Role of Suppressor of cytokine signalling 1 and 3

in Rhinovirus infection *in vitro* and *in vivo*

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Abstract

Rhinovirus (RV) infection is responsible for most asthma exacerbations. Defective RV induced interferon (IFN)-β and IFN-λs has been observed in asthmatic bronchial epithelial cells (BECs). Suppressors of cytokine signalling (SOCS) are negative regulators of cytokine signalling including IFNs. This study investigated the role of SOCS in the regulation of RV infection *in vitro* and *in vivo* using human BECs (HBECs) and mouse models of RV infection to determine whether SOCS were responsible for impaired IFN *ex vivo.* This was accomplished by measuring SOCS and IFN-β/λ mRNA and protein expression and IFN promoter activation.

RV induced SOCS1 and SOCS3 mRNA and protein in HBECs and in lung from C57/Bl6 mice and SOCS1 mRNA in mouse BAL macrophages *ex vivo*. SOCS1 and SOCS3 acted as negative regulators of RV induced IFN-β and IFN-λ1 promoter activation in HBECs. SOCS1 protein was increased in bronchial biopsies from mild asthmatics compared to non-asthmatics. SOCS1 mRNA expression was increased in HBECs from severe asthmatic children compared to non-asthmatics. Increased SOCS1 mRNA levels negatively correlated with RV1B induced IFN-λ mRNA and positively correlated with RV16 release. RV1B induced IFN-β mRNA was increased in BAL macrophages from $SOCS1^{-/-}$ IFN-$\gamma^{-/-}$ mice compared to IFN-$\gamma^{-/-}$ mice. Upon induction of SOCS1 by IL-13 in IFN-$\gamma^{-/-}$ mice and subsequent RV1B infection, reduced IFN-α and IFN-λ protein in BAL was observed compared to $SOCS1^{-/-}$ IFN-$\gamma^{-/-}$ mice.

This is the first report investigating SOCS1 and SOCS3 in relation to RV induced IFN responses. RV induced SOCS1 and SOCS3 *in vitro, ex vivo* and *in vivo.* SOCS3 negatively regulated RV induced IFN responses *in vitro*. SOCS1 negatively regulated RV induced IFN responses *in vitro, ex vivo* and *in vivo.* This is the first report finding an association between increased expression of a gene, SOCS1, with impaired IFN production and increased RV release in asthma.

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Abbreviations

A549	Alveolar lung cell line
AA	Atopic asthmatics
ACQ	Asthma control questionnaire
ACT	Asthma control test
AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ASMC	Airway smooth muscle cell
ATF2	Activating transcription factor 2
Atg	Autophagic protein
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BDR	Bronchodilator responsiveness
BEAS2B	Bronchial epithelial cell line
BEBM	Bronchial epithelium basal medium
BEC	Bronchial epithelial cell
BEGM	Bronchial epithelial growth medium
b-ME	b-mercaptoethanol
BSA	Bovine Serum Albumin
CARD	Caspase recruitment domain
CARDIF	CARD inducing IFN-β
CF	Cystic fibrosis
CHIP	Chromatin immunoprecipitation
CISH	Cytokine induced SH2-containing protein
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CPE	Cytopathic effect
CYLD	Cylindromatosis
d	day
DAK	Dihydroxyacetone kinase
DMEM	Dulbeccos modified eagles medium
DNA	Deoxyribonucleic acid
ds	Double stranded
DUB	Deubiquitinating
E.Coli	Escherichia Coli
ELISA	Enzyme Linked Immunosorbent Assay
ENA78	Epithelial-derived neutrophil-activating peptide 78/CXCL5/LIX
FADD	Fas-Associated protein with Death Domain
FCS	Foetal calf serum
FeNO	Fractional exhaled nitric oxide
FERM	Four point one, ezrin, radixin,moesin
FEV1	Forced expiratory volume in 1 second
FVC	Forced expiratory vital capacity

g	Gram
GAF	IFN-γ stimulated GAS binding transcription factor
GCSF	granulocyte colony stimulating factor
GTP	Guanosine triphosphate
h	Hours
h	Human
HBEC	Human BEC
HBS	Hepes buffered saline solution
HRP	Horseradish peroxidase
i.n.	Intranasal
i.p.	Intraperitoneal
ICAM-1	Intercellular adhesion molecule-1
ICS	Inhaled corticosteroids
ICS	Inhaled corticosteroids
IFN	Interferon
IFNAR	IFN-α receptor
Ig	Immunoglobulin
IKK	IkB kinase
IL	Interleukin
IP-10	IFN-γ-induced protein 10 kDa/CXCL10
IPS-1	IFN-β promoter stimulator-1
IRAK	IL-1 receptor activated kinase
IRF	Interferon regulatory factor
ISG	IFN stimulated gene
ISGF-3	IFN-α stimulated IRF binding factor-3
ISRE	IFN stimulated response element
JAK	Janus Kinases
KIR	Kinase inhibitory region
L	Liter
LABA	Long acting beta agonists
LB	Luria-Bertani
LDL	Low density lipoprotein
LIX	LPS-induced CXC chemokine/ ENA-78/CXCL5
LPS	Lipopolysaccharide
M	Molair
m	murine
MAVS	Mitochondrial antiviral signalling
MDA-5	Melanoma differentiation-associated protein-5
MDC	Monocytes-derived chemokines/CCL22
MHC	Major histocompatibility complex
mRNA	Messenger RNA
min	Minutes
MIP-2	Macrophage inflammatory protein/CXCL2
MOI	Multiplicity of infection
MyD88	Myeloid differentiation primary response gene 88
n/a	Not available

NA	non-atopic non-asthmatics
NaCL	Sodium chloride
NBD	Nucleotide-binding domain
NEMO	NF-κB essential modulator
N-ESS	N-extended SH2 domain
NF-κB	Nuclear factor-κB
NLR	Nucleotide-binding domain and leucine rich repeat containing
NLS	Nuclear localisation sequence
OAS	Oligoadenylate synthesases
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC20	Histamine provocative concentration causing a 20% drop in FEV1
PCR	Polymerase chain reaction
PEF	Peak expiratory flow
PEG	Poly(Ethylene glycol)
PIAS	Protein inhibitor of STAT
PKR	Protein kinase R
PolyIC	Polyionosinic-polycytidylicacid
Pro	Proline
PRR	Pathogen recognition receptor
RANTES	Regulated upon activation, normal T-cell expressed, and secreted/CCL5
RAST	Radio allergosorbent test
RD	Repressor domain
RIG-I	Retinoic acid inducible gene-1
RIP-1	Receptor-interacting protein 1
RLU	Relative light units
RNA	Ribonucleaic acid
RNaseL	Endoribonuclease L
RNF	RING finger family protein
RPM	Rotations per minute
RPMI	Roswell park memorial institute
RSV	Respiratory synctial virus
RT	Reverse transcriptase
RV	Rhinovirus
sec	Seconds
SEM	Standard error of the mean
Ser	Serine
SIKE	suppressor of IKKε
SOCS	Suppressors of cytokine signalling
SPT	Skin prick test
ss	Single stranded
STAT	Signal transducers of transcription
STRA	severe therapy resistant asthmatic

TAK-1	TGF-β activated kinase-1
TANK	TRAF family member-associated NF-κB activator
TARC	Thymus and activation-regulated chemokines/CCL17
TBK-1	TANK binding kinase-1
TCID50	Tissue culture infective dose 50%
TE	Trypsin/EDTA
TGF	Tumour growth factor
TIR	Toll/interleukin-1 receptor
TLR	Toll like receptor
TMB	Tetramethyl benzidine chromogen
TNF	Tumour necrosis factor
TNS	Trypsin neutralising solution
TRAF	TNF associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSLP	Thymic stromal lymphopoietin
TYK2	Tyrosine kinase 2
UV-RV1B	UV-inactivated RV1B
VISA	Virus-induced signalling adaptor
VPg	Genome virion protein
vRNA	viral RNA
WHO	World health organisation
wt	wild type

Acknowledgments and statement of work

I declare that the work presented in this thesis was carried out by myself unless stated otherwise. Specifically, Bronchoscopies to obtain HBECs and bronchial biopsies from atopic asthmatics and non-atopic non-asthmatics were performed by Dr.Anny Sykes or Dr Jonathan Macintyre (adult asthmatics study) and Professor Andrew Bush or paediatric consultants (severe asthmatic kids study). Culturing and infecting of HBECs obtained from atopic asthmatics and non-atopic non-asthmatics was performed by Dr. Anny Sykes, Dr. Michael Edwards and Dr. Nicholas Regamey. RNA isolation and cDNA synthesis on these samples was performed by Dr. Anny Sykes, Dr. Michael Edwards or myself. Staining of bronchial biopsies was performed by Dr. Jie Zhu.

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1 Introduction

1.1 Asthma

1.1.1 Definition and diagnosis

There is no clear, universally accepted definition of asthma. The global initiative for asthma (GINA) defines it based on the functional consequences of airway inflammation. Asthma is a chronic inflammatory disease of the airways with many cells and molecules playing a role. Inflammation is associated with airway hyperresponsiveness (AHR) leading to recurrent episodes of breathlessness, wheezing, coughing and chest tightness, particularly early in the morning or at night. These episodes are associated with variable, but widespread, airflow obstruction in the lung which is often reversible spontaneously or with treatment [1].

The diagnosis of asthma is based on recognition of a pattern of symptoms, signs of variability in airflow and reversible airflow obstruction. Symptoms can be triggered by exercise, infection, allergen exposure, cold air and drugs such as non-steroidal anti-inflammatories or β-blockers. Variability in airflow obstruction is measured by increase in forced expiratory volume in 1 second (FEV_1) of 15% after taking a dose of salbutamol, and variation (>20%) in peak expiratory flow (PEF) over time. PEF is defined as maximum speed of expiration over time. Asthmatics exhibit a variation in PEF, particularly with a daily pattern of variation. When diagnosis in allergic asthma is difficult, PC_{20} can be measured. PC_{20} stands for the concentration of histamine which causes a 20% drop in FEV_1. In research, spirometry is used to measure airflow obstruction. Spirometry measures forced vital capacity (FVC, the volume of air which is forcibly exhaled following maximum inspiration) and FEV_1. Airflow obstruction is then determined by the FEV_1/FVC ratio with < 70% indicating obstruction [2]. Asthma has many phenotypes, with atopy being the major association with 70-90% of adult asthmatics having atopy [3]. Other phenotypes include severe steroid-resistant asthma[4] and asthma induced by exposure to air pollution, cigarette smoke, diesel exhaust particles, obesity, aspirin and exercise [3].

1.1.2 Epidemiology

The reported prevalence of asthma has increased substantially around the world [4], with a wide variation in prevalence rates. Some areas have a stable or slightly decreased prevalence, whilst in others prevalence has substantially increased [5]. This increase could be a result of better recognition of symptoms and awareness of asthma. Approximately 400 million people are predicted to be affected by asthma in 2025 [6]. Studies of both adults and children have seen high prevalence in most westernized countries with New Zealand, Australia, Canada and United Kingdom having the highest prevalence with 15-20%, compared to 2-4% prevalence in Asian countries such as China and India [5, 7-

[9]. Migration studies have suggested prevalence to be dependent both on genetic and environmental factors (allergen exposure, lifestyle factors) and their interactions [10-12]. The World Health Organization (WHO) has estimated that annually, 15 million disability-adjusted life years are lost due to asthma which is 1% of the total global disease burden. Worldwide an estimated 250,000 people die of asthma a year [6].

1.2 Rhinoviruses: Classification, structure and lifecycle

Rhinoviruses (RVs) are picornaviruses. They have a single stranded, linear, positive sense, non-enveloped, ribonucleic acid (RNA) genome contained in a icosahedral protein capsid of about 28 to 30nM in size. They are closely related to enteroviruses which are also picornaviruses. RVs are subclassified based on receptor binding, into the major group which bind to intercellular adhesion molecule-1 (ICAM-1) receptor or the minor group, which bind to the low density lipoprotein receptor (LDL) receptor. Another subclassification of human RV into RV-A and RV-B is based on genetic analysis and responses to some antivirals. Recently approximately 50 previously unclassified strains have been identified of which most have been classified into the newly discovered group RV-C. Genomes of this new group suggest that they bind to unique cellular receptors [13].

