treatment of PBECs with MAPK inhibitors decreases cytokine release in response to RV16 infection.

PBECs were treated with MAPK inhibitor (20 μM); PD98059 (ERK), SB203580 (p38), SB202190 (p38) or SP600125 (JNK); vehicle control; or untreated (media) for one hour prior to RV16 infection (MOI 4). RNA was collected and qRT-PCR performed for CXCL8 and a GAPDH control. Data shown mean ± SEM of n=3 normalised to the GAPDH control and media control (A). Supernatant was collected and ELISA used to measure the release of CXCL8. Data shown are mean ± SEM of n=3, normalised to media control. Significance is indicated by * $p \leq 0.05$ verses RV16 + DMSO, as measured by two-way ANOVA, Dunnett's post-test.

3.2.3 The Roles of the MAPKs in the Viability of PBECs in Response to Rhinoviral Infection

The MAPK pathways are known to be involved in many cellular processes, including apoptosis and proliferation (Wada and Penninger, 2004). Induction of apoptosis is an important component of the innate immune response, and rhinoviral infection of PBECs has previously been shown to induce apoptosis (Wark et al., 2005). Thus, the effect of inhibiting each of the MAPKs on cell viability in rhinoviral infection of PBECs was determined. Infection with either serotype of rhinovirus did not affect cell viability, and this was unaffected by treatment with any of the MAPK inhibitors (Figure 3.5).

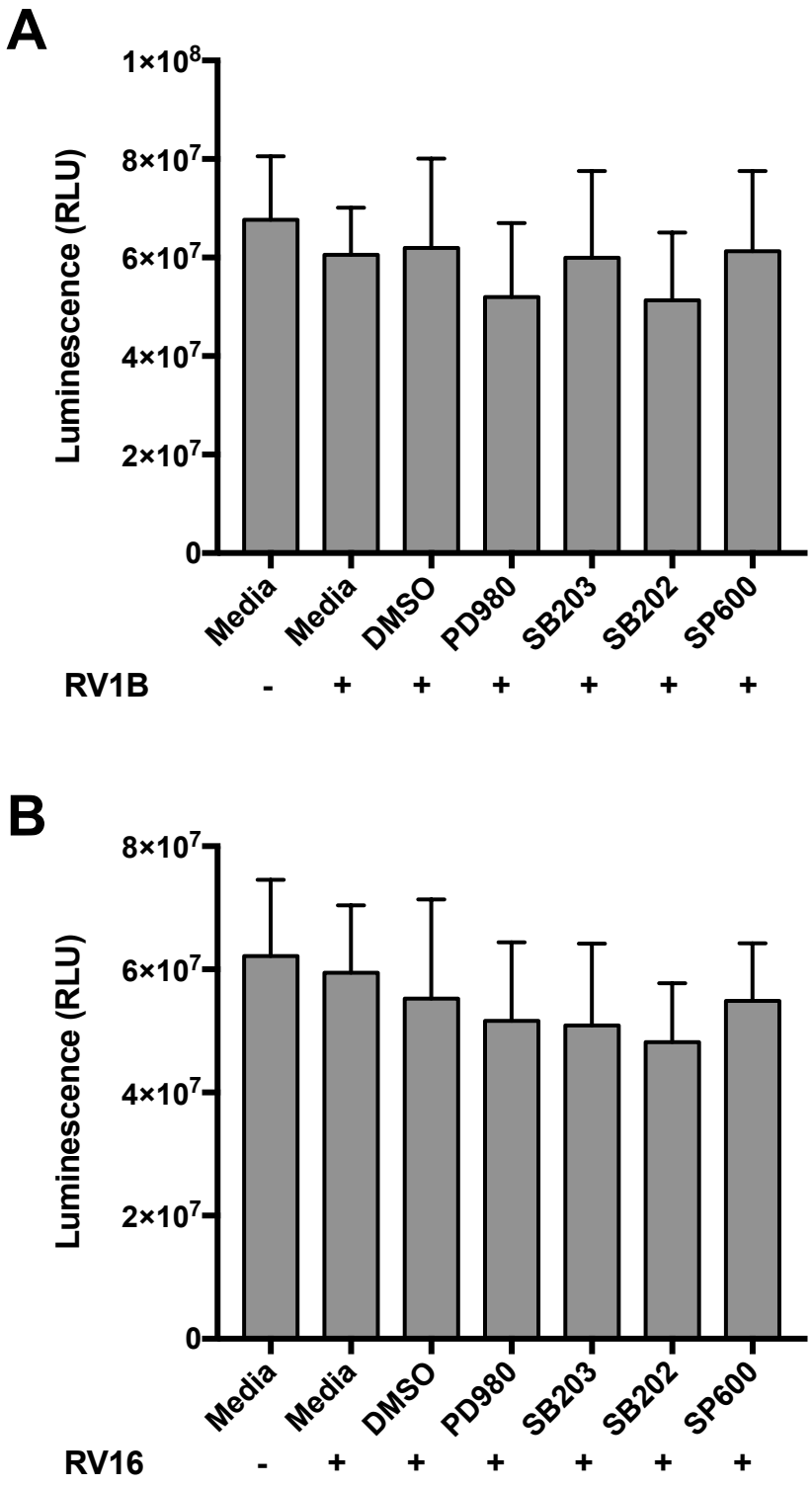

Figure 3.5 Treatment of PBECs with MAPK inhibitors does not affect cell viability.

PBECs were treated with the indicated MAPK inhibitors (20 μM), vehicle control (DMSO), or untreated (media), for one hour prior to 24 h infection with RV1B (MOI 3) (A) or RV16 (MOI 4) (B). Cell titre glo cell viability assay was performed. Data shown are mean ± SEM of relative light units, n=3. Significance was measured using one-way ANOVA, Dunnett's post-test.

3.2.4 The Roles of the MAPK Pathways in Rhinoviral Replication

The MAPKs control many different processes in the cell and therefore may affect the viral life cycle. For example, p38 can regulate the endocytosis pathway, and enteroviruses have been shown to utilise endocytosis machinery to harvest cholesterol (Ilnytska et al., 2013). Therefore, the effect of MAPK inhibition on rhinoviral replication was investigated by qRT-PCR for rhinoviral RNA levels. Treatment with the ERK inhibitor PD98059 did not have any effect on rhinoviral replication levels, as measured by RV RNA (Figure 3.6). Both p38 inhibitors consistently caused a decrease in replication of both strains of rhinovirus at 24 and 48 h post infection, although this was not statistically significant. Intriguingly, inhibition of JNK caused a large increase in the level of RV1B and RV16 replication at both time points (Figure 3.6).

Due to the role of IFNs in limiting the replication of rhinovirus, it was hypothesised that the effect of MAPK inhibition on rhinoviral replication seen in figure 3.6 was due to an effect on IFN production. Thus, the effect of each MAPK inhibitor on IFN-β mRNA production in response to RV1B and RV16 infection was investigated by qRT-PCR. Very little IFN-β was present in all samples, less than 0.1 copies of RNA per μg, and infection with RV1B or RV16 did not cause any changes (Figure 3.7). In RV16 infected samples, treatment with any of the MAPK inhibitors did not affect IFN-β mRNA levels. However, in RV1B infected samples, treatment with the JNK inhibitor SP600125 consistently caused around a 5-fold increase in IFN-β mRNA levels (Figure 3.7 A).

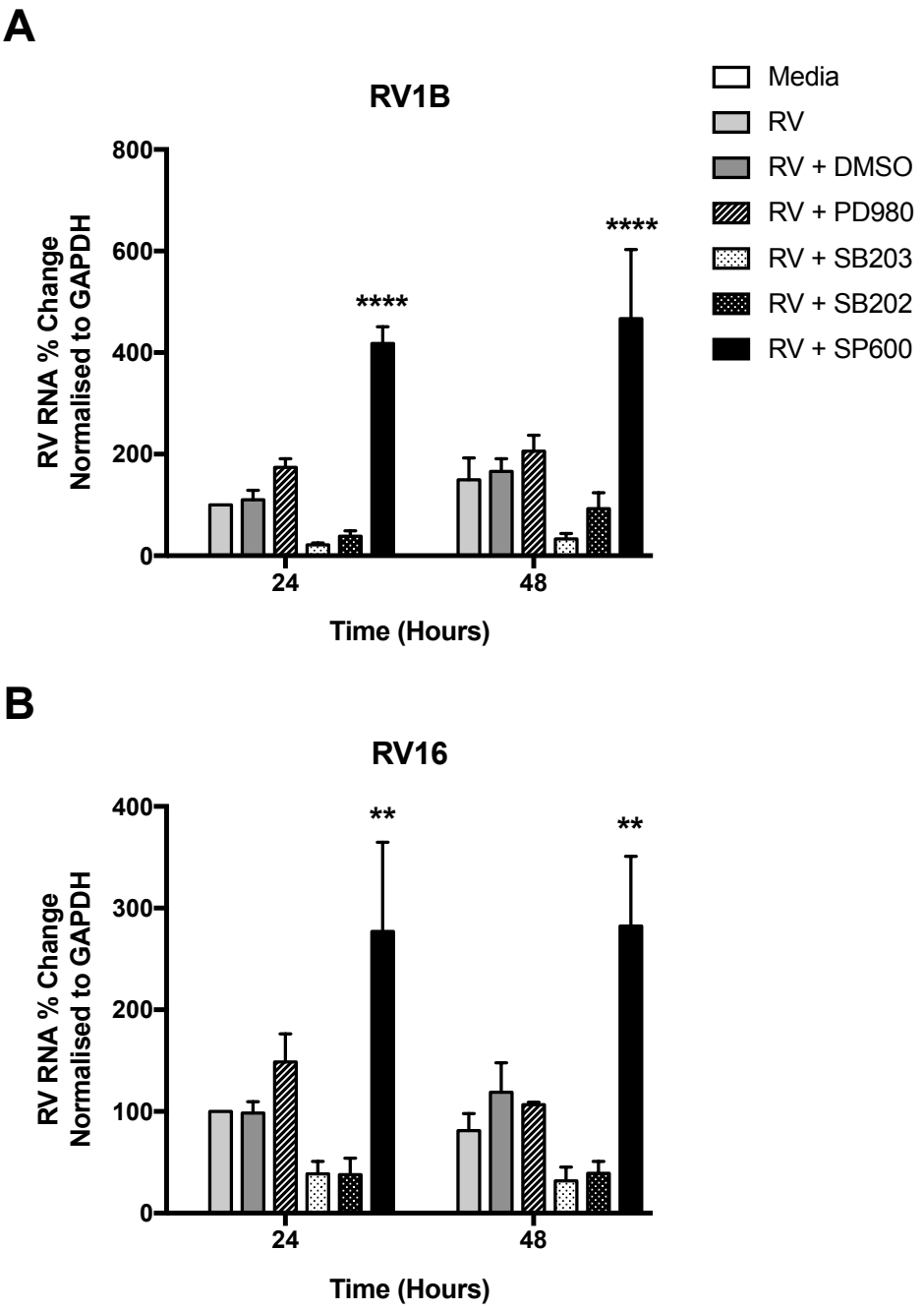

Figure 3.6 RV replication is decreased by p38 inhibition and increased by JNK inhibition.

PBECs were treated with the indicated MAPK inhibitors (20 μM), vehicle control (DMSO), or untreated (media), for one hour prior to 24 h infection with RV1B (MOI 3) (A) or RV16 (MOI 4) (B). RNA was collected and qRT-PCR performed for RV RNA and a GAPDH control. Data shown mean ± SEM normalised to the GAPDH control and RV only 24 h samples, n=4, except PD98059 which is n=3 (A) and n=2 (B). Significance is indicated by ** $p \leq 0.01$ and **** $p \leq 0.0001$ versus RV only as measured by two-way ANOVA with Dunnett's post-test (excluding PD98059 in B).

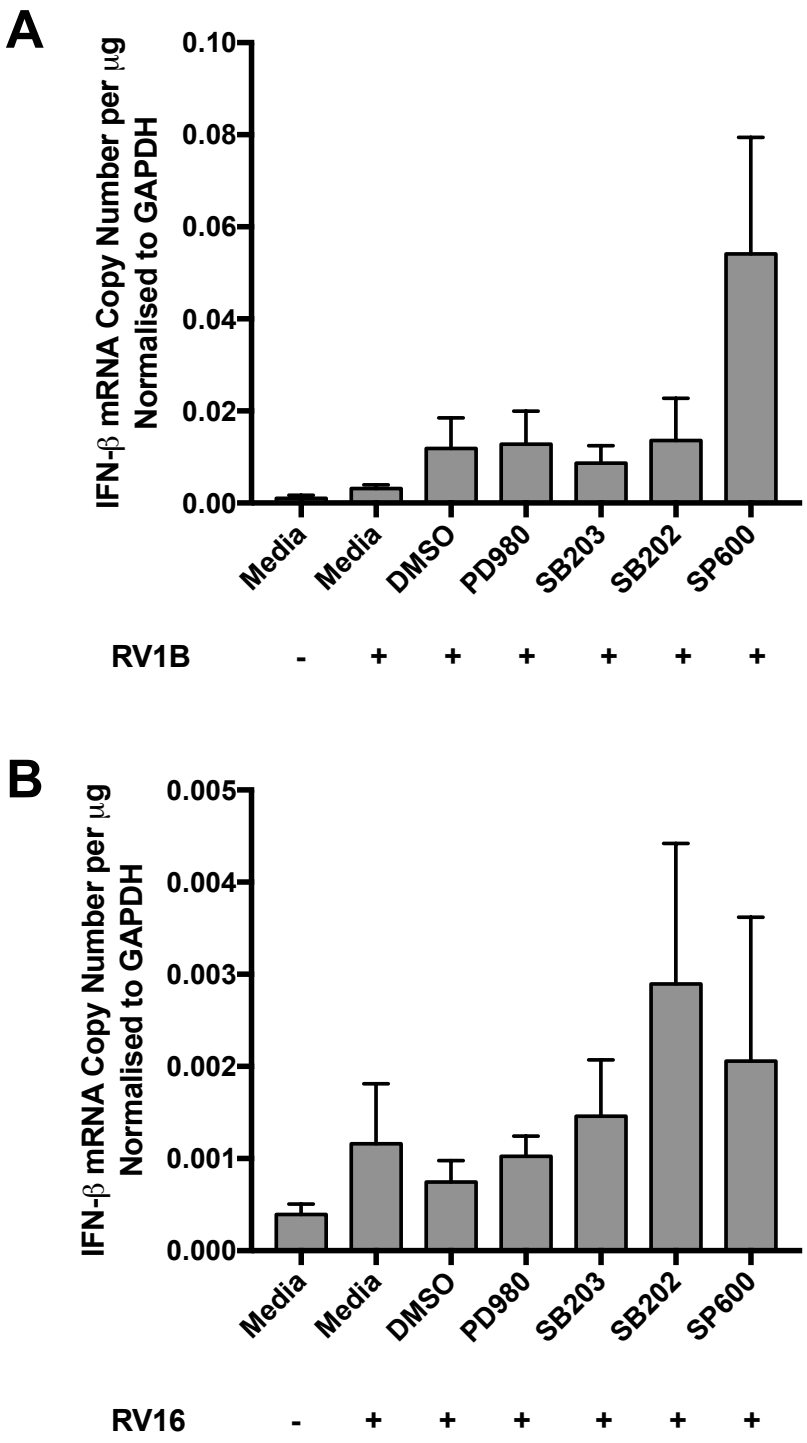

Figure 3.7 RV infection does not induce measurable IFN-β mRNA levels.

PBECs were treated with the indicated MAPK inhibitors (20 μM), vehicle control (DMSO), or untreated (media), for one hour prior to 4 h infection with RV1B (MOI 3) (A) or RV16 (MOI 4) (B). RNA was collected and qRT-PCR performed for IFN-β and a GAPDH control. Data shown mean ± SEM of n=4, normalised to the GAPDH control. Significance compared to the RV + DMSO control was measured using one-way ANOVA with Dunnett's post-test.

3.3 Activation of the MAPK Pathways in PBECs

As discussed above, NF-κB is activated by the MAPK pathways, and the NF-κB dependent cytokine CXCL8 was secreted in response to infection with rhinovirus (Figure 3.1). However, it is known that the IKK pathway also plays a major role in activating NF-κB transcription and cytokine release in response to TLR or IL-1RI activation (Figure 1.1). Therefore, the specific activation of the MAPK pathways in PBECs in response to viral infection was examined. MAPK proteins are thought to be present but inactive in unstimulated cells, and phosphorylated and activated in response to infection.

The level of phosphorylated, active, MAPK proteins was measured over 24 h in response to stimulation with IL-1β or poly(I:C), or infection with RV1B or RV16 using western blot. Some of the presented western blots are segmented, this is due to samples being run on separate gels, however, blots were all treated the same and blots for each antibody exposed for the same amount of time. Phosphorylated ERK protein was present in all media control samples (Figure 3.8). In response to stimulation with IL-1β or poly(I:C), or infection with RV1B, the levels of phospho-ERK increased slightly at 0.5 h then gradually declined over the rest of the time course, to below the level seen in media controls (Figure 3.8 A, B, C). Infection with RV16 did not cause the initial increase in phospho-ERK levels, but from 1 h post infection levels gradually declined to an almost undetectable level (Figure 3.8 D). This work is inconclusive, as it is only an n of 1. As inhibition of ERK did not have an effect on cytokine release, cell viability, or rhinoviral replication (Section 3.2), the activation of ERK MAPK was not investigated any further.

Activation of the p38 MAPK was then examined. No, or very little, p38 phosphorylation was observed in untreated cells, whilst stimulation with IL-1β caused phosphorylation of p38 within 30 minutes before declining back to baseline (Figure 3.9 A). Phosphorylation of p38 in response to stimulation with poly(I:C) occurred much more gradually, peaking at around 2 h, before declining again. The increase was not significant but was detected consistently (Figure 3.9 B). No p38 phosphorylation was observed in response to RV1B, although low background levels can be seen in the densitometry (Figure 3.9 C), whilst p38 phosphorylation in response to RV16 occurred late, at 8 and 24 h (Figure 3.9 D). As MAPKs are generally activated rapidly this indicated that p38 may have been activated prior to the time points examined, with the late peak detected in response to RV16 potentially due to a second round of infection.

To investigate this further, PBECs were infected with RV1B and RV16 and cell lysates were taken within the initial hour of virus addition, during viral attachment. p38 activation was observed in response to both viruses within this hour, peaking at around 20-30 min in response to RV1B (Figure 3.10 A) and 40 min in response to RV16 (Figure 3.10 B), although this only reached statistical significance for RV1B.

The final MAPK pathway, JNK, was then examined using the same technique. Similarly to p38, JNK was phosphorylated in response to IL-1β and poly(I:C), peaking at 30 min (Figure 3.11 A) and 2 hours (Figure 3.11 B) respectively. However, no activation was detected in response to either of the rhinoviral serotypes over 24 h (Figure 3.11 C and D). Therefore, JNK activation was investigated during the one hour viral attachment period. During this hour JNK was phosphorylated by RV1B, gradually increasing up to 40 min, although this did not reach statistical significance, before declining back to baseline (Figure 3.12 A). Phosphorylation was also observed in response to RV16 infection, occurring at 30 min and remaining up to 60 min (Figure 3.12 B).

As rhinoviral infection activated the MAPKs much earlier than poly(I:C) this early signal is unlikely to be dependent on TLR3 activation. UV-inactivated RV1B was used to determine whether the early MAPK activation was dependent on rhinoviral replication. Infection with RV1B again caused activation of both JNK and p38 by 20 minutes post viral addition which declined by 60 minutes. Stimulation with UV-inactivated RV1B also caused activation of both MAPKs (Figure 3.13). These data must be interpreted with caution as they are only n=1, however they are supported by previous work showing activation of p38 by UV-inactivated rhinovirus (Dumitru et al., 2006, Wang et al., 2006).

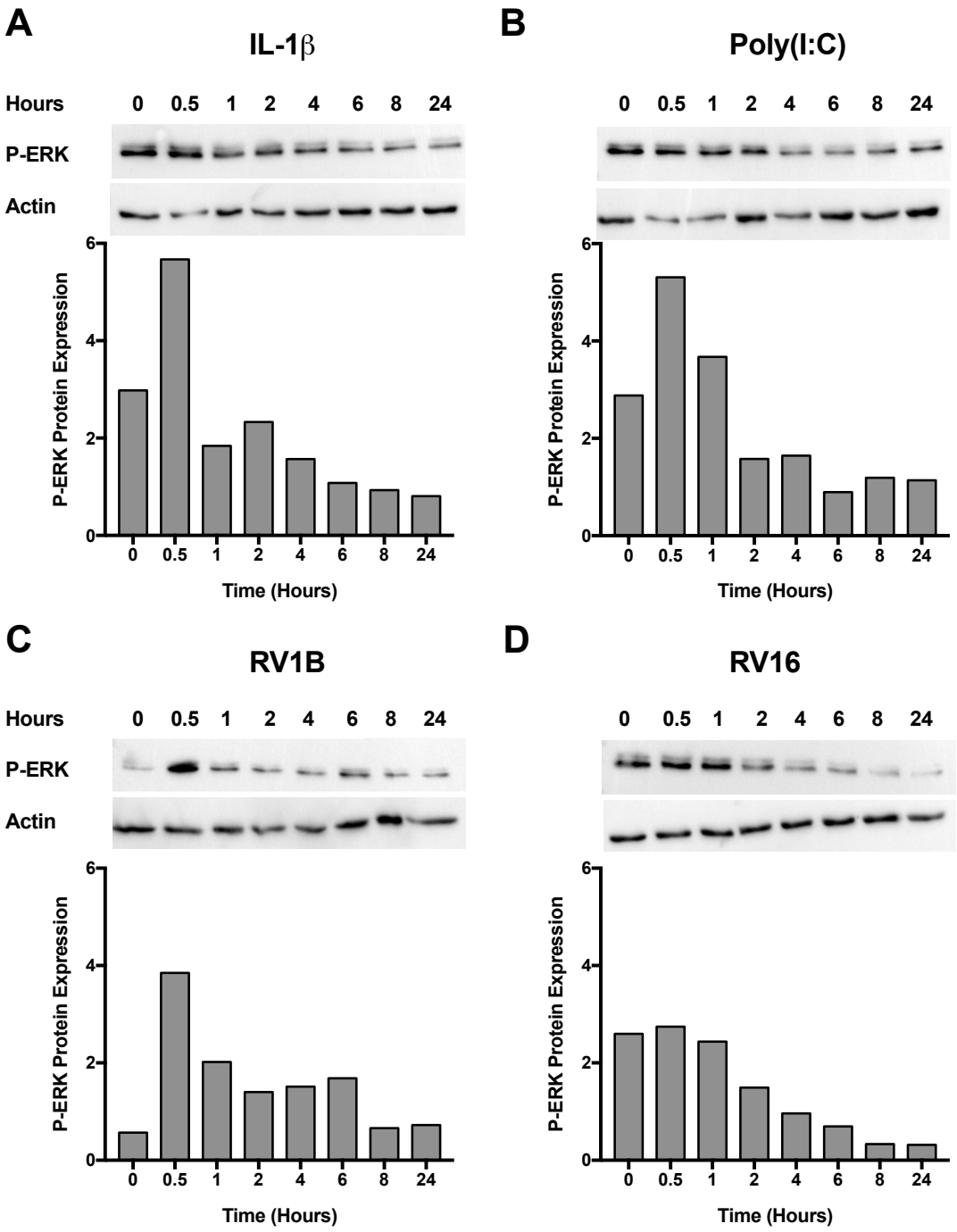

Figure 3.8 Levels of ERK activation gradually decline over 24 h.

PBECs were stimulated with IL-1β (100 ng/ml) (A), poly(I:C) (25 μg/ml) (B), RV1B (MOI 3) (C), or RV16 (MOI 4) (D) and cell lysate collected at the indicated time points. Phosphorylated ERK and total actin levels were measured using western blot. Blots are shown with densitometry below. Data shown are band density normalised to the actin control for each sample, n=1.

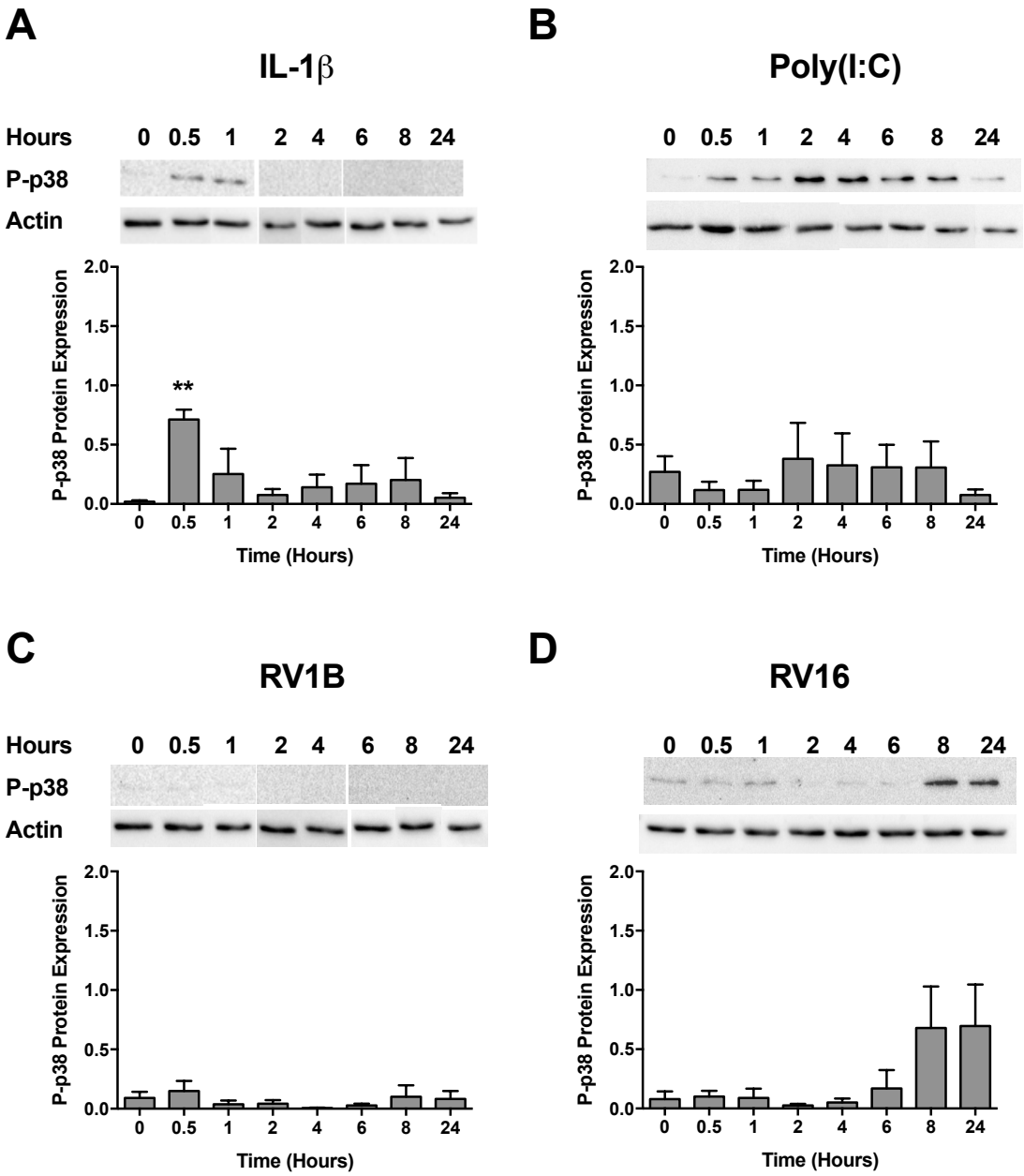

Figure 3.9 p38 is activated by stimulation with poly(I:C) and IL-1β.

PBECs were stimulated with IL-1β (100 ng/ml) (A), poly(I:C) (25 μg/ml) (B), RV1B (MOI 3) (C) or RV16 (MOI 4) (D) over 24 hours and cell lysate collected at the indicated time points. Phosphorylated p-38 and total actin levels were measured using western blot. Representative blots are shown with densitometry below. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3. Significance is indicated by ** $p \leq 0.01$ verses unstimulated control (0 h) as measured by one-way ANOVA, with Dunnett's post-test.

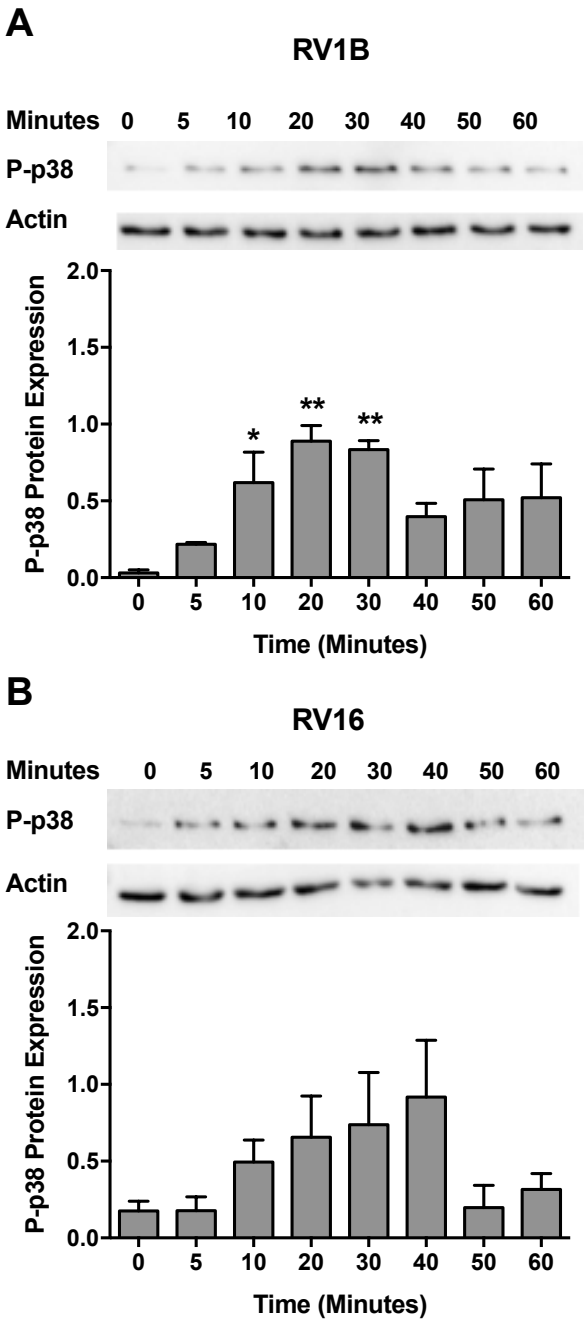

Figure 3.10 p38 is activated by major and minor rhinoviral infection during viral attachment.

PBECs were stimulated with RV1B (A) and RV16 (B) for one hour during viral attachment and cell lysate collected at the indicated time points. Phosphorylated p-38 and total actin levels were measured using western blot. Representative blots are shown with densitometry below. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3. Significance is indicated by * $p \leq 0.05$ and ** $p \leq 0.01$ verses unstimulated control (0 h) as measured by one-way ANOVA, with Dunnett's post-test.

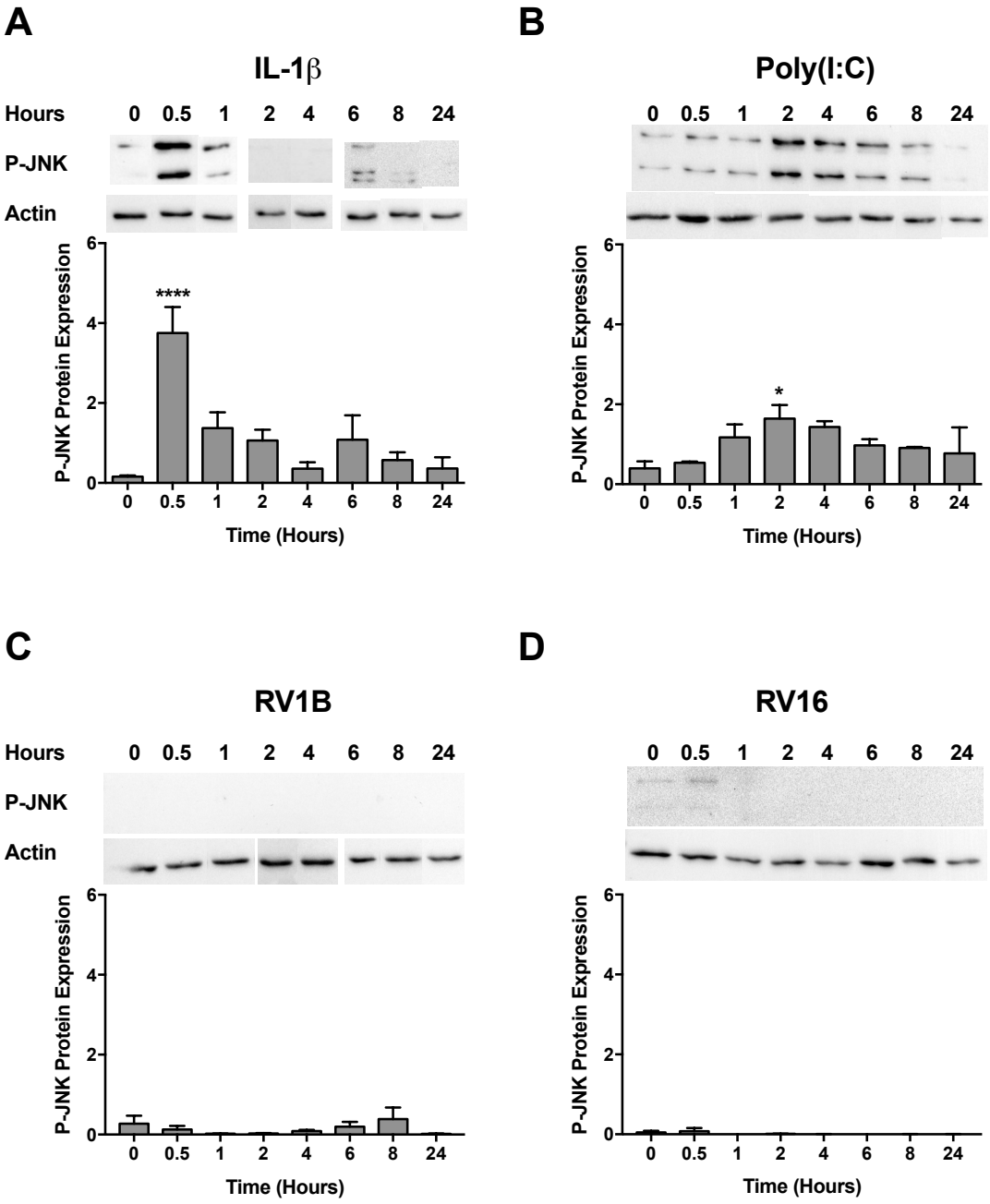

Figure 3.11 JNK is activated by stimulation with poly(I:C) and IL-1β.

PBECs were stimulated with IL-1β (100 ng/ml) (A), poly(I:C) (25 μg/ml) (B), RV1B (MOI 3) (C) or RV16 (MOI 4) (D) over 24 hours and cell lysate collected at the indicated time points. Phosphorylated JNK and total actin levels were measured using western blot. Representative blots are shown with densitometry below. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3. Significance is indicated by * $p \leq 0.05$ and **** $p \leq 0.0001$ verses unstimulated control (0 h) as measured by one-way ANOVA with Dunnett's post-test.

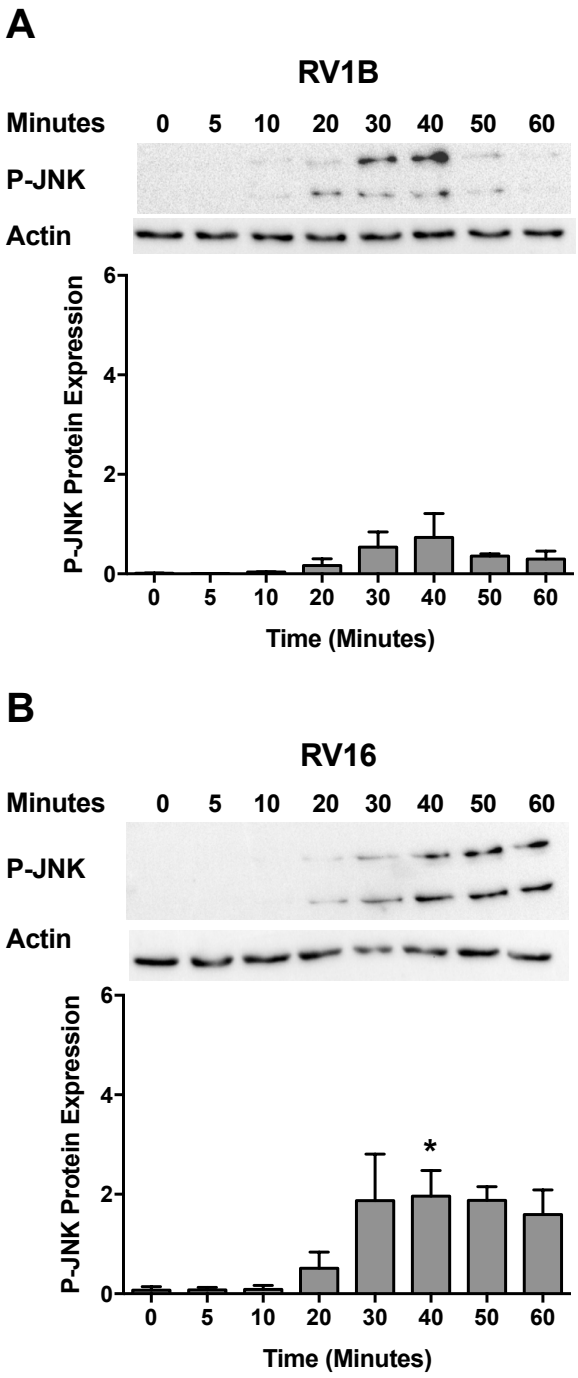

Figure 3.12 JNK is activated by major and minor rhinoviral infection during viral attachment.

PBECs were stimulated with RV1B (A) and RV16 (B) for one hour during viral attachment and cell lysate collected at the indicated time points. Phosphorylated JNK and total actin levels were measured using western blot. Representative blots are shown with densitometry below. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3. Significance is indicated by * $p \leq 0.05$ verses unstimulated control (0 h) as measured by one-way ANOVA, with Dunnett's post-test.

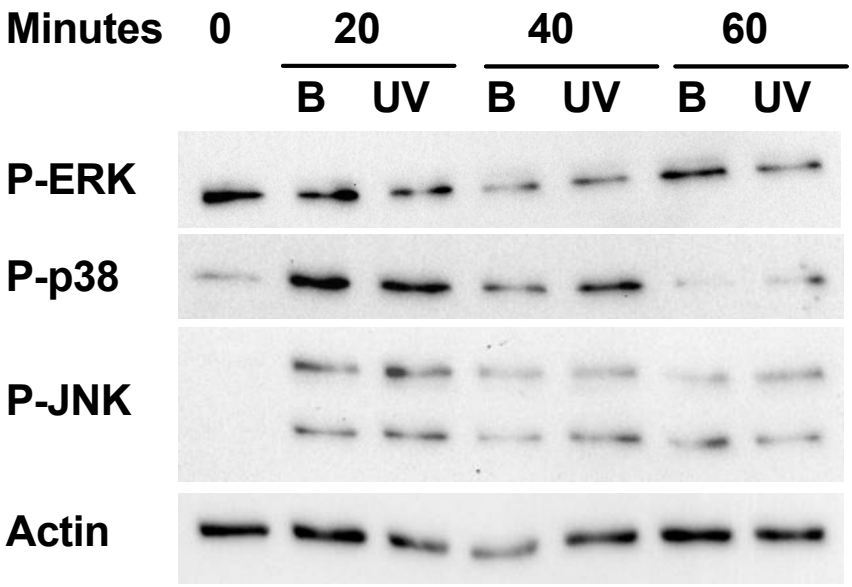

Figure 3.13 UV-inactivated rhinovirus activates the MAPK proteins.

PBECs were infected with RV1B (MOI 3) or UV-inactivated RV1B and cell lysate collected during viral attachment at the time points indicated. Phosphorylated p38, phosphorylated JNK and total actin levels were measured using western blot. Blots are shown, n=1.