The protein shell of the capsid contains 60 separate identical units (protomers). A protomer contains four proteins, viral protein 1A, 1B, 1C and 1D. 1B, 1C and 1D are on the surface of the capsid and 1A is internal next to the viral RNA. Varying amino acid sequences in the surface proteins defines the different RV serotype of which approximately 100 have been identified so far. The RNA is polyadenylated at the 3'-terminus and contains a small protein at the 5'-terminus, the genome virion protein (VPg), which serves as an initiator of RNA synthesis. The RV genome is divided into region P1 encoding the capsid proteins and the P2 and P3 regions which encode non-structural proteins including two viral proteases (2A and 3C), VPg and RNA-dependent RNA polymerase.

Replication of RVs takes places in the cytoplasm of infected cells. Major group RVs enter through the endosome and require acidic environment for uncoating. LDL receptors exhibit a negative charge which excludes binding of major group RVs. The capsid binds to the ICAM-1 or LDL receptor which causes loosening and expansion of the capsid pentamers. RNA then polarizes to the top of the capsid shell where the 1D protein expands and opens. Release of the RNA in the cytosol initiates translation, followed by RNA replication. The positive sense viral RNA acts as mRNA binding to host cell ribosomes causing translation of the RNA into a single polyprotein which is then cleaved by RV 3C protease into individual proteins. The positive sense viral RNA then acts as a template for the synthesis of negative sense RNA, which is transcribed by viral RNA polymerase, and in turn is used as a template for the synthesis of progeny viral genomes. The viral proteins and progeny viral RNA genomes aggregate and mature into virions. Host cell mRNA translation is inhibited after viral replication starts, which alters the integrity of cellular structure causing cytopathic effects (CPE). Cells undergoing RV infection release virions after cell lysis. Replication cycle times takes between 6h and 12h. Viral yield is approximately $2.5x10^4$ to $1x10^5$ virus particles per cell.

1.3 RV and asthma exacerbations

1.3.1 Asthma exacerbations

Transient worsening of asthma symptoms can occur as a result of exposure to risk factors, such as infection, air pollutants and exercise. Prolonged worsening of symptoms trigger asthma exacerbations, which are the major cause of morbidity, mortality and healthcare costs related to asthma [14]. The majority of exacerbations (approximately 80%) are associated with respiratory viral infections in both adults [15, 16] and children [17, 18]. RVs are the major viruses associated with asthma exacerbations and are responsible for approximately 60% of virus induced exacerbations [15, 18].

The pathogenesis of virus induced asthma exacerbations is incompletely understood, but includes an increase in levels of lymphocytes, granulocytes, mainly neutrophils, pro-inflammatory cytokines (interleukin (IL)-1β, IL-6 and IL-8), mucus production, CD8+ T-cell activation and oxidative stress. What causes this exacerbation of disease by viruses is unknown [19]. Maintenance therapy of asthma is based on anti-inflammatory medication; inhaled corticosteroids (ICS). Corticosteroid use is associated with reduced asthma exacerbation frequency [20-22]; however, this effect is only partial, even in combination with long-acting β_2-agonists [23]. This suggests that asthma exacerbations arise through distinct mechanisms than inflammation and bronchoconstriction which is treated by currently used medication and other more effective therapies are necessary.

1.3.2 RV and asthma exacerbations

Viruses are responsible for up to 80% of asthma exacerbations in adults and children [18]. It is not fully understood how and under what conditions a respiratory infection can cause an asthma exacerbation. Many factors can determine whether a virus induces an exacerbation, including the atopic status of the host, allergen exposure, host genetic factors and virus type. Some respiratory viruses are more often associated with asthma exacerbations than other strains. RVs account for up to 60% of asthma exacerbations in adults and children with other viruses such as influenza, respiratory syncytial virus (RSV), adenoviruses, coronoviruses and metapneumovirus being the other viruses most commonly associated with asthma exacerbations [24]. Differences in pathogenicity between RV strains have not yet been identified, probably because of the large number of serotypes. The new RV-C strains are often detected in children with wheezing episodes, and may be more prominent in asthma requiring hospitalisation [25], indicating that RV-C serotypes might be more pathogenic than RV-A and RV-B serotypes. The number of circulating strains per season makes determination of virulence of a specific strain difficult. There is little evidence that asthmatic patients have more frequent RV infection than healthy individuals, but RV causes longer duration of

illness and increased severity of lower respiratory symptoms in asthmatics [26, 27]. A positive correlation has been reported between allergy/allergic sensitisation and severity of RV infection. Presence of allergic sensitisation during infection causes more severe disease in children and non-atopic asthmatic children have less severe disease compared to allergic asthmatic children [28].

1.3.3 Pathogenesis of RV infection is related to synergistic or additive actions of allergen exposure

Poorly understood differences between asthmatics and healthy subjects upon RV infection are changes in lower airway inflammation and pulmonary physiology. One study shows that inoculation with RV induced similar illness in asthmatics and healthy subjects, but increased lower respiratory symptoms, lung function impairment, bronchial hyperreactivity, and eosinophilic lower airway inflammation in asthmatics compared to healthy controls. Virological and clinical outcomes in asthmatics were strongly related to deficient Th1 responses and to augmented Th2 responses, suggesting a possible link between allergic sensitisation and susceptibility to RV infection [29]. Other clinical studies have shown that there is a synergistic interaction between allergy and viral infections in increasing risk of asthma exacerbations. Studies in children and adults have indicated that allergic subjects who are exposed to allergens have the highest risk for virus-induced wheezing and asthma exacerbations [20, 30, 31].

In September there is a marked increase in the rate of children being hospitalized with asthma exacerbations. A correlation was observed between the hospitalization peak and detection of respiratory viral infection, with picornaviruses detected in the majority (62%) of cases of which >80% are RV. This frequent detection of respiratory viruses during September probably indicates a high prevalence of the virus at this time of the year, also, this is the beginning of the school term and the return of children to school highlights the role of children as vectors for RV associated diseases. This time is also associated with increased concentrations of aeroallergens in the environment. Although a viral infection can be the immediate trigger for an asthma exacerbation, allergic factors were thought likely to act together to cause the September epidemic [21, 32].

Several theories have emerged describing how RV could potentially interact with allergic inflammation to cause exacerbations. Both allergic inflammation and virus infection damage the epithelium. Damaged epithelium facilitates allergen crossing the epithelial barrier [33] and *in vitro* studies show that RV replication is greater in damaged epithelium [34, 35]. Other mechanistic studies have shown evidence for an antagonistic effect between allergic inflammation and antiviral immunity [36, 37]. Both RV infection and allergic inflammation induce epithelial cells to secrete thymic stromal lymphopoietin (TSLP), a cytokine which promotes Th2 differentiation and enhancement of

allergic inflammation [38]. *In vivo* animal studies have demonstrated that respiratory virus infection early in life can cause overproduction of Th2 cytokines leading to lower antiviral responses, increased allergy risk and changes in airway structure [39]. Finally, a clinical study in children showed that interactions between allergic induced inflammatory pathways and RV infection play a role in asthma exacerbations [40].

The above studies suggest that specific components of the host response to virus infections play a role in the pathogenesis of virus-induced wheeze and asthma. Atopic asthmatics react differently to RV infection than non-topic non-asthmatics, which has been shown by increased LRT illness ([15, 29]). This suggests that asthmatic have a higher susceptibility to virus infection. Allergic disease is either a risk factor for asthma exacerbation or infection of the airway epithelium enables the immune response to RV to interact with immune cells that also play a role in allergic sensitisation. Allergy and allergen exposure interacts with the host response to virus infections, and these two variables likely impact on the severity and duration of the other.

1.4 Innate immune signalling in viral infection

1.4.1 Interferons are the first line of defense against viral infection

Antiviral interferons (IFNs) are induced in response to virus infection. Type I IFNs consist of the single gene IFN-β and IFN-α which has 13 subtypes. The more recently discovered type III IFNs consist of IFN-λ1, 2, 3 (respectively IL-29, IL-28A and IL-28B). The receptor complex for type I IFNs contains 2 subunits; IFN-α receptor-1 (IFNAR-1) and IFNAR-2 [41]. The receptor complex that binds IFN-λ contains the IL-28Rα chain and the IL-10Rβ chain [42]. Despite having a different receptor, both types of IFNs have functional similarities, much more is known about the mechanism of action of IFN-α/β.

1.4.2 Deficiencies in IFN production in asthmatics

Several studies have investigated deficiencies in antiviral activity in asthmatics. In peripheral blood mononuclear cells (PBMCs) from asthmatics an inverse correlation was observed between RV induced IFN-γ and viral shedding. RV induced IFN-γ was positively correlated with pulmonary function and there was impaired RV induced IFN-α and IFN-γ production[43-45]. In airway sputum cells an increased Th1 response is associated with less severe colds and more rapid clearance of the virus [46]. These studies suggest that IFN-γ may play a beneficial role in RV infection and that reduced IFN- γ levels may be a marker for more severe RV associated illness.

Defective RV induced IFN production has also been observed in bronchial epithelial cells (BECs) in asthma. BECs are the major site of RV infection in the lower airway and cultured BECs from atopic asthmatics had exaggerated viral replication and impaired apoptosis compared to non-atopic non-asthmatics [47] with both major group virus (RV16) [48], and minor group virus (RV1B). This indicates that increased susceptibility is observed regardless of receptor utilization. In the same study, RV induced IFN-β production was deficient compared to the cells of the NA controls [47]. An inverse correlation between virus load and IFN-β production was observed and signalling pathways downstream of the IFN-β response remain intact confirming that an impaired IFN-β response was related to increased RV replication [47].

Comparable results were seen in a study examining type III IFNs. A deficient IFN-λ production was observed in BECs of asthmatics cultured *in vitro* and this inversely correlated with viral replication *in vitro*. Defective RV induced type III IFNs was also observed in bronchoalveolar lavage (BAL) cells (> 90% macrophages) from asthmatics. RV induced IFN-λ production in BAL cells *in vitro* was also inversely correlated with clinical outcomes related to asthma exacerbation severity in the same study [49]. Defective IFN was independent of ICS treatment. No differences in IFN responses were

observed in ICS treated asthmatics and asthmatics treated with inhaled β-agonist only [47, 49]. The above data suggests that IFN-β and IFN-λ production, and their downstream anti-viral responses are important in the pathogenesis of RV induced asthma exacerbations. Defective RV induced IFNs has not been observed by all investigators [50, 51] and experimental infection with RV does not always show clear differences in RV shedding between asthmatics and healthy subjects [29, 52, 53]. Many factors could potentially explain the differences in current study results, such as severity of asthma or subgroups of asthmatics having the deficiency. Further research is needed to understand these differences.

1.4.3 Innate immune signalling leads to IFN and proinflammatory cytokine expression

The production of IFN is mediated, in part, by recognition of replicating viral RNA or double stranded RNA (dsRNA). Viral dsRNA is recognized by toll-like receptor (TLR)-3 or the cytosolic RNA helicases, retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated protein-5 (MDA-5). dsRNA is a pathogen associated molecular pattern (PAMP) and a type of non-self RNA produced during infections by both RNA and deoxyribonucleic acid (DNA) viruses. RIG-I also recognizes 5' triphosphorylated single stranded RNA (ssRNA) [54]. Single stranded RNA (ssRNA) may also induce IFN via recognition by TLR7/8. Activation of TLRs leads to dimerization of the receptor and recruitment of myeloid differentiation primary response gene 88 (MyD88) and toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF) via their TIR domains. This results in activation of signalling cascades involving TNF associated factor (TRAF)3 and 6, ultimately resulting in the phosphorylation of transcription factors such as interferon regulatory factor (IRF)-3 and-7, nuclear factor-κB (NF-κB), activating transcription factor 2 (ATF2) and c-Jun. These activated transcription factors translocate to the nucleus and induce transcription of IFNs. RNA helicases are able to unwind dsRNA by ATPase activity, which makes the caspase recruitment domain (CARD) able to access a CARD containing intermediate, identified as either CARD inducing IFN-β (CARDIF), virus-induced signalling adaptor (VISA), mitochondrial antiviral signalling (MAVS) or IFN-β promoter stimulator-1 (IPS-1) [55]. ssRNA may also induce IFN via recognition by TLR7/8. Activation of TLRs leads to dimerization of the receptor and recruitment of the adaptor proteins MyD88 and TRIF via their TIR domains. The actions of MyD88 and TRIF or the RNA helicase adaptor CARDIF, results in activation of signalling cascades involving various kinases, ubiquitin ligases and other proteins.

Signalling via TLR3 and the RNA helicases utilize two ubiquitin ligases, TRAF3 and TRAF6, which are essential in recruiting two different kinase complexes which serve to phosphorylate the transcription factors required for IFN transcription. TRAF3 or TRAF6 activation recruits the scaffold protein TRAF family member-associated NF-κB activator (TANK), and its kinases TANK binding kinase-1 (TBK-1) and

IκB kinase (IKK)-ι/ε. A second complex, composed of IKK-α, IKK-β and the scaffold protein NF-κB essential modulator (NEMO/ IKK-γ) is also recruited. The IPS complex can also interact with other proteins such as fas-associated protein with death domain (FADD), receptor-interacting protein 1 (RIP-I) and tumour growth factor (TGF)-β activated kinase-1 (TAK-1) which activates the kinase IKK-β, phosphorylating the IκB/NF-κB complex to activate NF-κB. The kinases TBK-1, IKK-ι/ε, IKK-β are essential in phosphorylating the transcription factors IRF-3 and IRF-7, NF-κB, ATF2 and c-Jun. These activated transcription factors translocate to the nucleus to bind to the promoters of IFN genes and induce transcription. IRF-3, c-Jun, ATF2 and NF-κB have all been shown to bind the promoter of IFN-β [56-59]. IRF3, IRF7 and NF-κB have also been implicated in IFN-λ promoter activation [60]. NF-κB is also implicated in the transcription of a number of pro-inflammatory cytokines including IL-8/CXCL8, IL-6, epithelial-derived neutrophil-activating peptide 78 (ENA-78/CXCL5) and IFN-γ-induced protein 10 kDa (IP-10/CXCL10) [61-65].

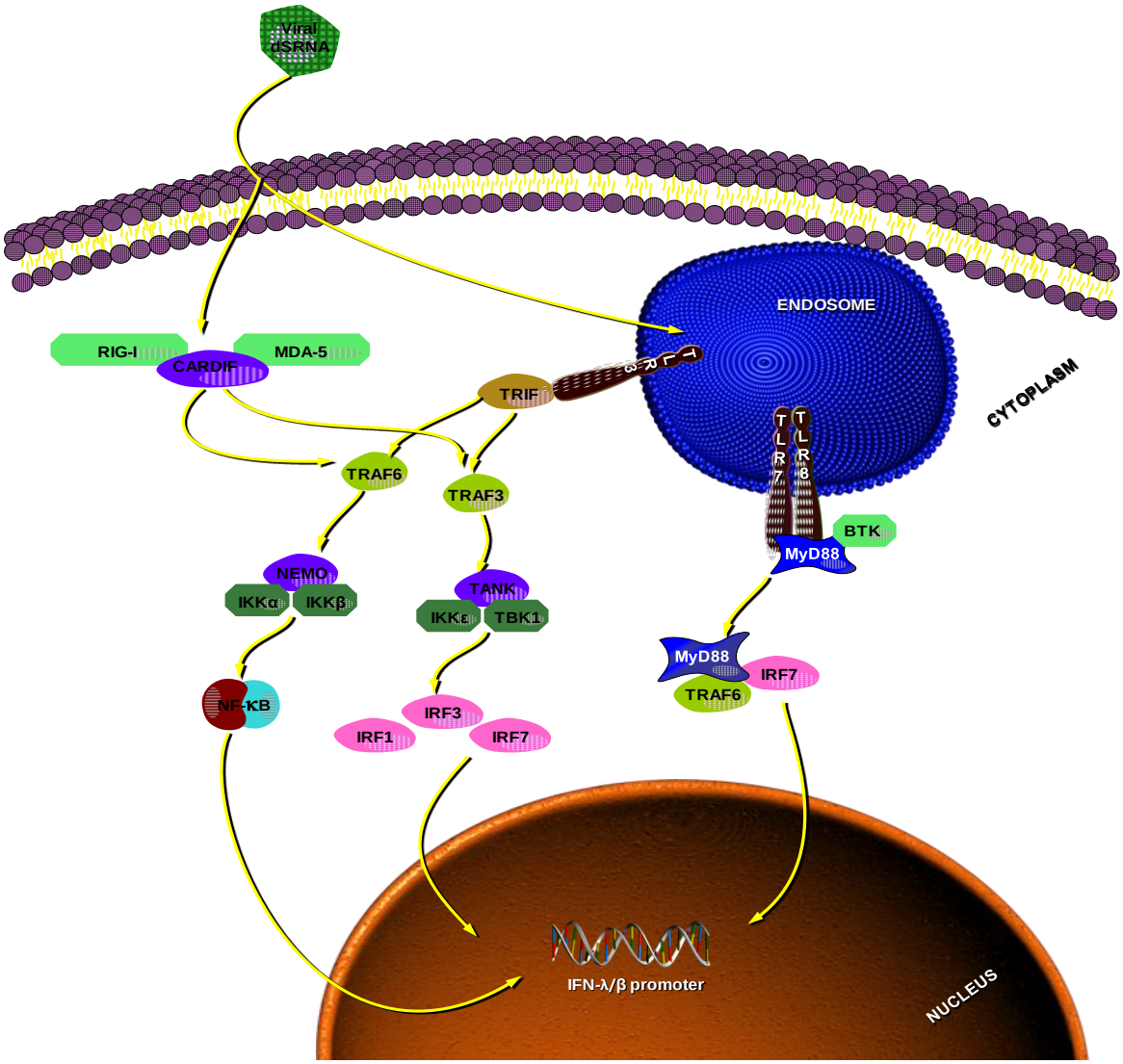

Figure 1.1: Signalling pathway leading to activation of IFN related promoters by TLR3 pathway and RIG-I/MDA-5 pathway.

1.4.4 Negative regulation of innate immune signalling leading to reduced IFN expression

The mechanism behind the IFN deficiency in asthmatics is currently unknown. A possible cause of this could be increased levels of negative regulators of virus induced IFN signalling in asthmatics. Cytokine expression is transient and tightly regulated to prevent unwanted cellular responses. Under steady state conditions RIG-I signalling is tightly regulated by an autonomous mechanism [66-70]. Recently several proteins have been identified that can attenuate or negatively regulate dsRNA signalling and are discussed below.

LGP2 is similar in structure to RIG-IC, a CARD deficient c-terminal fragment of RIG-I which acts as a dominant negative inhibitor of downstream RIG-I signalling [68, 71-73]. Evidence suggests that LPG2 can

be a positive regulator of antiviral signalling during virus infection [74]. LGP2's potential role in positive regulation makes the exact role LPG2 might play during infection unclear.

A20 has been shown to block RIG-I mediated signalling to IRF-3, IRF-7 and NF-κB pathways downstream of IPS-1 but upstream of TBK-1 and IKK-ι/ε [75, 76]. A20's mechanism of action is most likely through two ubiquitin related domains, leading to proteosomal degradation of TRIF and RIG-I [77, 78].

Pin1 is a peptidyl-prolyl isomerase which associates with a phosphorylation site of IRF-3, Ser339 [79]. This Pin1 association has shown to interfere with stability of the substrate, facilitating ubiquitin mediated proteosomal degradation. Pin1 deficiency causes prolonged viral infection [80, 81]. PIN1 can inhibit TLR3, RIG-I and MDA-5 mediated signalling. Pin1 has also been described as a positive regulator of NF-κB subunit p65 [82].

SIKE (suppressor of IKK-ι/ε) can associate with TBK1 and IKK-ι/ε and can inhibit TLR3, and RIG-1 mediated IFN-β and IFN stimulated response element (ISRE) promoter activation. It disrupts interaction between the kinases and IRF3, TRIF and RIG-I with no effect observed on the NF-κB pathway [83].

Atg5-Atg12 conjugate (autophagic protein 5- autophagic protein 12 conjugate) causes autophagy. After conjugation of Atg5 and Atg12 they associate directly with the CARD domain of RIG-I, MDA-5 and IPS-1, assumed to cause a bridge between RIG-I or MDA-5 and IPS-1 which inactivates this complex [84].

RNF125 is a RING finger family protein which has recently been shown to regulate RIG-I by ubiquitin conjugation. RNF125 associates with RIG-I through its N-terminal region, binding to the CARD and RNA helicase domain of RIG-I. RNF125 also associates with MDA-5 and IPS-1. dsRNA and virus induced IFN-β production are inhibited by expression of RNF125 and enhanced by its knockdown [85].

Deubiquitinating family protein A (DUBA) has been shown to interact with Lys63 linked ubiquitin chains on TRAF3 preventing interaction between TRAF3 and TBK-1. DUBA seems to have a more important role in TLR3, TLR4, TLR7, RIG-I and MDA-5 mediated IFN activation compared to NF-κB [86-89].

CYLD (cylindromatosis) is also part of the OTU DUB family of proteins and experiments suggest that it targets both RIG-I and TBK-1 for deubiquitination. CYLD associates with the RIG-I CARD domain and removes K63 linked ubiquitin form the CARD domain [90, 91].

NLRX1 is a member of the nucleotide-binding domain and leucine rich repeat containing (NLR) protein. A nucleotide-binding domain (NBD) of NLXR1 associates with the CARD-like domain of IPS-1, competing with RIG-I and disrupting the possibility of a CARD-CARD interaction between IPS-1 and RIG-I. NLRX1 causes inhibition of RIG-I, MDA-5 and IPS-1 mediated signalling, but not TLR3 [92].

ISG (IFN stimulated gene) 15 system contains ISG15, UBEL1 and UbcM8. When expressed together in a cell, the ubiquitin like ISG15 modifies RIG-I. The ISG15 system reduces ISRE at basal levels and IFN-β production upon virus infection [93, 94].

Dihydroxyacetone kinase (DAK) associates with MDA-5 using its CARD domain containing fragment, causing inhibition of MDA-5 mediated ISRE and IFN-β but not RIG-I, IPS-1 or TLR3. DAK does not have an effect on MDA-5 mediated NF-κB activation, making it specific for IRF-3 [95].

Optineurin is a Nemo-related protein (NRP/FIP2) that associates with TBK1 and the ubiquitin ligase TRAF3, causing decreased virus induced IFN-β production upon over-expression [96].

1.5 IFN induced signalling/ the JAK/STAT pathway

Newly produced cytokines, such as IFNs, interact with specific transmembrane receptor units of the hemopoietin receptor family inducing subunit dimerization leading to intracellular activation of the janus kinases/ signal transducers of transcription proteins (JAK/STAT) pathway. The receptor units are either bound as homodimers, e.g. growth hormone and erythropoietin receptors or heteromultimers, e.g. IFN and IL receptors (including IL-4 and IL-13 receptor).

1.5.1 JAKs

The cytoplasmic domains of receptor subunits must be associated with JAK to continue the signal transmission. In humans there are 4 JAKs known; JAK1, JAK2, JAK3 and Tyrosine kinase 2 (Tyk2) [97, 98], each having two kinase-homologous domains at the C-terminus and a non-catalytic regulatory domain which regulates the tyrosine kinase activity of the kinase domains. Multimerization of receptor subunits brings two JAKs in close proximity causing activation of JAK by transphosphorylation [99].

All JAKs exhibit broad patterns of expression, except JAK3, where expression is restricted to leukocytes. JAKs range in size from 120 to 140 kDa and feature seven conserved JAK homology (JH) domains. The two carboxyl-terminal JH regions represent the kinase and pseudo kinase domains. Activation of JAKs is driven by phosphorylation of critical tyrosines. The four amino-terminal JH domains (JH7–5 and half of JH4) constitute a FERM (four point one, ezrin, radixin, moesin) domain that mediates association with receptors. Specifically, JAKs associate with the proline-rich, membrane-proximal box1/box2 domain on cytokine receptors. An SH2-related domain of unknown function lies between the pseudokinase and FERM domains [100].

Tyk2 associates with receptors for type I IFNs, IL-6, IL-10 and IL-12/23 cytokine families [97, 101]. In Tyk2-deficient humans, the combined defects in the response to their target receptors are associated with enhanced allergic and impaired antimicrobial responses [101].

JAK1 associates with the type I IFN, type II IFN, IL-2, and IL-6 receptors. Evidence that the two type I IFN receptor chains, IFNAR1 and IFNAR2, associated with Tyk2 and Jak1, respectively, led to the concept that JAKs activate each other through transphosphorylation [97, 102, 103].