3.3.1 The Role of Phosphatases in MAPK Regulation

The phosphorylated, activated, p38 and JNK MAPK signals were lost not long after activation in response to stimulation with IL-1β or poly(I:C) or infection with rhinovirus (Figures 3.9 – 3.12). Phosphorylated proteins can be regulated through dephosphorylation or degradation. To determine the role of phosphatases in the regulation of p38 and JNK signalling in response to rhinovirus, PBECs were treated with a general phosphatase inhibitor, okadaic acid, prior to RV1B infection. Inhibition of dephosphorylation consistently caused an increase in the phosphorylated p38 and JNK signals at both 30 and 60 minutes after virus addition, although this was not statistically significant (Figure 3.14).

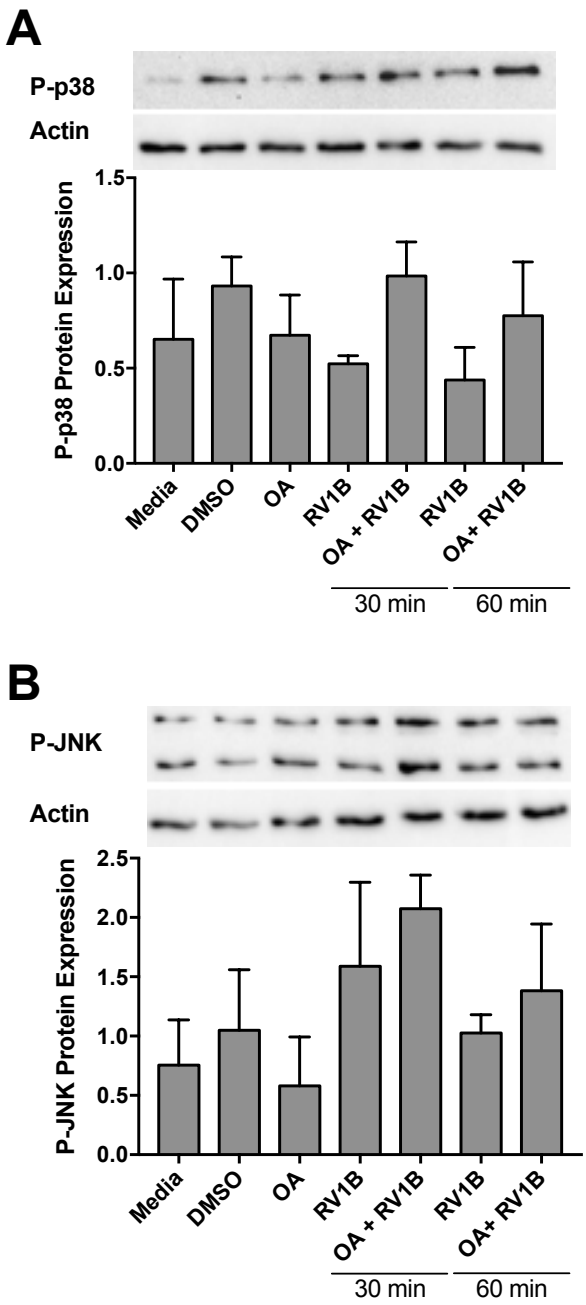

Figure 3.14 MAPKs are dephosphorylated.

PBECs were pre-treated with 1 μM okadaic acid (OA) or vehicle control (DMSO) for 30 min prior to RV1B infection and cell lysate collected at the indicated time points during viral attachment. Phosphorylated p38 (A) and phosphorylated JNK (B) and total actin levels were measured using western blot. Representative blots are shown with densitometry below. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3. Significance was measured using one-way ANOVA with Sidak's post-test.

3.3.2 The Effect of Viral Filtrate on MAPK Activation

In order to ensure that the MAPK activation seen in response to infection with RV1B or RV16 (Figure 3.10 and 3.12) is due to viral infection, PBECs were treated with viral filtrate and western blot used to measure MAPK activation. Phosphorylated ERK protein was present in media control and addition of RV1B or RV16 did not cause any difference in the level of activated ERK. Phospho-ERK was also present in samples treated with viral filtrate, but to a lesser extent (Figure 3.15 A and B). Addition of RV1B or RV16 to PBECs for 30 min caused an increase in levels of phosphorylated p38 and JNK. Addition of filtrate from both serotypes of virus caused increases in JNK and p38 activation to the same level (Figure 3.15 A, C and D).

To determine whether the activation of the MAPKs by the filtrate is causing any of the downstream effects seen in response to RV infection, the effect of viral filtrate on cytokine production was investigated. Infection with RV1B caused an increase in CXCL8 mRNA and secreted protein levels at 24 and 48 h, with a much larger effect at the later time point (Figure 3.16). Addition of filtrate from RV1B to PBECs did not increase CXCL8 mRNA or protein levels, with significant differences in CXCL8 production between RV1B and RV1B filtrate at 48 h post stimulation. Stimulation with RV16 virus or RV16 filtrate did not cause increases in CXCL8 mRNA levels or CXCL8 protein release (Figure 3.16).

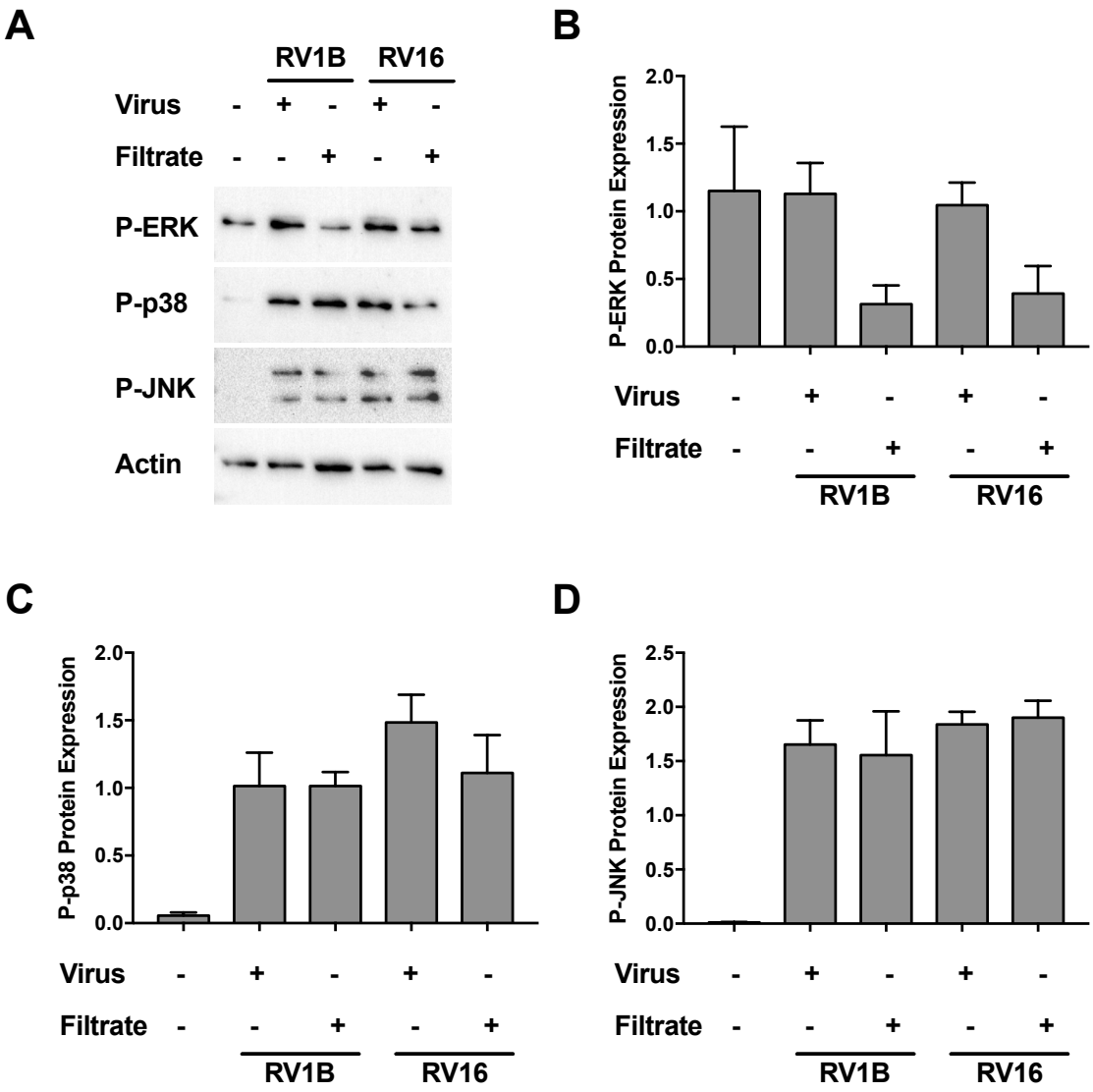

Figure 3.15 Rhinoviral filtrate activates the MAPK proteins.

PBECs were stimulated with RV1B (MOI 3) or RV16 (MOI 4) or with filtrate from either virus for 30 minutes. Cell lysate was collected during viral attachment. Phosphorylated ERK, p38, and JNK and total actin levels were measured using western blot. Representative blots are shown in A with densitometry in B, C and D. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3. Significance was measured using one-way ANOVA with Sidak's post-test.

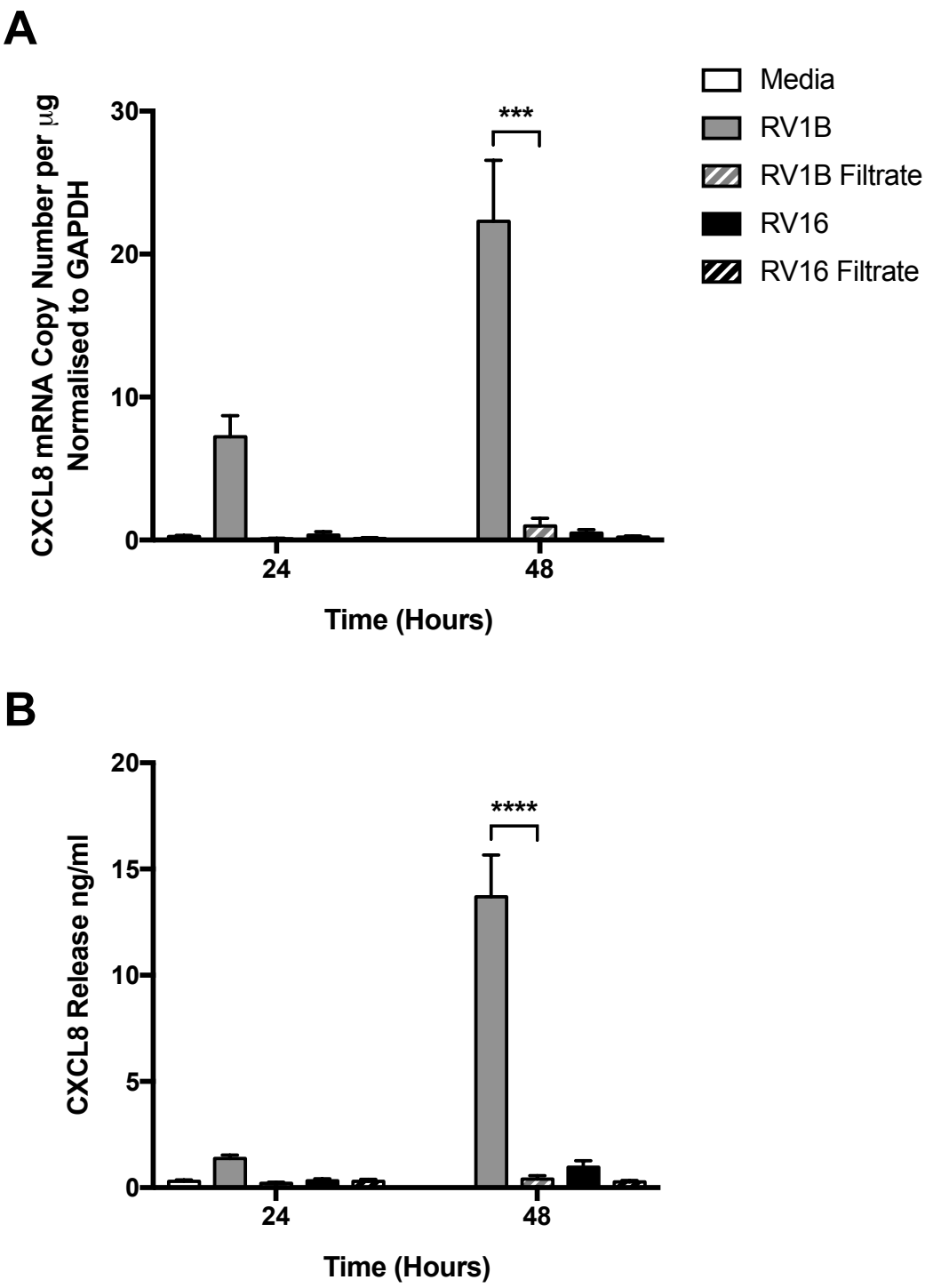

Figure 3.16 Rhinoviral filtrate does not induce cytokine production.

PBECs were infected with RV1B (MOI 3) or RV16 (MOI 4) or stimulated with filtrate from both viruses for 24 or 48 h. RNA was collected and qRT-PCR performed for CXCL8 and a GAPDH control. Data shown are mean ± SEM of n=3 normalised to the GAPDH control (A). Supernatant was collected and ELISA used to measure the release of CXCL8. Data shown are mean ± SEM of n=3. Significance is indicated by *** $p \leq 0.001$ or **** $p \leq 0.0001$ as measured by multiple t-tests.

3.4 Activation and Roles of the MAPK Pathways in PBECs - Summary

The data presented so far show that PBECs respond to rhinoviral infection or TLR3 activation by releasing inflammatory cytokines. This response is decreased when p38 or JNK are inhibited, but inhibition of ERK has no effect on cytokine production. The p38 and JNK pathways were also found to have roles in rhinoviral replication, with inhibition of p38 leading to decreased viral RNA production, and inhibition of JNK leading to the opposite, increased viral RNA. The increased viral replication in the absence of JNK signalling was not found to be due to changes in IFN-β production. These data suggest that the p38 and JNK pathways have important roles in the response of PBECs to rhinoviral infection.

JNK and p38 are activated in response to innate immune stimuli in PBECs. TLR3 activation, by poly(I:C), leads to activation of both pathways with similar timings, peaking 2 h post stimulation. Stimulation with the inflammatory cytokine IL-1β also leads to activation of both pathways, with an earlier peak activation of 30 minutes post stimulation. In response to rhinoviral infection, MAPK activation is undetectable, the phosphorylation seen by western blot being due to an unknown molecule present in the viral media. Inhibition of phosphatases increases the amount of activated MAPK, suggesting they are regulated by dephosphorylation.

3.5 Expression of DUSPs in PBECs

The p38 and JNK pathways have important roles in inducing cytokine release (Figures 3.2-3.4). It is therefore extremely important that these pathways are regulated in order to control and limit inflammation. As discussed in section 1.5, DUSPs 1, 2, 4 and 10 have all previously been shown to have roles in regulating the MAPK pathways in response to immune stimuli.

To the best of my knowledge, these proteins have not yet been studied in PBECs. Therefore, to ensure that the DUSPs were being expressed in PBECs, RT-PCR was used to measure the mRNA expression of DUSPs 1, 2, 4 and 10 and a GAPDH control. The regulation of expression of these DUSPs was investigated in response to stimulation with poly(I:C) or IL-1β over 24 h. All of the DUSPs studied were expressed at the mRNA level in PBECs, even at baseline (Figure 3.17), however, no obvious regulation was observed in response to either poly(I:C) or IL-1β.

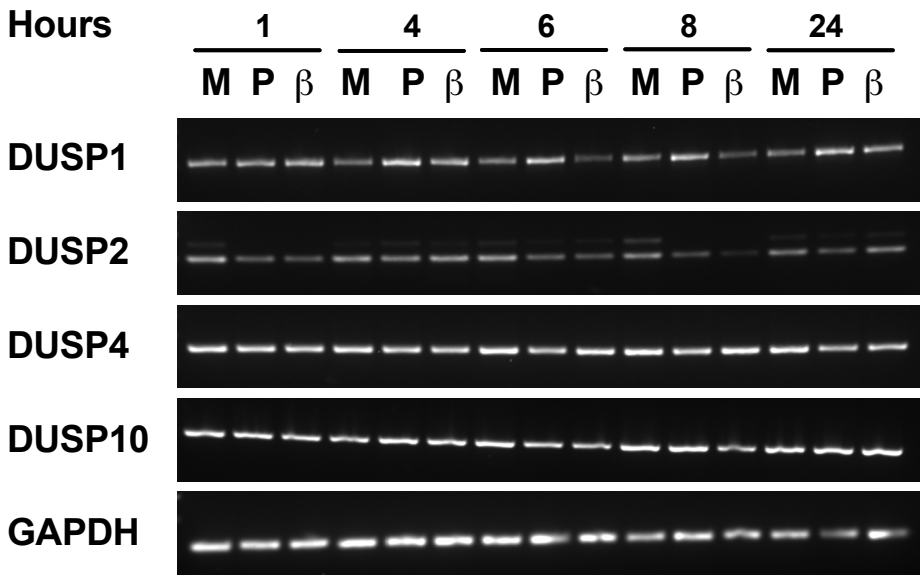

Figure 3.17 DUSPs 1, 2, 4 and 10 are expressed in PBECs.

PBECs were stimulated with poly(I:C) (25 μg/ml) (P), IL-1β (10 ng/ml) (β) or left untreated (M) over 24 hours. mRNA was collected at the time points indicated and RT-PCR performed using primers for DUSPs 1, 2, 4 and 10 and a GAPDH control. n=2 with a representative gel shown.

3.6 Expression and Regulation of DUSP1 in PBECs

3.6.1 DUSP1 mRNA Expression and Regulation in PBECs in Response to Rhinoviral Infection

As p38 and JNK had the biggest roles in inducing cytokine release in PBECs (Figures 3.2-3.4) one DUSP that has previously been shown to regulate these two pathways, DUSP1 (Chi et al., 2006), was taken forward for further investigation. The RT-PCR results (Figure 3.17) did not show any clear regulation of the DUSP1 mRNA levels in response to stimulation. Thus, a more sensitive technique, qRT-PCR, was utilised to investigate the expression in more detail to determine if the mRNA levels were regulated in response to either serotype of rhinovirus, poly(I:C) or IL-1β. Stimulation with poly(I:C) for 24 h induced approximately a 6-fold increase in DUSP1 mRNA expression (Figure 3.18 B). However, stimulation of PBECs with IL-1β, or infection with RV1B or RV16 did not alter the levels of DUSP1 mRNA expression (Figure 3.18).

As the MAPK proteins were mainly activated in the initial one-hour attachment phase of rhinoviral infection, and the MAPKs have been shown to be able to induce transcription of DUSP1 (Ananieva et al., 2008), the expression of DUSP1 over the attachment period was investigated. No changes in DUSP1 mRNA levels were observed in response to RV1B or RV16 over this hour (Figure 3.19).

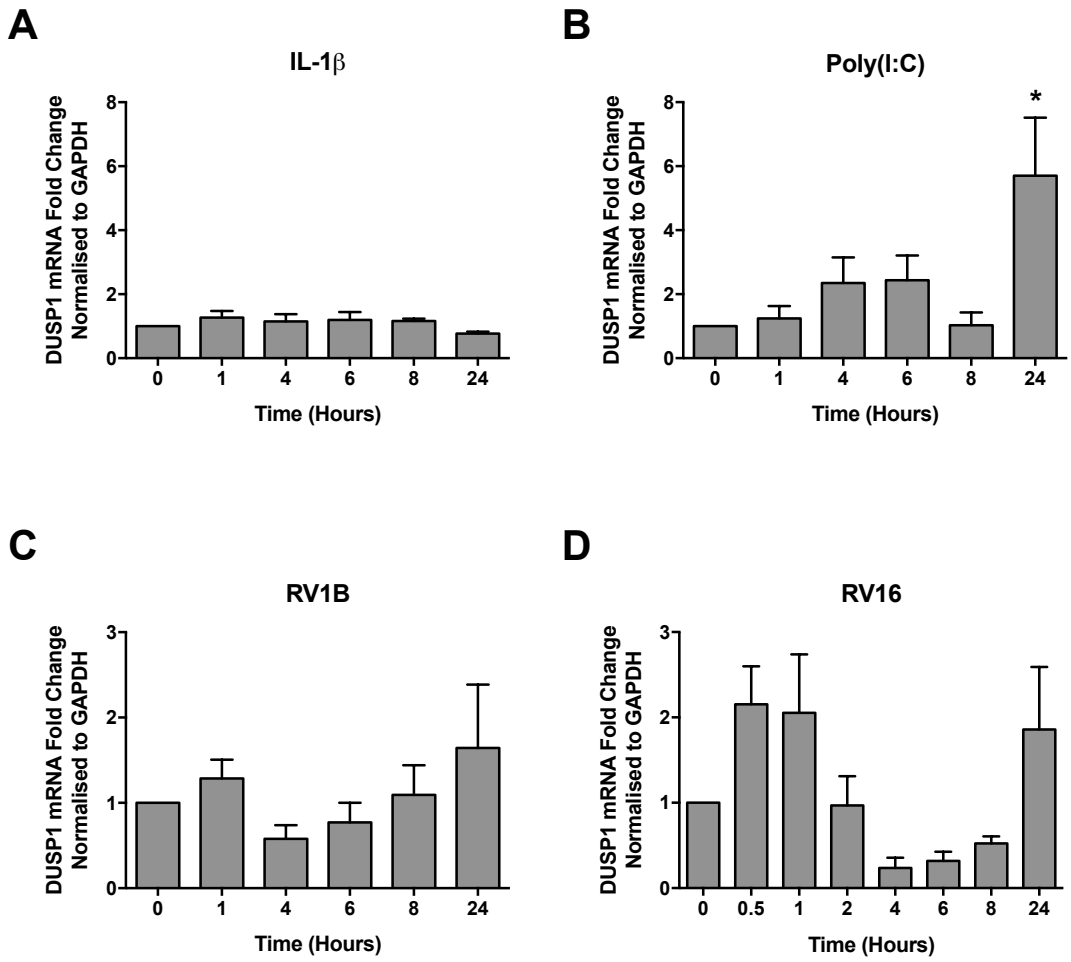

Figure 3.18 DUSP1 transcription is induced by poly(I:C) stimulation.

PBECs were stimulated with IL-1β (100 ng/ml) (A), poly(I:C) (25 μg/ml) (B), RV1B (MOI 3) (C) or RV16 (MOI 4) (D) over 24 hours. mRNA was collected at the time points indicated and ΔΔCt qRT-PCR performed for DUSP1 and a GAPDH control. Data shown are mean ± SEM, n=3, fold change compared to unstimulated control. Significance is indicated by * $p \leq 0.05$ verses unstimulated control (0 h) as measured by one-way ANOVA, with Dunnett's post-test, on the ΔΔCt values.

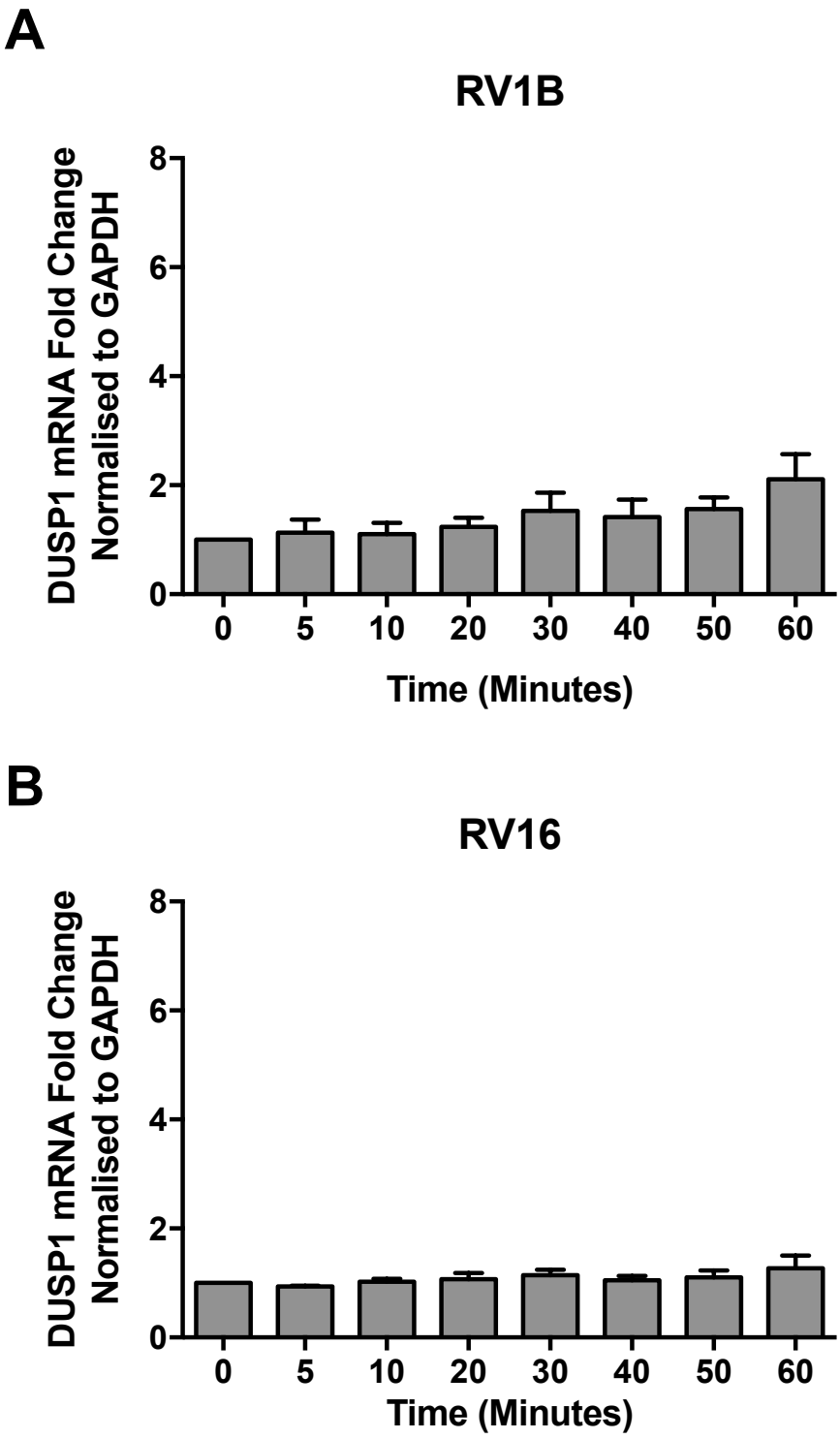

Figure 3.19 DUSP1 transcription does not change over the one-hour viral attachment.

PBECs were stimulated with RV1B (MOI 3) (A) or RV16 (MOI 4) (B) for one hour during viral attachment. mRNA was collected at the time points indicated and ΔΔCt qRT-PCR performed for DUSP1 and a GAPDH control. Data shown are mean ± SEM, n=3, fold change compared to unstimulated control. Significance was measured by one-way ANOVA, with Dunnett's post-test on the ΔΔCt values.

3.6.2 Role of the MAPK Pathways in Regulating DUSP1 Expression

Activation of two of the MAPK pathways, p38 and ERK, has previously been shown to lead to DUSP1 transcription (Ananieva et al., 2008). As poly(I:C) stimulation induced upregulation of DUSP1 mRNA expression at 24 h (Figure 3.18 B), PBECs were treated with a panel of MAPK inhibitors for 1 h prior to poly(I:C) stimulation to investigate the role of the MAPK pathways in this upregulation.

Inhibition of the p38 MAPK pathway led to a reduction in DUSP1 mRNA levels, to below the level in unstimulated cells, which is indicated by the dotted line (Figure 3.20). This difference was significant at 2 and 4 h post poly(I:C) stimulation and a similar pattern was observed at each time point. Treatment with JNK or ERK inhibitors did not have any effect on the level of DUSP1 mRNA.

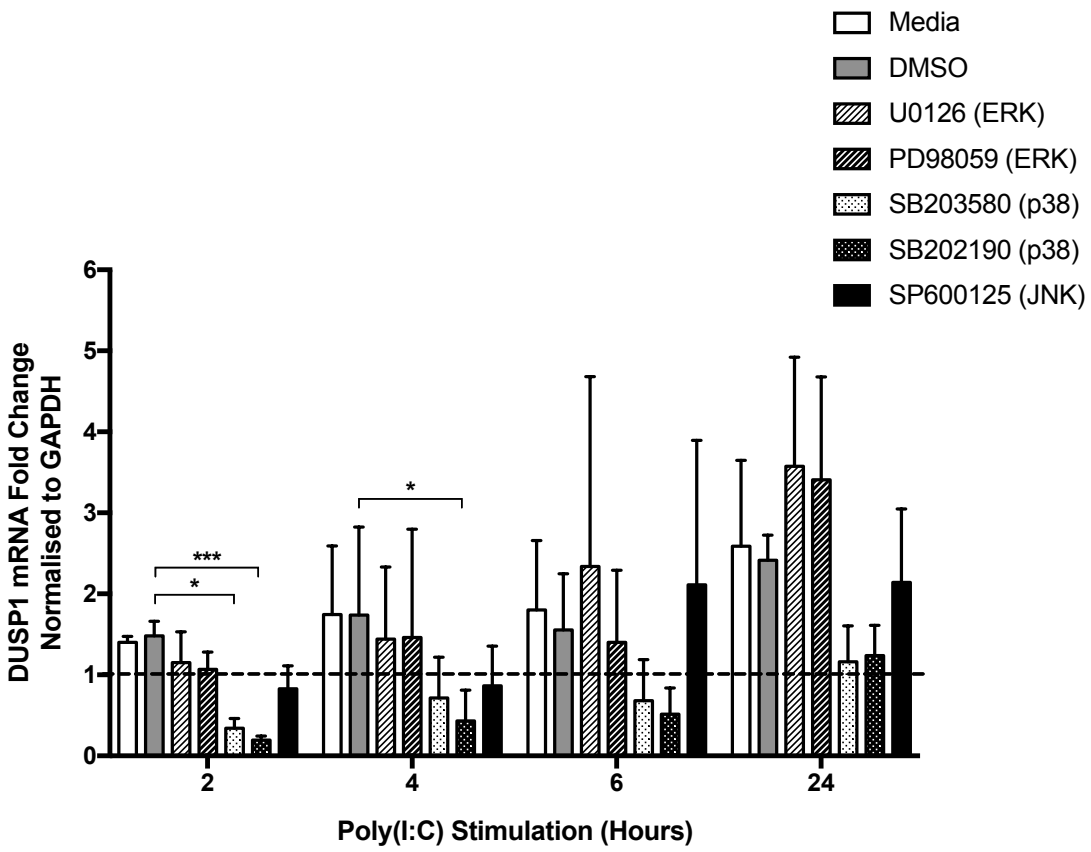

Figure 3.20 DUSP1 transcription is reduced by p38 inhibition.

PBECs were treated with MAPK inhibitor (20 μM), vehicle control or untreated for one hour prior to stimulation with poly(I:C) (25 μg/ml) over 24 hours. mRNA was collected at the time points indicated and ΔΔCt qRT-PCR performed for DUSP1 and a GAPDH control. Data shown are mean ± SEM, n=3, fold change compared to an unstimulated control, which is indicated by the dotted line. Significance is indicated by * $p \leq 0.05$ and *** $p \leq 0.001$ verses DMSO treated as measured by two-way ANOVA, with Dunnett's post-test, on the ΔΔCt values.

3.6.3 DUSP1 Protein Expression and Regulation in PBECs in Response to Rhinoviral Infection

Although DUSP1 mRNA was not regulated in response to rhinoviral infection (Figure 3.18), DUSP1 can be regulated post-translationally. For example, phosphorylation of serine residues 296 and 323 has been shown to induce degradation of DUSP1 protein (Lloberas et al., 2016) (Section 1.5.3). Therefore, the protein expression of DUSP1 was examined using western blot to determine if expression was regulated in response to infection with either rhinovirus serotype, or stimulation with poly(I:C) or IL-1β.

In accordance with the DUSP1 mRNA expression (Figure 3.18), PBECs expressed DUSP1 protein at baseline (Figure 3.21). The level of expression was not altered in response to stimulation with IL-1β or by infection with either rhinovirus strain, similarly to the qRT-PCR results. Although the expression of DUSP1 mRNA was increased around 6-fold in response to poly(I:C) stimulation (Figure 3.18 B), this was not observed at the protein level, with expression remaining constant over 24 h stimulation (Figure 3.21 B).

As discussed in section 1.5.3, the MAPK proteins have previously been shown to post-translationally regulate DUSP1 (Brondello et al., 1999). Therefore, the protein expression of DUSP1 was examined during the one-hour viral attachment, when the MAPK proteins were activated. DUSP1 protein levels were not altered over this hour in response to infection with either serotype of rhinovirus (Figure 3.22).

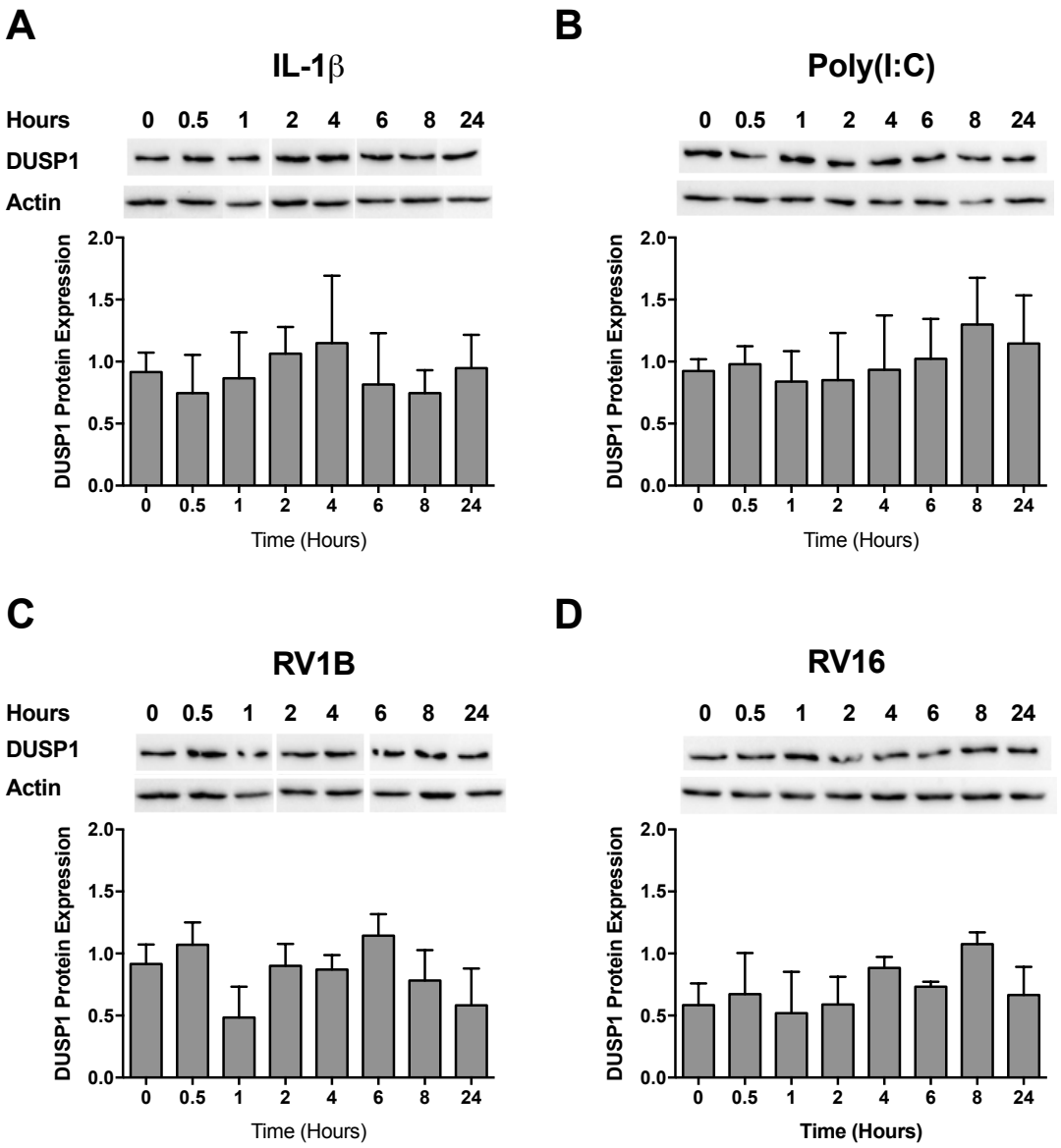

Figure 3.21 DUSP1 protein expression is not changed by major or minor rhinoviral infection or stimulation with poly(I:C) or IL-1β.

PBECs were stimulated with IL-1β (100 ng/ml) (A), poly(I:C) (25 μg/ml) (B), RV1B (MOI 3) (C) or RV16 (MOI 4) (D) over 24 hours and cell lysate collected at the indicated time points. Total DUSP1 and total actin levels were measured using western blot. Representative blots are shown with densitometry below. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3. Significance was measured verses unstimulated control (0 h) by one-way ANOVA, with Dunnett's post-test.

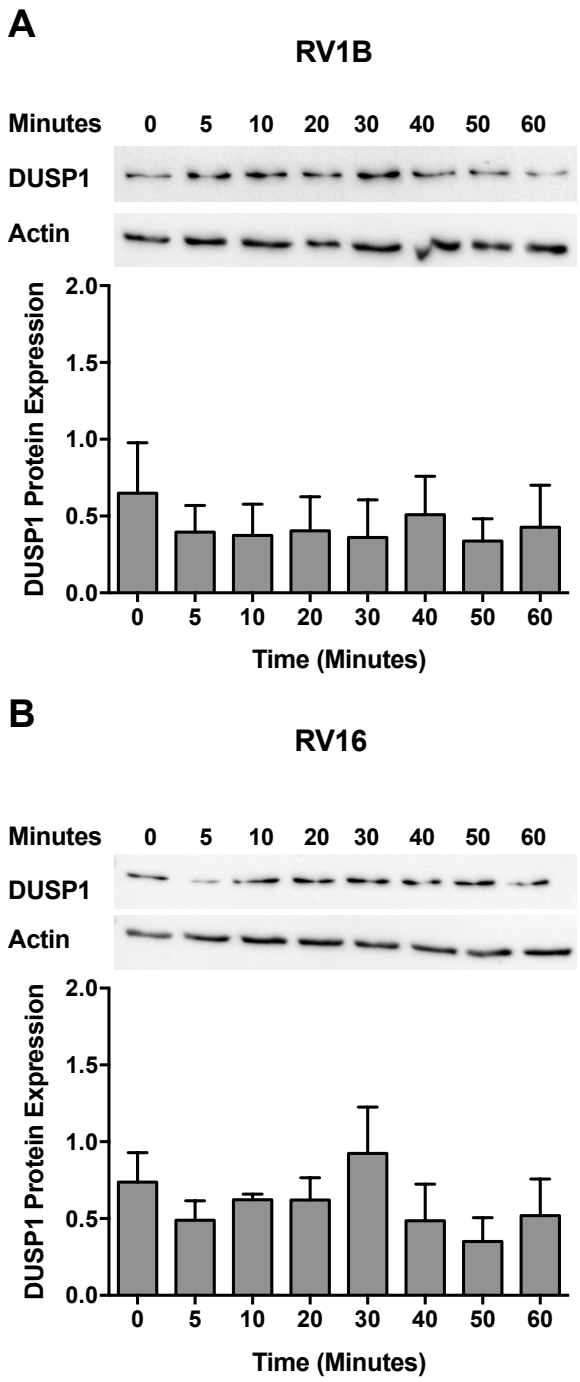

Figure 3.22 DUSP1 protein expression is not changed during the 1 hour viral attachment.

PBECs were infected with RV1B (A) and RV16 (B) for one hour during viral attachment and cell lysate collected at the indicated time points. Total DUSP1 and total actin levels were measured using western blot. Representative blots are shown with densitometry below. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3. Significance was measured verses unstimulated control (0 h) by one-way ANOVA, with Dunnett's post-test.

3.6.4 The Effect of Steroid Treatment on DUSP1 Expression

It has previously been shown that corticosteroids, such as dexamethasone, induce upregulation of DUSP1 in PBECs at the mRNA level (Papi et al., 2013). Three donors of PBECs were treated with a range of concentrations of dexamethasone over 24 h in order to determine the optimal conditions for upregulation of DUSP1 at the mRNA and protein levels. In two of the three donors, dexamethasone stimulation caused no change in the level of DUSP1 mRNA or protein (Figure 3.23). In the other donor, DUSP1 mRNA was upregulated by all concentrations of dexamethasone tested, with each incremental concentration inducing more DUSP1 transcription (Figure 3.23 A). The effect of 10 nM dexamethasone on mRNA expression in this donor did not translate across into protein expression, with an increase in DUSP1 levels at 2 h post stimulation which then declines back to baseline levels. The response of this donor to 100 nM dexamethasone is much more dramatic, with a maximum increase of 70-fold at 6 h post stimulation. Interestingly, the protein expression then declined, whereas the mRNA expression peaked at 24 h (Figure 3.23 B).