JAK2 is associated with receptors from the single-chain (i.e. Epo-R, GH-R, Prl-R) and IL-3 (IL-3R, IL-5R, and GM-CSFR) cytokine families, as well as the type II IFN receptor [97, 102, 103]. Ligand binding drives two receptor associated JAK2s into close proximity, enabling them to activate each other by transphosphorylation [104].

Leukocyte-specific JAK3 exclusively associates with the IL-2 receptor γ-chain. This chain also serves as a component for the receptors of several lymphotrophic cytokines, including IL-4, IL-7, IL-9, IL-15, and IL-21 [97, 102, 103].

1.5.2 STATs

Activated JAKs phosphorylate tyrosine residues on the receptor cytoplasmic domain, creating docking sites for proteins such as their major substrates, STATs. Seven STATs have been described in humans, STAT 1, 2, 3, 4, 5a, 5b and 6 [97, 105], ranging in size from 750 to 900 amino acids. STATs have a conserved tyrosine residue near the C-terminus, which gets phosphorylated by JAKs. STATs are normally located in the cytoplasm in their inactive form. The phosphorylated tyrosine allows dimerization of STATs by interacting with a conserved SH_2 domain. After dimerization STATs enter the nucleus and bind specific regulatory sequences to activate or inhibit transcription of target genes. STAT homodimers bind to members of the GAS family of enhancers. Type I IFNs promote the formation of STAT1-STAT2 heterodimers, which associate with IRF-9 to form IFN-α stimulated IRF binding factor-3 (ISGF-3) and bind to the ISRE enhancer family, leading to induction of ISGs. The JAK/STAT pathway is a direct signalling cascade enabling an extracellular signal to be translated into a transcriptional response [97, 106].

STAT1 was initially identified as a component of ISGF-3 [107]. Subsequently, it was discovered that GAF (IFN-γ stimulated GAS binding transcription factor) consists of STAT1 homodimers [108]. STAT1 plays a role in the biological response to both type I and II IFNs [109, 110]. Mutations in STAT1 in humans causes increased susceptibility to viral and bacterial infections [111]. STAT1 targeted genes promote inflammation and antagonize proliferation. STAT3 targeted genes have opposing actions to STAT1. The ability of several cytokines to activate both STAT1 and STAT3 (e.g. members of the type I IFNs and IL-6 families) may reflect an effort to achieve a more balanced response and maintain immune homeostasis.

STAT2 was also initially identified as a component of ISGF-3. STAT2 plays a pivotal role in the biological response to type I IFNs [112, 113]. There is little evidence that active STAT2 homodimers form or directly bind DNA. Rather, STAT2 heterodimerizes with STAT1. The mechanism by which STAT2 is recruited to IFNAR remains unclear.

STAT3 is known to transduce signals for the entire IL-6 and IL-10 families, as well as granulocyte colony stimulating factor (G-CSF), leptin, IL-21, IL-27, growth factors and oncogenes. STAT3 has been shown to have an important anti-inflammatory role and directs the expression of anti-apoptotic and pro-survival genes [102, 112].

STAT4 plays an important role in directing the biological response to IL-12 and IL-23, which share receptor components [97, 114]. The STAT4-dependent polarization of naive CD4+ lymphocytes into Th1 effector cells is directed by IL-12 [97, 102, 103]. STAT4 plays an analogous role in IL-12-dependent NK cell activation. Recently, STAT4 has been shown to play an important role in the IL-23-dependent expansion of Th17 cells and an associated autoimmunity [114].

STAT5a and STAT5b are 96% identical and produce many effects. They play a role in directing a biological response to IL-3, single-chain and γC receptor families [115, 116].

STAT6 transduces signals for the Th2 cytokines IL-4 and IL-13, which share receptor components [97, 102, 103]. Studies have confirmed an important role for STAT6 in both the IL-4/IL-13-dependent polarization of naive CD4+ lymphocytes into Th2 effectors and mast cell activation. Furthermore, these studies have shown that STAT6 is important in promoting B-cell function, including proliferation, maturation, and major histocompatibility complex (MHC)-II and immunoglobulin (Ig) E expression [117].

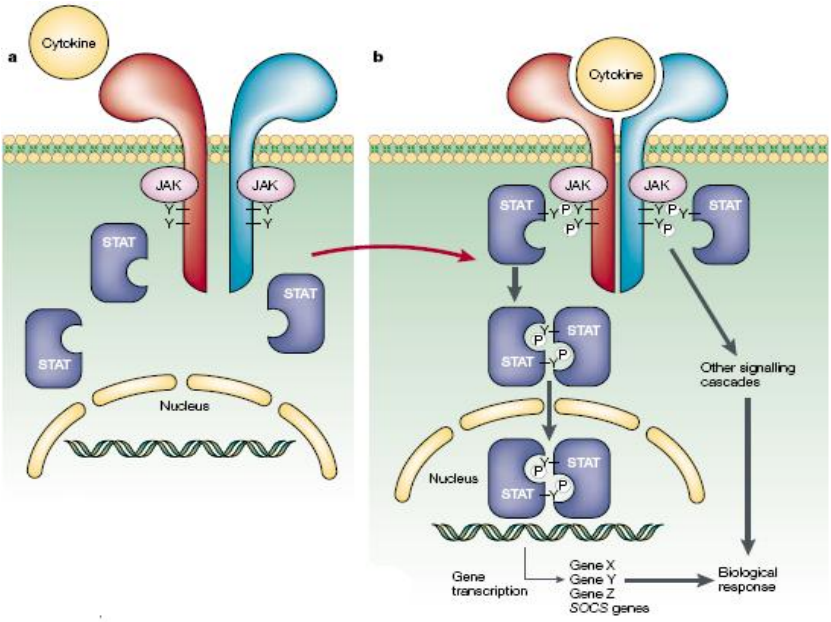

Figure 1.2: JAK/STAT signalling (adapted from [118]).

Cytokine-cytokine receptor interaction causes JAKS to get into close proximity and autophosporylate. JAKs then phosphorylate STATs, phosphorylated STATs form homodimers or heterodimers and translocate into the nucleus where they activate gene transcription.

1.5.3 IFN induced genes

IFN-α/β have an indirect antiviral effect through stimulation of innate and adaptive immune responses, and a direct effect through inhibition of viral replication in cells. The direct antiviral activity of type I IFNs is mediated by different mechanisms, e.g. blocking viral entry into a cell, control of viral transcription, cleavage of RNA, and preventing translation. Type I IFNs play several immunoregulatory roles and thereby influence innate and adaptive immune responses. For example, type I IFNs induce natural killer cell cytotoxicity [119] and up-regulation of the expression of MHC-I on many cells and of costimulatory molecules on antigen-presenting cells (APCs). IFN-α/β promotes T-cell proliferation [120] and enhances cross-presentation of exogenous antigen in MHC-I [121]. It has also been observed that IFN-λs induce expression of MHC-1 [122], but other cell-type specific immunomodulatory actions are yet unknown. Furthermore, IFN-α/β have a direct antiviral effect eventually leading to an antiviral state of the cell. The antiviral state of a cell has been defined as the ability of IFN inducible proteins to interfere with viral replication at a single cell level. After induction of IFN RNA and consequently secretion of IFN-α/β protein, IFNs binds to the surface of infected and neighbouring cells to initiate an intracellular signalling cascade, the JAK-STAT pathway, as explained above, which eventually leads to the induction of many ISGs through their ISRE on the ISG promoters.

Some ISGs are involved in establishing an antiviral state, but many of the mechanisms of action are not well defined. It is unclear what the role these gene products play in the therapeutic effects of IFN-β. Well characterized antiviral proteins are RIG-I, MDA-5, MxA GTPases, PKR and the 2'-5' oligoadenylate synthesases (OAS) that activate endoribonuclease L (RNase L). DsRNA can activate the OAS/RNase L system which then induces degradation of both viral and cellular RNA thereby blocking viral infections. DsRNA activates the pathogen recognition receptor (PRR), 2-5A synthetase (OAS), which results in production of 2-5A (oligoadenylates) from adenosine triphosphate (ATP). IFN signalling induces transcription of the OAS genes through ISREs in their promoters. Therefore, cells exposed to IFN as a result of a viral infection have increased levels of OAS that contribute to the IFN induced antiviral state [123]. 2-5A can activate catalytically-inactive RNase L monomers to form activated dimers with ribonuclease activity. Specifically, RNase L cleaves within single-stranded regions of viral RNA, resulting in inhibition of viral protein synthesis and viral replication. Mx proteins are interferon-induced guanosine triphosphatases (GTPases) that belong to the dynamin superfamily of large GTPases, they are not constitutively expressed in cells. The human MxA GTPase can accumulate in the cytoplasm of interferon-treated cells, partly associated with the endoplasmic reticulum. The mechanism of action of MxA is still not completely understood [124]. MxA proteins have an intrinsic GTPase activity and bind to essential viral components and block viral replication. Mx

GTPases appear to detect viral infection by sensing nucleocapsid-like structures. As a consequence, these viral components are trapped and sorted to locations where they become unavailable for the generation of new virus particles [124]. Protein kinase R (PKR) is an IFN inducible enzyme important in mediating antiviral effects of IFNs. PKR is a serine/threonine-specific protein kinase, which is autophosphorylated upon activation by double-stranded RNA. After phosphorylation, PKR phosphorylates the protein synthesis initiation factor eIF2, causing inhibition of translation. PKR may have additional substrates and can regulate signal transduction and gene transcription pathways [125, 126]. Another ISG is virus inhibitory protein, endoplasmatic reticulum associated, interferon inducible (Viperin/ Cig5 [127]). Viperin is an IFN-induced cytoplasmic protein of unknown function that inhibits production of human cytomegalovirus structural proteins [127], human immunodeficiency virus type 1 replication [128] and Hepatitis C virus replicon replication [129] and although its exact function not known, it plays a role in inhibiting RV replication [130].

1.5.4 Negative regulation of JAK/STAT signalling

JAK/STAT signalling is controlled at many levels both outside and inside the cell. Initiation, duration and magnitude regulation of the cytokine signalling occurs at multiple levels. The availability of cytokine can be limited to initiate a response either by negative feedback of its own signalling or it can be initiated by another cytokine such as Th1 and Th2 cytokines which suppress each other [131]. This regulates the expression and half life of cell surface receptor components and controls the duration of activation and half-life of intracellular signal transduction machinery.

Secreted and membrane associated receptors can act as antagonists by binding free cytokines. Activated receptors with bound cytokines are internalized and degraded, whereas activated signalling components can be dephosphorylated by a number of phosphatases. Specific STAT1 and STAT3 inhibitors (Protein inhibitor of STAT, 1 (PIAS1) and PIAS3) have also been reported [132, 133].

Phosphatases play an important role in regulating kinase-based signalling cascades. Genetic and biochemical approaches have implicated several phosphatases in the decay of cytokine receptors and JAKs, including SHP-1, SHP-2, and potentially CD45. Recruitment of SHP-1 (tyrosine phosphatase containing an SH2 domain) to a receptor complex terminates JAK activation by dephosphorylation of JAKs. SHP-1 binds directly to JAK1 and JAK2 [134-136]. Similar approaches have underscored a role for SHP-2, PTP1B, TC-PTP, and PTP-BL in STAT dephosphorylation. Although SHP-2 has been implicated in positive regulation of cell growth and development, several studies show a role for SHP-2 in negative regulation of both JAK/STAT signalling, e.g. GP130 and type I and II IFN induced signalling [137, 138]. Cells lacking SHP-2 show augmented suppression of cell viability, probably related to JAK and

STAT over activation [138]. Several other phosphatases have been described. SHIP (SH2-containing 5'-inositol phosphatase) is another tyrosine phosphatase implicated in negative regulation of JAK/STAT signalling in response to numerous cytokines [139-142]. c-Cbl could negatively regulate JAK/STAT by ubiquitin/ proteasome–dependent degradation of receptor tyrosine kinase [143, 144]. APS and c-Cbl might synergistically work together to bind JAK2 and result in negative regulation [145].