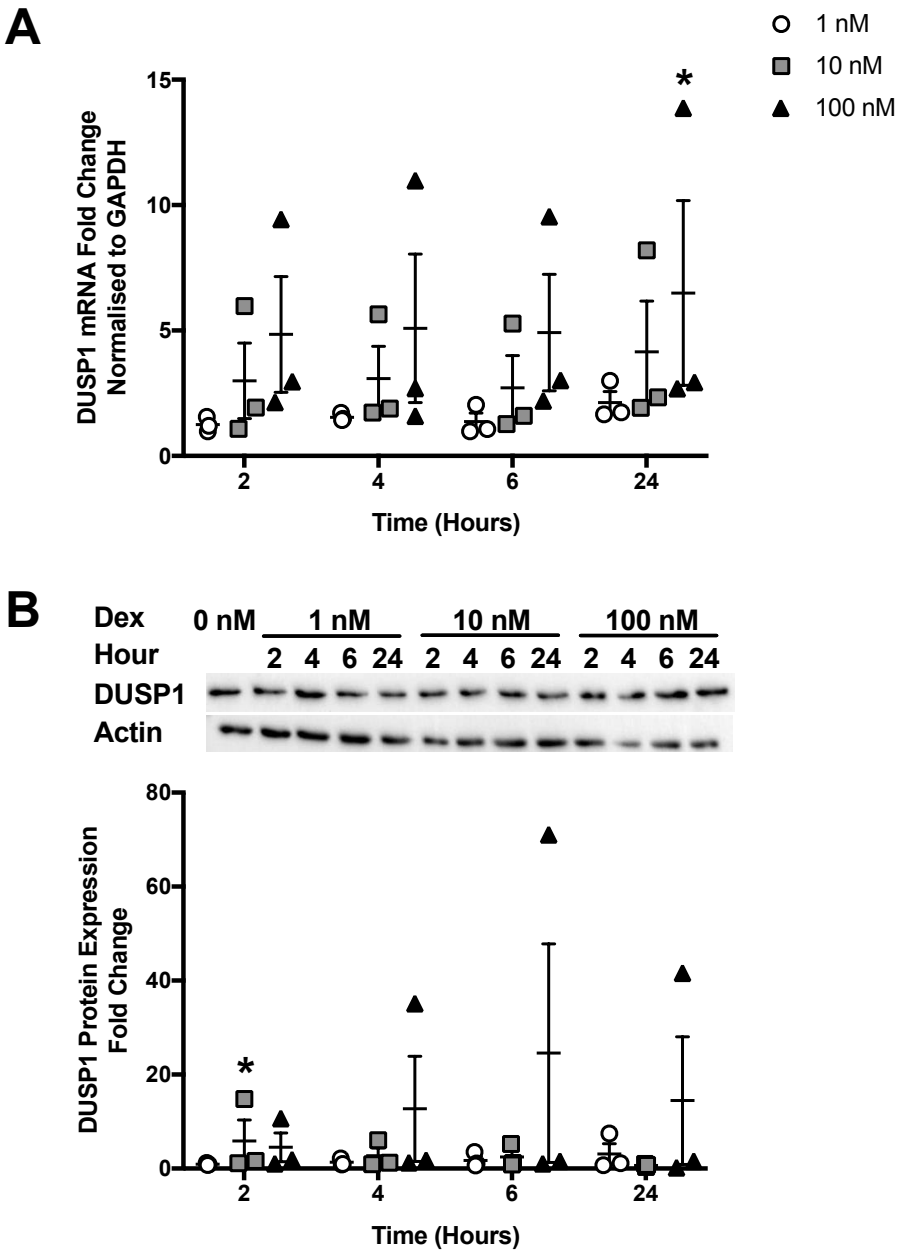

Figure 3.23 The effect of dexamethasone treatment on DUSP1 expression.

PBECs were treated with 1-100 nM dexamethasone (Dex.) over 24 h. mRNA or cell lysate was collected at the time points indicated. ΔΔCt qRT-PCR was performed for DUSP1 and a GAPDH control. Data shown are mean ± SEM, n=3, fold change normalised to GAPDH and untreated control (A). Total DUSP1 and actin protein levels were measured using western blot. Densitometry for each donor is shown below the blot for the responsive donor. Data shown are mean ± SEM band density normalised to actin control and untreated control for each sample (B). Significance is indicated by * $p \leq 0.05$ versus untreated control as measured by two-way ANOVA with Dunnett's post-test on the ΔΔCt values (A) or band density (B).

3.7 Phosphorylation of DUSP1

Phosphorylation of DUSP1 protein on serine residues 359 and 364 has previously been shown to stabilise the protein, prolonging its half-life (Brondello et al., 1999). Although rhinoviral infection did not alter expression of DUSP1 at the mRNA or protein level (Figures 3.18 and 3.21), DUSP1 could be regulated by post-translational modifications. Thus, the level of Ser-359 phosphorylated DUSP1 protein in PBECs was investigated using western blot to determine if DUSP1 was phosphorylated at baseline and whether this was regulated in response to infection with RV1B or RV16 or stimulation with poly(I:C) or IL-1β.

No, or little, phosphorylated DUSP1 was detected at baseline in PBECs (Figure 3.24). Phospho-DUSP1 levels gradually increased in response to stimulation with IL-1β, peaking around 4 h post-stimulation before declining back to baseline by 24 h (Figure 3.24 A). Poly(I:C) stimulation also caused a gradual rise in phospho-DUSP1 levels, peaking later, at 8 h post-stimulation, before declining back to baseline (Figure 3.24 B). Infection with either rhinoviral strain caused an early increase in phosphorylation of DUSP1, with a peak at 1 h before declining back to baseline, and a second peak at 24 h (Figure 3.24 C and D). As was suggested for the late peak in p38 activation in RV16 infection (Figure 3.9 D), this second peak could be due to a second round of infection by the nascent virions produced by the initially infected cells. Importantly, addition of viral filtrate to PBECs for one hour did not cause any upregulation of phosphorylated DUSP1 (data not shown).

As the MAPK proteins were found to be activated during the one-hour viral attachment, and these pathways have previously been shown to phosphorylate DUSP1 in negative feedback mechanisms (Brondello et al., 1999), the level of phospho-DUSP1 during this hour was investigated. No, or very little, phospho-DUSP1 was detected in response to either strain of rhinovirus during this hour (Figure 3.25).

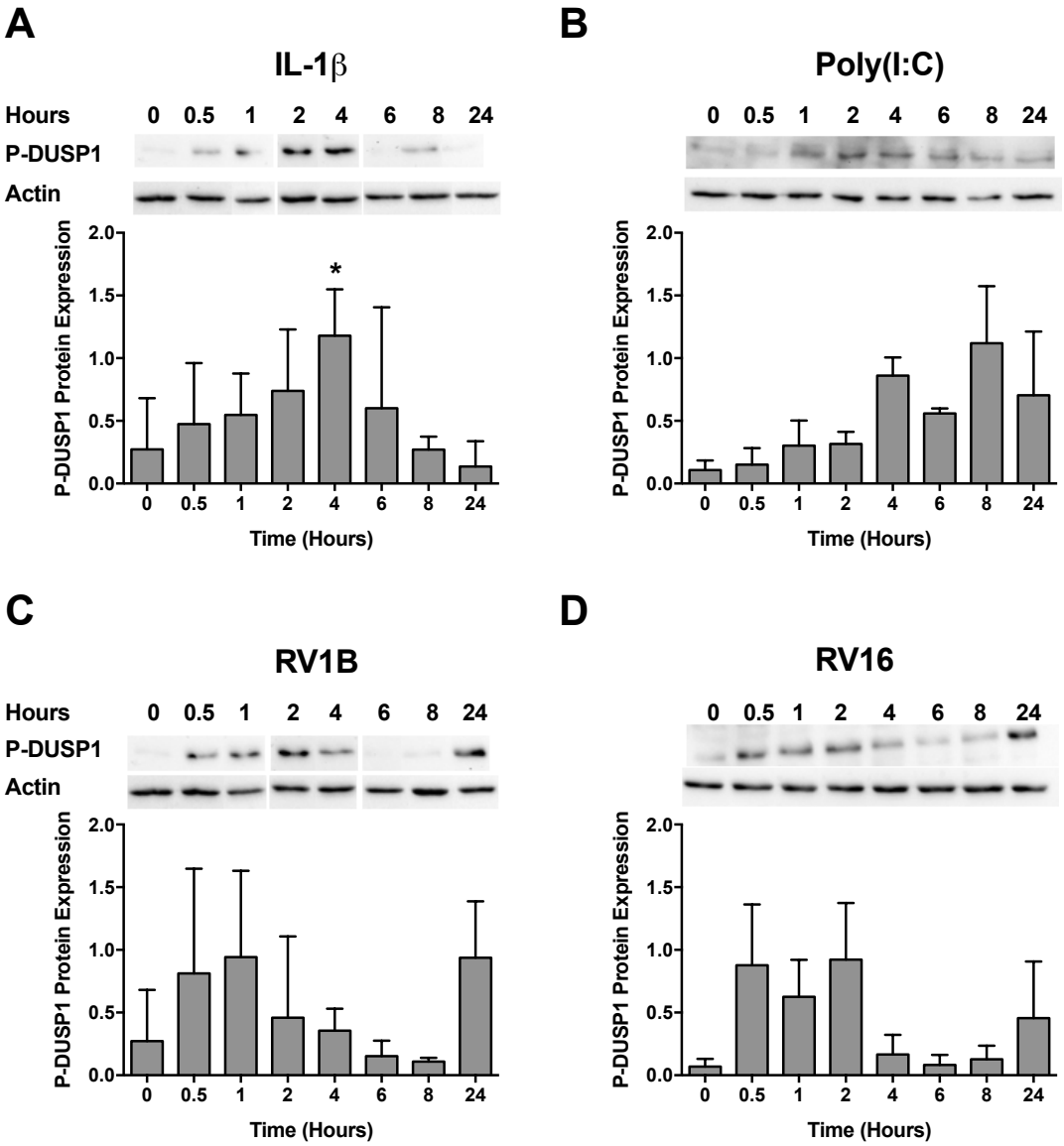

Figure 3.24 DUSP1 phosphorylation is induced by major and minor rhinoviral infection and stimulation with poly(I:C) and IL-1β.

PBECs were stimulated with IL-1β (100 ng/ml) (A), poly(I:C) (25 μg/ml) (B), RV1B (MOI 3) (C) or RV16 (MOI 4) (D) over 24 hours and cell lysate collected at the indicated time points. Phosphorylated DUSP1 and total actin levels were measured using western blot. Representative blots are shown with densitometry below. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3 for poly(I:C) and RV16, and n=4 for IL-1β and RV1B. Significance is indicated by * $p \leq 0.05$ verses unstimulated control (0 h) as measured by one-way ANOVA, with Dunnett's post-test.

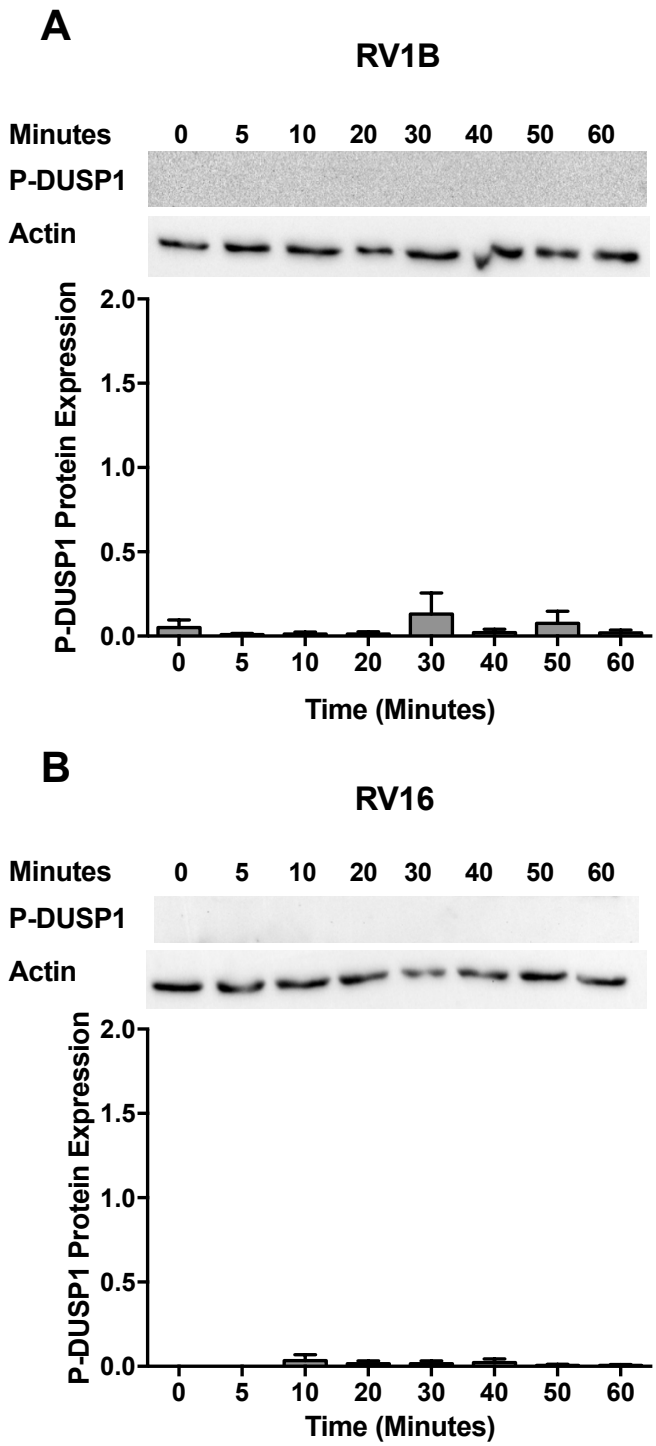

Figure 3.25 DUSP1 is not phosphorylated during the hour viral attachment.

PBECs were infected with RV1B (A) and RV16 (B) for one hour during viral attachment and cell lysate collected at the indicated time points. Phosphorylated DUSP1 and total actin levels were measured using western blot. Representative blots are shown with densitometry below. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3. Significance was measured verses unstimulated control (0 h) by one-way ANOVA, with Dunnett's post-test.

3.8 Expression and Regulation of DUSP10 in PBECs

Another DUSP previously found to preferentially regulate the p38 and JNK pathways, DUSP10 (Qian et al., 2009) (Section 1.5.4), was also taken forward for further investigation into the response to rhinoviral infection.

3.8.1 DUSP10 mRNA Expression and Regulation in PBECs in Response to Rhinoviral Infection

DUSP10 has previously been found to be present in macrophages at baseline, and this expression is increased in response to innate immune stimuli, such as LPS (Zhang et al., 2004). The RT-PCR data revealed that DUSP10 mRNA is expressed in PBECs, but no clear regulation was observed in response to poly(I:C) or IL-1β (Figure 3.17). Thus, the more sensitive qRT-PCR was used in order to investigate DUSP10 mRNA expression in more detail.

In accordance with the RT-PCR data, stimulation with poly(I:C) over 24 h did not alter DUSP10 mRNA levels (Figure 3.26 B). Infection with RV1B or RV16 or stimulation with IL-1β followed a similar pattern in the level of DUSP10 mRNA, although this was only statistically significant for RV16, with an early increase, peaking at 1 h for IL-1β and 2 h for RV infection, before declining to below the baseline level at 6 to 8 h, then coming back up to baseline level by 24 h (Figure 3.26 A, C and D). Interestingly, this is similar to the pattern seen in phosphorylated DUSP1 levels in response to RV infection (Figure 3.24 C and D).

To the best of our knowledge, the pathways responsible for inducing DUSP10 expression have not previously been identified. The MAPK pathways are capable of inducing transcription of DUSPs 1, 2 and 4 (Ananieva et al., 2008). Thus, to investigate whether they have a role in inducing transcription of DUSP10, mRNA levels of DUSP10 were examined during the one-hour viral attachment phase. Expression of DUSP10 was not altered over this hour in response to either serotype of rhinovirus (Figure 3.27).

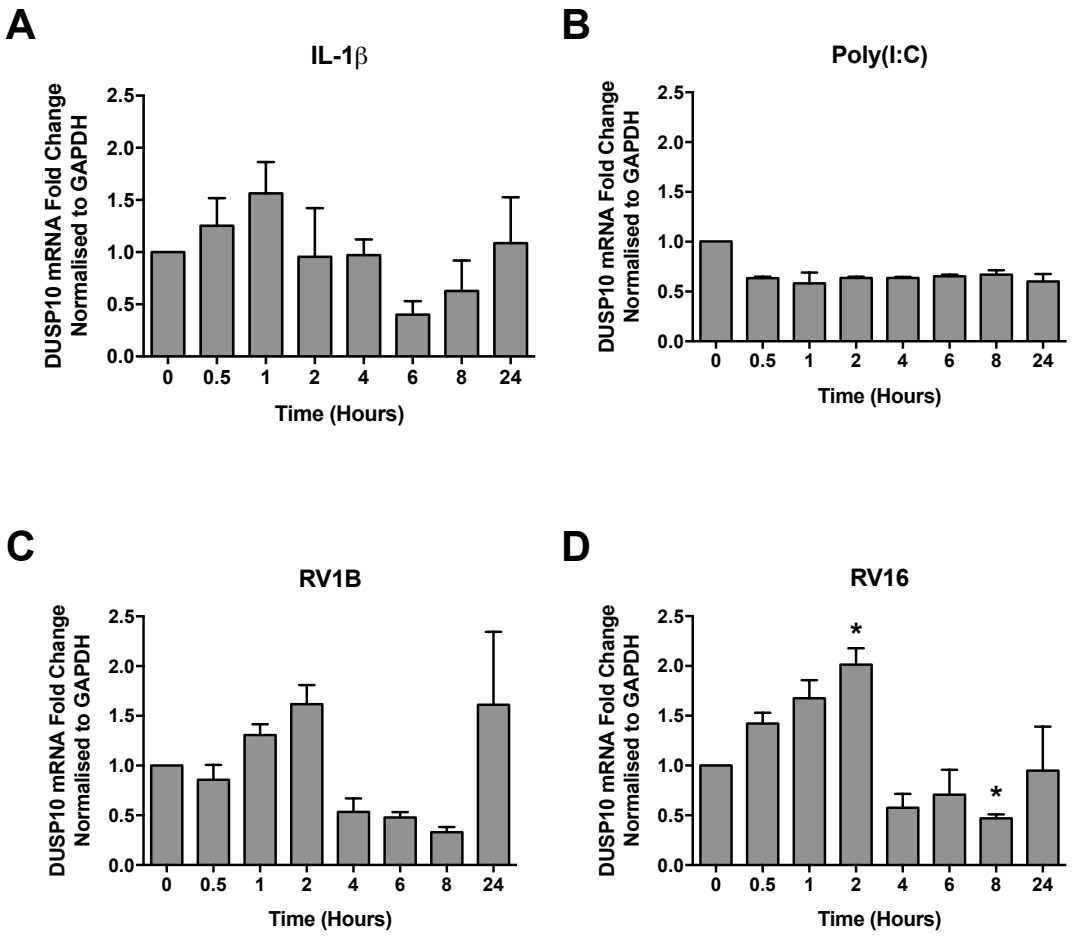

Figure 3.26 DUSP10 transcription does not change in response to major or minor rhinoviral infection or stimulation with poly(I:C) or IL-1β.

PBECs were stimulated with IL-1β (100 ng/ml) (A), poly(I:C) (25 μg/ml) (B), RV1B (MOI 3) (C) or RV16 (MOI 4) (D) over 24 hours. mRNA was collected at the time points indicated and ΔΔCt qRT-PCR performed for DUSP10 and a GAPDH control. Data shown are mean ± SEM, n=3, fold change normalised to GAPDH and unstimulated control. Significance is indicated by * $p \leq 0.05$ versus unstimulated control (0 h), as measured by one-way ANOVA, with Dunnett's post-test, on the ΔΔCt values.

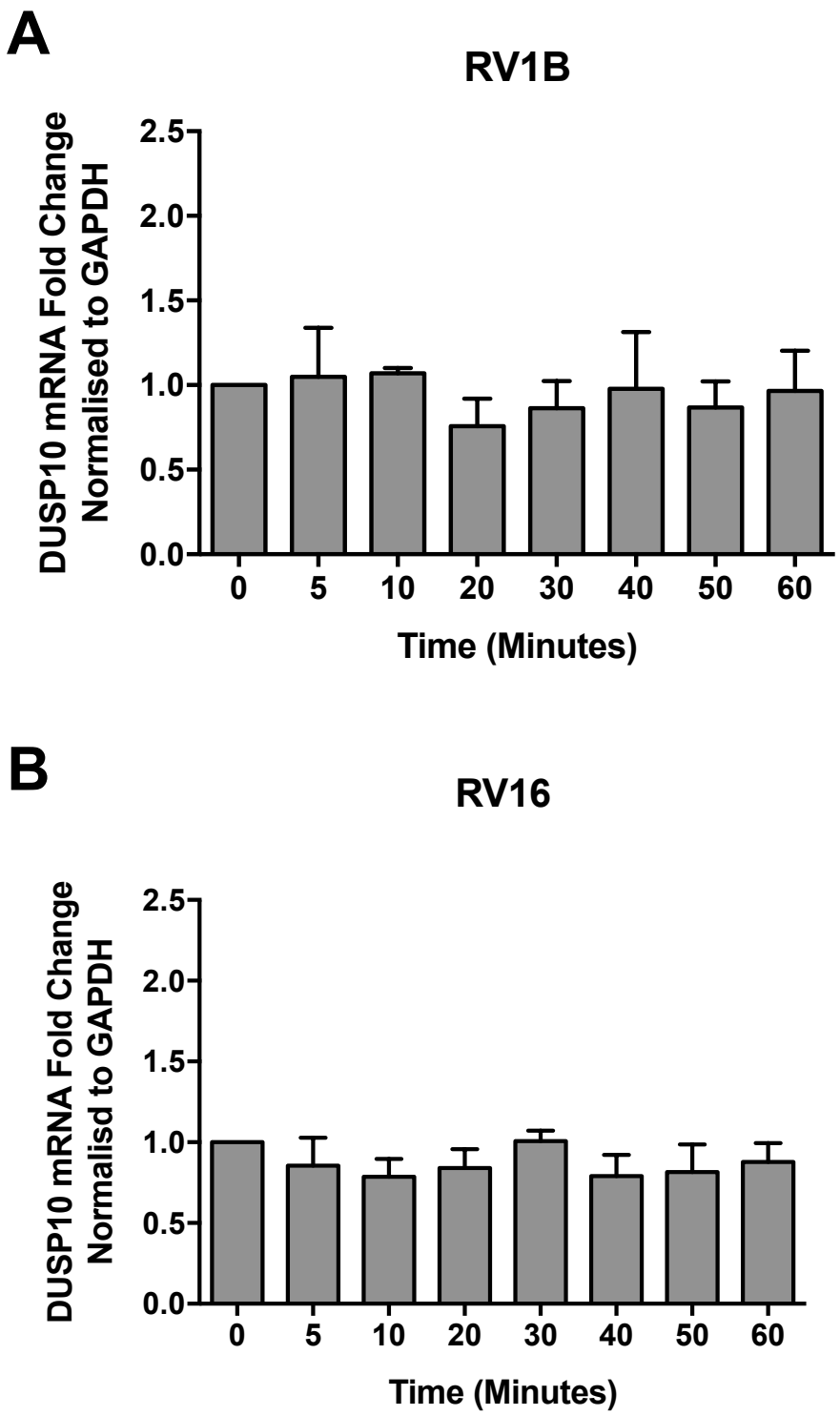

Figure 3.27 DUSP10 transcription does not change during viral attachment.

PBECs were stimulated with RV1B (A) and RV16 (B) for one hour during viral attachment. mRNA was collected at the time points indicated and ΔΔCt qRT-PCR performed for DUSP10 and a GAPDH control. Data shown are mean ± SEM, n=3, fold change normalised to GAPDH and unstimulated control. Significance was measured by one-way ANOVA, with Dunnett's post-test, on the ΔΔCt values.

3.8.2 DUSP10 Protein Expression and Regulation in PBECs in Response to Rhinoviral Infection

The expression of DUSP10 was then examined further, in order to determine whether the small, non-significant, changes in mRNA expression in response to rhinoviral infection or IL-1β stimulation (Figure 3.26) were observed at the protein level.

As in the qRT-PCR data, poly(I:C) stimulation did not alter DUSP10 expression over 24 h (Figure 3.28 B). Stimulation with IL-1β also did not cause any changes in DUSP10 protein level, despite the small changes in mRNA expression (Figure 3.28 A). DUSP10 protein expression remained steady in response to infection with RV1B or RV16 up to around 6 h post-infection (Figure 3.28 C and D). In accordance with the mRNA data (Figure 3.26), the expression then declined slightly, although this was not statistically significant. Unlike the mRNA expression, this decreased expression did not return to baseline by 24 h (Figure 3.28 C and D).

Expression of DUSP10 over the 1 h viral attachment phase was then investigated. Addition of RV16 did not affect the protein level of DUSP10 during this hour (Figure 3.29 B). Intriguingly, stimulation with RV1B consistently caused a reduction in the level of DUSP10 protein, decreasing to almost undetectable levels from 10 minutes before reappearing around 60 minutes after virus addition. However, this was not statistically significant (Figure 3.29 A).

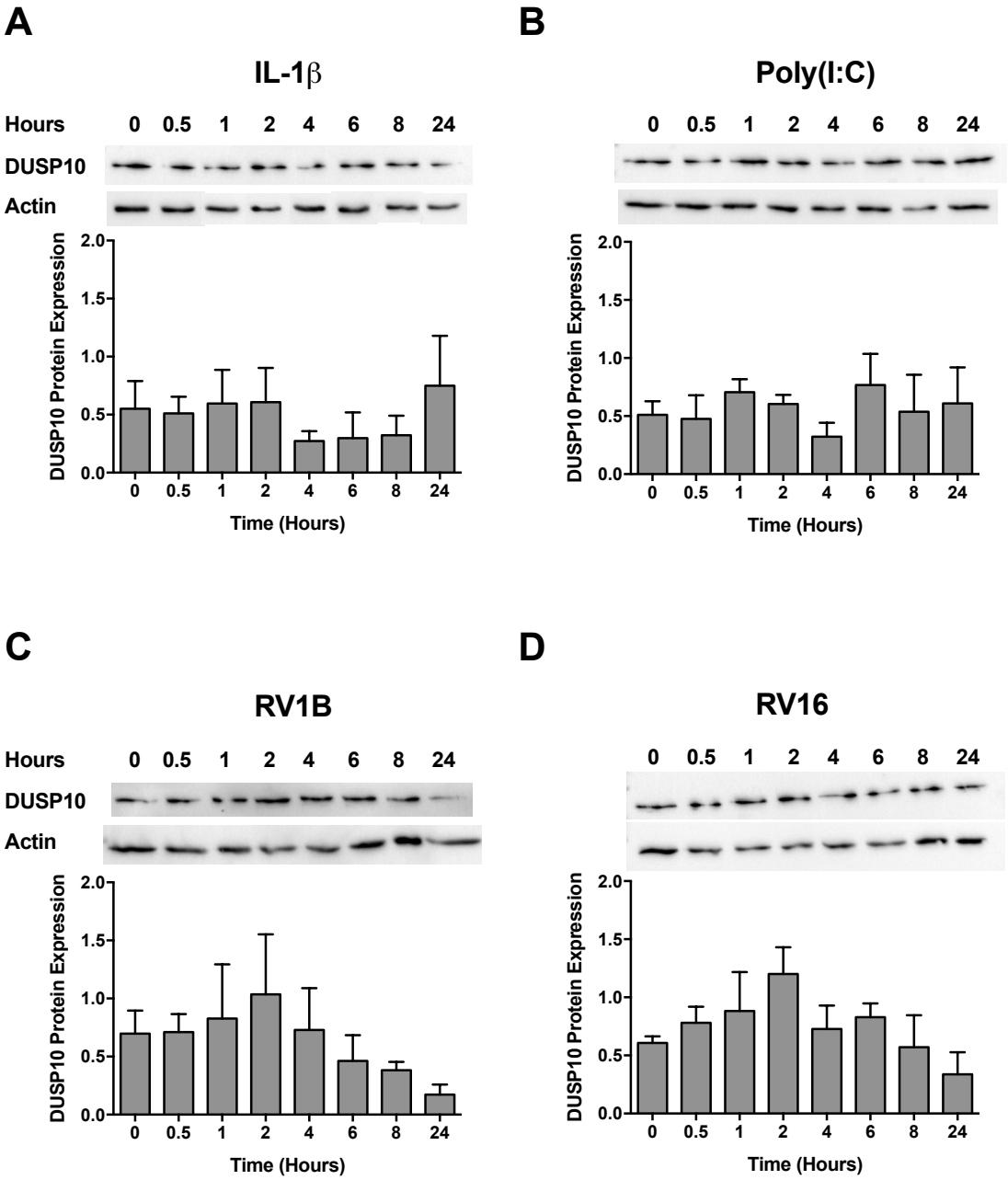

Figure 3.28 DUSP10 protein expression does not change in response to major or minor rhinoviral infection or stimulation with poly(I:C) or IL-1β.

PBECs were stimulated with IL-1β (100 ng/ml) (A), poly(I:C) (25 μg/ml) (B), RV1B (MOI 3) (C) or RV16 (MOI 4) (D) over 24 hours and cell lysate collected at the indicated time points. Total DUSP10 and total actin levels were measured using western blot. Representative blots are shown with densitometry below. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3. Significance verses unstimulated control (0 h) was measured by one-way ANOVA, with Dunnett's post-test.

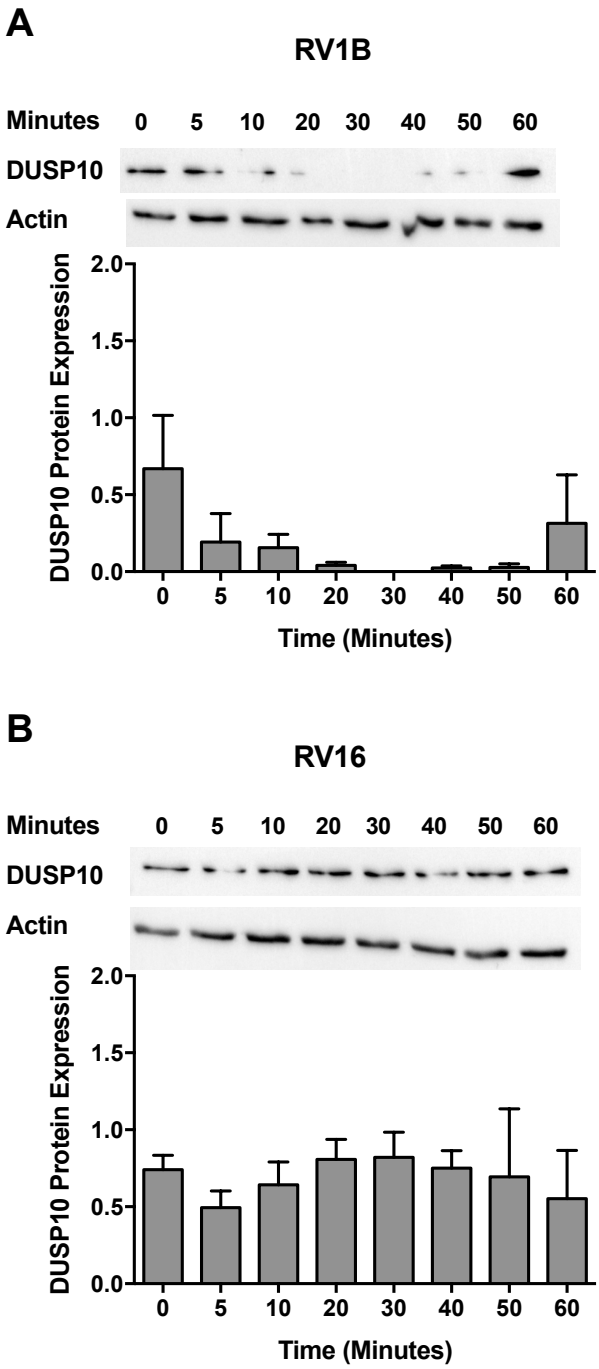

Figure 3.29 DUSP10 protein expression is decreased by RV1B but not RV16 during the hour viral attachment.

PBECs were stimulated with RV1B (A) and RV16 (B) for one hour during viral attachment and cell lysate collected at the indicated time points. Total DUSP10 and total actin levels were measured using western blot. Representative blots are shown with densitometry below. Data shown are mean ± SEM of band density normalised to the actin control for each sample, n=3. Significance verses unstimulated control (0 h) was measured by one-way ANOVA, with Dunnett's post-test.

3.8.3 The Effect of Steroid Treatment on DUSP10 Expression

Although the effect of steroid treatment on DUSP1 expression is well documented (Papi et al., 2013), to the best of our knowledge, the effect of steroids on DUSP10 expression has not previously been examined. The mRNA and protein expression of DUSP10 in PBECs were measured over 24 h of 1 to 100 nM dexamethasone treatment. Similarly to the results for DUSP1 expression (Figure 3.23), two out of three PBEC donors were unresponsive to dexamethasone treatment, with no change in DUSP10 levels. Very modest changes in DUSP10 expression were observed in response to the third donor, with 24 h treatment causing around a three-fold increase in DUSP10 mRNA levels in all three doses. A similar pattern is seen in the protein expression, with small increases at 24 h in one donor in response to each dose. In the 10 nM treatment, one donor had a large increase in protein expression at 2 h which then declined (Figure 3.30).

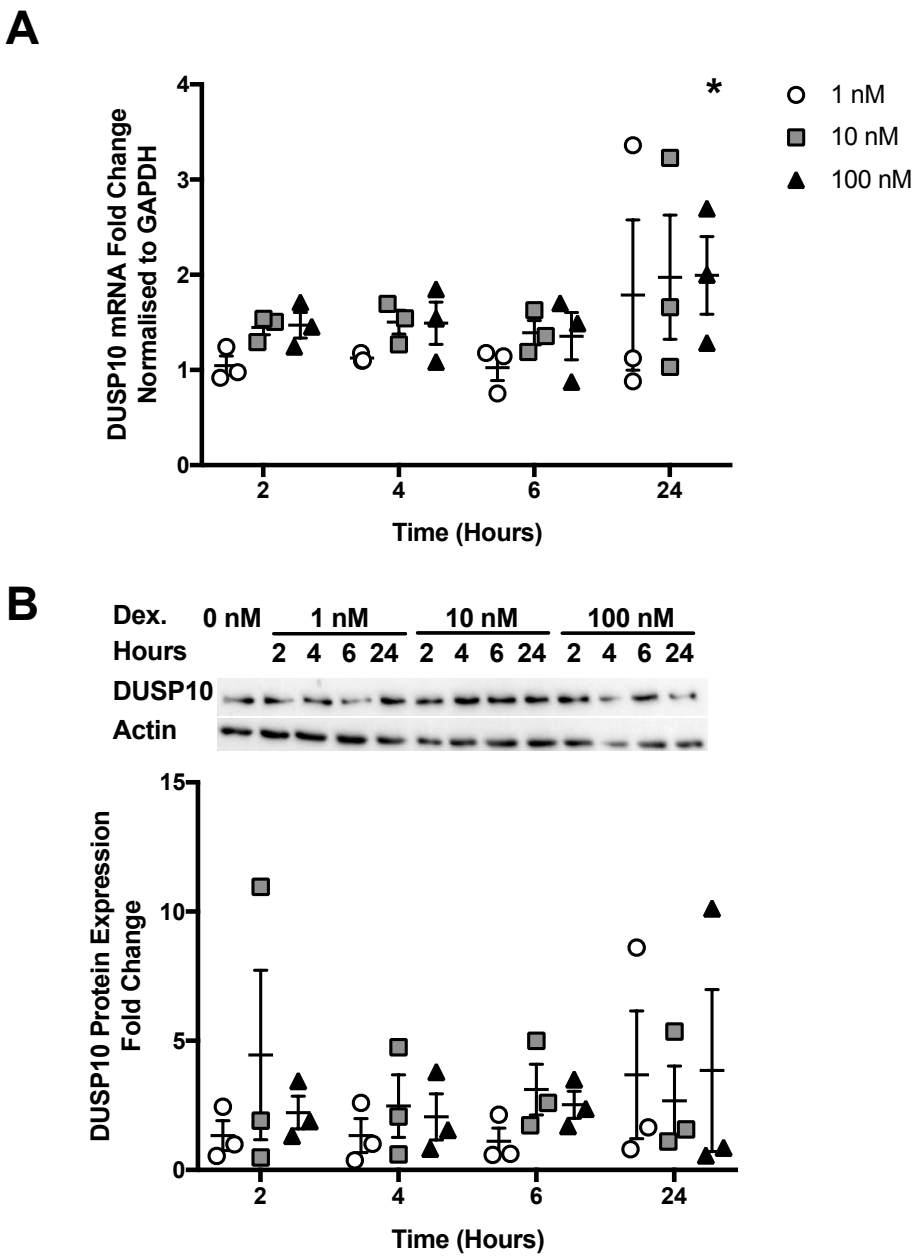

Figure 3.30 DUSP10 expression in response to dexamethasone treatment.

PBECs were treated with 1-100 nM dexamethasone (Dex.) over 24 h. mRNA or cell lysate was collected at the time points indicated. ΔΔCt qRT-PCR was performed for DUSP10 and a GAPDH control. Data shown are mean ± SEM, n=3, fold change normalised to GAPDH and untreated control (A). Total DUSP10 and actin protein levels were measured using western blot. A representative blot is shown with densitometry below. Data shown are mean ± SEM, n=3, band density normalised to actin control and untreated control for each sample (B). Significance is indicated by * $p \leq 0.05$ versus untreated control as measured by two-way ANOVA with Dunnett's post-test on the ΔΔCt values (A) or band density (B).

3.9 Expression and Regulation of DUSP4 in PBECs in Response to RV Infection

DUSP4 has also previously been found to have roles in regulating the innate immune response (Section 1.5.6). Although it is most commonly thought to act on the ERK pathway, its effect on innate immune signalling is controversial, with some suggesting it has a negative regulatory role and others suggesting it has a positive (Al-Mutairi et al., 2010, Cornell et al., 2012). In order to shed some light on this controversy, the expression of DUSP4 in PBECs, and its regulation in response to rhinoviral infection was investigated.

As discussed above, DUSP4 is expressed in PBECs at the mRNA level (Figure 3.17) but no clear regulation was observed in response to either poly(I:C) or IL-1β stimulation. Therefore, the more sensitive technique qRT-PCR was used to determine any changes in DUSP4 expression in response to stimulation with poly(I:C) or IL-1β or infection with RV1B or RV16.

DUSP4 mRNA was expressed by unstimulated PBECs, however, this expression was not altered in response to stimulation with IL-1β or poly(I:C) or by infection with either strain of rhinovirus (Figure 3.31).

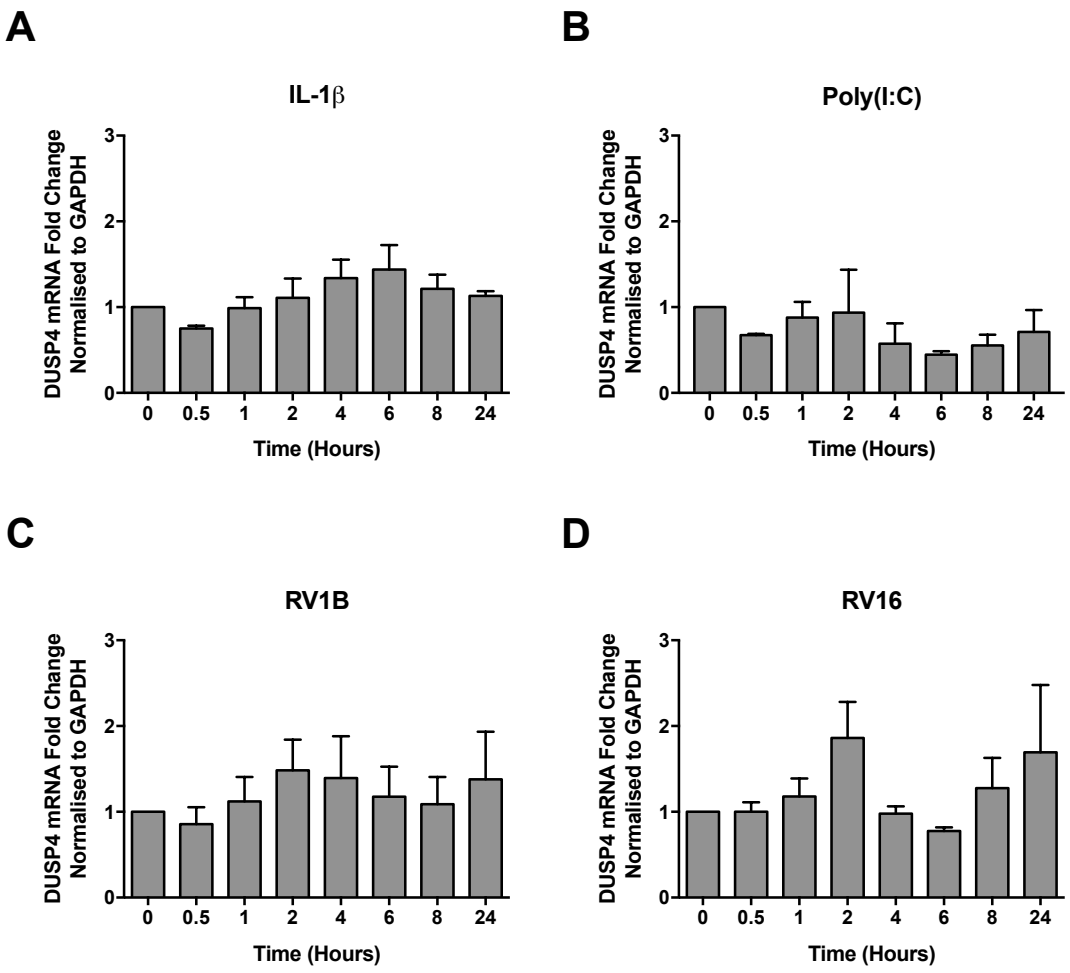

Figure 3.31 DUSP4 transcription does not change in response to major or minor rhinoviral infection or stimulation with poly(I:C) or IL-1β.

PBECs were stimulated with IL-1β (100 ng/ml) (A), poly(I:C) (25 μg/ml) (B), RV1B (MOI 3) (C) or RV16 (MOI 4) (D) over 24 hours. mRNA was collected at the time points indicated and ΔΔCt qRT-PCR performed for DUSP10 and a GAPDH control. Data shown are mean ± SEM, n=3, fold change compared to unstimulated control. Significance was measured by one-way ANOVA, with Dunnett's post-test, on the ΔΔCt values.

3.10 Discussion

3.10.1 Aims

The aim of this chapter was to characterise the response of PBECs to rhinoviral infection. The MAPK pathways have been shown to be important inducers of inflammation in the innate immune response. Therefore, it is important to characterise these pathways and their regulation in the response to rhinoviral infection.

The first aim was to describe the activation of the ERK, p38 and JNK MAPK pathways in response to infection with two strains of rhinovirus, RV1B and RV16, or stimulation with the synthetic TLR3 ligand poly(I:C), or the inflammatory cytokine IL-1β. Inhibitors of these pathways were utilised to determine their role in the response of PBECs to rhinoviral infection, and in rhinoviral replication.

Potential regulators of these pathways, the DUSP family, were then examined to determine whether they were expressed in PBECs and whether this expression was regulated by rhinoviral infection.

3.10.2 The Roles of the MAPKs in Viral Infection

The MAPK signalling pathways are known to be activated by rhinoviral infection (Dumitru et al., 2006, Griego et al., 2000, Lau et al., 2008, Newcomb et al., 2008, Wang et al., 2006), and previous inhibitor studies have shown the importance of p38 and ERK in the innate immune response to rhinovirus (Griego et al., 2000, Liu et al., 2008a). However, much of this work has been done in cell lines, and the roles of these pathways in the response of PBECs to rhinoviral infection have not been well characterised. Figure 3.1 demonstrates that the innate immune pathways are being activated by TLR3 stimulation and rhinoviral infection, leading to the secretion of inflammatory proteins. RV16 infection induced a much smaller response than RV1B, with no detectable CXCL8 release. However other work presented in this chapter, in particular figure 3.4, demonstrates that RV16 infection of PBECs does induce CXCL8 production. Differences in cytokine production in response to major and minor serotypes has been documented previously; in 2014 Schuler et al. found higher levels of CCL20, CCL2 and IP-10 released by primary MDMs infected with RV16 than RV1A (Schuler et al., 2014).

Inhibition of each of the MAPKs is a useful tool to delineate the role of each pathway and has been used extensively. Inhibition of either p38 or JNK MAPK led to a consistent decrease in CXCL8 release after 24 h of poly(I:C) stimulation. As discussed in section 1.3.4, Gern et al. have previously demonstrated that treatment of PBECs with SB203580 decreased CXCL8 release in response to 16 h of poly(I:C) treatment (Gern et al., 2003). The effect of JNK inhibition on cytokine release in PBECs has not been well studied. However, treatment of a human astroglioma cell line with SP600125 decreased CXCL8 release in response to poly(I:C) treatment (Park et al., 2006). Intriguingly, in this thesis MAPK inhibition did not have an observable effect on CXCL8 mRNA levels in response to 24 h of rhinoviral infection, although after 48 h infection CXCL8 mRNA and secreted protein was decreased by p38 or JNK inhibition. Previous studies have shown that treatment of BEAS-2B cells with SB203580 caused a decrease in CXCL8 release in response to rhinoviral infection at 40 h (Liu et al., 2008a) or 72 h (Griego et al., 2000). One potential reason that the effect of the inhibitors is not visible until later time points in response to rhinoviral infection could be due to the fact that much less CXCL8 is produced in response to rhinovirus than poly(I:C), making any differences caused by the inhibitors harder to determine. Previous work from our group and others suggests that rhinovirus only infects a small percentage, around 10%, of the PBEC monolayer (R. L. Roberts, Personal Communication, Jakiela et al., 2008, Lopez-Souza et al., 2009), whereas poly(I:C) stimulation would affect all cells, causing a much larger response. There are also other ways, other than mRNA upregulation, in which CXCL8 release could be induced. Some cytokines are stored in intracellular pools, ready for a quick response to infection (Cassatella et al., 2006, Vaday et al., 2006), and CXCL8 mRNA stability has previously been shown to be regulated by p38 (Winzen et al., 1999). These data suggest that both p38 and JNK play important roles in inducing inflammatory cytokine release in response to both poly(I:C) and rhinoviral infection.

Inhibition of p38 and JNK also caused a consistent decrease in CCL5 release. This was unexpected as CCL5 is generally thought of as an interferon-stimulated gene, with the IRF proteins inducing CCL5 transcription. Furthermore, a decrease in CCL5 release in response to poly(I:C) was not seen in the work of Gern et al. in 2003 when BEAS-2B cells were treated with SB203580 (Gern et al., 2003). However, this could be due to a difference between primary cells and a cell line. Indeed, the MAPKs have been found to have a role in inducing CCL5 release in response to infection with other viruses: with

ERK and p38 inducing CCL5 release in response to respiratory syncytial virus (RSV) (Pazdrak et al., 2002), and p38 and JNK inducing CCL5 in response to influenza infection (Kujime et al., 2000). CCL5 RNA levels also decrease in the presence of inhibitors of all three MAPKs in response to infection with the picornavirus, enterovirus-71, although this was in a glioblastoma cell line, U251 (Wang et al., 2015). NF-κB signalling has also previously been found to be capable of inducing CCL5 release (Moriuchi et al., 1997). Therefore, the decrease of CCL5 release with p38 or JNK inhibition suggests that either: p38 and JNK have important roles in regulating the interferon response to TLR3 as well as the inflammatory response; NF-κB has a larger role in inducing CCL5 production in PBECs than previously thought; or another pathway is leading to the release of CCL5 protein.

The effect of JNK inhibition on viral replication has not been well studied. The increase in rhinoviral replication with SP600125 treatment suggests that JNK has an important limiting role in viral replication. As mentioned above, inhibition of the MAPKs led to a decrease in CCL5 release. CCL5 is an interferon stimulated gene, upregulated by RV infection of PBECs (Khaitov et al., 2009, Lin et al., 1999). Addition of interferon-β to PBECs significantly reduces replication of RV16 (Wark et al., 2005). Thus, the increase in rhinoviral replication when JNK is inhibited could be due to a decrease in the IFN response. The JNK pathway has previously been reported to phosphorylate IRF3 in response to poly(I:C) signalling, although this study was performed in the 293T cell line using over expression techniques (Zhang et al., 2009). This is not supported by the results of this study, where JNK inhibition increased the expression of IFN-β in response to RV1B infection. This is likely to be due to the increased level of viral replication causing more IFN induction, rather than a direct effect of JNK. However, it is difficult to draw conclusions from these data due to the limited amount of IFN produced. It would be interesting to investigate this further by examining the expression of other IFNs, and determining whether MAPK inhibition affects it. IFN-α, IFN-β, and IFN-λ are all released by PBECs in response to infection with RV1B or RV16 (Khaitov et al., 2009). However, as p38 inhibition also led to a decrease in cytokine release a similar effect would be expected in response to p38 inhibition. It is possible that necessity for p38 in the viral life cycle may overcome this effect. Additional investigation into the effect of JNK

inhibition on IFN levels in rhinoviral infection is of importance to further elucidate these findings.

Inhibition of p38 consistently decreased the level of rhinoviral replication in PBECs. This indicates that the p38 pathway is utilised by the virus in the replication process. A study in 2010 showed that inhibition of p38 has a similar effect on the replication of several other respiratory viruses, including: adenovirus, RSV, coxsackie virus B3, and influenza virus (Marchant et al., 2010). Coxsackie virus B3 is a member of the Picornaviridae family, like rhinovirus, and therefore has a similar life cycle. The Marchant study went on to determine the mechanism of p38 involvement in influenza virus replication. They found that inhibition of p38 led to a retention of influenza virus particles in early endosomes, suggesting that p38 is involved in the release of virus from the endosome. Another possibility is that p38 is necessary for the accumulation of cholesterol within viral replication organelles, as discussed in section 1.3.4. This information is of extreme importance and requires further investigation. If p38 is necessary for rhinoviral replication, then regulation of p38 has the potential for use as a future anti-viral treatment. This work should be taken forward with $TCID_{50}$ assays to determine whether the difference in the level of viral RNA translates into a difference in the release of productive viral particles.

Although the MAPKs are known to have roles in processes such as apoptosis and proliferation (Wada and Penninger, 2004), inhibition of the MAPKs had no effect on the viability of PBECs in response to rhinoviral infection, suggesting that there was no MAPK-induced apoptosis or necrosis in response to rhinovirus. However, visual inspection of monolayers suggested that rhinoviral infection was causing some cell death (data not shown), and RV16 infection has previously been shown to decrease cell viability of PBECs, due to an increase in apoptosis (Wark et al., 2005). The assay used to detect cell viability measures the quantity of ATP present, from which the number of metabolically active cells can be inferred. PBECs infected with rhinovirus may be more metabolically active than uninfected cells, causing an increase in ATP levels which is not accounted for by this assay. Another limitation of this experiment is that no positive control was included to ensure the assay was working. Therefore, the effect of rhinoviral infection on cell viability and the role of the MAPKs in this process remains to be determined.

Overall, these findings suggest that the MAPKs p38 and JNK have important roles in the response of PBECs to rhinoviral infection, including inducing inflammatory cytokine release and possibly inducing IFN release. The p38 MAPK may also have an important role in the rhinoviral life cycle. Thus, the investigation into the regulation of these pathways is of importance. One limitation of this study is the use of small molecule inhibitors, which have some off-target effects (Bain et al., 2007). In future, it would be important to confirm these findings using siRNA knock down of the MAPK proteins. siRNA knock down in PBECs of p38 or c-Jun, the JNK target, have been published previously and were shown to reduce CXCL8 production in PBECs in response to lysophosphatidic acid (Saatian et al., 2006). It would also be of interest to determine whether growth of PBECs at air-liquid interface would affect these results, as differentiated cells have been found to be more susceptible to RV infection (Jakiela et al., 2008).

3.10.3 Activation of the MAPKs in Viral Infection

ERK phosphorylation in response to infection with RV39 has previously been shown in the bronchial epithelial cell line 16HBE14o-, with no phospho-ERK present at baseline, and appearing within 2 minutes of infection (Newcomb et al., 2007). This differs from the observations in this study; where activated ERK is present in all media controls but then declines over time. This could be due to treatment of the cells, by changing media and washing in PBS, activating ERK through stress pathways. The shortest time points would have the most recent media changes. There is a possible increase in ERK activation 30 minutes after stimulation with IL-1β, poly(I:C) and RV1B, however these data are preliminary and would need further repeats to investigate, comparing the level of activation with a control with media changes and washes done simultaneously. As inhibition of ERK had no effect on the response of PBECs to RV infection (Section 3.2) investigation of ERK activation was not continued.

Both p38 and JNK were activated by IL-1β and poly(I:C), as has been previously demonstrated in the literature (An et al., 2006, Gern et al., 2003, Jang et al., 2004, Jung et al., 2002, Wuyts et al., 2003). This activation in response to poly(I:C) demonstrates that TLR3 signalling can lead to activation of the MAPKs in primary bronchial epithelial cells, however it occurs later than previously shown. In 2003, Gern et al. showed that stimulation of PBECs with poly(I:C) activated p38, with peak phospho-p38 expression

seen by 70 minutes and persisting until 130 minutes (Gern et al., 2003), whereas in this study, peak expression was not observed until 2 h. This could be due to a much higher dose being used in the Gern study, of 100 μg/ml compared to 25 μg/ml.

Although MAPK activation is seen during the attachment phase of infection, this is not due to rhinoviral infection. No activation of p38 or JNK MAPKs is observed until 8 h RV16 infection, where p38 phosphorylation is observed. However, the effects of MAPK inhibition (Section 3.2) demonstrate that the MAPKs are being activated by infection. The lack of a visible band by western blot could be due to the fact that each cell is being infected at slightly different times, therefore the MAPKs are activated at different times, and there is not enough phosphorylated protein present at any one time to be detectable. Allowing the virus one hour to attach to the cells, before washing off any unbound viral particles is meant to ensure that infection occurs at similar times in the cell monolayer. However, this could be improved by incubating the virus with the cells at 4^{o}C to allow attachment but prevent internalisation. Then, when moved to 37^{o}C, internalisation occurs simultaneously. It would be interesting to investigate whether utilisation of this infection method would lead to a detectable MAPK signal. As discussed above, RV infects around 10% of PBECs. Therefore, the MAPK activation may not all be due to direct effects of viral infection, but due to molecules secreted by infected cells signalling to the surrounding cells. This could be further investigated using immunofluorescence techniques, imaging phosphorylated MAPKs and viral capsid or double-stranded RNA. This would demonstrate whether MAPK activation was only occurring in cells infected with rhinovirus.

Activation of the MAPKs by viral filtrate demonstrates that something released by HeLa Ohio cells during the viral propagation technique is activating the cells. This molecule is unaffected by UV treatment and must be smaller than 30 kDa. Many DAMPs would be too large to filter through, for example heat shock proteins are around 70 kDa and tenascin C is between 250 and 300 kDa, but some cytokines would still be present. IL-1β is 17 kDa so would pass through the filter easily, and previous work has shown that only a small amount of IL-1β is necessary to induce an immune response (Piper et al., 2013). The presence of these molecules may also vary between batches of virus, depending on the amount of cell death caused by infection. The MAPKs are known to be activated by binding of rhinovirus to cells (Dumitru et al., 2006, Lau et al., 2008,

Newcomb et al., 2008, Wang et al., 2006). Another possible cause for this early MAPK activation could be due to fragments of virus present in the solution binding to cells. HeLa Ohio cells may be lysed before all viral proteins are assembled into virions, and virions may be degraded by freeze-thawing. Viral proteins VP1 to VP3 are around 30 kDa so may not fit through the filter, but VP4 is 7 kDa so would still be present. Many single-stranded RNA viruses are known to produce defective viral particles during replication (Mercado-Lopez et al., 2013, Xu et al., 2017). Although the MAPKs were activated, this did not lead to any measurable effect on cytokine production or release, therefore, would not affect the results of the inhibitor experiments. In future, it will be important to use purified viral stocks, made using ultracentrifugation, which is currently being optimised in our group.

MAPKs can be regulated in many different ways, including subcellular localisation, availability of scaffold proteins and post-translational modifications, as well as dephosphorylation (Reviewed in: Raman et al., 2007). Treatment of PBECs with a general phosphatase inhibitor caused an increase in activation of both p38 and JNK, suggesting that the main mechanism of MAPK regulation is by dephosphorylation. Although this activation was not due to RV infection, it still gives an indication of how the MAPK pathways are being regulated. Several phosphatases are capable of regulating the MAPKs, including protein phosphatases 2C and 5 (PP2C and PP5), however, DUSPs are thought to be the main regulators (Morita et al., 2001, Takekawa et al., 1998, Zhou et al., 2004).

3.10.4 Expression and Regulation of DUSPs in PBECs

Four members of the DUSP family, DUSPs 1, 2, 4 and 10 have previously been found to regulate innate immune signalling pathways, although their role in regulating rhinoviral infection of PBECs has not yet been studied. Previously DUSP2 was only thought to be expressed by innate immune cells, such as mast cells, eosinophils, neutrophils and macrophages, and no expression was detected in primary human bronchial epithelial cells (Jeffrey et al., 2006). This is in contrast to this study, where DUSP2 mRNA was expressed in PBECs, even at baseline. To the best of my knowledge the expression of DUSPs 1, 4 and 10 in PBECs has not yet been investigated. DUSPs 1 and 4 have previously been characterised as early-response genes, with no expression at baseline of DUSP1 in primary human airway smooth muscle cells, or DUSP4 in mouse BMDMs or embryonic

fibroblasts (Cornell et al., 2010, Lawan et al., 2011, Manetsch et al., 2012). In contrast, DUSP10 has previously been observed to be constitutively present in HeLa cells and murine BMDMs, although it can be upregulated by innate immune stimuli (Tanoue et al., 1999, James et al., 2015). All three of these DUSPs were found to be expressed at the mRNA level in PBECs at baseline. This may be a specific characteristic of bronchial epithelial cells, compared to macrophages or fibroblasts, or it could be due to some aspect of culturing the PBECs which is activating them, as discussed above with ERK activation levels. The ERK pathway has previously been found to induce transcription of DUSPs 1, 2 and 4 (Ananieva et al., 2008, Brondello et al., 1997, Grumont et al., 1996). Therefore, the constitutive expression of these DUSPs may be due to the activation of ERK, induced by culturing the cells. DUSP1 has been shown to be upregulated by the presence of foetal calf serum in HeLa cells (Kwak et al., 1994). Although serum is removed prior to infection or stimulation, this demonstrates that aspects of culturing the cells in vitro can activate these pathways. Therefore, utilisation of in vivo methods is necessary to gain a clearer picture of DUSP regulation.

DUSP1 mRNA expression has previously been found to be increased by poly(I:C) stimulation in a bronchial epithelial cell line, NCI-H292, with around a 2-fold increase 1 h post stimulation (Golebski et al., 2014). In this study, DUSP1 mRNA expression was significantly increased by poly(I:C) stimulation much later on, at 24 h, with non-significant, but consistent, increases of around 2-fold also observed at 4-6 h post stimulation. The changes in DUSP1 mRNA were not translated into an increase in DUSP1 protein within 24 h. However, it is possible that changes in DUSP1 at the protein level would occur post 24 h and this requires further study. DUSP1 expression has also previously been found to be increased by stimulation with IL-1β in A549 cells, and airway smooth muscle cells, with protein levels peaking 1 h post stimulation (Issa et al., 2007, Shah et al., 2016b). In this study, no significant changes in DUSP1 expression were seen in response to IL-1β stimulation or rhinoviral infection, although RV16 infection causes a consistent increase of around 2-fold at 0.5 h post infection, however again this was not observed at the protein level. This is consistent with results from a microarray study, comparing gene expression from nasal scrapings of patients infected with RV16 and sham infected, where infection did not alter DUSP1 expression (Proud et al., 2008).

p38 and ERK MAPKs have previously been shown to induce transcription of DUSP1 through the MSK proteins (Ananieva et al., 2008). In this study, inhibition of p38 consistently reduced levels of DUSP1 mRNA, whereas ERK inhibition had no effect. Intriguingly, treatment with p38 inhibitors reduced DUSP1 expression to below baseline levels. This would suggest that, although no activated p38 is detectable at baseline, it is having a role in inducing some DUSP1 expression.

Although no regulation of DUSP1 expression was observed, all the stimulants investigated regulated DUSP1 through phosphorylation. This has previously been shown to stabilise the protein, prolonging its half-life (Brondello et al., 1999). However, if the protein was more stable, an increase in total DUSP1 protein would be predicted, which was not observed. DUSP1 was phosphorylated much earlier in response to rhinoviral infection than poly(I:C) stimulation. This indicates that an early, TLR3 independent, signalling pathway is induced upon rhinoviral binding and internalisation, which is leading to DUSP1 phosphorylation. It has previously been shown that ERK is capable of phosphorylating DUSP1 (Brondello et al., 1999). As ERK activation was seen in media controls in this study, phosphorylation of DUSP1 may be due to another kinase. For poly(I:C) and IL-1β, the peak in DUSP1 phosphorylation occurs slightly after the peak in p38 and JNK activation, for example IL-1β stimulation causes MAPK activation around 1 h post stimulation, and DUSP1 is phosphorylated at 2 h post stimulation. This suggests that the MAPK pathways could be inducing DUSP1 phosphorylation, and investigation into whether inhibition of these pathways affects DUSP1 phosphorylation would be of interest. The inclusion of inhibitors of other serine kinases involved in the response to RV, for example the PI3K pathway, would be extremely interesting, as, to the best of our knowledge, this has not been investigated before.

Although no significant changes were observed in DUSP10 expression, both RV1B and RV16 caused a consistent decrease in DUSP10 mRNA expression to below baseline levels at around 6 h post infection. This decrease was also observed at the protein level, with the lowest protein expression detected at 24 h. Interestingly, stimulation with poly(I:C) or IL-1β did not have this effect. Thus, rhinoviral infection may be targeting DUSP10 for degradation or downregulation. RV1B infection also caused a downregulation of DUSP10 protein during the viral attachment phase, although no changes were seen at the mRNA level during this time. In 2008, Proud et al. performed a gene expression array

on nasal scrapings to identify any changes induced by rhinoviral infection, they found that at 8 h and 48 h post RV16 infection the expression of many genes were downregulated, but DUSP10 was unchanged (Proud et al., 2008). However, as the downregulation observed in this study was only transient, experimental differences may mean that any down-regulation of DUSP10 could have been missed in the Proud study as only two time points were investigated. As discussed in section 1.5.4, DUSP10 has been associated with regulating IRF3 phosphorylation and the antiviral response in influenza infection (James et al., 2015). If DUSP10 has a role in anti-viral pathways, rhinovirus may have developed a mechanism to interfere with this by degrading or down regulating DUSP10. Many viruses target host proteins for degradation through host pathways, utilising host ubiquitin ligases or expressing their own (Barro and Patton, 2005, Okumura et al., 2008). However, preliminary data using MG132 suggests that inhibition of proteosomal degradation does not stop the loss of DUSP10 protein 30 minutes after RV1B addition (data not shown). Rhinovirus also encodes its own proteinases 2A and 3C, which have previously been shown to degrade components of the IFN signalling pathway (Drahos and Racaniello, 2009).

DUSP10 has also previously been found to be phosphorylated on serine residues 224 and 230, leading to stabilisation of the protein (Benavides-Serrato et al., 2014). This event was linked to the mTORC family of kinases, a group of proteins involved in the PI3K signalling pathway. As discussed above, the PI3K pathway has been found to be activated upon rhinoviral infection (Section 1.3.4). It would be of interest to know whether DUSP10 is regulated by phosphorylation upon rhinoviral infection, similarly to DUSP1. Attempts were made to investigate this. As no antibody against phosphorylated DUSP10 is commercially available, immunoprecipitation of DUSP10 from PBECs was attempted, but was unsuccessful (data not shown). Utilisation of the PhosTag (Wako) molecule, which binds to phosphorylated proteins, increasing their weight, enabling separation of phosphorylated and non-phosphorylated proteins by western blot was also unsuccessful (data not shown).

DUSP1 has previously been shown to be upregulated in A549 cells and PBECs by dexamethasone (Keranen et al., 2017, Newton et al., 2010, Papi et al., 2013). In A549 cells, a time course of dexamethasone stimulation, 1 μM, showed peak DUSP1 protein expression 6 h post stimulation (Newton et al., 2010) and stimulation of PBECs with 10

nM dexamethasone for 4 h caused around a 5-fold increase in DUSP1 mRNA levels (Papi et al., 2013). DUSP10 expression in response to dexamethasone stimulation has, to the best of my knowledge, not been studied previously. In this study, changes in protein expression of DUSPs 1 and 10 in response to dexamethasone were only observed in one out of three donors, and only at the highest dose used, 100 nM. Higher doses may have caused a response in all donors, however, 100 nM caused some toxicity to PBECs, as can be observed by the decline in actin protein levels. It is well documented that some patients are naturally steroid resistant (Schwartz et al., 1968), therefore it is possible that the two donors which do not upregulate DUSP expression may be steroid resistant. Smoking is known to reduce the efficacy of corticosteroids (Chalmers et al., 2002), and the responsive donor is a non-smoker, whereas one of the non-responsive donors is a smoker and the smoking status of the other is unknown (Table 7.1). This could be investigated by the inclusion of a larger number of donors with an equal ratio of smokers and non-smokers in this experiment.

3.10.5 Conclusions

The data presented in this chapter demonstrate that two of the MAPK pathways, p38 and JNK, play potentially important roles in rhinoviral infection of PBECs. TLR3 stimulation induces activation of the p38 and JNK MAPK proteins, and, while activated protein is undetectable in response to RV, inhibition of the pathways has an effect, demonstrating that they are being activated. Both pathways contribute towards the inflammatory response, inducing production and release of neutrophil and eosinophil chemoattractants. They also play opposing roles in rhinoviral replication: p38 being necessary for replication and JNK limiting it. The mechanisms for this remain to be elucidated, although JNK does not seem to be acting through induction of IFN-β.

Two proteins previously found to have important roles in regulating p38 and JNK, DUSP1 and DUSP10, are expressed in PBECs. Infection with RV also regulates both proteins, although in different ways. DUSP1 is phosphorylated in response to infection, which has previously been demonstrated to stabilise the protein, and further work is necessary to determine whether DUSP10 is also. DUSP10 expression is downregulated in response to RV infection, possibly through protein degradation. There are many other ways DUSPs may be regulated, which remain to be investigated, for example oxidation and acetylation. The differences in regulation of these proteins suggests they may have

different roles in regulating the response to RV infection. This will be further investigated in chapter 4.

These findings are summarised in figure 3.32.

These data support the hypothesis that regulating the MAPK pathways is a potential therapeutic target for the management of asthma and COPD exacerbations.

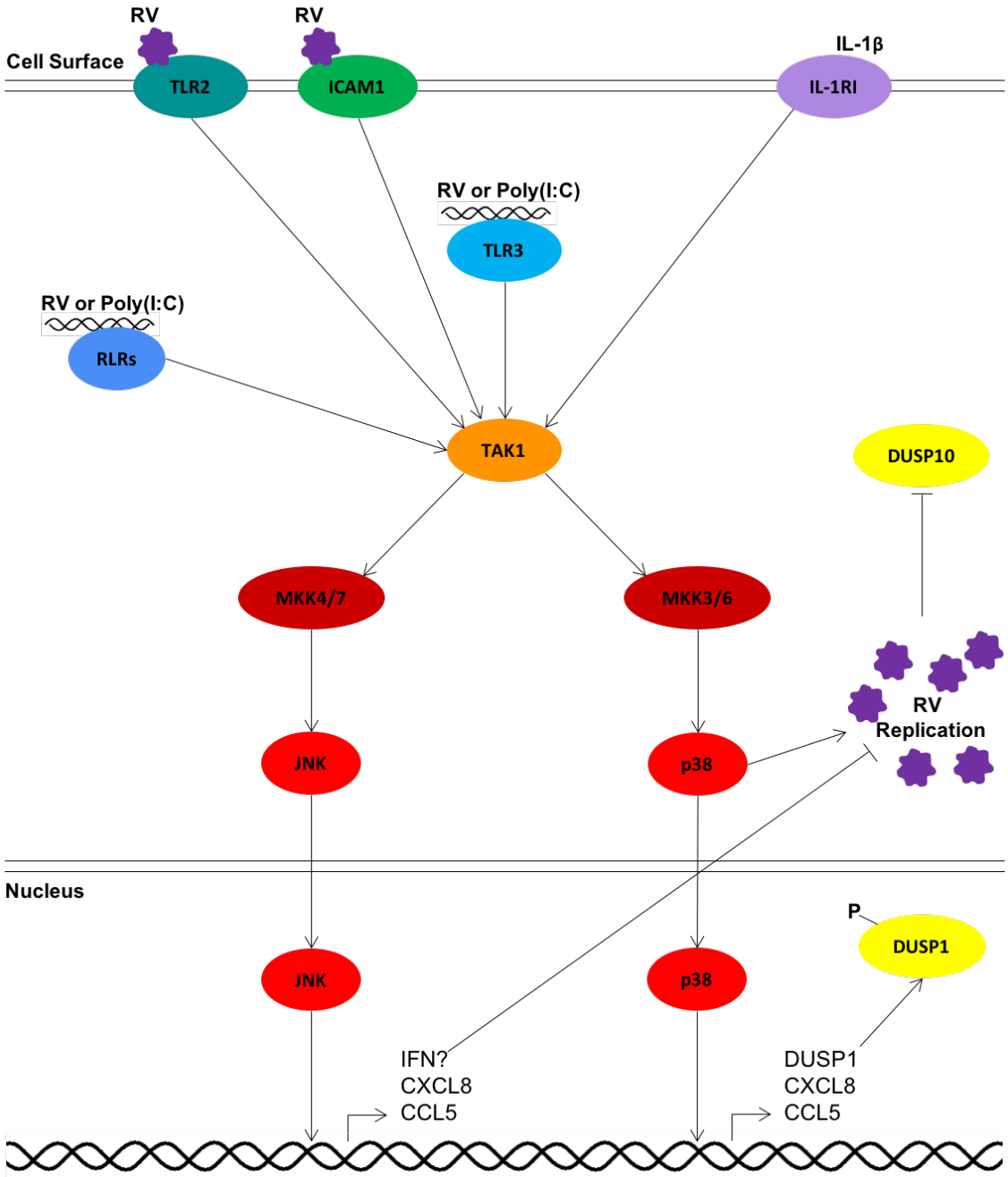

Figure 3.32 Summary of findings presented in chapter 3.

The p38 and JNK MAPK pathways are activated in PBECs in response to infection with rhinovirus and poly(I:C) through several pattern recognition receptors or ICAM-1 binding. IL-1β binding IL-1RI also leads to MAPK activation. The JNK pathway leads to production of inflammatory cytokines, and possibly interferon which limits rhinovirus replication. The p38 pathway leads to production of inflammatory cytokines, and DUSP1 which is subsequently phosphorylated. p38 activation positively regulates rhinovirus replication, which leads to downregulation of DUSP10.

4 Chapter Four – Results: The Roles of DUSPs in Rhinoviral Infection

4.1 Introduction

The results presented in chapter 3 suggest that p38 and JNK play important roles in the inflammatory response of PBECs to rhinoviral infection. Therefore, two DUSPs previously shown to regulate these pathways, and reduce inflammatory responses, were taken forward for investigation into their role in rhinoviral infection: DUSP1 and DUSP10.

As discussed in chapter 1, DUSP1 has been shown to regulate p38 and JNK activation, and thus inflammatory cytokine release, in the response to bacterial infection of macrophages (Chi et al., 2006, Hammer et al., 2006, Frazier et al., 2009, McGuire et al., 2017, Talwar et al., 2017, Zhao et al., 2005). DUSP1 has also been shown to regulate inflammatory cytokine release in response to poly(I:C) and vaccinia virus and to regulate the interferon response to HCV (Caceres et al., 2013, Choi et al., 2015, Golebski et al., 2015).

In 2015, James et al. studied the role of DUSP10 in viral infection. They found that DUSP10 knock out mice showed increased viral titres when infected with influenza virus compared to wild-type mice. This was associated with increased inflammatory cytokines and type I IFNs in BAL, BMDMs and bone marrow derived dendritic cells (BMDCs). When the MAPK activation was examined, ERK phosphorylation in response to influenza was prolonged in knock out BMDMs, but activated p38 and JNK levels were comparable. Type I IFN expression was also increased in knock out BMDMs when stimulated with poly(I:C), or the RIG-I substrate 5'-tri-phosphorylated double-stranded RNA, or infected with vesicular stomatitis virus or sendai virus. This increase in IFN production correlated with an increase in phosphorylated IRF3 expression, and, using immunoprecipitation, they showed a direct interaction between DUSP10 and IRF3 (James et al., 2015). This suggests that in RNA virus infection, DUSP10 dephosphorylates IRF3, regulating the anti-viral IFN response.

4.1.1 Aims

The p38 and JNK MAPK pathways are important inducers of inflammation in response to rhinoviral infection. Previous work has demonstrated that two proteins, DUSPs 1 and 10, regulate inflammation through dephosphorylating p38 and JNK, although their roles in rhinoviral infection have not yet been studied. The results presented in chapter three show that both DUSPs 1 and 10 are expressed in PBECs and are regulated by rhinoviral infection. The aim of this chapter was to determine the roles of DUSPs 1 and 10, using siRNA knock down, in rhinoviral infection of PBECs. This included their roles in rhinoviral replication, cytokine production and MAPK activation.

4.2 Knock Down of DUSP1 Expression

To the best of my knowledge DUSP1 knock down has never been successfully attempted in PBECs, although it has been accomplished in airway epithelial cell lines, such as A549 and NCI-H292 (Golebski et al., 2015, Shah et al., 2016a). In order to determine the role of DUSP1 in rhinoviral infection of PBECs, siRNA knock down of DUSP1 protein was attempted. BEAS-2B cells were initially used to optimise the siRNA knock down. Transfection of BEAS-2Bs with four different siRNA targeting DUSP1, listed in table 2.7, did not lead to measurable differences in DUSP1 protein levels after 24 h treatment (data not shown) or 48 h treatment (Figure 4.1 A). DUSP1 siRNA number 4 had been optimised in BEAS-2B cells previously by another group (S. V. Shah, Personal Communication), however in this study, this was unsuccessful in BEAS-2Bs, HeLa Ohio, and PBECs. DUSP1 mRNA levels were also measured and no differences were detected between control and DUSP1 siRNA treatments (data not shown). CXCL8 release in response to poly(I:C) stimulation was also quantified, to investigate whether the DUSP1 siRNA treatment had measurable consequences despite uncertain knock down. No differences in CXCL8 production were observed between control and DUSP1 siRNA treatments (Figure 4.1 B). DUSP1 knock down was also attempted in PBECs using all four siRNAs, with Lipofectamine transfection reagent, using a variety of conditions (Table 4.1), with no observable knock down (Figure 4.1 C).

In order to optimise the DUSP1 knock down, BEAS-2B cells were treated with a variety of concentrations of siRNA and Lipofectamine transfection reagent. Lipofectamine was chosen initially as it is regularly used to successfully transfect PBECs. As knock down was unsuccessful, different transfection reagents were then tested. Sigma-Aldrich Mission siRNA transfection reagent was chosen as it has been validated in a variety of cell lines and primary cells. Viromer Blue was also selected as it has been designed for transfection of hard-to-transfect primary cells. However, neither led to measurable knock down of DUSP1 mRNA or protein levels (data not shown). All conditions tested are listed in table 4.1.

Dexamethasone treatment causes upregulation of DUSP1 protein expression (Figure 3.23). Any small differences in DUSP1 expression caused by siRNA treatment may be more detectable when DUSP1 is upregulated by dexamethasone. However, DUSP1

siRNA treatment did not knock down DUSP1 expression in cells treated with vehicle control or dexamethasone (data not shown).

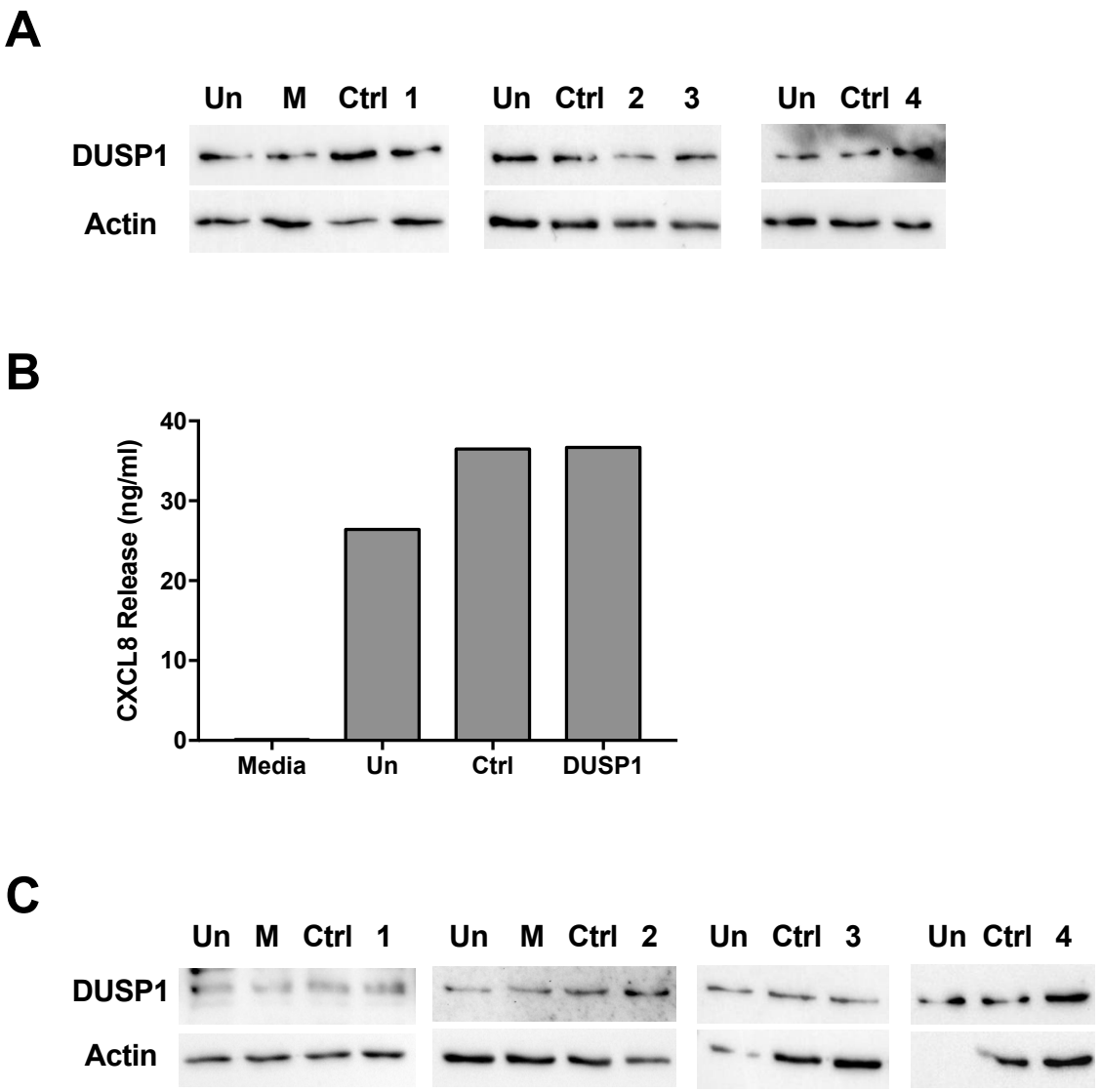

Figure 4.1 Attempted DUSP1 knock down.

BEAS-2B cells were untransfected (U), mock transfected (M) or transfected with control siRNA (Ctrl) or four different DUSP1 siRNA: 1-3 at 0.1 μM and 4 at 0.075 μM, and cell lysate collected at 48 h. Total DUSP1 and total actin levels were measured using western blot. A representative blot is shown, n=1-2 (A). After 48 h knock down with DUSP1 siRNA 4, BEAS-2Bs were stimulated with poly(I:C) (25 μg/ml) for 24 h and supernatant collected. CXCL8 was detected using ELISA, n=1 (B). PBECs were untransfected, mock transfected or transfected with control siRNA or DUSP1 siRNA 1 and cell lysate collected at 24 h, or transfected with control or DUSP1 siRNA 2-4 for 48 h, n=1-2 (C).