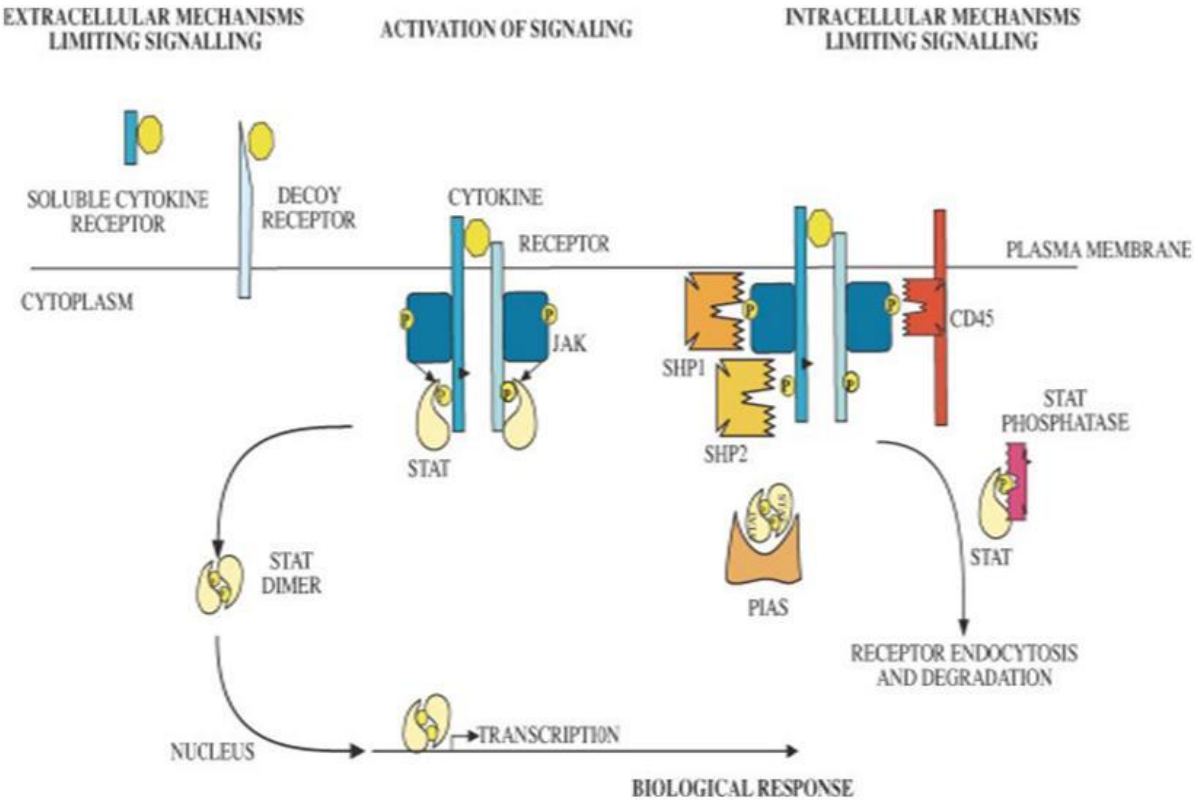

Figure 1.3: Negative regulation of JAK/STAT signalling (adapted from [146]).

Soluble cytokine receptors and decoy membrane bound receptors can act as antagonists for free cytokines. SHP1 and SHP2, CD45 and STAT phosphatases can dephosphorylate activated signalling components and activated receptors can be internalized and degraded.

1.6 Suppressors of cytokine signalling

In addition to JAK/STAT pathway effectors, there are negative regulators of this pathway such as suppressors of cytokine signalling (SOCS). The SOCS family of proteins consists of 8 members, SOCS1-7 and cytokine induced SH2-containing protein (CISH). The SOCS complete a negative feedback loop in the JAK/STAT pathway. Activated STATs stimulate the transcription of SOCS genes. The newly formed SOCS proteins perform their negative regulatory actions in three ways. Different members of the SOCS family are associated with negative regulation of different cytokines.

The three mechanisms of action are based on SOCS structure. The family is defined by similar structural motifs including a SOCS box motif at the C-terminus and a centrally located SH2 domain. In addition SOCS1 and SOCS3 have a kinase inhibitory region (KIR) at the N-terminus [118].

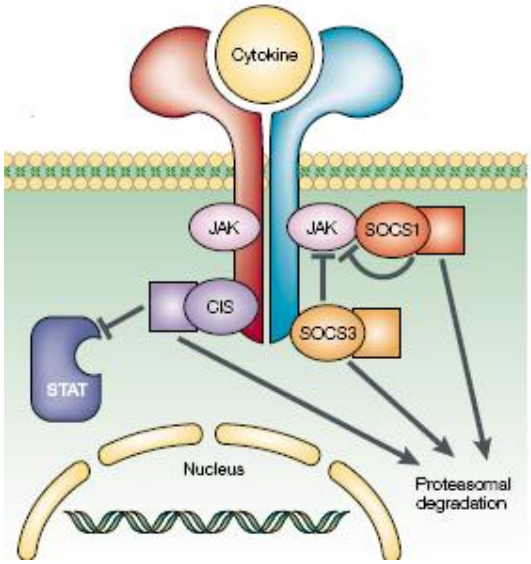

Figure 1.4: Negative regulation of JAK/STAT signalling by SOCS family (adapted from [118]).

SOCS act as negative regulators of JAK/STAT signalling in 3 ways; binding directly to the receptor blocking STAT binding, interacting with phosphorylated JAK sites and interacting with E3 ubiquitin ligase causing proteasomal degradation of receptor signalling components.

1.6.1 SOCS box and ubiquitination of target proteins

The SOCS box is a 40 amino acid module located at the carboxy terminus and it plays a role in target protein ubiquitination which results in regulation of the half life of a wide range of proteins. Protein ubiquitination is mediated by the subsequent action of an E1 ubiquitin-activating enzyme, an E2 ubiquitin conjugating enzyme and an E3 ligating enzyme. The E3 ligases define specificity to the substrate and attach ubiquitin to lysine side chains of the substrate. SOCS box containing proteins are able to form complexes with Elongin B and C, cullin-5 and RING-finger containing protein, Rbx [147,

[148]. This complex then functions as an E3 ubiquitin ligase with the SOCS box motif acting as substrate recognition site [149], which then recruits E2 ubiquitin transferase. The SOCS box contains a B/C box which mediates interaction with Elongin C and a cullin box motif which supports the interaction with cullin-5 [150].

Besides playing an important role in the degradation of substrates the SOCS box also is involved in the regulation of SOCS proteins themselves, causing both degradation and stabilization of the proteins. Several reports have shown that the Elongin B/C complex links SOCS proteins to E3 ligase activity, which then promotes degradation. This has been shown for SOCS1 [151], SOCS3 [152] and CISH [153]. On the contrary, binding between Elongin B/C and the SOCS box has been shown to stabilize SOCS proteins [151, 154-156].

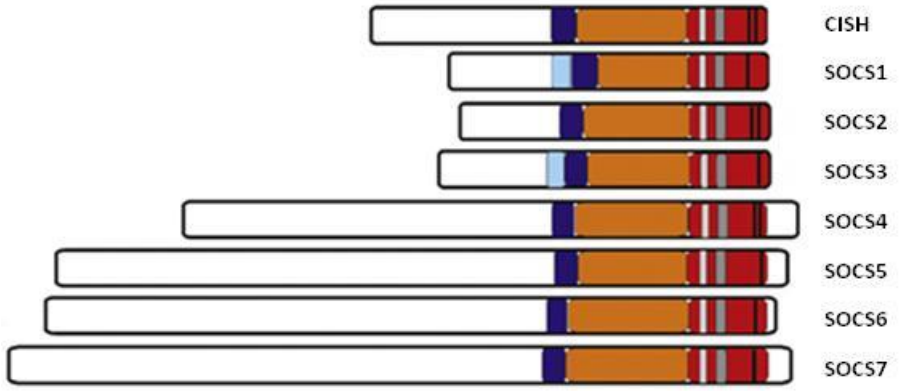

Figure 1.5: Structure of SOCS family of proteins (adapted from [157]).

Light blue denotes KIR region, dark blue denotes ESS region, orange denotes SH2 domain red denotes SOCS box, light grey denotes B/C box, dark grey denotes cullin box, black denotes conserved tyrosine residues

1.6.2 KIR of SOCS1 and SOCS3 acts as JAK pseudosubstrates

SOCS1 and SOCS3 can, in addition to their ability to suppress signalling using ubiquitin mediated degradation, inhibit JAK kinase activity directly. In contrast to the other SOCS family members, SOCS1 and SOCS3 have a KIR which can act as a JAK pseudosubstrate preventing substrates binding to the JH1 region of JAK. The KIR region is 12 amino acids long and is located towards the N terminus, near to the SH2 domain. The direct inhibition of kinase activity has been shown for JAK2 mediated STAT1 phosphorylation [158]. KIR point mutations have shown that despite intact SH2 domain, SOCS action is lost [158, 159] and recently a SOCS1-KIR peptide and a SOCS1 analogue (Tkip) have been shown to interact directly with JAK and inhibit IFN-γ signalling [160].

1.6.3 The centrally located SH2 domain determines specificity of SOCS family member to receptors

The centrally located Sh2 domain determines specificity by binding to phosphotyrosine on the receptors with small regions on this domain's N-and C-terminus being critical for this binding. The N-extended SH2 domain (N-ESS, Fig.1.5) forms a 15-residue alpha helix directly contacting the phosphotyrosine-binding [158, 159, 161].

1.6.4 Role of SOCS in allergic disease and asthma

Several studies have shown the importance of both SOCS1 and SOCS3 in regulating immune responses initiated by allergen sensitization and exposure. Increases in SOCS3 have been shown to play a role in Th2 mediated allergic immune diseases. SOCS3 mRNA levels were higher in asthmatics and atopic dermatitis patients and correlated with IgE levels for both diseases and disease severity in the asthmatics [162]. The same study demonstrated that SOCS3 is expressed in a Th2 environment in humans and mice. SOCS3 transgenic mice have an enhanced Th2 response in an ovalbumin (OVA) model with increased eosinophilia, lL-4, IL-5, IL-13 in BAL fluids and OVA specific IgE in serum Transgenic mice expressing a dominant negative form of SOCS3 and heterozygous $SOCS3^{+/-}$ mice showed the opposite [162]. All this information points to SOCS3 being a negative regulator of Th1 responses. Besides its role in Th1/Th2 responses, SOCS3 has also been shown to negatively regulate Th3 and Th17 subsets [163, 164]. Increased levels of SOCS3 are related to increased allergic responses in both human and mice, with mouse model data supporting the role SOCS3 plays in allergic diseases in humans.

The role of SOCS1 in allergy and asthma has been investigated using SOCS1 knockout mice. However SOCS1 knockout mice do not survive due to fatal inflammation, which can be rescued by deletion of IFN-γ, but also by removal of STAT6. Alterations in IL-4 and/or IL-13 signalling contribute to the fatal inflammation in SOCS1 deficient mice [165, 166]. Consistent with this, T cell-specific deletion of SOCS1 leads to enhanced production of IFN-γ and IL-4 and enhanced differentiation into Th1 and Th2 cells [167]. These studies indicate that SOCS1 is an inhibitor of Th1/Th2 polarisation. A functional polymorphism in the SOCS1 promoter has been associated with asthma in adults [168]. A promoter variant of this polymorphism enhanced SOCS1 mRNA expression levels in an alveolar lung cell line (A549) and reduced STAT1 phosphorylation after IFN-β stimulation [168]. Absence of SOCS1 in mice has been reported to result in elevated serum IgE levels, eosinophilia and Th2 cytokines upon OVA sensitization and challenge [169] or IL-13 [170] treatment. This again suggests that SOCS1 may negatively regulate Th2 cell polarization, with SOCS1 deficient mice showing enhanced Th2 responses (Table 1.1). In the IL-13 study SOCS1 RNA was measured in cultured airway smooth muscle cells (ASMCs) of

asthmatic and healthy subjects, with equal levels at baseline. However IL-13 induction of SOCS1 was impaired in asthmatics [170], which contradicts the previous mentioned study [168]. The role of SOCS1 in asthma is therefore less well defined, with indications of both increased levels of SOCS1 and failure of induction of SOCS1 mRNA in asthmatics [168, 170] .