Table 4.1: Conditions for Attempted DUSP1 Knock Down

Cell Type	Conditions	siRNA			
		1	**2**	**3**	**4**
PBECs	1X Lipo. 24 h	X		X	
	1X Lipo. 48 h		X	X	X
	2X Lipo. 24 h		X		
	2X Lipo. 48 h		X	X	
	1X siRNA 24 h				
	1X siRNA 48 h		X		X
	2X siRNA 48 h		X		X
	PIC Stimulation	X		X	
	Different Confluencies	X	X	X	
BEAS-2B	1X Lipo. 24 h	X			X
	1X Lipo. 48 h	X		X	X
	2X Lipo. 24 h	X	X		X
	2X Lipo. 48 h	X	X	X	X
	1X siRNA 24 h		X		X
	1X siRNA 48 h	X	X	X	X
	2X siRNA 24 h				X
	2X siRNA 48 h	X		X	X
	Mission 1X siRNA	X	X	X	
	Mission 2X siRNA	X	X	X	
	Viromer 1X siRNA	X	X	X	
	Viromer 2X siRNA	X	X	X	
	PIC Stimulation	X			X
	Dex. Treatment		X	X	X
	Different Confluencies	X	X	X	X
	Different Media	X	X	X	X
HeLa Ohio	2X Lipo. 24 h	X		X	X
	2X Lipo. 48 h			X	X
	1X siRNA 24 h	X		X	X
	1X siRNA 48 h			X	X
	Dex. Treatment			X	X

DUSP1 knock down was attempted in the cell types shown using four different siRNAs targeting DUSP1 (siRNA 1-4). The conditions attempted for each siRNA in each cell type are marked with an X. Lipo. = Lipofectamine, PIC = poly(I:C), and Dex. = dexamethasone.

4.3 Knock Down of DUSP10 Expression in PBECs

In order to determine the role of DUSP10 in regulating the response of PBECs to rhinoviral infection, DUSP10 expression was successfully knocked down using siRNA. DUSP10 expression was knocked down at both the protein level and the RNA level with an average knock down efficiency of 78.14% and 88.96% respectively (Figure 4.2).

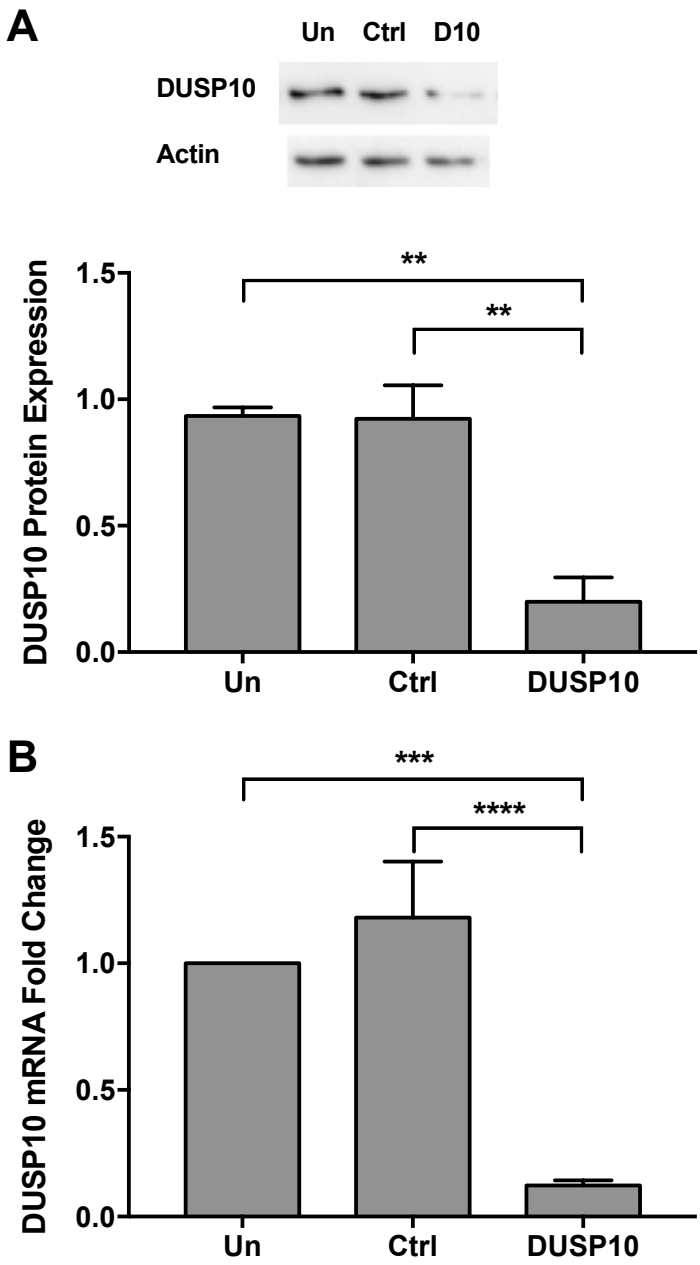

Figure 4.2 DUSP10 knock down in PBECs.

PBECs were untransfected (Un) or treated with DUSP10 siRNA (DUSP10) or control siRNA (Ctrl) (100 nM) and cell lysate collected at 48 h. Total DUSP10 and total actin levels were measured using western blot. A representative blot is shown with densitometry below. Data shown are mean ± SEM band density normalised to actin control for each sample (A). ΔΔCt qRT-PCR was performed for DUSP10 and a GAPDH control. Data shown are mean ± SEM of fold change normalised to GAPDH and untransfected (B). Significance is indicated by ** $p \leq 0.01$, **** $p \leq 0.0001$ as measured by one-way ANOVA, Dunnett's post-test to compare siRNA treatments, on band density, n=3 (A) or ΔΔCt values, n=7 (B).

4.4 The Role of DUSP10 in Rhinoviral Infection of PBECs

4.4.1 The Effect of DUSP10 Knock Down on RV Replication

A lack of DUSP10 protein has previously been associated with reduced influenza virus replication, and increased interferon production (James et al. 2015). The effect of reduced DUSP10 protein levels on replication of rhinovirus was investigated. After treatment with DUSP10 or control siRNA, replication of both RV1B and RV16 in PBECs was determined by qRT-PCR with primers specific for rhinoviral RNA. Both RV1B and RV16 replicated in PBECs, although the level of replication varied greatly between donors, and the level of replication was unaffected by treatment with either control or DUSP10 siRNA (Figure 4.3).

The effect of knocking down DUSP10 on IFN production was then investigated. Treatment with control siRNA caused increases in IFN-β mRNA levels and cytokine release compared to untransfected cells in some donors, suggesting that siRNA treatment was inducing an anti-viral response, as has been observed previously (Kariko et al., 2004) (Figures 4.4 and 4.5). Loss of DUSP10 did not cause any differences in IFN-β mRNA levels in response to infection with either serotype of rhinovirus or poly(I:C), although very small amounts of IFN-β were present (Figure 4.4).

Knock down of DUSP10 did not have an observable effect on release of the interferon-stimulated gene CCL5 in response to either strain of rhinovirus (Figure 4.5 A and B). However, in response to stimulation with poly(I:C), DUSP10 siRNA treatment caused a statistically significant decrease in CCL5 release compared to control siRNA treatment. Although this is statistically significant, the level of CCL5 released by DUSP10 knock down cells is very similar to untransfected cells (Figure 4.5 C).

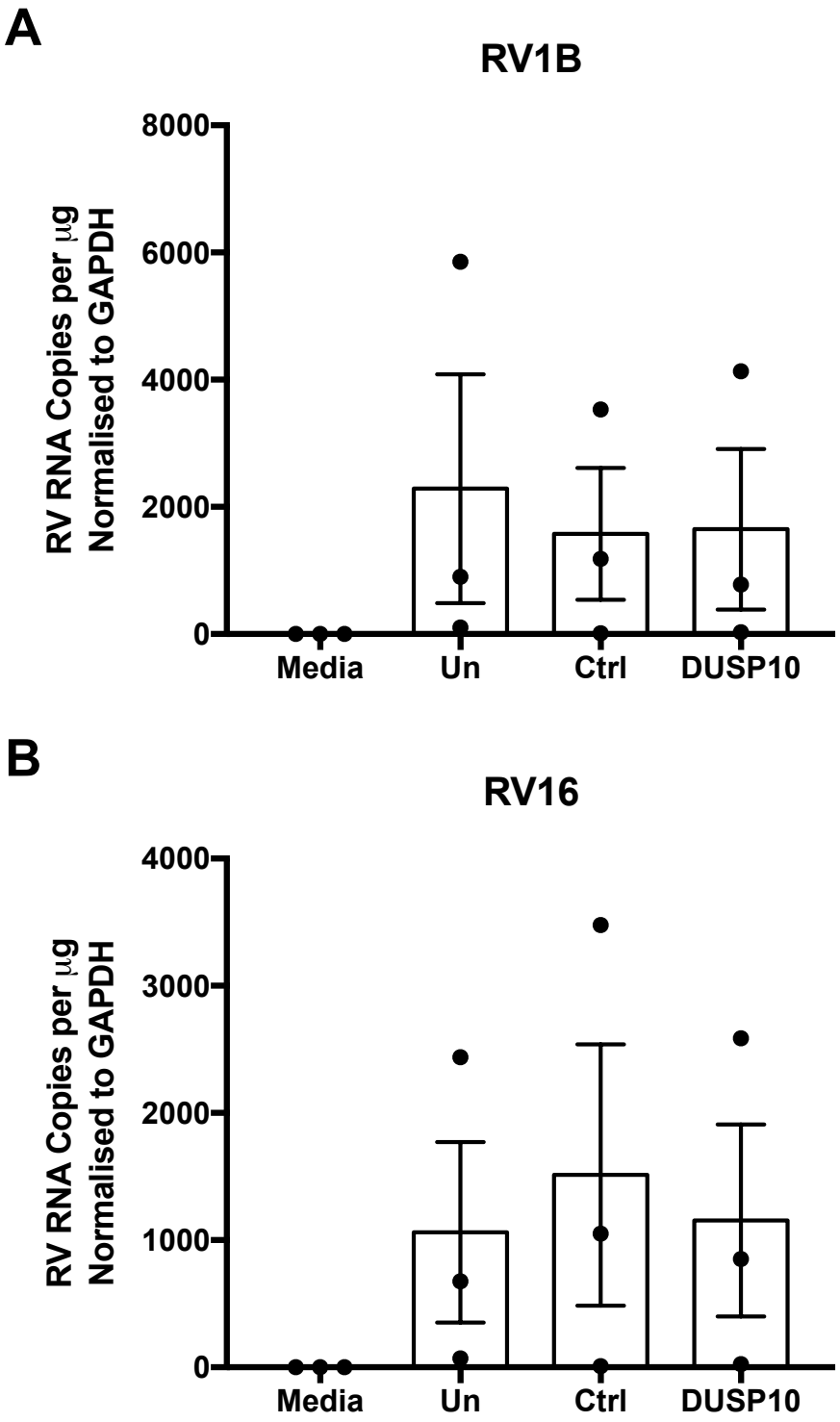

Figure 4.3 DUSP10 knock down does not affect RV replication.

PBECs were untransfected (Un) or treated with DUSP10 or control siRNA (Ctrl) (100 nM) for 48 h prior to infection with RV1B (MOI 3) (A) or RV16 (MOI 4) (B) for 24 h. RNA was collected and qRT-PCR performed for RV RNA and a GAPDH control. Data shown mean ± SEM normalised to the GAPDH control, n=3. Significance between control and DUSP10 siRNA was measured by paired t-test.

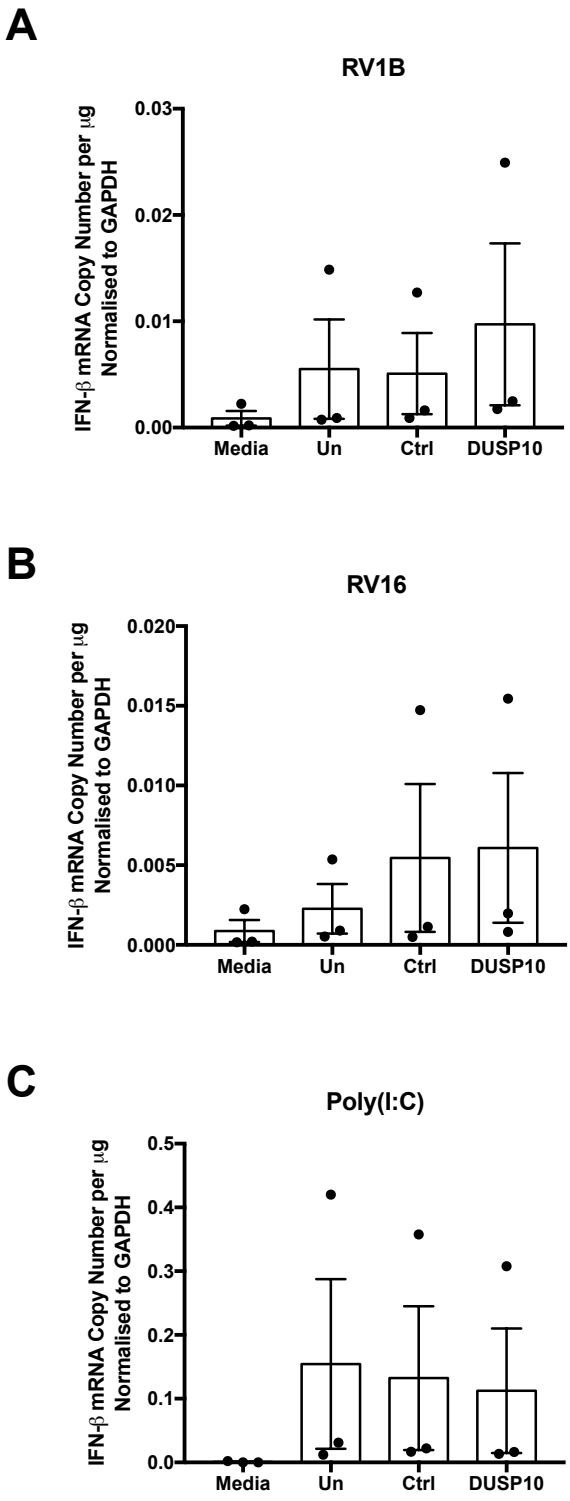

Figure 4.4 DUSP10 knock down does not affect IFN-β expression.

PBECs were untransfected (Un) or treated with DUSP10 or control siRNA (Ctrl) (100 nM) for 48 h prior to stimulation with poly(I:C) (25 μg/ml) (A), RV1B (MOI 3) (B) or RV16 (MOI 4) (C) for 4 h. mRNA was collected and qRT-PCR performed for IFN-β and a GAPDH control. Data shown are mean ± SEM normalised to the GAPDH control, n=3. Significance between control and DUSP10 siRNA was measured by paired t-test.

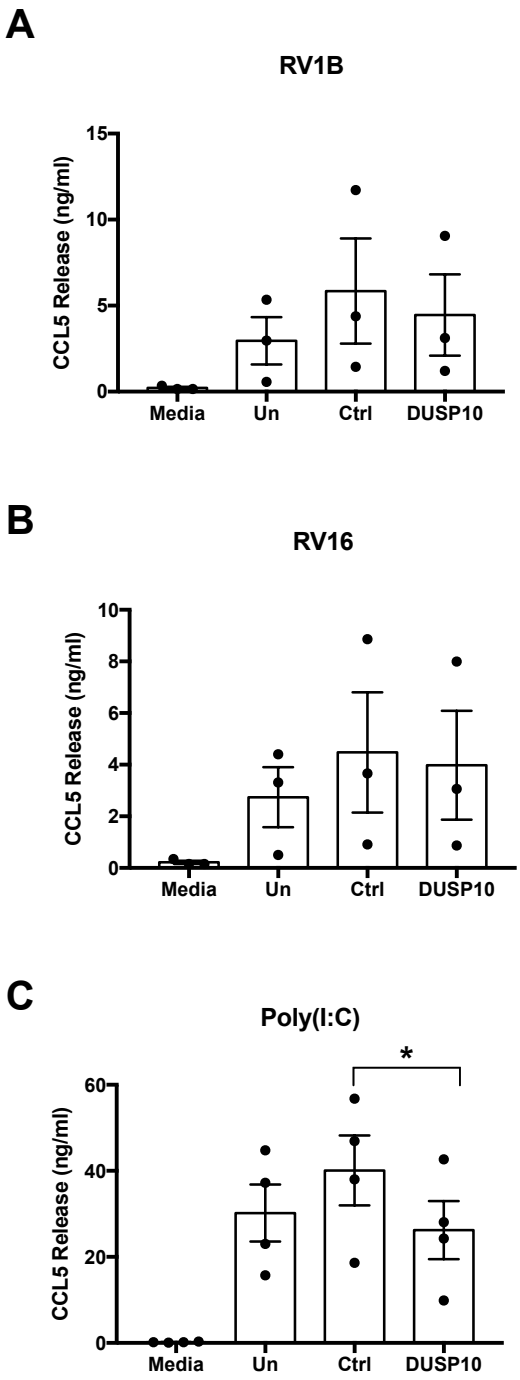

Figure 4.5 DUSP10 knock down decreases CCL5 release in response to poly(I:C) stimulation, but not RV infection.

PBECs were untransfected (Un) or treated with DUSP10 or control (Ctrl) siRNA (100 nM) for 48 h prior to stimulation with poly(I:C) (25 μg/ml) (A), RV1B (MOI 3) (B) or RV16 (MOI 4) (C) for 24 h. Supernatant was collected and ELISA used to measure the release of CCL5. Data shown are mean ± SEM, n=4 for poly(I:C) and 3 for RV1B and RV16. Significance between DUSP10 and control siRNA treatment is indicated by * $p \leq 0.05$ as measured by paired t-test.

4.5 The Role of DUSP10 in Cytokine Production

4.5.1 The Effect of DUSP10 Knock Down on CXCL8 Production in PBECs

DUSP10 knock out mice have previously been found to show increased cytokine generation in response to influenza infection, as well as other innate immune stimuli, such as poly(I:C), LPS, and bacterial infection (Section 1.5.4). Thus, the effect of DUSP10 knock down on cytokine generation in PBECs in response to rhinoviral infection, or poly(I:C), or IL-1β stimulation was investigated. There was a large amount of donor variation, making it difficult to draw conclusions from replicates of n= 3 or 4. In response to rhinoviral infection, loss of DUSP10 did not affect levels of CXCL8 mRNA or secreted protein, compared to cells treated with control siRNA (Figures 4.6 and 4.7 C and D). In response to poly(I:C) stimulation, the mean CXCL8 mRNA level increased in cells treated with DUSP10 siRNA, however, in two out of four donors the level decreased compared to control siRNA treatment (Figure 4.6 B). In addition, CXCL8 release is not increased with DUSP10 knock down in poly(I:C) treated cells (Figure 4.7 B).

In response to stimulation with IL-1β, cells treated with DUSP10 siRNA showed consistently higher CXCL8 mRNA levels than untransfected or control siRNA treated cells, although this was not statistically significant, likely consequent upon a large amount of donor variation (Figure 4.6 A). This difference was not evident in the levels of CXCL8 release (Figure 4.7 A).

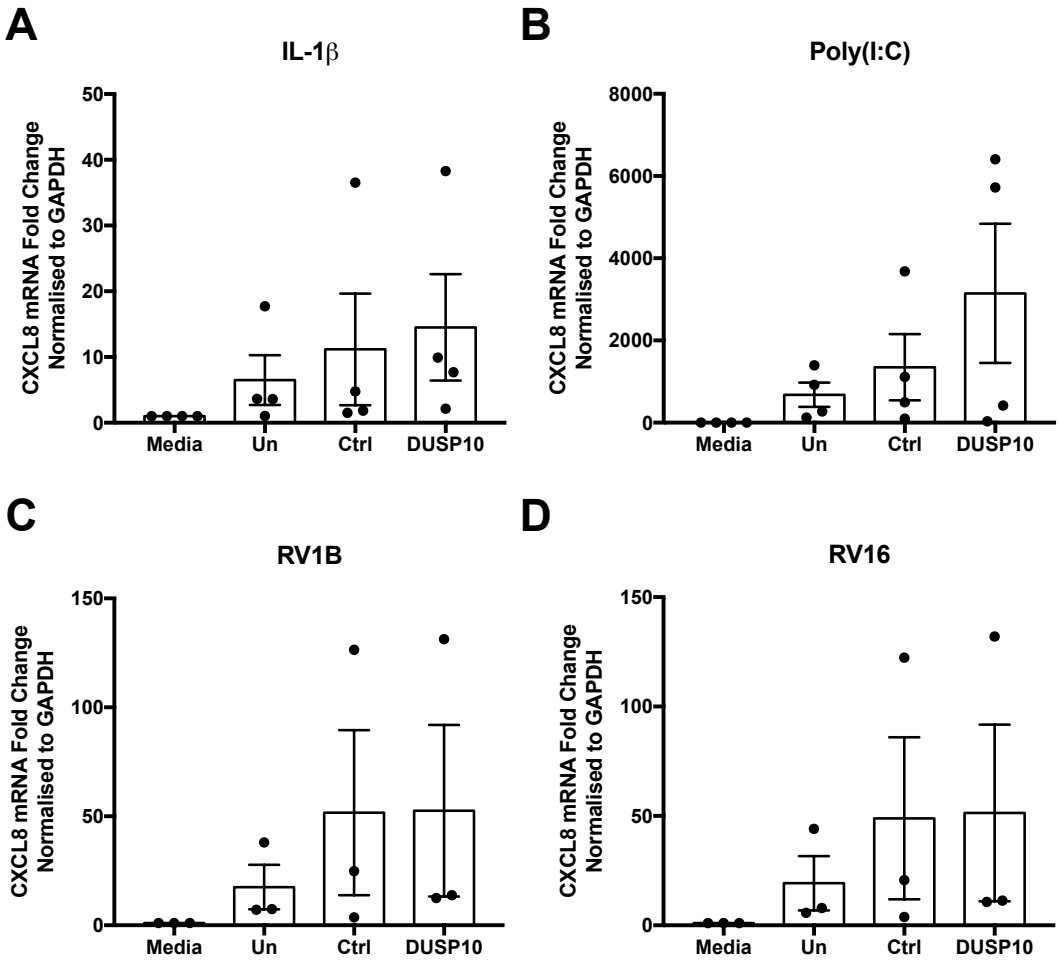

Figure 4.6 The effect of DUSP10 knock down on CXCL8 mRNA production.

PBECs were untransfected (Un) or treated with DUSP10 or control siRNA (Ctrl) (100 nM) for 48 h prior to stimulation with IL-1β (100 ng/ml) (A), poly(I:C) (25 μg/ml) (B), RV1B (MOI 3) (C) or RV16 (MOI 4) (D) for 24 h. RNA was collected and qRT-PCR performed for CXCL8 and a GAPDH control. Data shown mean ± SEM normalised to GAPDH and unstimulated (media) control, n=4 for A and B, and n=3 for C and D. Significance between DUSP10 and control siRNA treatments was measured by paired t-test.

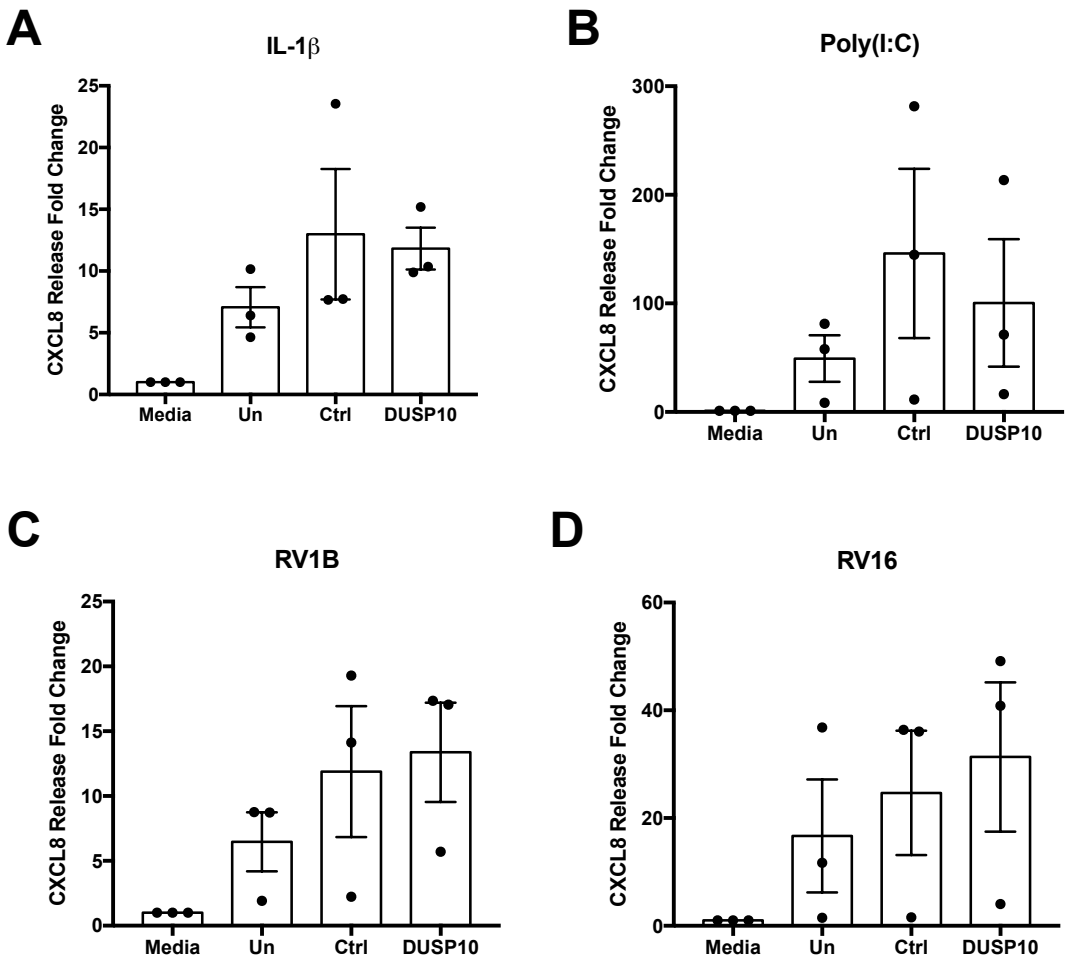

Figure 4.7 The effect of DUSP10 knock down on CXCL8 release.

PBECs were untransfected (Un) or treated with DUSP10 or control (Ctrl) siRNA (100 nM) for 48 h prior to stimulation with IL-1β (100 ng/ml) (A), poly(I:C) (25 μg/ml) (B), RV1B (MOI 3) (C) or RV16 (MOI 4) (D) for 24 h. Supernatant was collected and ELISA used to measure the release of CXCL8. Data shown are mean ± SEM, n=3, normalised to unstimulated (media) control. Significance between DUSP10 and control siRNA treatments was measured by paired t-test.

4.5.2 The Effect of DUSP10 Knock Down on CXCL8 Production in Response to IL-1β

In order to investigate the role of DUSP10 in the response to IL-1β in more detail, CXCL8 mRNA production was investigated at an earlier time point, of 6 h. Loss of DUSP10 caused an increase in the mean level of CXCL8 mRNA compared to control siRNA treated cells from 58.6 to 90.2 fold (Figure 4.8 A). In two out of three donors, DUSP10 knock down caused a large increase in CXCL8 mRNA levels (Figure 4.8 B). In the third donor, CXCL8 levels were much lower, with a small increase in cells treated with DUSP10 siRNA.

PBECs with confirmed DUSP10 knock down were treated with a range of IL-1β concentrations. At each concentration of IL-1β, DUSP10 knock down consistently resulted in an increase in CXCL8 mRNA levels, with significant differences in cells treated with 10 and 100 ng/ml (Figure 4.9 A). However, when measured by ELISA, this difference was not observed, with decreases in CXCL8 release in DUSP10 knock down cells observed in many of the donors (Figure 4.9 B). DUSP10 knock down had been observed to cause cell death, hence the different patterns in qRT-PCR and ELISA results could be due to the fact that qRT-PCR data is normalised to housekeeping RNA, whereas ELISA data is not normalised. Therefore, the number of cells per well at the time of supernatant collection was determined (Figure 4.10 A), and CXCL8 release normalised to cell number. Cells treated with DUSP10 siRNA had on average 40.5% less cells than those treated with control siRNA (Figure 4.10 A). At each concentration of IL-1β, DUSP10 knock down caused an increase in CXCL8 release per 10^5 cells (Figure 4.10 B).

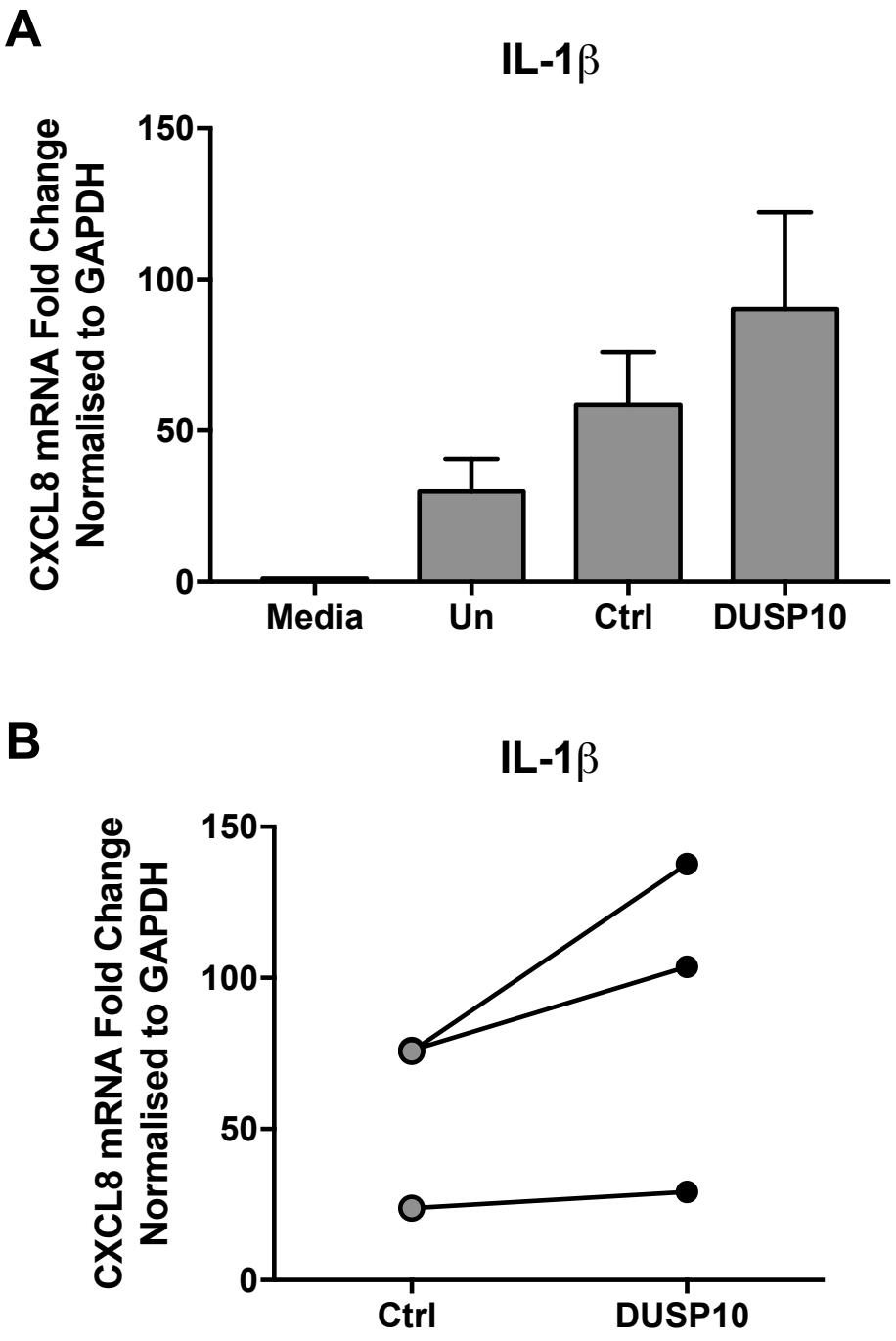

Figure 4.8 DUSP10 knock down increases CXCL8 mRNA production in response to IL-1β.

PBECs were untransfected (Un) or treated with DUSP10 or control siRNA (Ctrl) (100 nM) for 48 h prior to stimulation with IL-1β (100 ng/ml) for 6 h. RNA was collected and qRT-PCR performed for CXCL8 and a GAPDH control. Data shown mean ± SEM (A) and individual donors (B) normalised to GAPDH and media control, n=3. Significance was measured by one-way ANOVA, Dunnett's post-test to compare siRNA treatments (A) or paired t-test (B).

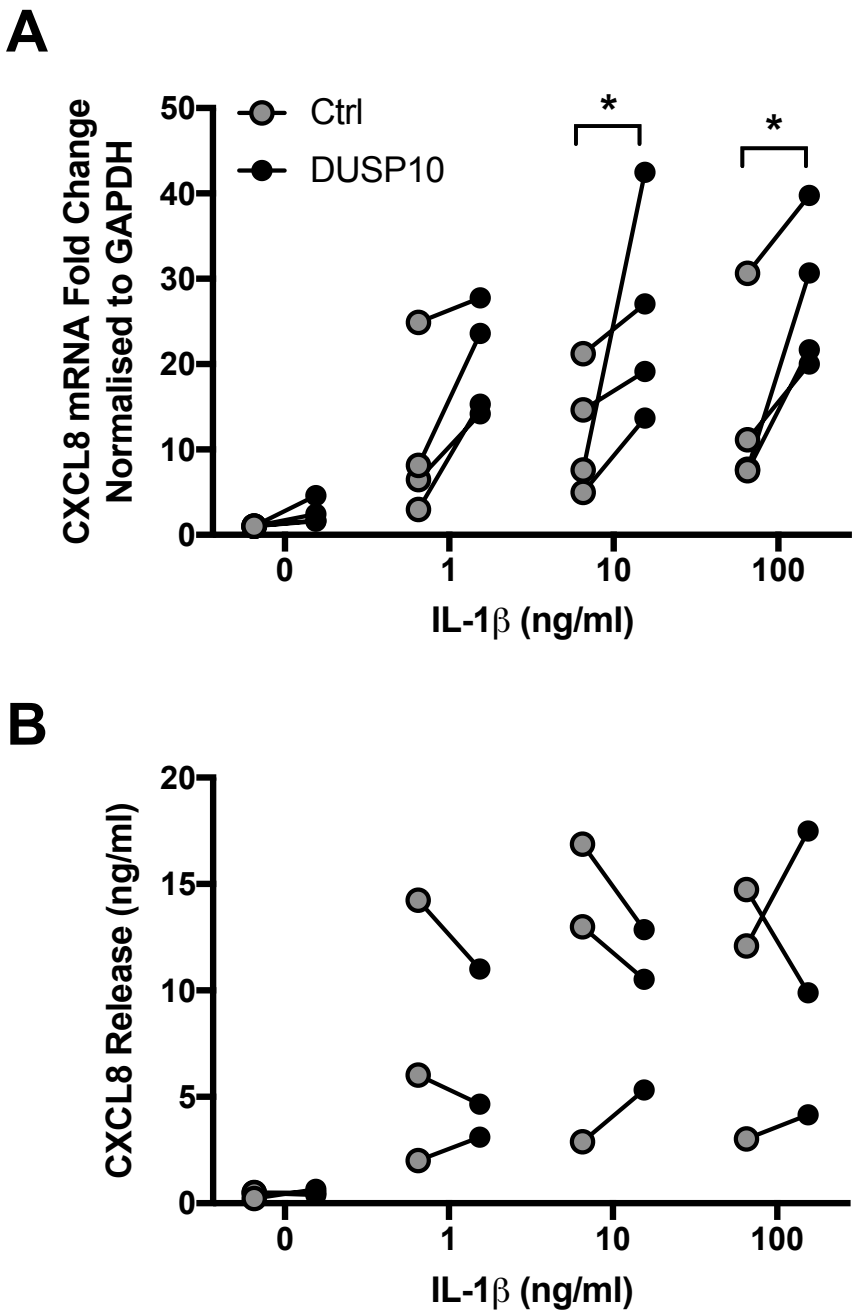

Figure 4.9 DUSP10 knock down increases CXCL8 mRNA production, but not release, in response to IL-1β.

PBECs were treated with DUSP10 or control siRNA (Ctrl) (100 nM) for 48 h prior to stimulation with IL-1β (1-100 ng/ml) for 24 h. mRNA was collected and qRT-PCR performed for CXCL8 and a GAPDH control. Data shown are each donor normalised to the GAPDH control and unstimulated (0 ng/ml) Ctrl cells n=4 (A). Supernatant was collected and ELISA used to measure the release of CXCL8, n=3 (B). Significance is indicated by * $p \leq 0.05$ as measured by two-way ANOVA with Sidak's post-test, to compare between control and DUSP10 siRNA at each dose.

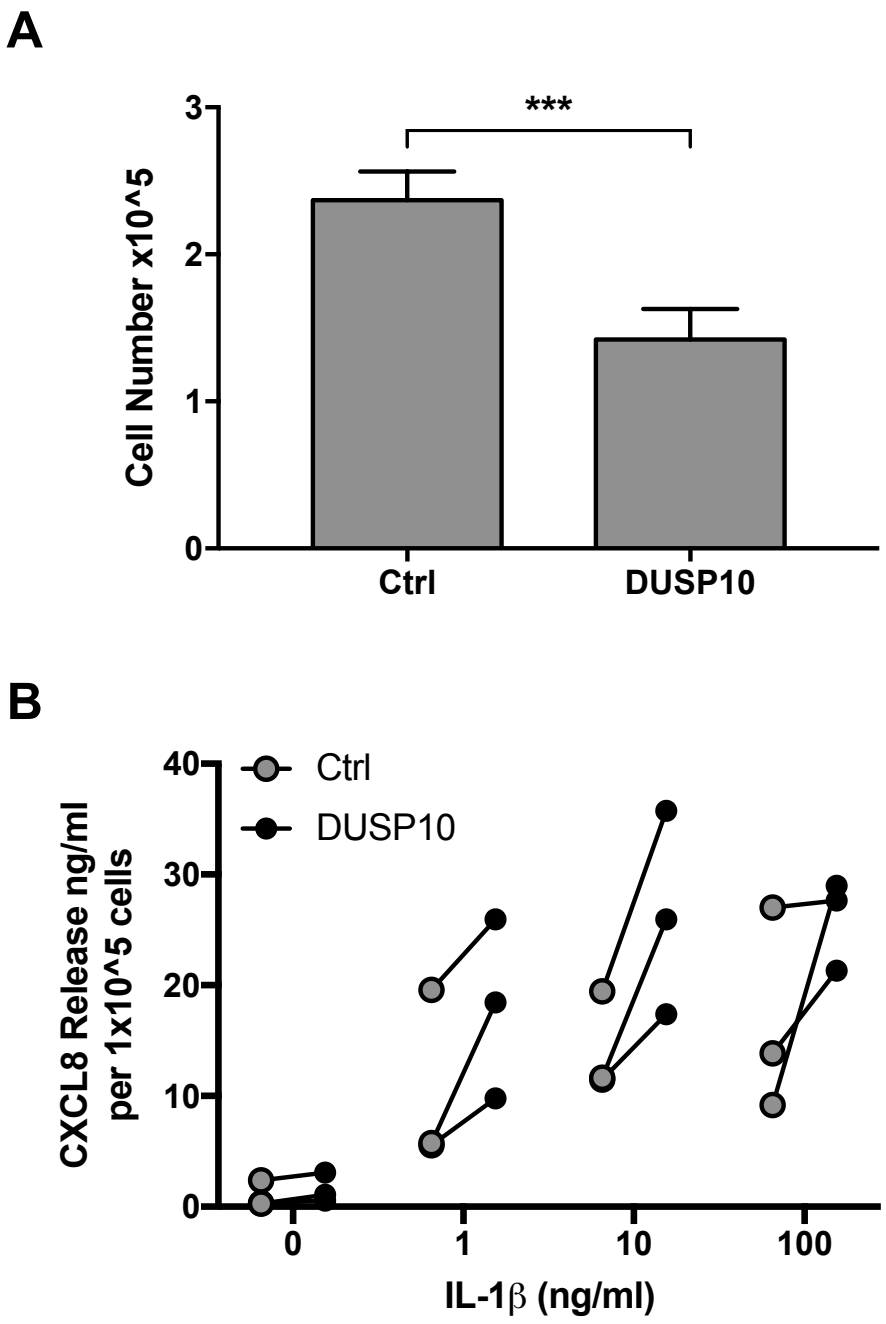

Figure 4.10 DUSP10 knock down increases CXCL8 release per cell in response to IL-1β.

PBECs were treated with DUSP10 or control siRNA (Ctrl) (100 nM) for 48 h prior to stimulation with IL-1β (1-100 ng/ml) for 24 h. Cells were detached and counted using a haemocytometer. Data shown are mean ± SEM, n=8. Significance is indicated by *** $p \leq 0.001$ as measured by t-test (A). Supernatant was collected and ELISA used to measure the release of CXCL8. Each donor is shown, normalised to cell counts, n=3. Significance was measured using two-way ANOVA, Sidak's post-test to compare between control and DUSP10 siRNA at each dose (B).

4.5.3 The Effect of DUSP10 Knock Down on MAPK Activation in Response to IL-1β

Cells taken from DUSP10 knock out mice have previously been found to have increased MAPK activation, in particular p38 and JNK, in response to a range of stimuli (Section 1.5.4). Inhibition of p38 and JNK caused a significant decrease in CXCL8 production in response to poly(I:C) and RV infection (Figures 3.2-3.4). Therefore, it was hypothesised that any increase in CXCL8 production in DUSP10 knock down in response to IL-1β (Figure 4.9 and 4.10) would be due to increased MAPK activation. This was investigated using western blot for the phosphorylated, activated p38 and JNK proteins. DUSP10 knock down did not affect the level of phosphorylated p38 or JNK 30 minutes post IL-1β stimulation (Figure 4.11).

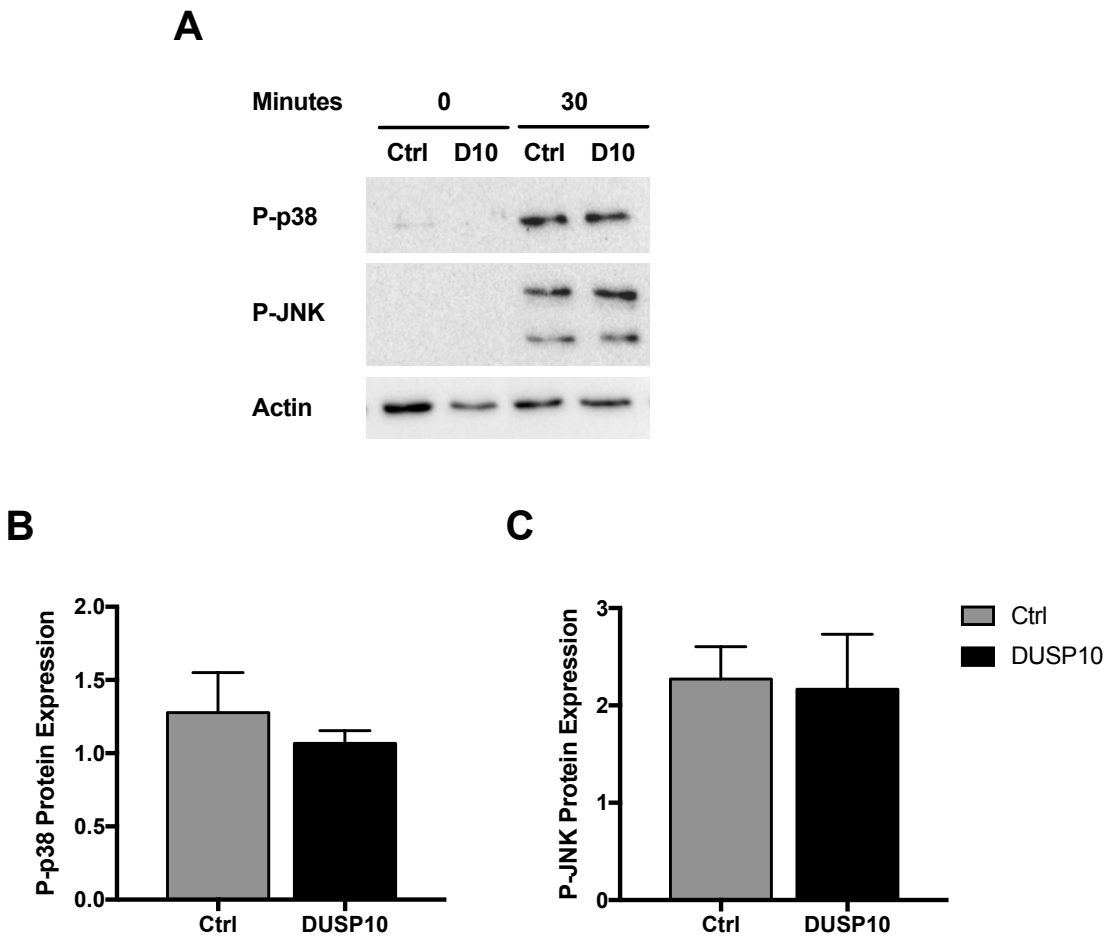

Figure 4.11 DUSP10 knock down does not affect MAPK activation in response to IL-1β.

PBECs were treated with DUSP10 siRNA (DUSP10) or control siRNA (Ctrl) (100 nM) for 48 h before stimulation with IL-1β (100 ng/ml) and cell lysate collected at 30 min. Phosphorylated p38 and JNK and total actin levels were measured using western blot. A representative blot is shown (A) with densitometry below (B and C). Data shown are mean ± SEM band density normalised to actin control, n=3. Significance between DUSP10 and control siRNA treatments was measured by paired t-test.

4.5.4 The Effect of DUSP10 Knock Down on Cytokine Release in Response to IL-1β

As DUSP10 knock down caused increases in CXCL8 production and release in response to IL-1β but not rhinoviral infection, the role of DUSP10 in IL-1β signalling was taken forward for further investigation. In order to gain a wider view of the role of DUSP10 in epithelial responses to IL-1β, an array was used to determine the effect of DUSP10 knock down on the release of a variety of cytokines. The chosen array contained antibodies for 36 human cytokines, chemokines and proteins known to be upregulated by inflammation, enabling a broad view of the effect of the loss of DUSP10.

IL-1β stimulation caused an upregulation in the release of several cytokines: CXCL1, CXCL10, G-CSF, GM-CSF, IL-1β, IL-6, and CXCL8. IL-1ra was secreted by PBECs, but the level was unaffected by IL-1β stimulation. Two molecules with roles in airway remodelling were secreted by PBECs in both stimulated and unstimulated cells: MIF and Serpin1 (Chen et al., 2010, Oh et al., 2002). CXCL12 release was decreased by IL-1β stimulation compared to media only controls. (Table 4.2). Some proteins that are known to be released by epithelial cells in response to rhinoviral infection or dsRNA were not released in response to IL-1β, including: CXCL1, ICAM-1, IL-1α, IL-2, IL-16, IL-17E and IL-18 (Beale et al., 2014, Papadopoulos et al., 2000, Piper et al., 2013, Schneider et al., 2010, Sha et al., 2004, Terajima et al., 1997, Xu et al., 2010). As observed previously, IL-1β stimulation did not induce the production of IFN or IFN-stimulated genes, such as CCL5 and CXCL11, which are known to be produced in response to RV infection (Chen et al., 2006, Lin et al., 1999, Piper et al., 2013).

DUSP10 knock down caused some changes in levels of cytokine release. Release of CXCL1 and IL-1β were both increased in DUSP10 knock down compared to control siRNA treated cells, with IL-1β levels almost doubling (Table 4.2). When normalised to cell number, CXCL8 levels were also increased by DUSP10 siRNA treatment (Figure 4.13). Intriguingly, CXCL10 levels were decreased in DUSP10 knock down.

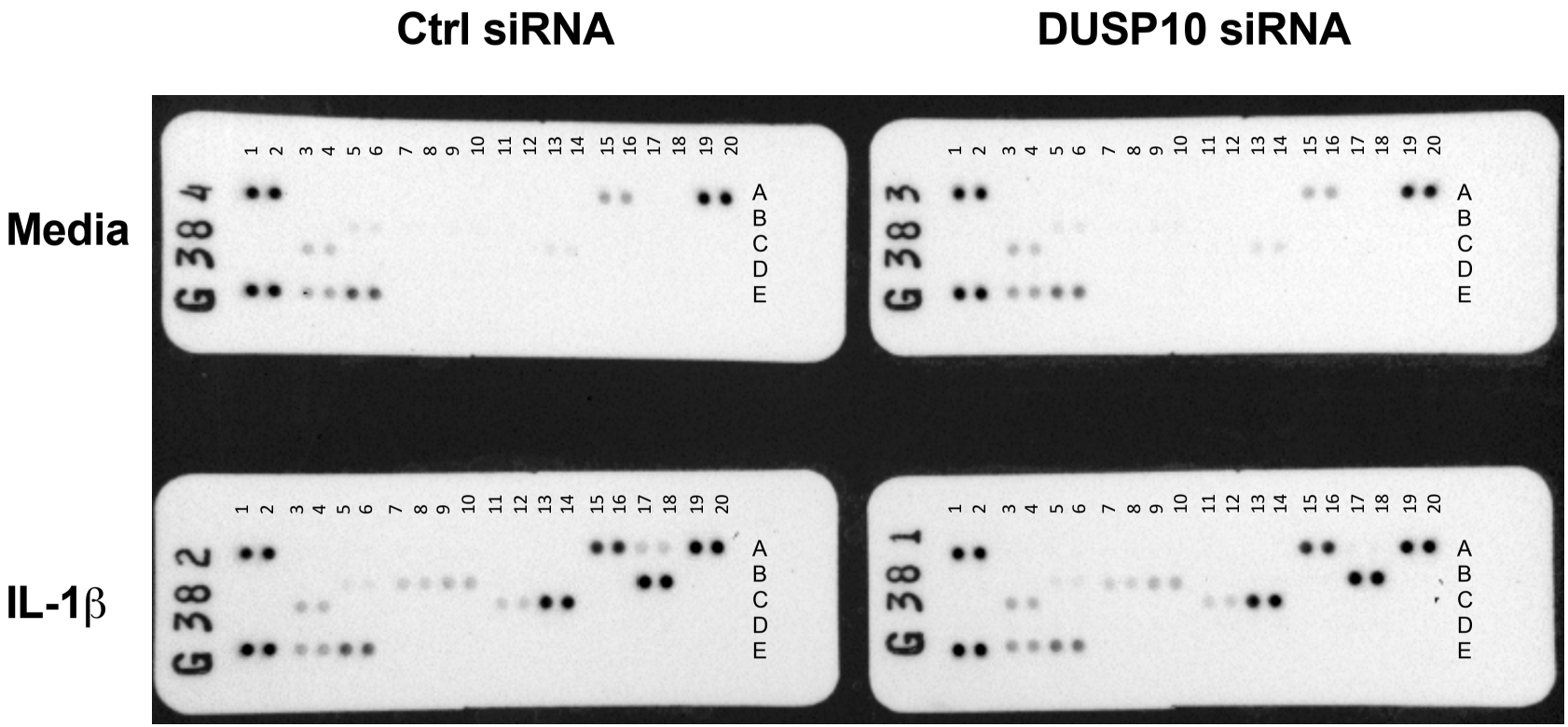

Figure 4.12 Stimulation of PBECs with IL-1β induces cytokine release.

PBECs were treated with DUSP10 siRNA (DUSP10) or control siRNA (Ctrl) (100 nM) for 48 h prior to stimulation with IL-1β (10 ng/ml) for 24 h. Cell supernatant was collected and a cytokine array performed. Arrays are shown. Co-ordinates A 1, 2, 19 and 20 and E 1 and 2 are reference points, n=1.

Table 4.2: The Effect of DUSP10 Knock Down on Cytokine Release in Response to IL-1β.

Coordinate	Cytokine	Spot Density				Role
		Media Ctrl	Media DUSP10	IL-1 β Ctrl	IL-1 β DUSP10	
A 3, 4	CCL1	0	0	0	0	Monocyte chemoattractant
A 5, 6	CCL2	0	0	0	0	Monocyte, T cell, NK cell and DC chemoattractant
A 7, 8	MIP-1 α	0	0	0	0	Activates monocytes, T cells and NK cells
A 9, 10	CCL5	0	0	0	0	Monocyte, eosinophil, basophil chemoattractant
A 11, 12	CD40 Ligand	0	0	0	0	B cell differentiation
A 13, 14	C5a	0	0	0	0	Complement component
A 15, 16	CXCL1	2953.7505	3022.7505	9701.196	10097.8525	Neutrophil chemoattractant
A 17, 18	CXCL10	0	0	1642.048	158.6065	Th1 chemoattractant
B 3, 4	CXCL11	0	0	0	0	T cell chemoattractant
B 5, 6	CXCL12	3873.3575	1316.805	569.006	425.4705	Neutrophil retention signal
B 7, 8	G-CSF	0	0	1387.9765	1338.719	Neutrophil development
B 9, 10	GM-CSF	0	0	2336.74	2508.4725	Enhances neutrophil activity
B 11, 12	ICAM-1	0	0	0	0	Leukocyte attachment
B 13, 14	IFN- γ	0	0	0	0	Induces antiviral responses
B 15, 16	IL-1 α	0	0	0	0	Induces inflammatory cytokine production
B 17, 18	IL-1 β	0	0	12953.3635	22122.4905	Induces inflammatory cytokine production
C 3, 4	IL-1ra	3335.7755	4431.1505	2508.9475	2418.033	IL-1 receptor antagonist
C 5, 6	IL-2	0	0	0	0	Differentiation of monocytes, NK, B and T cells
C 7, 8	IL-4	0	0	0	0	Th2 development
C 9, 10	IL-5	0	0	0	0	Proliferation and activation of eosinophils
C 11, 12	IL-6	0	0	1881.7905	1548.3405	B cell differentiation
C 13, 14	CXCL8	1130.8305	2097.008	12514.56	12289.474	Neutrophil chemoattractant

Table 4.2 Continued: The Effect of DUSP10 Knock Down on Cytokine Release in Response to IL-1β.

Coordinate	Cytokine	Spot Density				Role
		Media Ctrl	Media DUSP10	IL-1 β Ctrl	IL-1 β DUSP10	
C 15, 16	IL-10	0	0	0	0	Anti-inflammatory
C 17, 18	IL-12	0	0	0	0	Activates NK and T cells
D 3, 4	IL-13	0	0	0	0	Th2 development
D 5, 6	IL-16	0	0	0	0	T cell, monocyte and eosinophil chemoattractant
D 7,8	IL-17A	0	0	0	0	Secreted by T cells
D 9, 10	IL-17E	0	0	0	0	Promotes Th2 response
D 11, 12	IL-18	0	0	0	0	Stimulates Th1 cells
D 13, 14	IL-21	0	0	0	0	B cell differentiation
D 15, 16	IL-27	0	0	0	0	Expansion of CD4+ cells
D 17, 18	IL-32 α	0	0	0	0	Induces inflammatory cytokine production
E 3, 4	MIF	3217.4325	3833.529	2878.947	3154.0435	Induces inflammatory cytokine production
E 5, 6	SerpinE1	6870.3925	6562.807	6690.514	5748.807	Extracellular matrix - Fibrosis
E 7, 8	TNF- α	0	0	0	0	Induces inflammatory cytokine production
E 9, 10	TREM-1	0	0	0	0	Amplifies neutrophil and monocyte responses

PBECs were treated with DUSP10 siRNA (DUSP10) or control siRNA (Ctrl) (100 nM) for 48 h prior to stimulation with IL-1β (10 ng/ml) for 24 h. Cell supernatant was collected and a cytokine array performed. Average spot density, as analysed by ImageJ, for each co-ordinate on the array is presented. Cytokines with increased expression in response to IL-1β are coloured green, and those with decreased expression are coloured red.

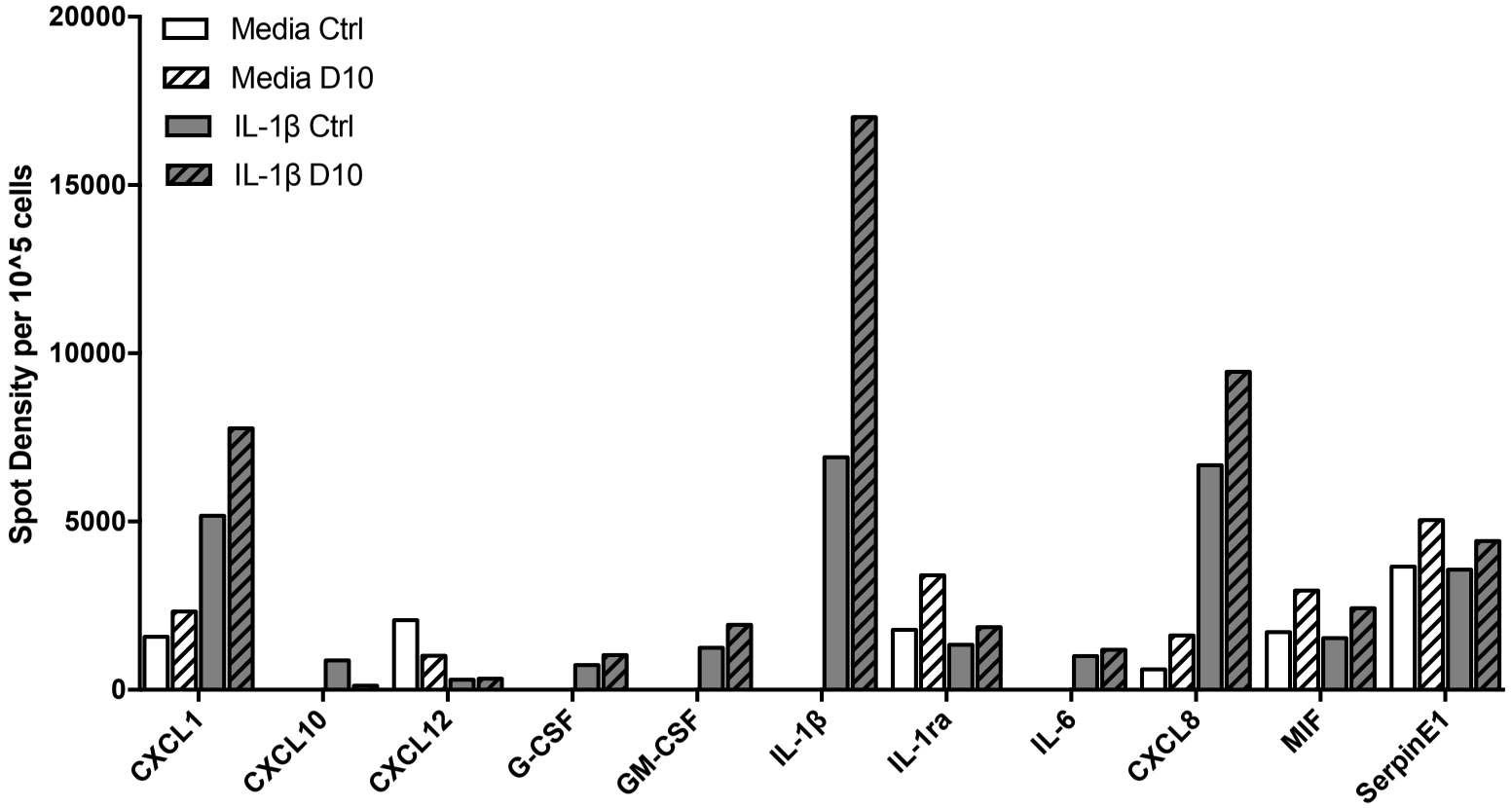

Figure 4.13 The effect of DUSP10 knock down on cytokine release in response to IL-1β.

PBECs were treated with DUSP10 siRNA (D10) or control siRNA (Ctrl) (100 nM) for 48 h prior to stimulation with IL-1β (10 ng/ml) for 24 h. Cell supernatant was collected and a cytokine array performed. Data presented are spot density normalised to cell number, n=1.

4.6 IL-1β Potentiation of the Response to RV Infection

IL-1β has previously been shown to potentiate the response of epithelial cells to rhinoviral infection (Stokes et al., 2011). Therefore, the effect of dual stimulation with IL-1β and rhinovirus on cytokine release by epithelial cells was investigated. PBECs were stimulated with 10 or 100 ng/ml IL-1β, with and without RV16 infection, and mRNA and secreted protein levels of CXCL8 measured. Infection with RV16 caused small increases in CXCL8, which was increased by stimulation with IL-1β. At the mRNA level, only the highest IL-1β concentration caused observable increases in CXCL8 production, compared to RV16 alone. However, as measured by ELISA, both IL-1β concentrations caused incremental increases in CXCL8 release. This response was further potentiated by DUSP10 knock down, with significantly higher CXCL8 levels released in cells treated with DUSP10 siRNA (Figure 4.14).

In order to determine whether the potentiation of the response to RV16 with IL-1β was due to an effect on rhinoviral replication, RV RNA levels were measured using qRT-PCR. Each concentration of IL-1β did not affect the amount of RV RNA present (Figure 4.15). As seen previously (Figure 4.3), this was unaffected by DUSP10 knock down.

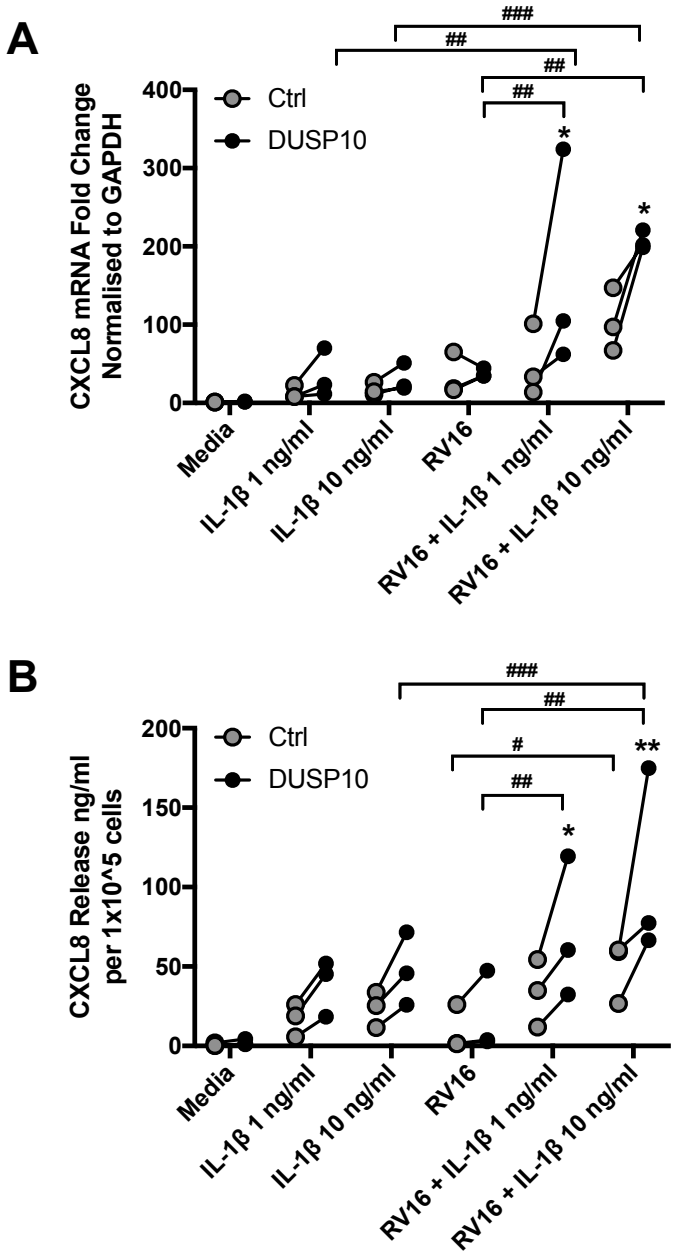

Figure 4.14 IL-1β potentiates CXCL8 production in response to RV16, and DUSP10 knock down potentiates it further.

PBECs were treated with DUSP10 siRNA (DUSP10) or control siRNA (Ctrl) (100 nM) for 48 h prior to stimulation with IL-1β (1-10 ng/ml) and/or RV16 infection (MOI 4). qRT-PCR was performed for CXCL8 and a GAPDH control. Data shown are fold change normalised to GAPDH control and media control (A). Supernatant was collected and ELISA performed for CXCL8. Data shown are ng/ml normalised to cell counts (B). Significance is indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ between control and DUSP10 siRNA, as measured by two-way ANOVA, Sidak's post-test, or # between stimulants, as measured by two-way ANOVA, Tukey's post-test, n=3.

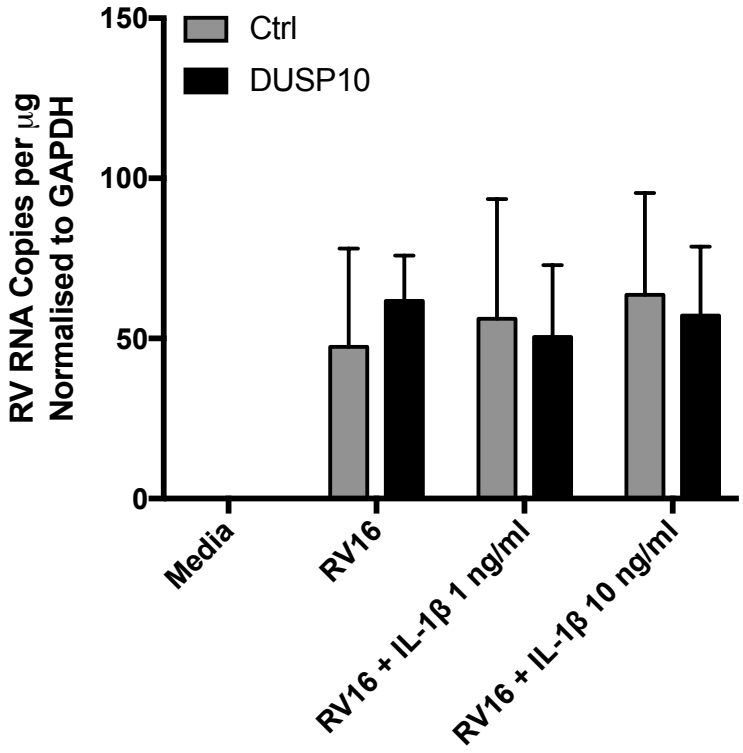

Figure 4.15 IL-1β stimulation does not affect RV replication.

PBECs were treated with DUSP10 siRNA (DUSP10) or control siRNA (Ctrl) (100 nM) for 48 h prior to stimulation with IL-1β (1-10 ng/ml) and/or RV16 infection (MOI 4). qRT-PCR was performed for RV RNA and a GAPDH control. Data shown are mean ± SEM, n=3, normalised to GAPDH control. Significance was measured by two-way ANOVA, Dunnett's post-test, to compare between RV16 and RV16+IL-1β treatments.

4.7 The Role of DUSP10 in Steroid Treatment

DUSP1 is known to contribute significantly to the restriction of cytokine release by dexamethasone (Papi et al., 2013). However, to the best of my knowledge, the role of DUSP10 in dexamethasone treatment has not previously been investigated. In order to determine whether DUSP10 contributes to the effect of dexamethasone, PBECs were treated with DUSP10 or control siRNA 48 h before simultaneous treatment with dexamethasone and poly(I:C), and the level of CXCL8 mRNA determined by qRT-PCR. As seen previously, in the absence of dexamethasone, poly(I:C) stimulation upregulated CXCL8 mRNA and secreted protein levels (Figure 4.16). In cells treated with control siRNA dexamethasone treatment significantly reduced CXCL8 mRNA production, whereas in DUSP10 knock down cells there was no difference in CXCL8 mRNA levels when dexamethasone was present (Figure 4.16 A). However, when CXCL8 release was measured by ELISA, dexamethasone treatment did not induce any differences between control or DUSP10 siRNA treatments (Figure 4.16 B).

As the dexamethasone treatment may not have had time to act before upregulation of CXCL8 mRNA by poly(I:C), the experiment was repeated with a 4 h dexamethasone pre-treatment before stimulation. As in figure 4.16, dexamethasone treatment consistently caused a small reduction in CXCL8 mRNA production in response to poly(I:C) in cells treated with control siRNA, although this was not statistically significant (Figure 4.17 A). This difference was not observed in cells treated with DUSP10 siRNA. The same pattern was observed in secreted protein levels, although also not statistically significant (Figure 4.17 B).

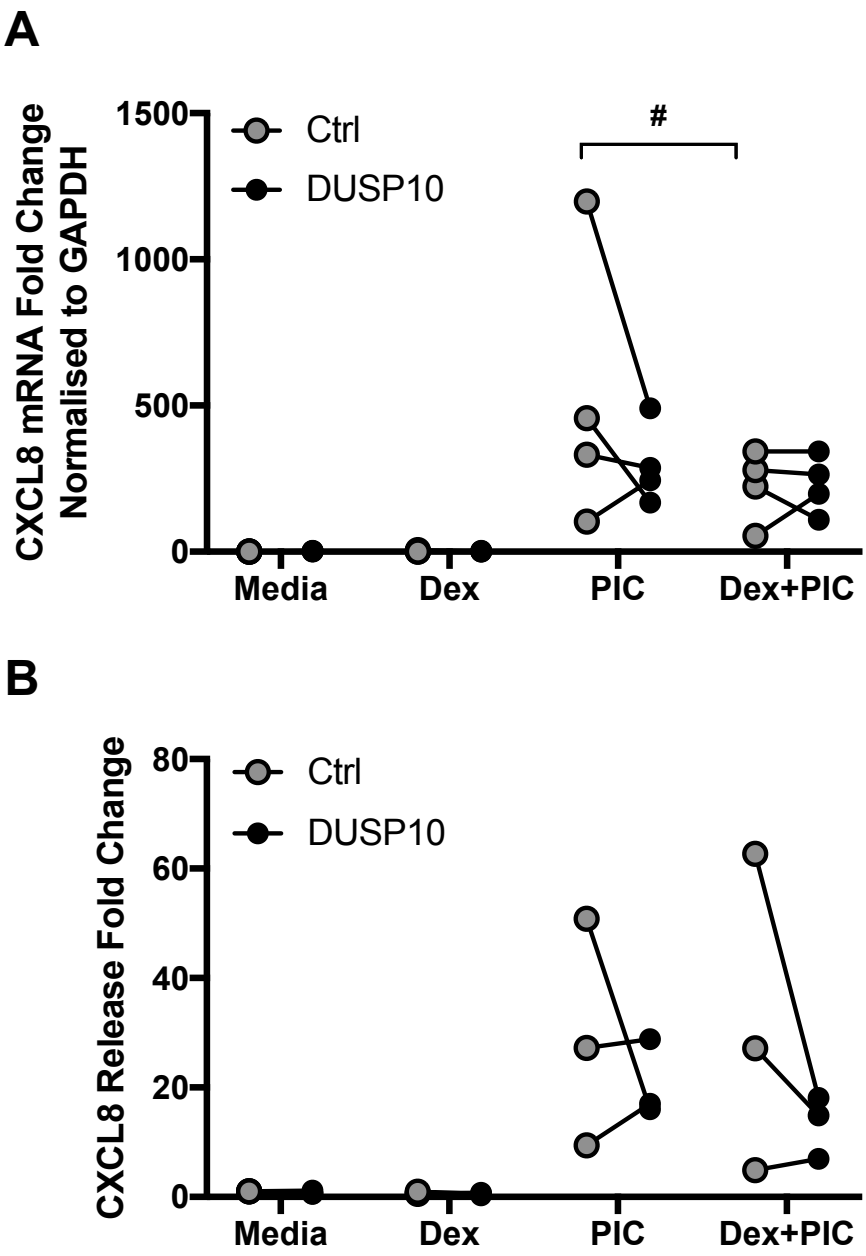

Figure 4.16 Dexamethasone treatment does not reduce CXCL8 production in response to poly(I:C).

PBECs were treated with DUSP10 siRNA (DUSP10) or control siRNA (Ctrl) (100 nM) for 48 h, prior to stimulation with poly(I:C) (PIC) (25 μg/ml) and dexamethasone (Dex) (10 nM). qRT-PCR was performed for CXCL8 and a GAPDH control. Data shown are mean ± SEM of fold change normalised to GAPDH and media control, n=4 (A). Supernatant was collected and ELISA performed for CXCL8. Data shown are fold change compared to media control, n=3 (B). Significance between treatments is indicated by # $p \leq 0.05$, as measured by two-way ANOVA, Dunnett's post-test. Significance between control and DUSP10 siRNA was measured using two-way ANOVA, Sidak's post-test.

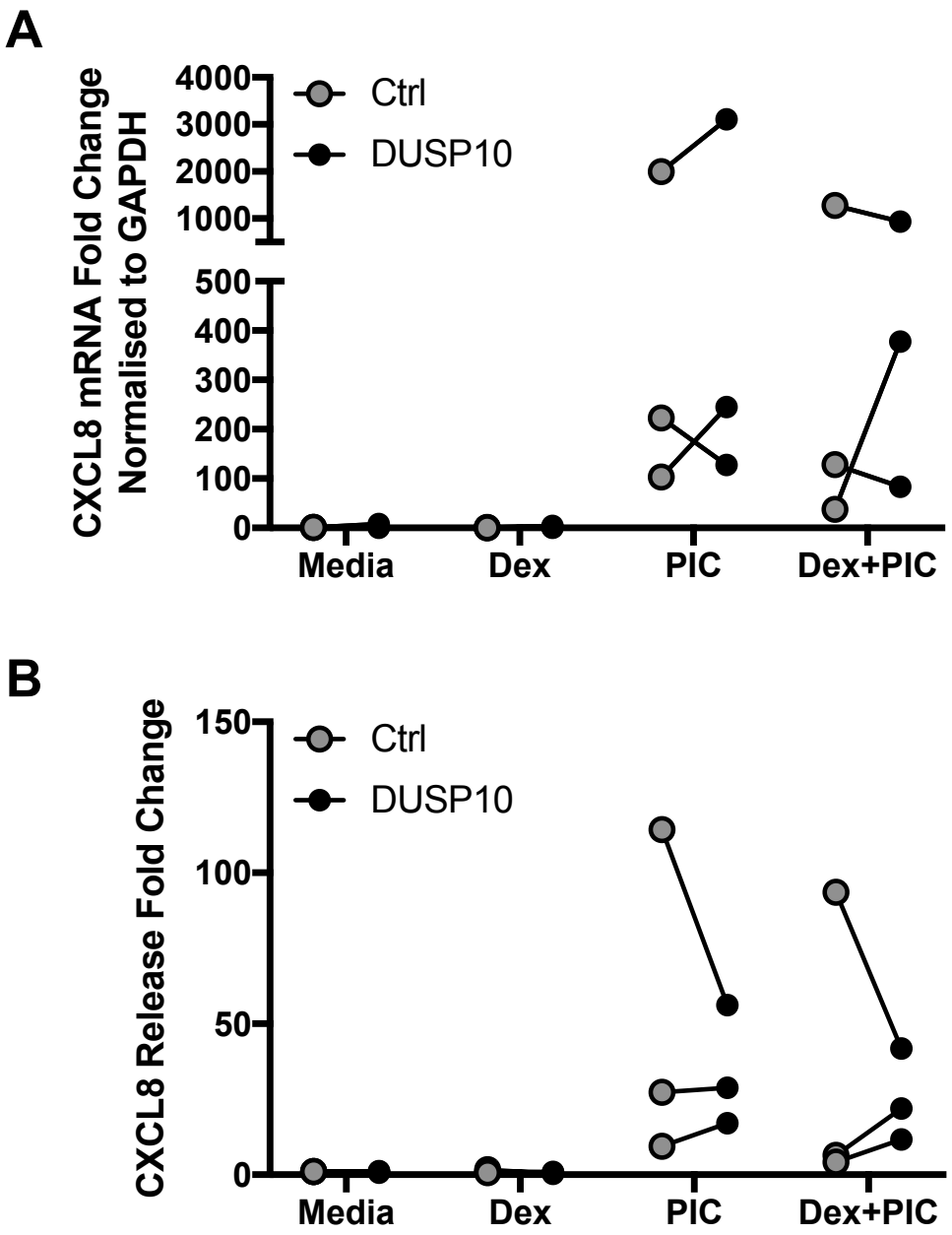

Figure 4.17 Dexamethasone treatment does not reduce CXCL8 production in response to poly(I:C) with 4 h pre-treatment.

PBECs were treated with DUSP10 siRNA (DUSP10) or control siRNA (Ctrl) (100 nM) for 48 h, then treated with dexamethasone (10 nM) for 4 h prior to stimulation with poly(I:C) (25 μg/ml). qRT-PCR was performed for CXCL8 and a GAPDH control. Data shown are mean ± SEM of fold change normalised to GAPDH and media control (A). Supernatant was collected and ELISA performed for CXCL8. Data shown are fold change compared to media control (B). Significance between treatments was measured by two-way ANOVA, Dunnett's post-test. Significance between control and DUSP10 siRNA was measured using two-way ANOVA, Sidak's post-test, n=3.

4.8 Discussion

4.8.1 Aims

The aims of this chapter were to determine whether DUSPs 1 and 10 had roles in regulating responses of airways epithelial cells to rhinoviral infection. Both DUSPs have previously been shown to have roles in regulating innate immune signalling pathways, in particular p38 and JNK, in response to infection with bacteria and other viruses. Their role in rhinoviral infection has not yet been studied, and it was deemed likely they would be important as the results presented in chapter three show p38 and JNK are strong inducers of inflammation in RV infection.

4.8.2 DUSP1

Although DUSP1 is the most well studied protein from the DUSP family, there is little known about its role in viral infection. Previous work has suggested it may have an important role in viral infection of the airway, as siRNA knock down in the epithelial cell line NCI-H292 increases production of IL-6 and CXCL8 in response to poly(I:C) stimulation (Golebski et al., 2015). In order to take this work further, and investigate the role of DUSP1 in rhinoviral infection of epithelial cells, siRNA knock down was attempted in PBECs and the epithelial cell lines BEAS-2B and HeLa Ohio.

Four different DUSP1 siRNA mixtures were utilised, with many different techniques attempted to optimise knock down of DUSP1. This included altering the concentrations of siRNA and Lipofectamine transfection reagent, incubation times, cell media, and cell confluencies. Although Lipofectamine is routinely used successfully in our group, other transfection reagents, shown to work well in hard to transfect cells, were also utilised. Under all conditions tested, DUSP1 knock down was unsuccessful. Knock down of DUSP1 in PBECs has never been published. Although it has been reported in BEAS-2B cells (S. V. Shah, Personal Communication), we were unable to replicate this.

Knock down of other genes using the same techniques were run alongside DUSP1 knock down attempts and were successful (data not shown), suggesting that the transfection was productive and the siRNA complexes were entering the cells. The knock down efficiency was measured at the mRNA level, as well as the protein level, and no differences were found. As the data presented in chapter three shows low levels of DUSP1 mRNA are constitutively expressed, the inability to knock DUSP1 expression

down in unlikely to be due to the protein having a long half-life. One possibility is that loss of DUSP1 protein is fatal, and any cells with a successful knock down died. DUSP1 has previously been found to inhibit apoptosis (MagiGalluzzi et al., 1997). Although no significant cell death was observed by eye when highly confluent monolayers, 80-90%, were transfected, when lower confluencies, 60-70%, were used, a large amount of cell death was seen. However, this was seen with both DUSP1 and control siRNA treatments, suggesting it was due to the transfection itself rather than the loss of DUSP1. Another common reason for inefficient knock down is due to secondary structures in the mRNA blocking siRNA binding (Bohula et al., 2003, Holen et al., 2002). However, the utilisation of four different siRNA mixtures, two of which were pools containing three or four different sequences, means a wide range of target sites were included.

In future, the role of DUSP1 in rhinoviral infection could be determined by other means, for example by over expressing DUSP1 in an airway epithelial cell line, or utilising DUSP1 knock out mice. Mice cannot be infected with major group strains of rhinovirus as they do not express the receptor ICAM-1. Infection models utilising minor group strains have been reported, but replication of the virus is variable (Newcomb et al., 2008). A more promising method could be to generate knock out human epithelial cells using CRISPR-Cas9 technology. CRISPR-Cas9 has been widely used to make knock outs in cell lines, and has been recently reported to have worked in primary nasal epithelial cells (Chu et al., 2015).