1.6.5 Effects of SOCS on antiviral immunity

Currently no studies have investigated the potential role of SOCS proteins as negative regulators in RV induced innate immune responses. Studies using viruses other than RV or IFNs as stimuli have shown a clear role for SOCS in inhibiting IFN signalling causing a negative feedback on antiviral activity. IFN-α, IFN-λ1 and IFN-λ2 induced pSTAT1 and pSTAT3 are inhibited in HepG2 cells by overexpressing SOCS1 and SOCS3. SOCS1 and SOCS3 overexpression caused inhibition of the IFN-α, IFN-λ1 and IFN-λ2 induction of the ISGs, MxA and OAS [171, 172]. Semliki Forest virus infected SOCS1 knockout mice have a lower viral load than control animals, suggesting an important role for SOCS1 in inhibiting antiviral activity. It was shown that this effect was not caused by increased type I IFN levels, but is dependent upon type I IFN signalling (Table 1.1). The knockout mice have prolonged STAT phosphorylation, causing prolonged activation of the ISG OAS which is regulated by STAT through the IFNAR complex. SOCS1 specifically interacted with IFNAR1 independently of JAK1 [173]. Confirmation of the negative regulation of SOCS1 on antiviral activity was shown by using a SOCS1 antagonist which also showed antiviral effects[174].

Several studies have suggested SOCS may be important in negatively regulating antiviral responses induced by respiratory virus infection including influenza and RSV. All SOCS mRNA are constitutively expressed in a bronchial epithelial cell line (BEAS2Bs) with the exception of CISH [175] and bronchial epithelium is also the major site of RV infection [23]. SOCS1 and SOCS3 are induced upon Influenza and RSV infection [175-178] and SOCS3 induction by influenza is independent of type I IFN expression and dsRNA (polyIC) but dependent on 5' triphosphate RNA, which is dependent on the NF-κB pathway [179]. On the contrary, RSV induced SOCS1 and SOCS3 is regulated by TLR3 signalling, indicated by IRF-3 activation and nuclear translocation [178]. This indicates that different virus induced pathways may be involved in regulating SOCS induction by different respiratory viruses. SOCS1 and SOCS3 overexpression caused inhibition of RSV and influenza induced IFN signalling through suppression of phosphorylation of STAT1 and STAT2 [176, 179]. This gives an indication of the importance of SOCS1 and SOCS3 in inhibiting IFN induced signalling in bronchial epithelium in the context of respiratory viruses. These studies however did not show that SOCS1 and SOCS3 could inhibit virus induced IFN directly.

A possible role of SOCS in suppressing virus induced IFN has only recently been recognised. This is supported by a study showing that overexpression of both SOCS1 and SOCS3 leads to reduction of influenza induced IFN-β promoter activity. The mechanism appears to be different for both SOCS proteins, with SOCS1 reducing IRF3 activity and SOCS3 reducing NF-κB and also IL-8 promoter activity [175] (Table 1.1). IRF3 and NF-κB are crucial for IFN-β production and can be negatively regulated by SOCS1 and/or SOCS3. This suggests that SOCS not only play a role in inhibiting IFN signalling but also IFN production. Also, SOCS1 has been shown to degrade the adaptor molecule Mal which is specifically involved in TLR2 and TLR4 signalling by polyubiquitination upon activation. This shows a role for SOCS1 as a negative regulator of a different signalling pathway [180] (Table 1.1). Whether SOCS1 can play a role in negatively regulating other TLR signalling intermediates has not yet been investigated. Furthermore, SOCS1 may prevent NF-κB p65 mediated activation of NF-κB dependant genes, such as IL-8/CXCL8 and macrophage inflammatory protein (MIP-2)/CXCL2 [181] (Table 1.1). Besides the role of p65 in regulating NF-κB dependent genes, it has also been shown to be important in regulating virus induced IFN dependent genes [182]. Collectively, the data support the hypothesis that SOCS proteins are more than negative regulators of cytokine-cytokine receptor signalling events, and may be involved in the specific regulation of virus induced responses including IFN production.

Table 1.1: Evidence that supports a role for SOCS1 in virus induced asthma exacerbations

Study results	Reference
Polymorphism in SOCS1 promoter in asthmatics increases the levels of SOCS1.	[168]
SOCS1 knockout mice have increased eosinophilia, serum IgE and Th2 cytokines, indicating that SOCS1 negatively regulates Th1.	[169, 170]
SOCS1 is a negative regulator of type I and III IFN induced JAK/STAT signalling through JAK1.	[171-174]
SOCS1 can negatively regulate other signalling pathways including TLR2 and TLR4 signalling through Mal degradation.	[180]
SOCS1 can translocate into the nucleus and prevent NF-κB p65 mediated activation of NK-κB dependant genes.	[181]
SOCS1 inhibits influenza induced IFN-β and IRF3 activation suggesting a role for SOCS1 in negatively regulating IFN-β production.	[175]

1.7 Rationale, hypothesis, aims and outline

1.7.1 Rationale

The mechanisms of RV induced asthma exacerbations are still poorly understood. More insight into the cellular mechanisms induced upon RV infection is necessary and differences in these responses between asthma and healthy patients need to be identified. Recent work has shown a possible role for type I and III IFN deficiency in RV induced asthma exacerbations. SOCS are negative regulators of JAK/STAT signalling. IFNs and Th2 cytokines both signal through JAK/STAT and SOCS may negatively regulate other signalling pathways. SOCS1 expression is elevated in some asthmatic tissues. Despite several studies investigating the inhibitory role of SOCS in the Th1/Th2 balance and virus and IFN signalling, the role of SOCS in RV infection has not yet been investigated.

1.7.2 Hypotheses

Modulation of SOCS1 and SOCS3 can affect RV induced IFN responses and Th2 induced responses which indicates a role for SOCS1 and SOCS3 in RV infection and the pathogenesis of RV induced asthma exacerbations.

Specific hypotheses to be tested in this study are:

1. SOCS1 and SOCS3 mRNA and protein are induced by RV infection and stimulation with type I and III IFNs, PolyIC, inflammatory mediators and Th2 cytokines in BECs.

2. Modulation of SOCS1 and SOCS3 *in vitro* affects RV induced type I and III IFNs and Th2 induced signalling. Overexpression of SOCS1 and SOCS3 leads to a decrease in RV and type I IFN induced type I and III IFN signalling and Th2 induced STAT6 signalling. Conversely, inhibition of SOCS1 and SOCS3 leads to an increase in the above.

3. SOCS1 and SOCS3 are increased in RV infected asthmatic BECs which positively correlate with RV replication and negatively correlate with type I and III IFN induction.

4. Studies using a RV infection model in SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ C57/Bl6 mice show increased IFN expression, a longer duration of IFN induction and reduced RV replication compared to negative controls.

1.7.3 Aims

To assess whether SOCS1 and SOCS3 play a role in RV induced IFN responses *in vitro* and *in vivo* and to determine whether SOCS1 and SOCS3 can play a role in RV infection and may contribute to IFN deficiency in asthmatics.

Specific aims to be tested in this study are:

1. To investigate the induction of mRNA and protein of SOCS family members in HBECs *in vitro*, upon RV infection and stimulation with type I and III IFN, PolyIC, Th2 cytokines and inflammatory mediators

2. To investigate whether SOCS1 and SOCS3 can modulate responses induced by RV infection, type I IFNs, Th2 cytokines and inflammatory mediators in HBECs; specifically, IFN, STAT6, proinflammatory cytokine and NF-κB activation.

3. To investigate whether SOCS1 and SOCS3 levels and responses to RV infection are different in HBECs from asthmatics compared to NAs and whether this correlates with both clinical and RV related study outcomes. RV related study outcomes that will be investigated are type I and III IFN mRNA expression and RV release and replication. Clinical parameters that will be investigated are IgE levels, skin prick test positivity, radio allergosorbent test (RAST) and lung function.

4. To investigate the role of SOCS1 in a RV infection model using $SOCS1^{-/-}$ IFN-$\gamma^{-/-}$ C57/Bl6 mice. Outcomes to be investigated are differential cell counts in BAL (e.g. macrophages, eosinophils, lymphocytes and neutrophils) and RV mRNA, type I and III IFN mRNA and protein and chemokine protein induction.

1.7.4 Outline

Chapter 1 is the introduction to the subject including background on RV and asthma, cellular signalling involved in RV infection and the negative feedback mechanism of this including the SOCS family. Chapter 2 will outline the material and methods involved in performing this study. This will be followed by 3 results chapters, starting with *in vitro* determination of SOCS functions, followed by a chapter investigating SOCS *ex vivo* in samples obtained from asthmatics and healthy subjects and finally investigating the role of SOCS1 *in vivo* in an RV infection and IL-13 pretreatment and RV infection model in mice. The IL-13 pretreatment model was designed as a surrogate for allergic

inflammation. Chapter 6 will be a discussion and conclusion about the obtained results in this PhD thesis and the plans for future work.

2 Materials and methods

2.1 Materials

2.1.1 Buffers and reagents

Table 2.1: Buffers and reagents.

Reagent	Composition	Supplier
ACK lysis buffer	0.15M Ammonium Chloride, 1.0mM Potassium Hydrogen Carbonate, 0.1mM Disodium EDTA in 500mL dH_20, Sterile Filtered	Sigma Aldrich, USA; Invitrogen (Gibco), UK
Ampicillin (100mg/mL)	Provided in 70% (v/v) ethanol	Sigma Aldrich, USA
BAL fluid	14mg Lidocaine and 400uL 0.5M EDTA in 40mL PBS	
β-mercaptoethanol (β-Me)	14.3M $C_2H_60_5$	Sigma Aldrich, USA
Bovine serum albumin powder (BSA)	1% BSA in 0.15M NaCl pH 7.0	Sigma Aldrich, USA
Dulbecco's-PBS (1X) (D-PBS)	Phosphate buffered saline: 0.0014M KH_2PO_4, 0.008M Na_2HP0_4-$7H_20$, 0.0026M KCl, 0.137M NaCl	PAA, Austria
ELISA (Enzyme Linked Immunosorbent Assay) diluent	1% w/v BSA in PBS	Invitrogen,UK; PAA, Austria
ELISA wash buffer	0.05% Tween20 in PBS	Sigma Aldrich, USA; PAA, Austria
ELISA stop solution	2M H_2SO_4	Sigma Aldrich, USA
Ethanol	99.6% Ethanol	VWR, UK

HEPES buffered saline solution	30mM HEPES	Lonza, USA
Isoflurane	Isoflurane-Vet 100% w/w	Merial, UK
Lipofectamine 2000 (LP2000)	No information given by supplier	Invitrogen, UK
Methanol	Methanol	VWR, UK
NuPAGE anti-oxidant	No information given by supplier	Invitrogen, UK
Pentobarbitone	'Pentoject' Pentobarbitone Sodium 20% w/v	AnimalCare Ltd, UK
Poly(Ethylene glycol) (PEG)	Poly(Ethylene glycol) 6,000	Sigma Aldrich, USA
PolyIC	used at a concentration of 10mg/mL in HBECs and 100mg/mL in BAL cells.	Sigma Aldrich, USA
Trypan Blue solution 0.4%	0.4% $C_{34}H_{24}N_6Na_4O_{14}S_4$ in 0.81% sodium chloride and 0.06% potassium phosphate	Sigma Aldrich, USA
Quantitect probe Polymerase chain reaction (PCR) master mix	HotStarTaq DNA polymerase, quantitect probe PCR buffer (Tris-HCl, KCl, $(NH_4)_2SO_4$, 8mM $MgCl_2$ pH 8.7) dNTP mix, ROX (passive reference dye)	Qiagen, UK
Random hexamers	Random hexadeoxynucleotides (0.5μg/mL)	Promega, USA
SeeBlue Plus2 pre-stained standard	Containing: Tris-HCl, formamide, sodium dodecyl sulphate, phenol red No quantities specified by manufacturer	Invitrogen, UK
Sodium Chloride (NaCL) 5M	146g NaCl in 500mL water (5M)	Sigma Aldrich, UK
Streptavidin – HRP (horseradish peroxidase)	ELISA grade Streptavidin – HRP conjugate	Biosource, USA

Superfect transfection reagent	3 mg/mL Superfect transfection reagent	Qiagen, UK
TMB Substrate	Tetramethyl benzidine chromogen solution	Invitrogen, UK
Tris-glycine SDS sample buffer (2X)	0.5M Tris-HCl (pH 6.8), glycerol, 10% (w/v) SDS, 0.1% bromophenol blue in distilled water	Invitrogen, UK
Trypan Blue	0.4% trypan blue in 0.85% saline solution	Invitrogen, UK
Trypsin-EDTA (TE) (1X) molecular grade	0.025% trypsin, 0.01% EDTA	Invitrogen, UK
Trypsin-EDTA (1X)	0.025% trypsin, 0.01% EDTA	Lonza, USA
Trypsin-EDTA (10X)	0.5% trypsin, 5.3mM EDTA	Invitrogen, UK
Trypsin neutralising solution (TNS)	No information given by supplier	Lonza, USA
Tween 20 (polyoxetylene-sorbitan monolaureate)	2 ethylene oxide units, 1 sorbitol unit, 1 lauric acid unit	Sigma Aldrich, USA
Western blot blocking solution	5g w/v milk proteins in western blot washing solution	Sigma Aldrich, USA
Western blot running buffer	50mL Novex® Tris-Glycine SDS Running Buffer (10X), 950mL water	Invitrogen, UK
Western blot stripping buffer	2% sodium dodecyl sulphate (SDS), 62.5 mM Tris-HCL (pH 6.7), 100mM 2-mercaptoethanol	Sigma Aldrich, USA
Western blot transfer buffer	For 1 gel: 40mL Novex® Tris-Glycine Transfer Buffer (25X), 100mL methanol (10% v/v), 860mL water, 1mL NuPAGE	Invitrogen, UK

	anti-oxidant. For 2 gels 20% v/v	
Western blot washing solution	0.1% Tween20 in PBS	Sigma Aldrich, USA, PAA, Austria

2.1.2 Commercially available kits

Table 2.2: Commercially available kits.