4.8.3 DUSP10 and Viral Replication

Knock down of DUSP10 protein in PBECs was successful, with around 80% of the protein removed. This allowed the investigation into the role of DUSP10 in rhinoviral infection of PBECs. Loss of DUSP10 did not affect replication of rhinovirus or production of IFN-β. This is in contrast to the results of a study from 2015, where DUSP10 knock out mice had much lower levels of influenza replication, due to an increase in IFN levels (James et al., 2015). The difference between these two studies could be due to DUSP10 having different roles in response to different viruses, for example both viruses have very different replication cycles and influenza is thought to signal through TLR7 more than TLR3 (Diebold et al., 2004). However, the James et al. paper also stimulated BMDMs from DUSP10 knock out mice with poly(I:C) and found an increase in IFN production; whereas in this study, there was no difference in IFN-β levels in PBECs in response to

poly(I:C) between cells treated with control or DUSP10 siRNA. This could be due to the difference in cell types, the influenza paper examining murine BMDMs rather than human epithelial cells. DUSP10 may have specific roles in different cell types, as has been suggested for DUSP1 (Zhang et al., 2015, Zhao et al., 2017). It could also be a species dependent phenotype, it would be interesting to determine the effect of DUSP10 knock down on influenza replication in human PBECs.

IFN-β mRNA levels were low in response to infection with RV or stimulation with poly(I:C), making any differences caused by DUSP10 knock down difficult to determine. However, DUSP10 knock down also caused no change in the level of CCL5, an interferon-stimulated gene, in response to RV and caused a significant downregulation in response to poly(I:C). This indicates that DUSP10 knock down is not upregulating ISG expression, and thus IFN production, in response to rhinoviral infection. Consequently, investigation into the effect of DUSP10 knock down on other IFNs may not be worthwhile. The significant decrease in CCL5 production in DUSP10 knock down cells seen in response to poly(I:C) is likely due to the cell death observed in cells treated with DUSP10 siRNA (discussed below), rather than a positive regulatory role for DUSP10 in CCL5 production, although other DUSPs have been shown to have positive regulatory roles by acting as scaffold proteins (Ju et al., 2016, Takagaki et al., 2004, Zama et al., 2002). One study has previously suggested that DUSP10 can act as a scaffold protein for ERK signalling in the fibroblast cell line COS-7 (Nomura et al., 2012).

4.8.4 The Role of DUSP10 in Cell Viability

DUSP10 knock down caused cell death in around 40% of the PBEC monolayer. DUSPs have been shown to have roles in cell-cycle pathways, with many of them implicated in several cancers. DUSP10 itself is upregulated in tumours taken from colon cancers and glioblastomas, and an SNP in the DUSP10 gene is associated with colorectal cancer (Benavides-Serrato et al., 2014, Nomura et al., 2012, Zhang et al., 2014). However, it seems that DUSP10 has a protective role in cancer, with overexpression of DUSP10 decreasing proliferation of an adenocarcinoma cell line, BxPC3 (He et al., 2014). Some cancer therapies upregulate DUSP10 expression, contributing to the anti-tumour effect through inactivating p38 (Krishnan et al., 2007, Nonn et al., 2006). Similarly to this study, MacKeigan et al. performed an siRNA screen for proteins important for survival of HeLa cells, and found that siRNA knock down of DUSP10 reduced cell survival (MacKeigan et

al., 2005). This demonstrates that DUSP10 has an important role in regulating the cell cycle, and knocking down its expression may be detrimental to the cell. This is supported by the fact that DUSP10 is phosphorylated and stabilised by mTORC2, an important regulator of cell growth (Benavides-Serrato et al., 2014). The role of DUSP10 in the cell cycle is thought to be through regulation of the MAPKs (He et al., 2014). JNK is known to be involved in cell growth, as inhibition of the protein leads to cell cycle arrest (Takahashi et al., 2013). However, DUSP10 knock down did not affect activation of the MAPKs. Therefore, more work is necessary to investigate how loss of DUSP10 is causing cell death, the first step being to determine the type of cell death: apoptosis, necrosis or pyroptosis (discussed below).

The MAPKs also have roles in regulating tight junctions between the epithelial cells. Inhibition of either ERK or JNK has been shown to prevent tight junction disassembly in response to various stimuli (Cohen et al., 2010, Petecchia et al., 2012). Disruption of tight junctions is another possible mechanism by which loss of DUSP10 leads to cell death; DUSP10 knock down increasing MAPK activity leading to the loss of tight junctions. This has implications in rhinoviral infection, as infection also leads to disruption of tight junctions (Sajjan et al., 2008, Yeo and Jang, 2010). Rhinoviral infection causes significant damage to the airway epithelium (Bossios et al., 2005, Wark et al., 2002). The role of DUSP10 in regulation of the cell cycle or tight junctions may be important in recovery of the epithelium after infection.

4.8.5 The Role of DUSP10 in Inflammatory Cytokine Release

DUSP10 knock down did not affect inflammatory cytokine production in response to infection with either strain of rhinovirus, or poly(I:C) stimulation, suggesting that DUSP10 is not negatively regulating the TLR3 signalling pathway in PBECs. It is possible that there could be redundancy between the DUSP proteins, with another protein compensating for the loss of DUSP10. Preliminary data suggests that DUSP10 knock down does not affect the mRNA expression of DUSP1 (data not shown), but other studies have found altered expression of other DUSPs, including DUSPs 2 and 4, in response to loss of DUSP1 (Maier et al., 2007). It could also be due to this being a knock down rather than a knock out. Small amounts of DUSP10 protein may be enough to regulate signalling pathways, knock out models would have to be utilised to investigate this further. However, the effect of knock down on cell viability suggests enough protein

has been removed for the effect to be seen. The variability between donors also makes results difficult to interpret, and greater n numbers would be necessary to make a firm conclusion. These were not included in this study as the results did not suggest DUSP10 caused a difference in CXCL8 expression in RV infection.

In response to IL-1β stimulation, DUSP10 knock down increased CXCL8 mRNA production and protein release, suggesting an important role in the regulation of inflammatory cytokine release. In order to gain a wider picture of the effect of DUSP10 knock down on IL-1β signalling, a cytokine array was used. Although this technique is only semi-quantitative, and only includes samples from one donor, it gives an indication of which cytokines are good candidates to follow up in future. IL-1β stimulation of PBECs induced release of several cytokines. DUSP10 knock down did not affect the release of many of these cytokines, unless the results were normalised to cell number, as observed previously. This array confirmed the upregulation of CXCL8 in DUSP10 knock down cells, and showed increased release of another neutrophil chemoattractant, CXCL1 (Nagarkar et al., 2009). This endorses the anti-inflammatory function of DUSP10, and suggests an important role in regulating damaging neutrophilia in the airway. Other cytokines with differential expression in DUSP10 knock down cells included IL-1β itself and CXCL10, each of which are discussed in more detail below.

The greatest difference between control and DUSP10 siRNA treated cells was in IL-1β release. This implies that, in addition to regulating a pathway that leads to production of inflammatory cytokines, DUSP10 is also regulating a pathway specific to IL-1β production. IL-1β is produced in response to infection or stimulation by transcription of pro-IL-1β, which is then cleaved into the active protein by the inflammasome. The inflammasome consists of a sensor protein, the adaptor protein ASC, and caspase-1. There are several different sensor proteins, most being members of the Nod-like receptor (NLR) family. Rhinoviral infection of PBECs is known to induce IL-1β release in a caspase-1 dependent manner (Piper et al., 2013). Triantafilou et al. demonstrated that the RV protein 2B induces inflammasome activation in PBECs by forming pores in the endoplasmic reticulum and Golgi, releasing Ca^{2+} ions (Triantafilou et al., 2013). The NLRs, NLRP3 and NLRC5 sense the changes in ion concentration, leading to activation of caspase-1, and IL-1β production. As IL-1β is differentially expressed in DUSP10 knock down cells, one possibility is that DUSP10 is regulating inflammasome activation.

DUSP10 has previously been linked to the inflammasome adaptor, ASC, as THP-1 cells treated with ASC shRNA had increased DUSP10 mRNA levels, suggesting that ASC supresses DUSP10 production (Taxman et al., 2011). The interplay between DUSP10 and the inflammasome requires further investigation, for example measuring levels of caspase-1 activation in DUSP10 knock down cells. The levels of IL-1β in DUSP10 knock down in response to rhinovirus should also be determined, to see whether this phenotype is a specific response to activation with IL-1β.

Prolonged stimulation of the inflammasome can lead to cell death through pyroptosis (Brough and Rothwell, 2007). Pyroptosis is a mechanism of cell death with aspects of both apoptosis and necrosis. It is highly inflammatory, resulting from cell lysis, unlike apoptosis, but is dependent on caspase-1 activation, unlike necrosis (Fink and Cookson, 2006). Infection of the neuroblastoma cell line SK-N-SH with the picornavirus, enterovirus-71 has been found to activate the inflammasome and lead to cell death through pyroptosis (Yogarajah et al., 2017). If DUSP10 does regulate inflammasome activation, pyroptosis could be another mechanism by which loss of DUSP10 leads to cell death. One way in which to investigate whether DUSP10 knock down induces pyroptosis is utilisation of the stain 7-aminoactinomycin D (7-AAD) which binds to extracellular DNA.

Although DUSP10 knock down increased production of IL-1β, it did not affect release of the receptor antagonist IL-1ra, nor did stimulation with IL-1β. The balance between IL-1β and IL-1ra is important in preventing excessive, damaging inflammatory responses. Stimuli that induce production of IL-1β generally also induce IL-1ra, and IL-1β stimulation has been shown to induce release of IL-1ra (Gabay et al., 1997). Experimental human RV16 infection showed a positive correlation between levels of IL-1β and IL-1ra in nasal lavage (de Kluijver et al., 2003). The fact that IL-1ra is not upregulated by DUSP10 knock down, but IL-1β is, suggests that loss of DUSP10 leads to an excessive IL-1β mediated inflammatory response.

CXCL10 was released in response to IL-1β stimulation. CXCL10 has been shown to be induced in PBECs by RV16 infection, and levels in nasal lavage correlate with symptom severity, viral titre and lymphocyte number (Spurrell et al., 2005). It has also been associated with asthma and COPD, with cells taken from these patients expressing higher protein levels (Miotto et al., 2001, Saetta et al., 2002). CXCL10 has been shown

to be an important chemoattractant for Th1 cells (Hyun et al., 2005, Sallusto et al., 1998, Xie et al., 2003). Knock down of DUSP10 decreased release of CXCL10 in response to IL-1β stimulation. This suggests that DUSP10 positively regulates its release, and could have an important role in regulating the Th1/Th2 axis in airway inflammation. The balance between Th1 and Th2 responses is an important component of asthma exacerbations, and therapies targeting this have had promising results in recent clinical trials (Corren et al., 2017). Inhibition of the ERK or p38 pathways, using inhibitors U0126, PD98059 and SB203580, has previously been shown to increase CXCL10 production in PBECs in response to RV16 infection (Zaheer et al., 2009). This increase in CXCL10 transcription was due to increased activity of the transcription factor IRF1. IL-1 signalling has previously been found to induce IRF1 activation and production of CXCL10 in primary human astrocytes (Harikumar et al., 2014). The decrease in CXCL10 in cells treated with DUSP10 siRNA suggests that DUSP10 is negatively regulating this pathway. It would be important to determine the effect of DUSP10 knock down on the activation of IRF1 in PBECs, as this could help identify the point at which DUSP10 is acting.

Interestingly, IL-1β stimulation was found to decrease production of CXCL12. CXCL12 has an important role in the neutrophil life cycle, regulating their release from the bone marrow, and their return once senescent (Martin et al., 2003, Petit et al., 2002, Suratt et al., 2004). CXCL12 has also been shown to have a role in the lung. Experimental RV16 infection increased CXCL12 levels in nasal secretions (Branigan et al., 2014), and blocking CXCL12 signalling reduced neutrophil numbers in the murine lung in response to LPS (Petty et al., 2007). In 2011, Yamanda, et al. demonstrated that CXCL12 expression increased lung neutrophil numbers by acting as a retention signal, rather than a chemoattractant, and protected neutrophils from apoptosis (Yamada et al., 2011). This implies that IL-1β signalling in PBECs reduces the production of a neutrophil retention signal, perhaps as a negative feedback mechanism to reduce the amount of damage done by inflammation.

The fact that DUSP10 regulated the response to IL-1β, but not to RV infection suggests that DUSP10 has a role in regulating part of the pathway induced by IL-1β which is not involved in the TLR3 signalling cascade. For example, IL-1β activates the MyD88 pathway, which involves phosphorylation of IRAK1 and/or IRAK2, which are not involved in the TLR3-TRIF pathway. DUSP10 could be dephosphorylating these IRAK proteins,

blocking this signalling pathway. However, activation of p38 and JNK, which are downstream of the IRAK proteins, in response to IL-1β was unaffected by DUSP10 knock down. DUSP10 could also be regulating these pathways in ways other than dephosphorylation; DUSP16 has been demonstrated to negatively regulate ERK by anchoring it in the cytoplasm (Masuda et al., 2010).

These findings, and the potential sites at which DUSP10 may be acting, are summarised in figure 4.18.

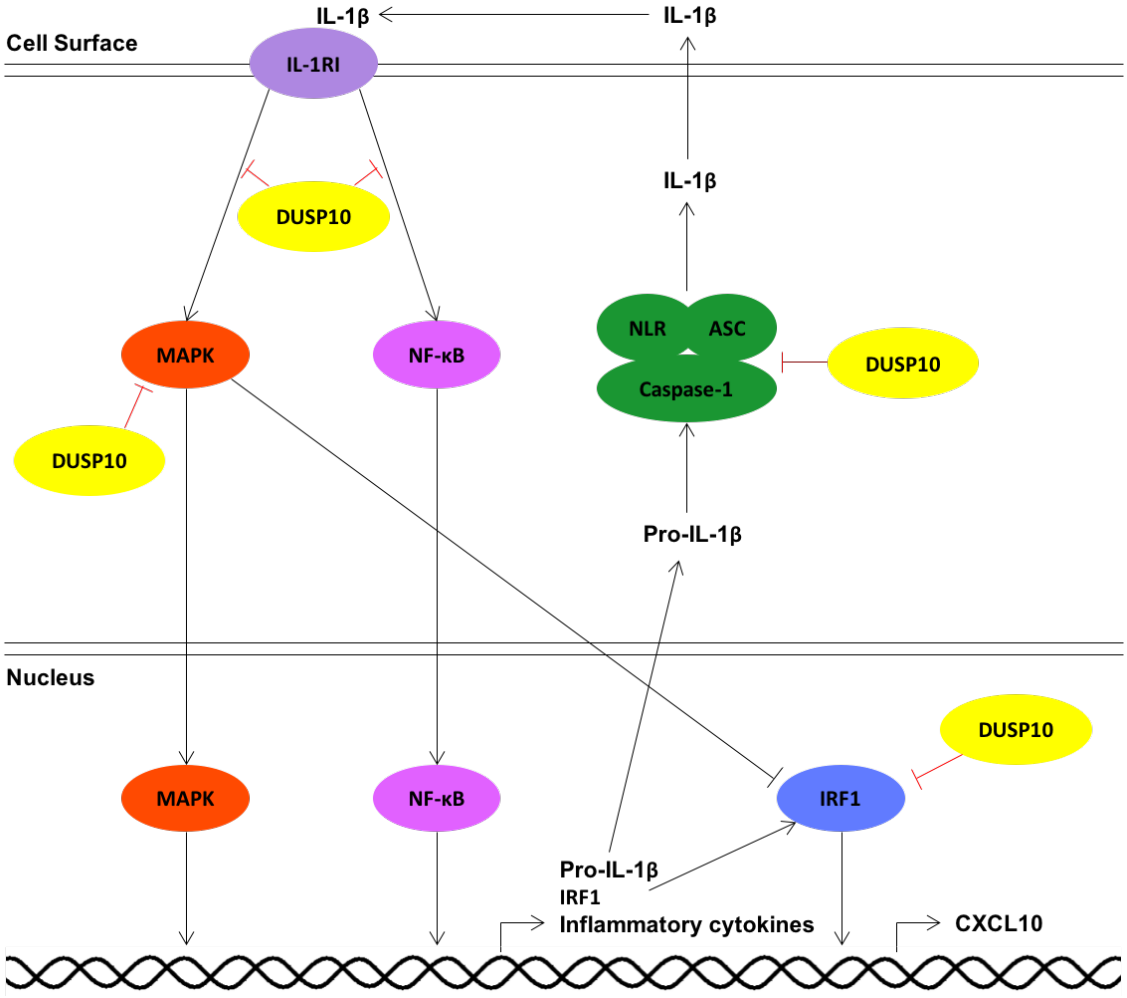

Figure 4.18 The Role of DUSP10 in IL-1β signalling in PBECs.

IL-1β binds IL-1RI on the epithelial surface triggering activation of the MAPK and NF-κB pathways. These pathways lead to the production of pro-IL-1β, IRF1 and inflammatory cytokines. Pro-IL-1β is cleaved to mature IL-1β by the inflammasome, composed of an NLR sensor, ASC and caspase-1. Mature IL-1β is secreted. IRF1 induces transcription of cytokines, including CXCL10, this is negatively regulated by the MAPKs. Red lines indicate potential sites at which DUSP10 may be negatively regulating these pathways.

Co-stimulating PBECs with RV and IL-1β dramatically potentiated the response to RV alone. As monocytes release IL-1β in response to infection with RV (Ganesan et al., 2016); monocytes present in the infected airway would potentiate the response of PBECs to rhinoviral infection. The response was further increased by loss of DUSP10. This suggests that DUSP10 would have a role in the response to rhinoviral infection in the airway; RV inducing IL-1β release by monocytes, which would act on epithelial cells to induce cytokine release, which is regulated by DUSP10. As discussed above, the results obtained using a cytokine array suggest DUSP10 also has an important role in regulating release of IL-1β in PBECs, therefore loss of DUSP10 would further potentiate the inflammatory response.

4.8.6 The Role of DUSP10 in Steroid Treatment

Dexamethasone treatment is known to reduce the production of inflammatory cytokines in response to a range of stimuli (Abraham et al., 2006, Goleva et al., 2013, Harada et al., 2011, Keranen et al., 2017, Newton et al., 2010, Papi et al., 2013, Rahman et al., 2016, Rider et al., 2013, Shah et al., 2016a). In response to poly(I:C), dexamethasone reduces the production of the inflammatory cytokines TSLP and CXCL8 in PBECs and BEAS-2Bs respectively (Harada et al., 2011, Rider et al., 2013). This is in contrast to the results found in the present study, as dexamethasone treatment did not have much of an effect on CXCL8 production or release, with or without a 4 h pre-treatment. This is unexpected as dexamethasone is a commonly used steroid with well documented anti-inflammatory effects. In future, this experiment could be repeated using a higher concentration of dexamethasone than 10 nM. The dose of 10 nM was selected as the higher concentration of 100 nM led to cell death in PBECs (Figure 3.23), however inclusion of a dose response curve could indicate whether dexamethasone was having an effect. Many of the papers discussed above used 1 μM dexamethasone, including those studying PBECs, the effect of this on cell death was not presented (Harada et al., 2011, Papi et al., 2013). If this experiment was to be repeated, the inclusion of a positive control, to ensure dexamethasone was working, would be useful. For example, reductions in cytokine release in response to LPS by dexamethasone have been widely published (Abraham et al., 2006, Keranen et al., 2017, Newton et al., 2010, Papi et al., 2013, Shah et al., 2016a). Therefore, the inclusion of cells treated with LPS alone and in combination with dexamethasone would indicate whether the

dexamethasone was having an effect. This could also be repeated using a different steroid, for example budesonide has been shown to reduce CXCL8 production in BEAS-2B cells and PBECs in response to RV infection (Skevaki et al., 2009).

Once steroid treatment has been established to reduce inflammatory cytokine release, the effect of DUSP10 knock down can be investigated. Although there is a slight trend towards less CXCL8 mRNA with dexamethasone treatment in cells treated with control siRNA, it is small and variable. This makes it difficult to conclude whether the loss of DUSP10 affects it.

4.8.7 Conclusions

The work presented in this chapter suggests that DUSP10 has a regulatory role in PBECs. Although not directly regulating the release of inflammatory cytokines or interferons to rhinoviral infection of PBECs, DUSP10 does regulate cytokine production in response to IL-1β, an important molecule in inflammation. DUSP10 knock down increased secretion of two neutrophil chemoattractants, CXCL8 and CXCL1, in response to IL-1β signalling, suggesting it may contribute to the regulation of neutrophil influx into the lung, a damaging response to rhinoviral infection. DUSP10 may also play a role in regulating the Th1/Th2 axis, with decreased secretion of a Th1 cytokine in response to IL-1β in cells treated with DUSP10 siRNA. DUSP10 also negatively regulates the release of IL-1β itself, suggesting a possible role in inflammasome regulation.

These data demonstrate that DUSP10 has an important role in regulating inflammatory cytokine release in response to IL-1β stimulation. Previous work has demonstrated an important role for IL-1β in cross-talk between macrophages and epithelial cells in rhinoviral infection (Stokes et al., 2011), and IL-1β was found to potentiate the inflammatory response to RV. Thus, DUSP10 may have a role in regulating inflammation in response to rhinoviral infection of the airway.

5 Chapter Five: Rhinoviral Infection of Monocytes and Macrophages

5.1 Introduction

During rhinoviral infection, many different cell types are present in the inflamed airway. Therefore, to understand the pathogenesis of rhinoviral infections, it may be important to study the responses of these different cells to RV and their interactions with each other. The results presented in chapter four suggest an important role for DUSP10 in the response to IL-1β. An important source of IL-1β in the inflamed airway are macrophages and monocytes (Netea et al., 2009). Therefore, in this chapter, I will be focusing on the roles of macrophages and monocytes in rhinoviral infection, and the role of DUSP10 in their interactions with PBECs.

Resident macrophages are present in the airway, capable of phagocytosing and killing pathogens, and secreting inflammatory mediators. During an immune response, monocytes are recruited from the blood vessels into the respiratory tract by chemotactic cytokines. These monocytes play important roles in pathogen control by secreting inflammatory cytokines and nitric oxide (Jagannath et al., 1998, Jakubzick et al., 2013). They have a greater ability to release some pro-inflammatory mediators, such as IL-1β, than macrophages (Netea et al., 2009). These infiltrating monocytes can also differentiate into recruited macrophages, including M1 cells, which release pro-inflammatory mediators, and M2 cells which are thought to have an anti-inflammatory resolution role and play a part in tissue remodelling (Van den Bossche et al., 2012, Verreck et al., 2004). Monocytes can also differentiate into antigen presenting cells and migrate to the lymph nodes (Jakubzick et al., 2013).

Some of the cytokines released by macrophages and monocytes can act on epithelial cells and potentiate their response. In vitro co-culture models have demonstrated that this cooperative signalling between epithelial cells and monocytes can dramatically exacerbate the inflammatory response to many different stimuli (Chaudhuri et al., 2010, Haller et al., 2000, Ishii et al., 2005, Morris et al., 2006, Stokes et al., 2011, Tsutsumi-Ishii and Nagaoka, 2003). Previous work from our group has shown that addition of monocytes to BEAS-2B cultures considerably increases the release of CXCL8 in response to RV1B infection, when the monocytes are activated by LPS (Stokes et al., 2011). Co-

cultures of primary monocytes and PBECs have also been found to have increased production of CCL2 and CXCL10 in response to RV16 infection (Korpi-Steiner et al., 2010). IL-1β has been shown to be particularly important in this signalling network, as blocking IL-1β signalling using blocking antibodies or the IL-1 receptor antagonist (IL-1Ra) inhibited this increased cytokine generation (Chaudhuri et al., 2010, Morris et al., 2005, Stokes et al., 2011, Tsutsumi-Ishii and Nagaoka, 2003).

Monocytes produce a wide range of inflammatory cytokines in response to stimulation with rhinovirus, including: IL-1β, IL-6, CXCL8, CXCL10, and TNFα (Ganesan et al., 2016, Gern et al., 1994, Johnston et al., 1997, Karta et al., 2014, Saba et al., 2014). Much of this expression is not affected by UV inactivation of the virus, suggesting replication is unnecessary for the response. Indeed, many studies suggest that rhinovirus does not replicate within monocytes or macrophages. In 1994, Gern et al. infected macrophages taken from BAL with RV16, they found viral RNA within the macrophages, suggesting the virus was entering the cells, but the viral titres declined over time (Gern et al., 1994). Saba et al. found a similar result in response to infection with the minor group rhinovirus, RV1B, with bone marrow macrophages, and monocytes isolated from BAL both showing no increase in viral titre over 25 h (Saba et al., 2014). Although rhinovirus does not seem to replicate in primary monocytic cells, some replication of major group rhinoviruses, RV9 and RV16, is detectable in the monocyte cell line THP-1 (Johnston et al., 1997, Laza-Stanca et al., 2006).

5.1.1 Aims

The results summarised in chapter four show that DUSP10 negatively regulates the inflammatory response to IL-1β stimulation of epithelial cells. IL-1β has been shown to be an important molecule in the cross-talk between monocytes/macrophages and epithelial cells in response to rhinoviral infection (Stokes et al., 2011). Therefore, it was hypothesised that DUSP10 would have an important role in the response to rhinovirus in the airway, where epithelial cells, monocytes and macrophages are all present.

The aim of this chapter was to determine the role of DUSP10 in rhinoviral infection in a multi-cellular environment. The first aim was to determine whether rhinovirus replicated in monocytes or macrophages and characterise their response to the virus. The second aim was to determine the effect on PBECs of factors released by

macrophages or monocytes in response to rhinovirus, and the role of DUSP10 in this response.

5.2 Rhinoviral Replication in Monocytes and Macrophages

As discussed in section 5.1, macrophages and monocytes have previously been found to respond to rhinoviral infection, but replication of the virus in these cells has not been detected. Therefore, replication of both serotypes of rhinovirus was measured in monocytes and monocyte-derived macrophages (MDMs).

Rhinovirus did not replicate well in MDMs cultured for either 7 or 14 days, with very low copy numbers of rhinoviral RNA per μg, although these data are preliminary (Figure 5.1). In monocytes, rhinovirus replication was more productive, with both RV1B and RV16 reaching around 1,500 copies per μg by 48 h when cells were infected in 0% serum media (Figure 5.2 A). However, when monocytes were infected in 2% serum media rhinovirus did not replicate well, with the highest level reached 136 copies per μg in 48 h RV1B infection (Figure 5.2 B).

This was confirmed by measuring the number of virions released by monocytes infected in 0% and 2% media, using the $TCID_{50}$ assay. In 0% media RV1B reached a $TCID_{50}$/ml of 35933.33 by 24 h, and RV16 reached the slightly lower titre of 250633.33 (Figure 5.3 A). At 48 h this decreased, in contrast to the qRT-PCR results which showed higher titres at the later time point (Figure 5.2 A). In 2% media, the rhinoviral titres are much lower, only reaching 8000-9000 $TCID_{50}$/ml by 24 h, and again decreasing by 48 h post infection (Figure 5.3 B).

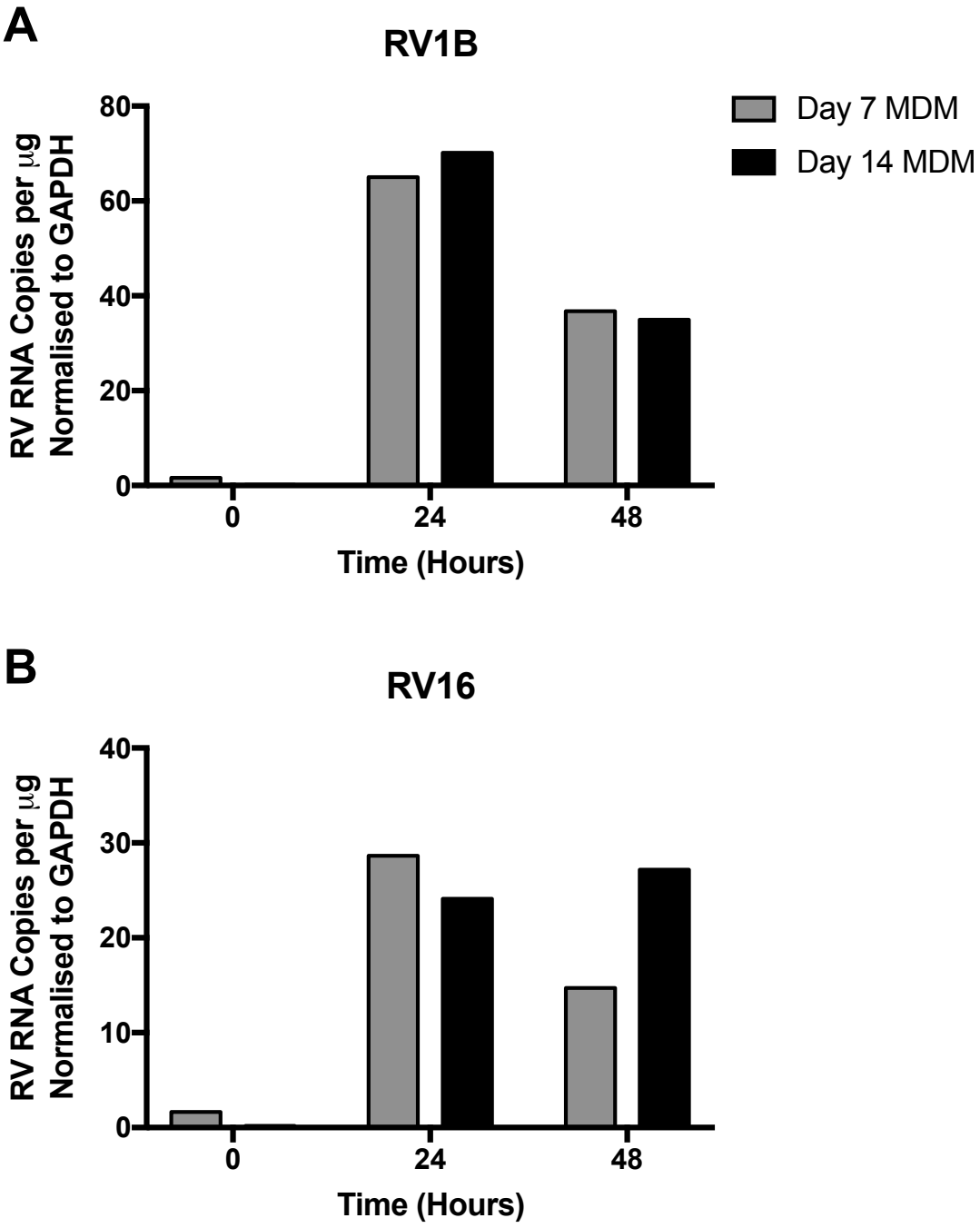

Figure 5.1 RV does not replicate well in MDMs.

MDMs were differentiated for 7 or 14 days prior to RV1B (MOI 4) (A) or RV16 (MOI 4) (B) infection in serum free media. RNA was collected at 24 and 48 h and qRT-PCR performed for RV RNA and a GAPDH control. Data shown normalised to the GAPDH control, n=1.

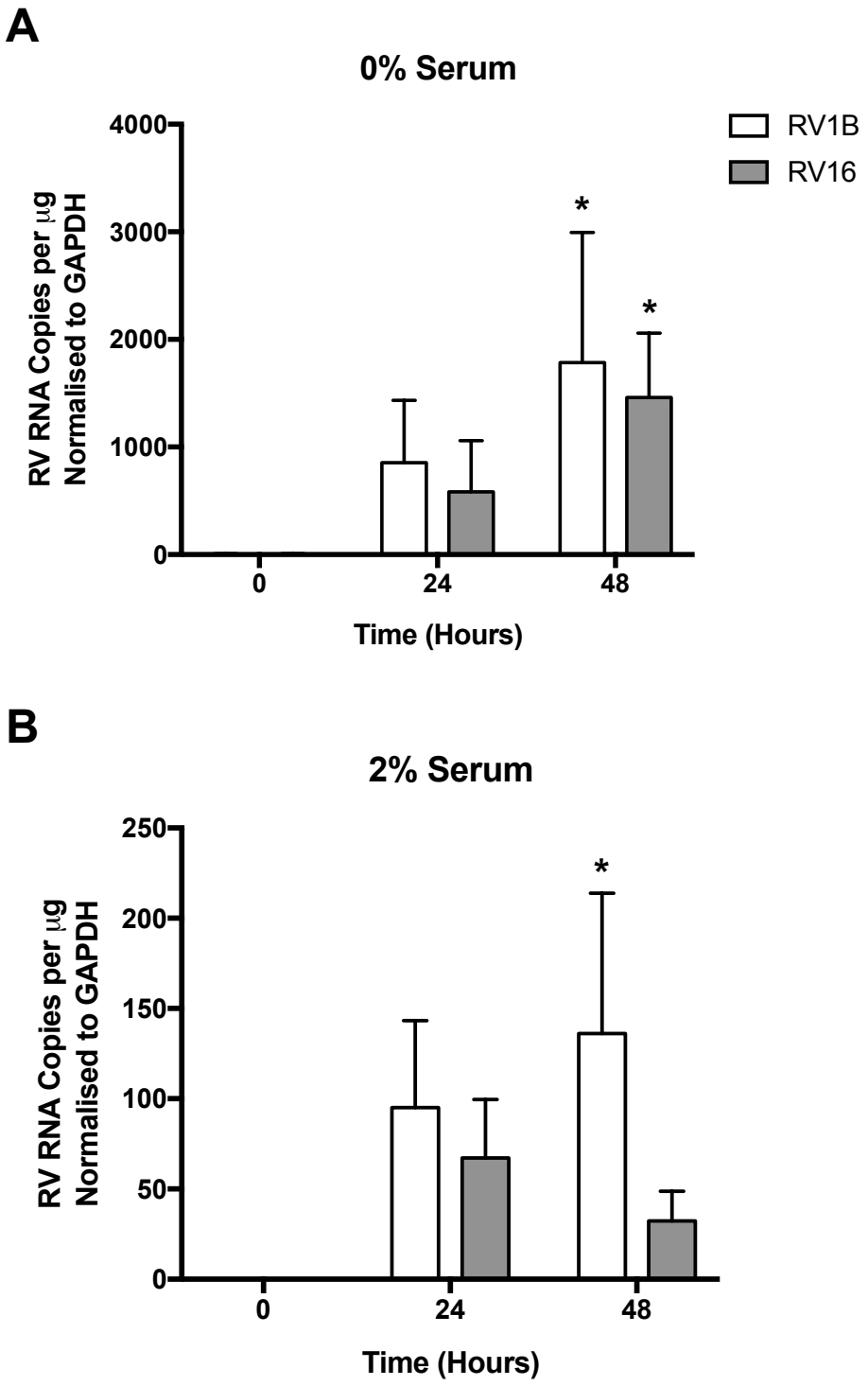

Figure 5.2 RV replicates in monocytes when no serum is present.

Monocytes were infected with RV1B (MOI 4) or RV16 (MOI 4) infection in 0%, n=3 (A) or 2% serum, n=4 (B). RNA was collected at 24 and 48 h and qRT-PCR performed for RV RNA and a GAPDH control. Data shown mean ± SEM normalised to the GAPDH control. Significance is indicated by * $p \leq 0.05$ compared to uninfected (0 h) control as measured by two-way ANOVA, Dunnett's post-test.

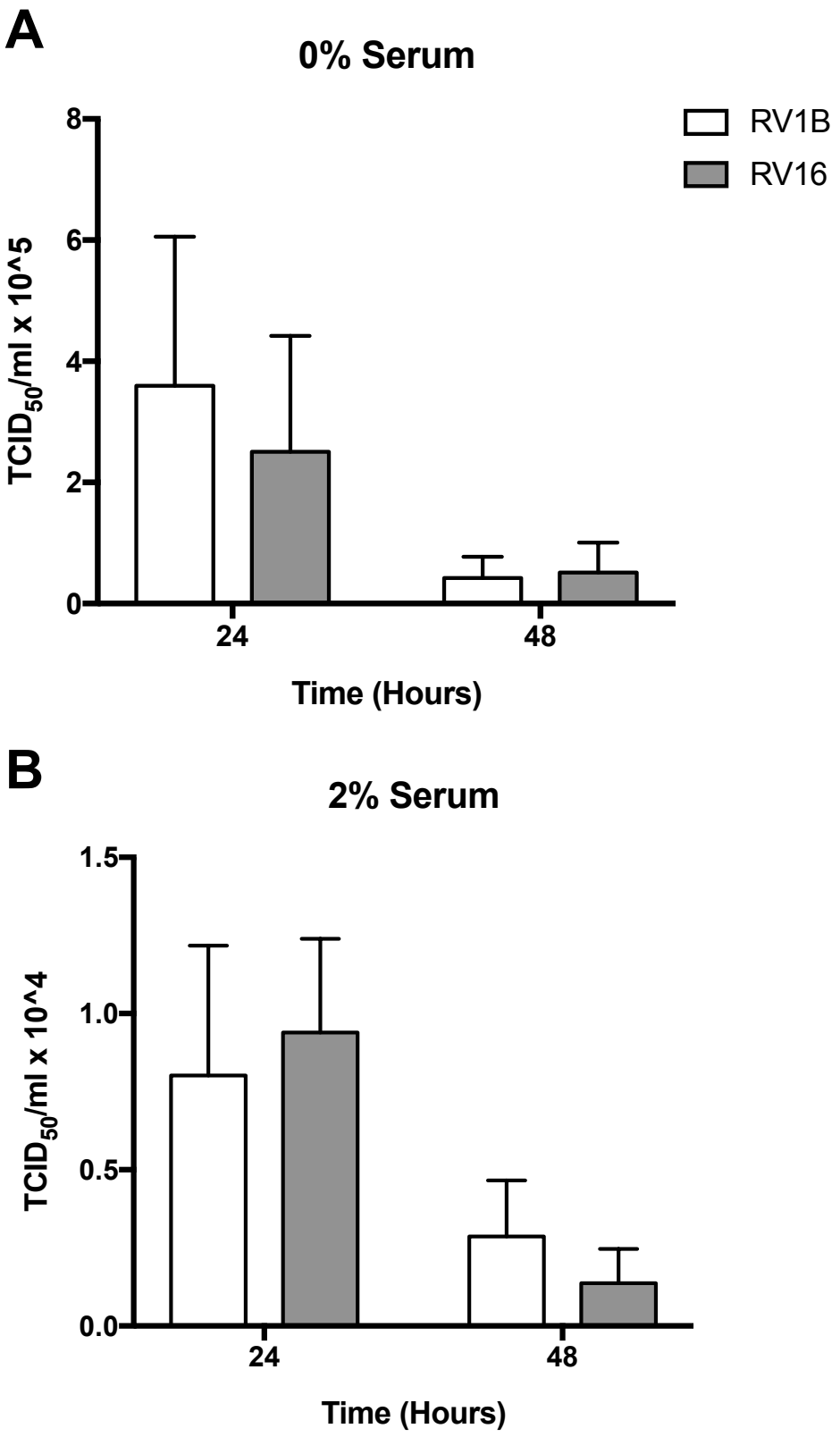

Figure 5.3 RV replicates in monocytes when no serum is present.

Monocytes were infected with RV1B (MOI 4) or RV16 (MOI 4) infection in 0% (A) or 2% serum (B). Supernatant was collected at 24 and 48 h and $TCID_{50}$ assay performed. Data shown mean ± SEM, n=3.

5.2.1 Cytokine Production in Monocytes in Response to RV Infection

As rhinovirus appeared to replicate more efficiently in monocytes, these cells were taken forward for investigation into cytokine release in response to rhinoviral infection. CXCL8 and IL-1β release in response to infection with RV1B and RV16 in both 0% and 2% media was measured by ELISA.

Monocytes released high amounts of CXCL8 at baseline, to comparable levels in both 0% and 2% media. The amount of CXCL8 released was not altered by infection with either RV1B or RV16 (Figure 5.4).

IL-1β was also released by monocytes at baseline, this was slightly lower in cells infected in 2% media than 0% media (Figure 5.5). In 0% media, infection with RV1B or RV16 did not alter the level of IL-1β released by monocytes. In 2% media, both RV1B and RV16 infection significantly increased IL-1β release at 24 h post infection, this was slightly higher in response to RV1B than RV16 (Figure 5.5 B). All samples were above the minimum level of detection.

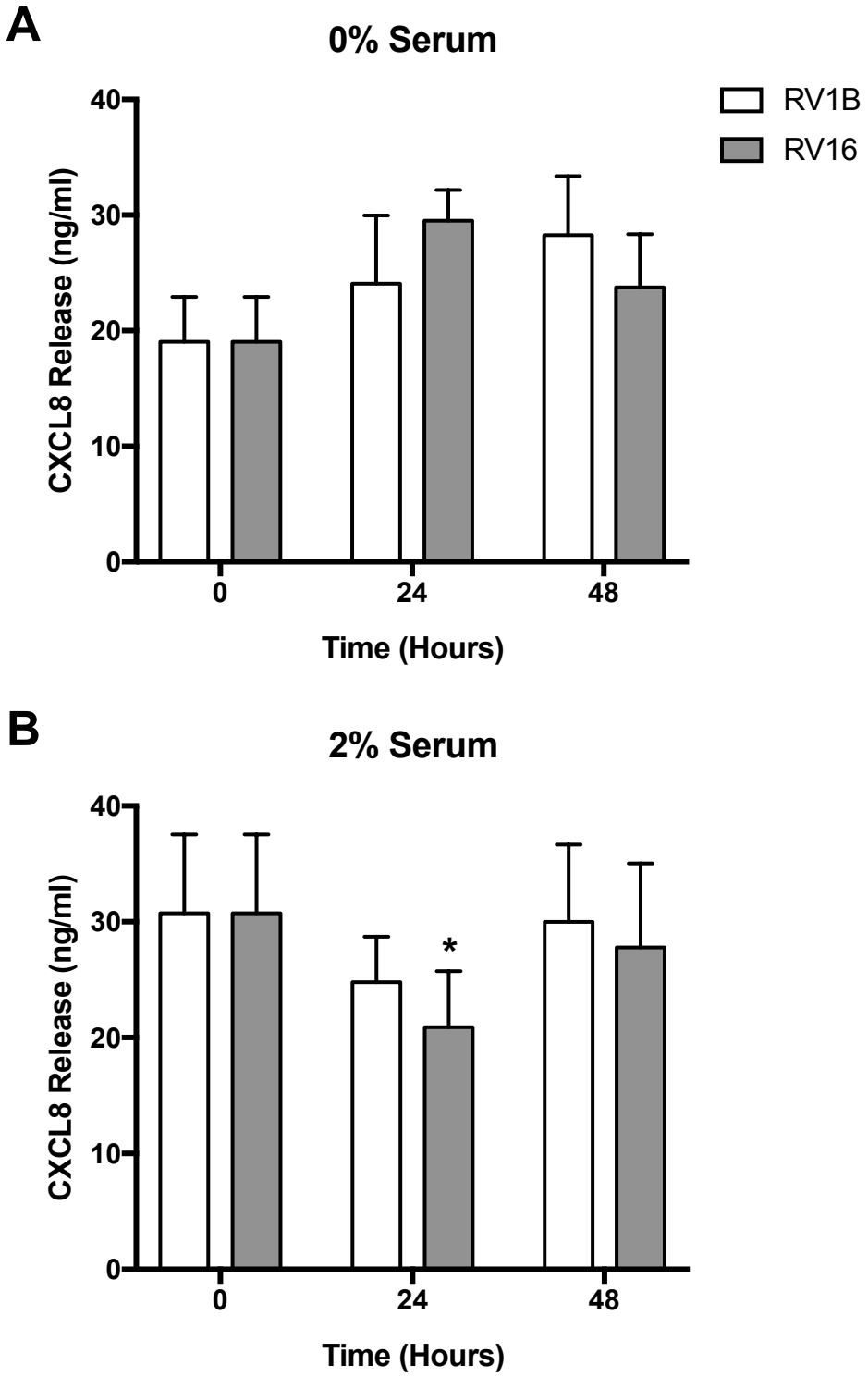

Figure 5.4 CXCL8 is constitutively released by monocytes in culture.

Monocytes were infected with RV1B (MOI 4) or RV16 (MOI 4) infection in 0% (A) or 2% serum (B). Supernatant was collected at 24 and 48 h and ELISA used to measure the release of CXCL8. Data shown are mean ± SEM, n=3. Significance is indicated by * $p \leq 0.05$ compared to uninfected (0 h) as measured by two-way ANOVA, Dunnett's post-test.

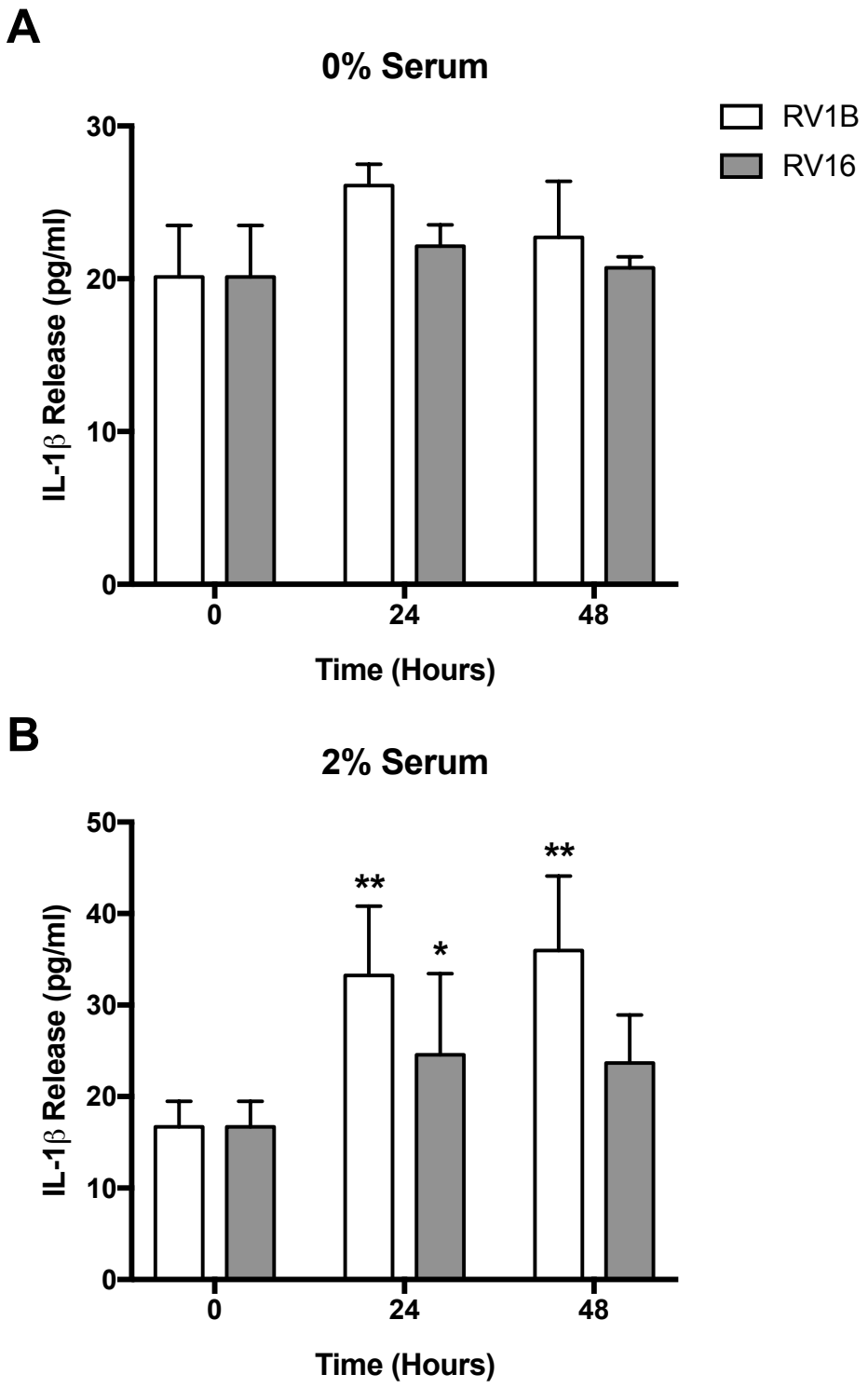

Figure 5.5 IL-1β is constitutively released by monocytes in culture.

Monocytes were infected with RV1B (MOI 4) or RV16 (MOI 4) infection in 0% (A) or 2% serum (B). Supernatant was collected at 24 and 48 h and ELISA used to measure the release of IL-1β. Data shown are mean ± SEM, n=3. Significance is indicated by * $p \leq 0.05$, ** $p \leq 0.01$ compared to uninfected (0 h) as measured by two-way ANOVA, Dunnett's post-test.

5.3 The Activation of PBECs by Monocytes in RV Infection

The cross-talk between monocytes and epithelial cells in response to rhinoviral infection was then investigated. Supernatant from infected monocytes was transferred to PBECs to determine whether factors released by monocytes, in response to rhinoviral infection, could activate epithelial cells. Monocytes were infected with RV and supernatant collected at 24 and 48 h. Supernatant was filtered to remove viral particles before diluting 1:2 with PBEC recovery media, hereafter called conditioned media. PBECs were pre-treated with DUSP10 or control siRNA before addition of conditioned media for 24 h, methodology is shown in figure 5.6 A. CXCL8 mRNA production and protein secretion was measured.

Transfer of supernatant from uninfected monocytes to PBECs caused some CXCL8 mRNA production and protein release (Figure 5.6 and 5.7). This level was not affected by infection of the monocytes with RV1B (Figure 5.6) or RV16 (Figure 5.7). CXCL8 mRNA production was consistently higher in DUSP10 knock down cells than cells treated with control siRNA, and this was statistically significant in RV16 infection (Figure 5.7). This effect was not seen by ELISA; however, the results were not normalised by cell number. The CXCL8 release measured by ELISA will include CXCL8 released by the monocytes, carried over in the conditioned media. However, mRNA expression data indicates that PBECs are producing CXCL8.

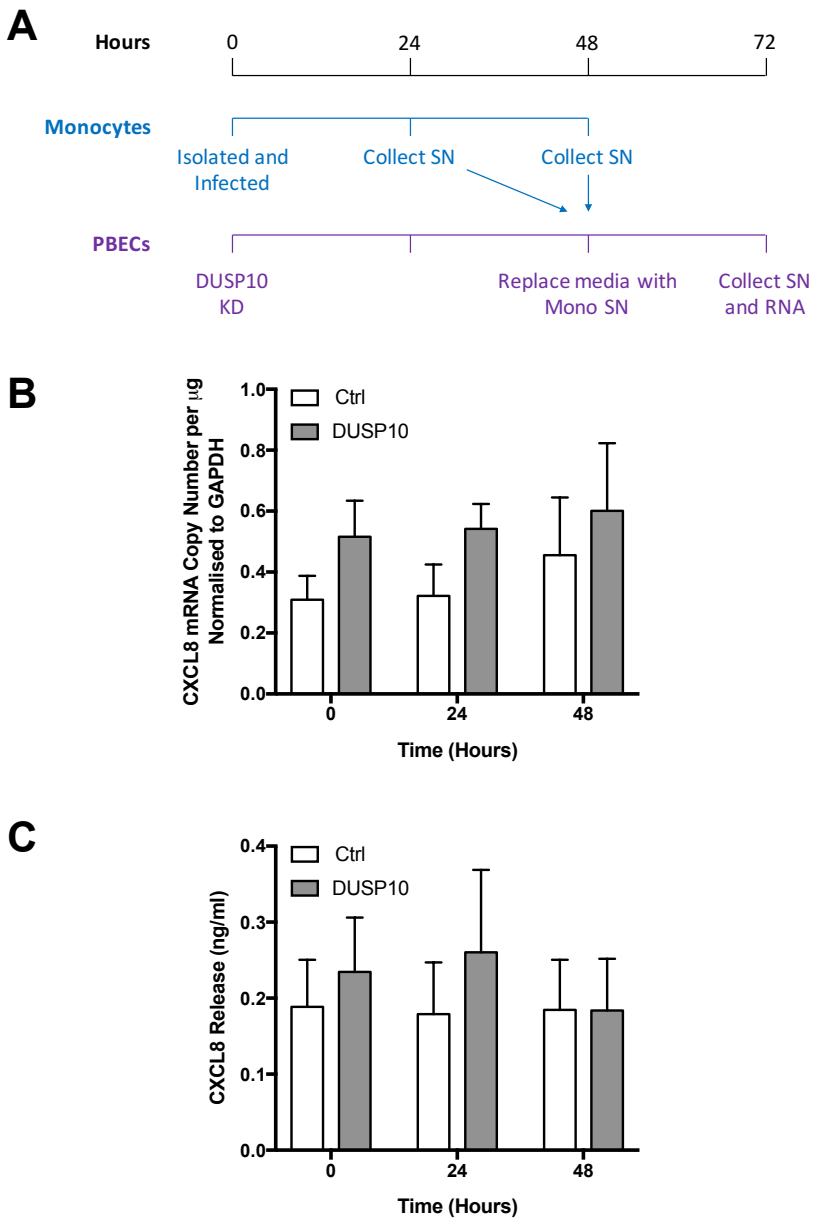

Figure 5.6 Supernatant from monocytes infected with RV1B can induce CXCL8 production in PBECs.

Monocytes were infected with RV1B (MOI 4) for 0, 24 or 48 h, in 0% serum, and supernatant collected. Supernatant was filtered and diluted 1:2 in recovery media before addition to PBECs pre-treated with DUSP10 or control (Ctrl) siRNA (100 nM) for 48 h. The time line of the experiment is shown in A. RNA was collected and qRT-PCR performed for CXCL8 and GAPDH. Data shown are mean ± SEM normalised to GAPDH control (B). Supernatant was collected and ELISA used to measure the release of CXCL8. Data shown are mean ± SEM (C). Significance between siRNA treatments was measured by two-way ANOVA, Sidak's post-test. Significance between time points was measured by two-way ANOVA, Dunnett's post-test. n=3.

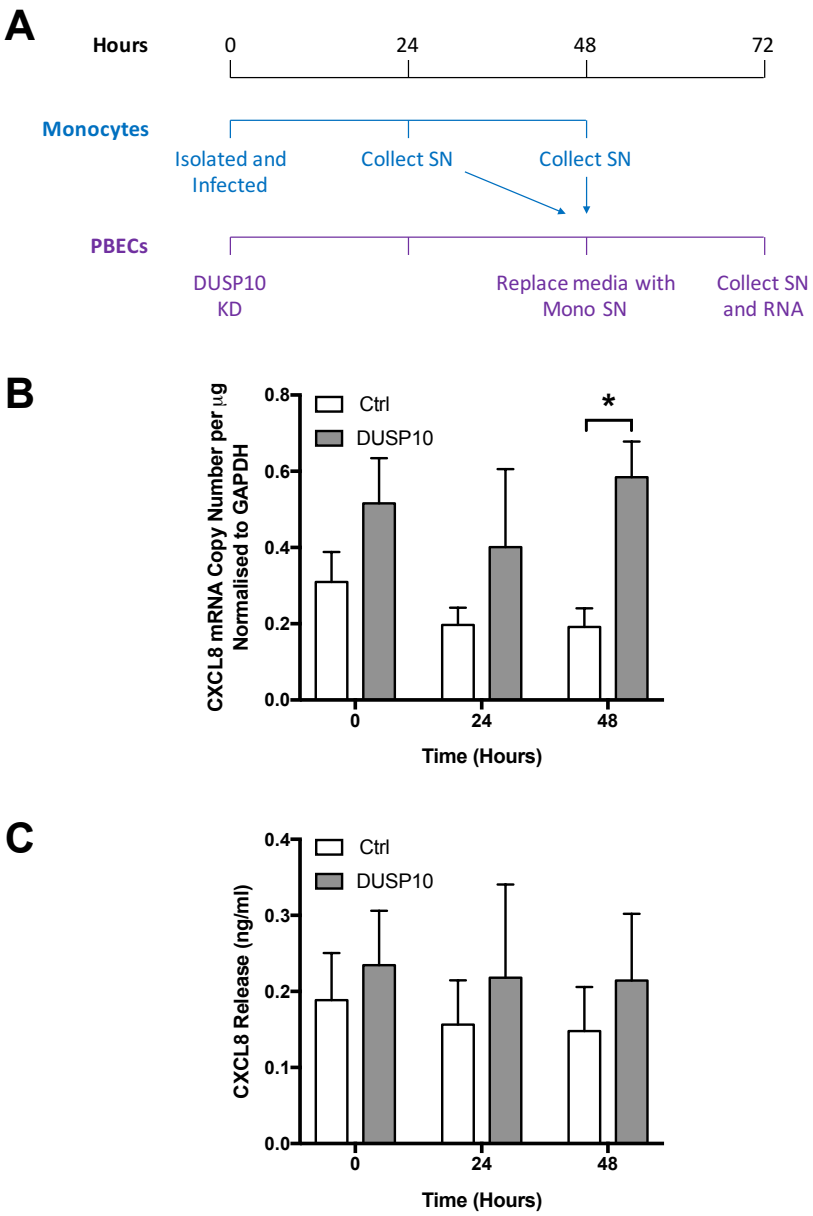

Figure 5.7 Supernatant from monocytes infected with RV16 can induce CXCL8 production in PBECs.

Monocytes were infected with RV16 (MOI 4) for 0, 24 or 48 h, in 0% serum and supernatant collected. Supernatant was filtered and diluted 1:2 in recovery media before addition to PBECs pre-treated with DUSP10 or control (Ctrl) siRNA (100 nM) for 48 h. The time line of the experiment is shown in A. RNA was collected and qRT-PCR performed for CXCL8 and GAPDH. Data shown are mean ± SEM normalised to GAPDH control (B). Supernatant was collected and ELISA used to measure the release of CXCL8. Data shown are mean ± SEM (C). Significance between siRNA treatments is indicated by * $p \leq 0.05$ as measured by two-way ANOVA, Sidak's post-test. Significance between time points was measured by two-way ANOVA, Dunnett's post-test. n=3.

5.4 Discussion

5.4.1 Aims

The aim of this chapter was to characterise the response of monocytes and macrophages to rhinoviral infection, in terms of cytokine release and viral replication in these cells. The role of these cells in the airway was then investigated, by examining the effect of factors released by these cells in response to RV on PBECs, and determining the role of DUSP10 in this setting.

5.4.2 Rhinovirus Replication in Monocytes and Macrophages

Rhinovirus was found to replicate well in monocytes, as demonstrated by increases in rhinoviral RNA and released virions. Rhinovirus replicated much more effectively when no serum was present. As discussed in section 4.1, several groups have shown that rhinovirus does not replicate in monocytes (Gern et al., 1994, Johnston et al., 1997, Laza-Stanca et al., 2006, Saba et al., 2014). In each of these studies, serum was present, at different concentrations, during infection. The decreased replication in the presence of serum could be due to factors within the serum which prevent replication. Although FCS is heat inactivated to remove immune factors such as complement, there may still be anti-viral factors present. For example, FCS is known to contain low levels of IgG (ThermoFisher). Cows and humans come into close contact so it could be speculated that they make antibodies to human rhinovirus which are present in the FCS.

The lack of serum would cause stress in the cells, as is seen by the release of cytokines at baseline. It may be that stressed cells are more permissive to infection. Recent work in keeping with my findings has just been published. Zhou et al. demonstrated that RV16 is capable of replicating in PBMCs or THP-1 cells when co-cultured with A549 cells. This was thought to be due to a product secreted by the epithelial cells, as infecting THP-1 cells in “conditioned media” taken from A549 cells or PBECs also had this effect (Zhou et al., 2017). The conditioned media was generated by incubating serum free RPMI with A549 cells for 24 h. This was in comparison to non-conditioned media: RPMI containing 10% FCS. Therefore, the increased rhinoviral replication in conditioned media observed by Zhou et al. may be due to the lack of serum, rather than a product secreted by epithelial cells. In the Zhou paper, they postulate that the increased replication is due to an increase in ICAM-1 expression. It would therefore be of interest to examine whether

culturing monocytes without serum increases expression of ICAM-1. However, in this study, both RV1B and RV16 replicated in the absence of serum. RV1B belongs to the minor group rhinoviruses, and therefore does not bind ICAM-1. Cellular stress has been demonstrated to upregulate both ICAM-1 and LDLR (Ma et al., 2008, Tumur et al., 2010). Perhaps the lack of serum induces stress pathways which upregulate receptors for both major and minor group rhinoviruses. To the best of our knowledge this is the first study to demonstrate replication of a minor group rhinovirus in monocytes.

Rhinovirus did not replicate well in MDMs, even though these were infected in 0% serum media. It would also be of interest to examine ICAM-1 and LDLR expression in MDMs, as this may also be the reason for these cells being less permissive to rhinoviral infection. Differentiation of monocytes into MDMs has previously been shown to change expression levels of many adhesion molecules (Prieto et al., 1994). Prieto et al. showed similar expression levels of ICAM-1 on the surface of monocytes and MDMs, however they utilised a different method of macrophage differentiation to that used this study, which may affect results. For example, they treated monocytes with concanavolin A during the differentiation process, which has previously been found to affect expression of surface ligands (Krause et al., 2001). Replication of human cytomegalovirus in MDMs has previously been found to differ depending on whether the cells were differentiated in the presence of concanavolin A (Soderberg-Naucler et al., 2001).

Rhinovirus has been shown to induce secretion of CXCL8 and IL-1β in monocytes or macrophages previously. Infection of PBMCs with RV9 induced CXCL8 release, and infection of MDMs with RV16 induced IL-1β release (Ganesan et al., 2016, Johnston et al., 1997). In this study, monocytes released both cytokines at baseline, and expression was not altered by RV16 or RV1B infection, except for slight increases in IL-1β when infected in 2% serum. This implies that due to the stress caused by reduced serum levels, as discussed above, cytokine expression is already at its peak and cannot be increased by infection. The amount of CXCL8 released by monocytes was similar to the expression previously found at 48 h post infection, of between 20 and 30 ng/ml (Johnston et al., 1997). The release of IL-1β in this study was much lower than that found by Ganesan et al., with around 30 pg/ml compared to 100 ng/ml (Ganesan et al., 2016). This could be due to the examination of different time points, 16 h compared to 24 or 48, or the use

of differentiated cells. However, in this study, preliminary data found release of IL-1β by MDMs to be undetectable, with or without RV infection (data not shown).

5.4.3 Activation of PBECs by Monocytes

As the data presented in chapter four demonstrated that IL-1β potentiates the response of PBECs to rhinoviral infection, the cross-talk between monocytes and PBECs in the response to RV was then investigated. Supernatant transfer from monocytes to PBECs induced small amounts of CXCL8 production and release. This showed that molecules released by the monocytes are able to stimulate cytokine production by PBECs, and CXCL8 mRNA levels were consistently higher in cells treated with DUSP10 siRNA. However, this CXCL8 production was not upregulated by rhinoviral infection, likely due to the high baseline levels of cytokine production in monocytes. This experiment demonstrates that the presence of monocytes could potentiate the response of PBECs to RV, however more optimisation is needed to minimise stress in the monocytes at baseline.

5.4.4 Conclusions

As IL-1β is known to play an important role in the cross-talk between epithelial cells and monocytes, the role of DUSP10 in the interplay between the two cells types was investigated. Rhinovirus replicated well in monocytes, although under stressed conditions, and supernatants taken from monocytes did induce cytokine production by epithelial cells, which was increased by DUSP10 knock down.

6 Chapter Six: Final Discussion

6.1 How Does This Thesis Add to the Current Knowledge?

This is the first study to investigate DUSP regulation of epithelial inflammatory responses to respiratory viral infection. Respiratory inflammation is a damaging response to rhinoviral infection and can cause severe complications, especially in patients with asthma or COPD. DUSPs represent a possible future therapeutic target for limiting excessive inflammation.

The MAPK pathways are known to induce the production of inflammatory cytokines in response to a variety of stimuli, including rhinovirus. However, previous studies have focused on the roles of p38 and ERK (Griego et al., 2000, Liu et al., 2008a). This study reinforces the role of p38 in the inflammatory response to rhinoviral infection, but also demonstrates a similar role for JNK, with inhibition of either dramatically reducing CXCL8 production.

Although the role of the MAPKs in cytokine induction has been investigated previously, their impact on rhinoviral replication has not. This was the first study to demonstrate that the p38 pathway is necessary for replication of RV, although this has previously been shown for other viruses (Marchant et al., 2010). In opposition to this, JNK was found to limit rhinoviral replication, although the mechanism by which it acts is unknown. These findings have important implications for any future therapeutics, if JNK was to be targeted to reduce inflammation, this may have detrimental effects on the immune control of rhinoviral replication.

DUSPs 1 and 10 were expressed in PBECs at baseline. The expression of these proteins in PBECs, and their regulation in response to RV have not previously been investigated. Rhinoviral infection did not alter expression of DUSP1 but did induce phosphorylation of the protein, previously shown to stabilise it (Brondello et al., 1999). DUSP10 was also regulated in response to rhinoviral infection, although in a different way: RV1B addition caused a rapid downregulation of DUSP10 protein which recovered by 60 minutes. Both serotypes of rhinovirus also caused a later decrease in DUSP10 by around 4 h post infection. These data demonstrate that both proteins are being regulated by infection, suggesting an important role in the response of PBECs to RV.

Although DUSP10 did not have a direct role in regulating the response of PBECs to RV infection, in contrast to previous work with influenza virus (James et al., 2015), it did regulate the response to IL-1β. IL-1β has previously been shown to have an important role in potentiating the inflammatory response of epithelial cells to rhinoviral infection (Morris et al., 2005, Stokes et al., 2011). This suggests that DUSP10 would have an important anti-inflammatory role in rhinoviral infection of the airway. This is reinforced by the data showing that co-stimulation of PBECs with RV and IL-1β increases the production of CXCL8, and loss of DUSP10 potentiates this still further.

Activation of epithelial cells by IL-1β is not only important in rhinoviral infection but has roles in many different settings. For example, IL-1β is known to have roles in asthma and COPD, with levels correlating to exacerbation severity (Ackerman et al., 1994, Botelho et al., 2011). In murine asthma models, knock out of the IL-1β receptor leads to much lower levels of pulmonary eosinophilia (Schmitz et al., 2003). IL-1β also has an important role in other respiratory viral infections, for example blocking IL-1β signalling decreases inflammation in response to influenza virus (Kim et al., 2015, Thomas et al., 2009). Clinical trials have demonstrated the effect of the IL-1β antagonist IL-1Ra in treating several inflammatory disorders (Abbate et al., 2010, Cohen et al., 2002, Hawkins et al., 2004). This demonstrates that the role of DUSP10 in regulating the epithelial response to IL-1β would have import in a wide range of settings, and could be utilised as a target for future anti-inflammatory treatments.

The data put forward in this work propose a new mechanism by which inflammation is regulated within the infected airway. This is summarised in figure 6.1. In this model, rhinovirus infects epithelial cells lining the airway where it activates pattern recognition receptors and innate immune signalling pathways, including the MAPK pathways. This leads to the production of inflammatory cytokines which are secreted, recruiting immune cells such as monocytes to the site of infection. Rhinovirus infects and replicates within these monocytes, causing them to release inflammatory cytokines, including IL-1β. IL-1β binds to the receptor IL-1Ra on the surface of epithelial cells activating innate immune signalling pathways and the production of cytokines, potentiating the inflammatory response to rhinovirus. The response to IL-1β is negatively regulated by DUSP10. Therefore, DUSP10 has an important role in limiting the inflammatory response in rhinoviral infected airways.

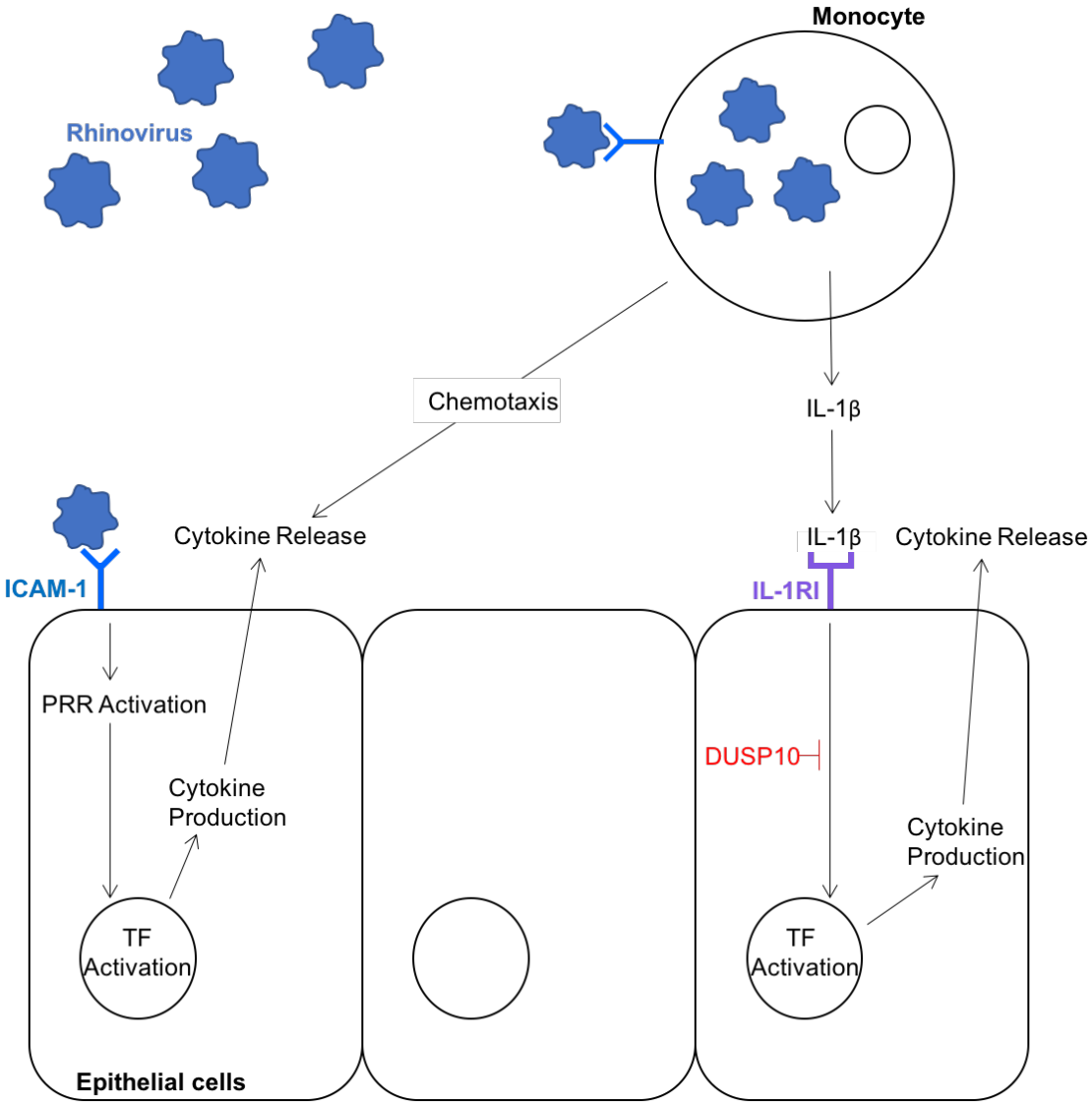

Figure 6.1 The role of DUSP10 in the rhinovirus infected airway.

Rhinovirus binds to the epithelial cell surface triggering internalisation. Pattern recognition receptors recognise aspects of RV and trigger signalling pathways leading to transcription factor (TF) activation and the production and secretion of inflammatory cytokines. Immune cells, including monocytes, move towards the released cytokines by chemotaxis. Rhinovirus infects and replicates within monocytes. Infected monocytes release IL-1β which binds IL-1RI on the epithelial surface. This triggers activation of signalling pathways leading to the production and release of inflammatory cytokines, which is regulated by DUSP10.

6.2 Limitations of the Study

The major limitation of this work is the inclusion of only three to four donors in each figure as the donors are so variable. The variability between donors is expected, and represents the differences you would predict within the population. Although a wide age range was included, the majority of donors were between 50 and 70 years of age and 11 out of 13 donors were male. The smoking status was known for 8 donors and out of these, only one was a non-smoker (Table 7.1). This makes it very difficult to determine any patterns caused by donor characteristics. This donor variability makes the data difficult to interpret and changes may be missed due to large error bars. The application of statistical tests to small numbers of replicates is also difficult and statistical significance is less likely to be found. Therefore, the data presented here would be made much more robust by the inclusion of a larger number of donors. However, the interesting findings within this study, for example the increase in CXCL8 production in response to IL-1β when DUSP10 is knocked down, are clear.

Another limitation is the use of small molecule inhibitors against the MAPKs. As discussed in section 3.10.2, these inhibitors have many off-target effects. The concentrations used are high, well above the IC_{50} values. This could have been improved by the inclusion of dose response curves, as lowering the concentrations would reduce off-target effects and allow the delineation of each pathway more successfully.

This study could also be improved by the use of purified rhinovirus. The effect of viral filtrate on MAPK activation demonstrates that other molecules within the solution are having effects on the cells, although this does not lead to cytokine release. This effect may also vary between batches of virus. Although each figure was completed using the same batch of virus, this adds more variability into the study.

6.3 Future Work

If DUSPs are to be used as future therapeutic targets for rhinoviral infection, it is important that they are fully understood. This includes characterising the pathways DUSPs regulate and how they regulate them, and how these pathways impact on the expression and regulation of the DUSPs themselves. Therefore, validation of the results presented in this thesis, for example the data generated using MAPK inhibitors, using alternative techniques and purified viral stocks is of importance.

The regulation of DUSPs 1 and 10 in response to rhinoviral infection should be further investigated. DUSPs can be post-translationally regulated in a number of ways, including phosphorylation, acetylation and oxidation (Benavides-Serrato et al., 2014, Cao et al., 2008, Kamata et al., 2005). Therefore, the effect of rhinoviral infection on DUSPs has only been partially investigated so far. RV infection induces an earlier phosphorylation of DUSP1 than poly(I:C), suggesting that this is independent of TLR3. The pathway leading to this phosphorylation event and the kinase responsible are unknown. An early signalling pathway is also responsible for the loss of DUSP10 in RV1B infection. To investigate both of these events, a panel of small molecule inhibitors could be used to block a range of signalling pathways to narrow down the protein responsible.

As discussed in chapter four, siRNA knock down of DUSP1 was unsuccessful. Therefore, the role of DUSP1 in the response to rhinoviral infection remains to be understood. The utilisation of overexpression techniques or generation of CRISPR knock out cells could be used to investigate this in the future.

Rhinovirus was found to replicate best in monocytes when no serum was present. The reason for this remains to be explored. Initially the expression of ICAM-1 and LDLR on the cell surface should be determined. It would also be of interest to learn whether the monocytes infected in 2% serum are acting as a reservoir of rhinovirus. This could be determined by infecting in 2% serum for a period of time before removing the serum and measuring whether rhinovirus replication then increases.

The major findings of this thesis suggest a new mechanism by which DUSP10 regulates the inflammatory response to rhinovirus. This mechanism is presented in figure 6.1, however, the point in the IL-1β signalling pathway at which DUSP10 is acting remains unknown. Potential sites at which DUSP10 may act are presented in figure 4.18. Data presented in this thesis suggests that DUSP10 is not dephosphorylating the MAPK proteins p38 or JNK, as DUSP10 knock down did not increase p38 or JNK activation levels, however it is possible that the activation could be prolonged. While this should be further investigated, it would also be interesting to investigate whether DUSP10 is acting at a different, novel, point. If funding were available, it would be exciting to utilise a commercially available antibody screen, specific to phosphorylated proteins. This would enable the identification of any unknown targets of DUSP10. Expression of CXCL10 in response to IL-1β was decreased when DUSP10 was knocked down. CXCL10 is

transcriptionally induced by IRF1. Although this finding should be confirmed using a more quantitative technique, such as ELISA, and samples from multiple donors, it suggests a point at which DUSP10 may be acting and investigation into the effect of DUSP10 knock down on IRF1 activation would be of interest. Knock down of DUSP10 also affected expression of IL-1β itself. This identifies another potential role for DUSP10 in inflammasome regulation. This also links in with the fact that DUSP10 knock down causes significant cell death. Further investigation is required to determine the mechanism of cell death: pyroptosis, apoptosis or necrosis.

IL-1β is known to have an important role in the cross-talk between epithelial cells and monocytes in rhinoviral infection (Section 5.1). This suggests that DUSP10 has an important role in regulating the response to rhinovirus in the airway. Transfer of supernatants from monocytes to PBECs was found to induce some cytokine secretion, suggesting that factors secreted by monocytes are able to induce inflammation in PBECs. It would be of interest to move this forward into a co-culture model, as previous studies have shown that it is not only secreted factors that influence cellular communication, but cell-cell contacts (Lee and Rannels, 1996, Tao and Kobzik, 2002). However, monocytes released high levels of cytokines at baseline, likely due to the stress caused by a lack of serum. In order to move forward into co-culture models, optimisation is necessary to reduce this stress. Once optimised, the role of DUSP10 in rhinoviral infection can be investigated in a multi-cellular environment, more representative of the infected airway.

This work has identified DUSP10 as an important regulator of airway inflammation. This could lead to the future development of new treatments for viral induced asthma and COPD exacerbations. However, more work is required in order to fully understand these pathways before they can be manipulated for therapeutic use. This includes identification of the proteins targeted by DUSP10, further characterisation of regulation of DUSP10 expression, and investigation into the role of DUSP10 in a multi-cellular environment.

6.4 Conclusions

Exacerbations of asthma and COPD caused by rhinoviral infections represent a significant cause of morbidity and mortality. It is therefore important to develop new therapeutics aimed at limiting the excessive inflammatory response to RV. In order to do this, the pathways leading to this response and their regulation need to be fully characterised. This study has identified two pathways, p38 and JNK, as important inducers of inflammation in response to RV, and characterised the expression of two potential regulators, DUSPs 1 and 10. Both proteins were expressed in PBECs and regulated by RV infection. A novel role for DUSP10 was characterised in limiting cytokine production in response to IL-1β alone, and in combination with RV. Therefore, DUSP10 plays an important negative regulatory role in the inflammatory response of airway epithelial cells. This study has identified DUSP10 as a potential future therapeutic target for exacerbations of asthma and COPD.

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Table 7.1: PBEC Donors Used in Study

Donor	Age	Gender	Smoker	Figure																	
				3.1 A	3.1 B	3.2	3.3	3.4	3.5	3.6	3.7	3.8	3.9	3.10	3.11	3.12	3.13	3.14	3.15	3.16	3.17	3.18
111001.11	51	Male	Yes	X	X		X	X		X					X			X		X		
111001.12	51	Male	Yes											X		X						
1101701.37	28	Male	?						X	X												
2032705.12	55	Male	?																			
3110701	51	Male	Yes	X	X		X	X	X	X	X							X	X			X
395Z012.1	81	Male	No								X							X				
397Z013.2	64	Male	Yes																			
4032402	69	Female	Yes	X	X				X		X											X
4081203.3	79	Male	Yes	X	X								X						X		X	
4100601.2	73	Female	Yes			X							X		X					X		
6031301.14	26	Male	?		X									X		X			X	X		
8121902.18	21	Male	?			X																
9082701.12	68	Male	?		X	X	X	X		X	X	X	X	X	X	X	X				X	X

Table 7.1 Continued: PBEC Donors Used in Study

Donor	Age	Gender	Smoker	Figure																		
				3.19	3.20	3.21	3.22	3.23	3.24	3.25	3.26	3.27	3.28	3.29	3.30	3.31	4.1	4.2 A	4.2 B	4.3	4.4	4.5	4.6
111001.11	51	Male	Yes						X				X			X	X						
111001.12	51	Male	Yes				X			X				X									
1101701.37	28	Male	?																				
2032705.12	55	Male	?					X							X								
3110701	51	Male	Yes					X							X	X		X	X	X	X	X	X
395Z012.1	81	Male	No					X							X		X		X			X	
397Z013.2	64	Male	Yes																X	X	X		
4032402	69	Female	Yes						X		X					X			X	X	X	X	X
4081203.3	79	Male	Yes			X			X														X
4100601.2	73	Female	Yes	X	X	X					X	X	X					X	X				
6031301.14	26	Male	?	X			X			X		X		X				X	X				
8121902.18	21	Male	?		X																		
9082701.12	68	Male	?	X	X	X	X		X	X	X	X	X	X					X			X	X

Table 7.1 Continued: PBEC Donors Used in Study

Donor	Age	Gender	Smoker	Figure												
				4.7	4.8	4.9	4.10	4.11	4.12	4.13	4.14	4.15	4.16	4.17	5.6	5.7
111001.11	51	Male	Yes				X				X	X	X	X	X	X
111001.12	51	Male	Yes													
1101701.37	28	Male	?			X	X									
2032705.12	55	Male	?													
3110701	51	Male	Yes	X				X					X			
395Z012.1	81	Male	No				X									
397Z013.2	64	Male	Yes			X										
4032402	69	Female	Yes	X										X		
4081203.3	79	Male	Yes		X	X					X	X				
4100601.2	73	Female	Yes		X	X		X	X	X	X	X	X		X	X
6031301.14	26	Male	?		X										X	X
8121902.18	21	Male	?													
9082701.12	68	Male	?	X				X					X	X		

The characteristics of each donor used in this study. For five donors the smoker status is unknown, represented by ?. The donors included in each figure are indicated by X.