Name	Components	Supplier
CelLytic NuCLEAR Extraction Kit	10X hypotonic Lysis Buffer (100mM HEPES, pH 7.9, 15mM MgCl2, 100mM KCl), Protease Inhibitor Cocktail (4-(2-Aminoethyl) benzenesulfonyl fluoride, Pepstatin A, Bestatin, Leupeptin, Aprotinin, and trans-EpoxysuccinylL-leucyl-amido(4-guanidino)-butane), 1M Dithiothreitol, Extraction Buffer (20mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.42M NaCl, 0.2mM EDTA, and 25% (v/v) Glycerol)	Sigma Aldrich, USA
Dual-Luciferase® Reporter Assay System	Luciferase Assay Buffer II, Luciferase Assay Substrate (Lyophilized Product), Stop & Glo Buffer, 50X Stop & Glo Substrate, 5X Passive Lysis Buffer	Promega, USA
ECL Advance western blotting detection kit	Solution A: 50mL ECL advance solution containing tris buffer in 3.2% (v/v) ethanol. Solution B: 50mL proprietary substrate in tris buffer	GE Healthcare, UK

ECL Plus western blotting detection kit	Solution A: 100mL ECL plus substrate containing tris buffer Solution B: 2.5mL Acridan solution in Dioxane and Ethanol	GE Healthcare, UK
Maxiprep Kit	Buffer P1, Buffer P2, Buffer P3, Buffer QBT, Buffer QC, Buffer QF, Qiagen-tip 500	Qiagen, UK
Mouse Eotaxin/ CCL11 DuoSet	Capture antibody (rat anti-mouse Eotaxin), detection antibody (biotynylated goat anti-mouse Eotaxin), standard (recombinant mouse Eotaxin), streptavidin-HRP	R&D Systems, USA
Mouse IFN-α ELISA kit	Pre-coated microtiter Plate, wash Concentrate, mouse IFN-α standard 10,000 pg/mL, sample buffer, antibody concentrate, HRP conjugate concentrate, concentrate diluent, assay diluent, TMB substrate Solution, stop solution	PBL Interferon Source, USA
Mouse IFN-β ELISA kit	Pre-coated microtiter Plate, 10x wash Concentrate, mouse IFN-β standard 500,000 pg/mL, sample diluent, antibody concentrate, HRP conjugate concentrate, concentrate diluent, TMB substrate Solution, stop solution	PBL Interferon Source, USA
Mouse IFN-λ2/3/ IL28AB DuoSet	Capture antibody (rat anti-mouse IL-28AB), detection antibody (biotynylated goat anti-mouse IL-28AB), standard (recombinant mouse IL-28AB), streptavidin-HRP	R&D Systems, USA

Mouse IP-10/CXCL10 DuoSet	Capture antibody (rat anti-mouse IP-10), detection antibody (biotynylated goat anti-mouse IP-10), standard (recombinant mouse IP-10), streptavidin-HRP	R&D Systems, USA
Mouse KC/CXCL1 DuoSet	Capture antibody (rat anti-mouse KC), detection antibody (biotynylated goat anti-mouse KC), standard (recombinant mouse KC), streptavidin-HRP	R&D Systems, USA
Mouse LPS-induced CXC chemokine(LIX)/CXCL5/ ENA-78 Duoset	Capture antibody (rat anti-mouse LIX), detection antibody (biotynylated goat anti-mouse LIX), standard (recombinant mouse LIX), streptavidin-HRP	R&D Systems, USA
Mouse monocytes-derived chemokines (MDC)/CCL22 Duoset	Capture antibody (rat anti-mouse MDC), detection antibody (biotynylated goat anti-mouse MDC), standard (recombinant mouse MDC), streptavidin-HRP	R&D Systems, USA
Mouse MIP-2/CXCL2 Duoset	Capture antibody (rat anti-mouse MIP-2), detection antibody (biotynylated goat anti-mouse MIP-2), standard (recombinant mouse MIP-2), streptavidin-HRP	R&D Systems, USA
Mouse regulated upon activation, normal T-cell expressed, and secreted (RANTES)/ CCL5 DuoSet	Capture antibody (rat anti-mouse RANTES), detection antibody (biotynylated goat anti-mouse RANTES), standard (recombinant mouse RANTES), streptavidin-HRP	R&D Systems, USA
Mouse thymus and activation-regulated chemokines (TARC)/CCL17 DuoSet	Capture antibody (rat anti-mouse TARC), detection antibody (biotynylated goat anti-mouse TARC), standard (recombinant mouse TARC), streptavidin-HRP	R&D Systems, USA

Omniscript RT (Reverse Transcription) Kit	dATP, dCTP, dGTP, dTTP,(10mM each) in water, 10X Buffer RT, Omniscript reverse transcriptase, RNase-free water	Promega, USA
PCR Purification Kit	Spin columns, Buffer PB, Buffer PE, Buffer EB, pH Indicator, Loading Dye , collection tubes	Qiagen, UK
REASTAIN Quick-Diff Kit	Reastain Quick-Diff Blue: Azur II 0,09% Glycerol 5% Sodiumazide <0,1%; Reastain Quick-Diff Fix: Methanol 100%, Methyleneblue <0,01%; Reastain Quick-Diff Red,Eosin Y 0,12%, Sodiumazide <0,1%; Phosphate buffer: Potassium dihydrogen phosphate/ disodium hydrogen phosphate x $2H_2O$ 67mmol/L	Reagena, Finland
RNAase-free DNase set	1500 units RNase-free DNase I, RNase-free buffer RDD	Qiagen, UK
RNeasy Mini Kit	Buffer RLT, Buffer RW1, Buffer RPE, RNase-free water, RNeasy mini spin columns, Collection tubes	Qiagen, UK
TOPO TA cloning kit (with pCR 2.1- TOPO vector)	Components of this kit used in this study include: pCR 2.1-TOPO (10ng/μL) in 50% glycerol, 50mM Tris-HCl (pH 7.4), 1mM EDTA, 1mM DTT, 0.1% trition X-100, 100μg/mL BSA, phenol red; salt solution (1.2M NaCl, 0.06M $MgCl_2$), One Shot Chemically Competent TOP10 *E. coli*	Invitrogen, UK

Refer to manufacturers' component formulations for detailed component composition.

2.1.3 Media and media supplements

Table 2.3: Media and media supplements for human cells.

Name	Composition	Supplier
BAL cell culture medium	50mL (10%) FCS, 10mL (2%) Hepes buffer, 5mL (1%) NaHCO3, 5mL p/s in 500mL RPMI medium	
BEAS2B cell growth medium	50mL (10%) FCS, 10mL (2%) Hepes buffer, 5mL (1%) $NaHCO_3$in 500mL RPMI medium	PAA, Austria; Invitrogen, UK
BEAS2B cell infection medium	10mL (4%) FCS, 10mL (2%) Hepes buffer, 5mL (1%) $NaHCO_3$in 500mL RPMI medium	PAA, Austria; Invitrogen, UK
BEAS2B serum free medium	10mL (2%) Hepes buffer, 5mL (1%) $NaHCO_3$in 500mL RPMI medium	PAA, Austria; Invitrogen, UK
Bronchial epithelium basal medium (BEBM)	Refer to manufacturers' medium formulation	Lonza, USA
Bronchial epithelial growth medium (BEGM)	BEBM with BEGM single quot additives	Lonza, USA
BEGM single quot additives	13g/L bovine pituitary extract, 5g/L insulin, 50g/L gentamicin, 0.1mg/L retinoic acid, 10g/L transferrin, 6.5mg/L triiodothyronine, 0.5g/L hydrocortisone & epinephrine, 0.5mg/L human epidermal growth factor	Lonza, USA
Dulbeccos modified eagles medium (D-MEM)	With high glucose (4,5g/L)and L-glutamine Refer to manufacturers' medium formulation	PAA, Austria
Fibronectin	Lyophilizate from human plasma	Invitrogen, USA

Foetal calf serum (FCS)	Heat inactivated foetal calf serum	Invitrogen, USA
HeLa cell growth medium	50mL (10%) FCS, 10mL (2%) Hepes buffer, 5mL (1%) $NaHCO_3$in 500mL D-MEM	PAA, Austria; Invitrogen, UK
HeLa cell infection medium	10mL (2%) FCS, 10mL (2%) Hepes buffer, 5mL (1%) $NaHCO_3$ in 500mL D-MEM	PAA, Austria; Invitrogen, UK
HeLa cell medium for virus titrations	20mL (4%) FCS, 10mL (2%) Hepes buffer, 5mL (1%) $NaHCO_3$, pen/strep in 500mL D-MEM	PAA, Austria; Invitrogen, UK
HEPES buffered solution	238.3g (1M) HEPES in 1L distilled water, pH 7.2-7.5	Invitrogen, UK
Optimem-1 medium	Refer to manufacturers' medium formulation	Invitrogen, UK
Penicillin/ Streptomycin	10,000 units/mL penicillin, 10,0000µg/mL streptomycin	PAA, Austria
Recombinant human like collagen	>95% human collagen from *E. coli*	Biovision, USA
RPMI (Roswell park memorial institute) Medium	with L-glutamine and phenol red Refer to manufacturers' medium formulation	PAA, Austria
Sodium bicarbonate	7.5% $NaHCO_3$ solution	Invitrogen, UK
Ultroser-G BioSpera	Serum substitute – lyophilized Ultroser G	Pall Life Sciences, UK

Table 2.4: Media and media supplements for bacteria.

Name	Composition	Supplier
Escherichia coli (*E.coli*) Fast medium Amp agar (powdered)	LB based agar medium supplemented with ampicillin (100mg/mL) No quantities given by supplier	InvivoGen, USA
E. coli Fast medium Amp liquid (powdered)	TB based medium (tryptone, yeast extract, K_2HPO_4, glycerol) supplemented with ampicillin No quantities given by supplier	InvivoGen, USA
Luria-Bertani (LB) agar	14g of agarose, in 1L LB media	Sigma- Aldrich, USA
LB medium	10g tryptone, 5g yeast extract, 5g NaCl, in 1L distilled water, adjusted to pH 7.0	Sigma- Aldrich, USA

2.1.4 Cells

The human cervix carcinoma cell line HeLa (Ohio and H1) and the transformed human bronchial epithelial cell line BEAS2B were obtained from the American Type Culture Collection (ATCC), and primary Human Bronchial Epithelial Cells (HBEC) were obtained from Lonza (USA). Three different sources of HBECs were used throughout this study with each obtained from NA, non-smoking, non-alcoholic subjects. A summary of the individual characteristics of each HBEC source is shown in Table 2.4. HeLa cells, BEAS2Bs and HBECs were provided in frozen cryovials and stored in liquid nitrogen until resuscitated in pre-warmed D-MEM, RPMI and BEGM complete medium respectively (Table 2.3). HeLa cells and BEAS2B cells were grown in 175cm^2 tissue culture flasks and HBECs in 80cm^2 in a humidified 37°C incubator with 5% CO_2. Once resuscitated, HBECs were given the passage number 1 and were typically cultured and used in experiments until passage 6-7, beyond which point cell growth rate and transfection efficiency was reduced and cells were no longer used.

Table 2.5: HBECs and their sources used in this project.

	HBEC source number		
	4F1604	7F3081	7F3143
Age	27 Y	49 Y	27 Y
Sex	M	M	F
Race	AA	C	C
Smoking	N	N	N
Alcohol	N	N	N
Cell passage	1	1	1
Viability-blue exclusion (%)	83	86	86
Cell count (cells/mL)	955000	685000	682500
Doubling time (h)	21	24	24
Population doubling	15	19	18

Y=Years; M=Male;F=Female; AA=African American; C=Caucasian; N=Non

2.1.5 Antibodies for western blotting

Table 2.6: Antibodies used in western blotting and their working concentrations used in this project.

Antibody	Isotype	Working concentration	Supplier
Anti-SOCS1	Mouse IgG_1	500ng/mL	Abcam, UK; Millipore, UK
Anti-SOCS3	Mouse IgG_{2b}	200ng/mL	Santa Cruz, USA
Anti-CISH	Mouse IgG_1	200µg/mL	Santa Cruz, USA
Anti-α tubulin	Mouse IgM	200ng/mL	Santa Cruz, USA
Anti-rabbit	Sheep IgG-HRP	200ng/mL	AbD Serotec, UK
Anti-mouse	Goat IgG-HRP	80ng/mL	Santa Cruz, USA

2.1.6 Primers and probes for Realtime RT-PCR

All Realtime RT-PCR primers were purchased from Invitrogen (UK) in a desalted, dry pellet form, and all probes purchased from Qiagen (UK) in a HPLC purified, dry pellet form. All handing of primers and probes was in a designated DNA template free, nuclease-free flow hood. Primers and probes were resuspended using sterile TE buffer (Table 2.1) at a stock concentration of 100µM and stored at -20°C. Primer and probe working stocks were prepared by diluting to 5µM in nuclease-free water (Promega, Madison, WI, USA) and stored at -20°C. Forward and reverse primer and probe sequences, and their optimised concentration (nM) for use in Realtime RT-PCR are shown in Table 2.7.

Table 2.7: TaqMan Realtime RT-PCR primers and probes and their optimal concentrations used in this project.

h=human, m=murine.

Name	Sequence (FAM 5'-TAMRA 3')
hCISH forward (900nM)	tggtagtccagagaccccaac
hCISH reverse (900nM)	agatgtctcaggatgggcttt
hCISH probe (100nM)	acagggaagaaccagagaagccaggg

hIFN-β forward (300nM)	cacggatacagaacctatgg
hIFN-β reverse (900nM)	acgaacagtgtcgcctacta
hIFN-β probe (100nM)	tcagacaagattcatctagcactggctgga
hIFN-λ1/IL-29 forward (300nM)	ggacgccttggaagagtcact
hIFN-λ1/IL-29 reverse (900nM)	agaagcctcaggtcccaattc
hIFN-λ1/IL-29 probe (100nM)	agttgcagctctcctgtcttccccg
hIFN-λ2/3/IL-28AB forward (300nM)	ctgccacatagcccagttca
hIFN-λ2/3/IL-28AB reverse (900nM)	agaagcgactcttctaaggcatctt
hIFN-λ2/3/IL-28AB probe (100nM)	tctccacaggagctgcaggccttta
hMxA forward (900nM)	cagcacctgatggcctatcac
hMxA reverse (900nM)	catgaactggatgatcaaagg
hMxA probe (100nM)	aggccagcaagcgcatctccag
hOAS forward (900nM)	ctgacfctgacctggttgtct
hOAS reverse (900nM)	ccccggcgatttaactgat
hOAS probe (100nM)	cctcagtcctctcaccacttttca
hRIG-I forward (900nM)	ccaagccaaagcagttttcaa
hRIG-I reverse (900nM)	cacarggattccccagtcatg
hRIG-I probe (100nM)	ttgaaaaaagagcaaagatattctgtgcccgac
hRV forward (50nM)	gtgaagagccscrtgtgct
hRV reverse (300nM)	gctscagggttaaggttagcc
hRV probe (100nM)	tgagtcctccggcccctgaatg

hSOCS1 forward (900nM)	ccctcggtgttgtagcagctt
hSOCS1 reverse (900nM)	ggtttgtgcaaagatactgggtatat
hSOCS1 probe (100nM)	aggtaggaggtgcgagttcaggtcctg
hSOCS2 forward (900nM)	gaacggcactgttcacctttatc
hSOCS2 reverse (900nM)	gcctacagagatgctgcagaga
hSOCS2 probe (100nM)	ggtgctgacgtgtagagcggtttggt
hSOCS3 forward (900nM)	tgggacgatagcaaccacaa
hSOCS3 reverse (900nM)	cgaagtgtcccctgtttgga
hSOCS3 probe (100nM)	tggattctccttcaattcctcagcttccc
hSOCS4 forward (900nM)	gtagcgatggtgtttcactac
hSOCS4 reverse (300nM)	tcttggccgggcacagt
hSOCS4 probe (100nM)	ctgacctcaggtgaccacccgcc
hSOCS5 forward (900nM)	cagggactctgcgcaagag
hSOCS5 reverse (300nM)	cgggcatgcagggatct
hSOCS5 probe (100nM)	agcggcggaagctcacagagaagag
hSOCS6 forward (900nM)	cctcctcagcacccatagtct
hSOCS6 reverse (900nM)	ggcgcgggactgtagtgat
hSOCS6 probe (100nM)	gctgcggagcgacgtggaccttat
hSOCS7 forward (300nM)	ccactcgcgggtccttct
hSOCS7 reverse (900nM)	caccgttgcagctcttggt
hSOCS7 probe (100nM)	ctccttcaaaatccgcctcagtcgc

hViperin forward (900nM)	cacaaagaagtgtcctgcttggt
hViperin reverse (900nM)	aagcgcatatatttcatccagaataag
hViperin probe (100nM)	cctgaatctaaccagaagatgaaagactcc
mIFN-β forward (900nM)	ccatcatgaacaggtggat
mIFN-β reverse (900nM)	gagagggctgtggtggagaa
mIFN-β probe (100nM)	ctccacgctgcgttcctgctgtg
mIFN-γ forward (900nM)	tcaagtggcatagatgtggaagaa
mIFN-γ reverse (900nM)	tggctctgcaggattttcatg
mIFN-γ probe (100nM)	tcaccatccttttgccagtt
mIL-28 forward (900nM)	aaaggattgccacattgctc
mIL-28 reverse (900nM)	tcaagcagcctcttctcgat
mIL-28 probe (100nM)	tccccaaaagagctgcaggc
mOAS forward (900nM)	tcctgggtcatgttaatacttcca
mOAS reverse (900nM)	ccccagggaggtacattcct
mOAS probe (100nM)	caagcctgatcccagaatctatgcc
mSOCS1 forward (900nM)	ccgtgggtcgcgagaac
mSOCS1 reverse (900nM)	aaggaactcaggtagtcacggagta
mSOCS1 probe (100nM)	tggcgcgcatccctcttaaccc
mSOCS3 forward (900nM)	gcgggcacctttcttatcc
mSOCS3 reverse (900nM)	tccccgactgggtcttgac
mSOCS3 probe (100nM)	ctcggaccagcgccacttcttca

mViperin forward (900nM)	cgaagacatgaatgaacacatcaa
mViperin reverse (900nM)	aattaggaggcactggaaaacct
mViperin probe (100nM)	ccagcgcacagggctcaggg
18S forward (300nM)	cgccgctagaggtgaaattct
18S reverse (300nM)	cattcttggcaaatgctttcg
18S probe (100nM)	accggcgcaagacggaccaga

2.1.7 Plasmids

The plasmids used in this study are listed in Table 2.8. They were either obtained commercially, non-commercially, or built in house. All plasmids were grown in One Shot Chemically Competent TOP10 *E. coli* (Invitrogen) and all plasmid DNA prepared using a maxiprep method, aliquoted and stored at -80°C at 1μg/mL. Plasmids were re-suspended in LB medium (Table 2.3), streaked onto plates containing ampicillin LB agar (Table 2.3), and an individual colony was picked and grown in Ampicillin LB medium, to produce a starter culture, according to manufacturers' instructions.

Table 2.8: Plasmids used in this study.

Plasmids were either obtained commercially, non-commercially, or built in house.

Plasmid	Description	Source
-546bp CXCL8 promoter reporter construct	A fragment of the CXCL8 promoter cloned into the pGL3-basic luciferase reporter gene vector (-546bp relative to the transcriptional start site)	Naofumi Mukaida
-651bp IL-6 promoter reporter construct	Full-length IL-6 promoter cloned into the pGL3-basic luciferase reporter gene vector (-651bp relative to the transcriptional start site)	Rey Pannetieri
CXCL5/ ENA-78 promoter reporter construct	-983bp fragment of promoter	Switchgear Genomics, USA

CXCL-10/IP-10 promoter reporter construct	-928bp fragment of promoter	Switchgear Genomics, USA
Cyclooxygenase (COX)2 promoter reporter construct	-928bp fragment of promoter	Switchgear Genomics, USA
IFN-β promoter reporter construct	Wild-type IFN-β promoter luciferase reporter construct containing PRDI-IV enhancer elements (-125bp relative to the transcriptional start site)	Takashi Fujita
IFN-λ1 promoter reporter construct	1Kb of the human IFN-λ1 promoter inserted into the *Mlu* I and *Xho* I restriction sites of the pGL3-basic luciferase reporter gene vector	Built in house
pEFBOS	ΔRIG-I empty vector control	T. Fujita
pEFBOS-ΔRIG-I	Constitutively active RIG-I, encoding the RIG-I CARD domain only	T. Fujita
pNF-κB Luciferase	NF-κB dependent luciferase reporter (containing consensus NF-κB-binding sites)	Clontech, France
pORF5-hSOCS1	Expression vector encoding hSOCS1 open reading frame	InvivoGen, USA
pORF5-hSOCS3	Expression vector encoding hSOCS3 open reading frame	InvivoGen, USA
pORF5-mcs	pORF5-hSOCS1 and 3 empty vector control	InvivoGen, USA
pUNO- hIKKβ	Expression vector encoding hIKKβ open reading frame	InvivoGen, USA
pUNO- hIKKε	Expression vector encoding hIKKε open reading frame	InvivoGen, USA
pUNO-hIRF3	Expression vector encoding hIRF3 open reading frame	InvivoGen, USA
pUNO1-mcs (pUNO1)	ΔTRIF empty vector control	InvivoGen, USA

pUNO1-sahTRIFΔ (ΔTRIF)	Super-activated human TRIF, encoding TRIF lacking the C-terminal 67aa	InvivoGen, USA
pUNO-mcs (pUNO)	pUNO-IRF3, pUNO-TBK-1, pUNO IKKβ and pUNO- IKKε empty vector control	InvivoGen, USA
pUNO-hTBK-1	Expression vector encoding hTBK-1 open reading frame	InvivoGen, USA
STAT6 promoter reporter construct	STAT6 dependent luciferase reporter , TPU474 (containing four repeats of the C/EBP- STAT6-binding sites from the human IgE promoter)	U. Schindler
TaqMan RT-PCR standard plasmids	TaqMan RT-PCR products were each ligated into a pCR 2.1-TOPO vector	Built in house

2.1.8 Proteins

Table 2.9: Recombinant Proteins used in this study

Protein	Working concentration	Supplier
IL-4	50ng/mL	R&D systems, USA
IL-13	50ng/mL	R&D systems, USA
IFN-β	10ng/mL	PBL Interferonsource, USA
IFN-λ1	10ng/mL	R&D systems, USA
IL-1β	10ng/mL	R&D systems, USA
TNF-α	10ng/mL	R&D systems, USA

2.2 Clinical methods

2.2.1 Bronchoscopy

Bronchoscopies were performed according to BTS guidelines [183] in the endoscopy unit at St. Mary's Hospital, Royal Brompton hospital or Bern children's hospital. Participants were monitored by a separate doctor or nurse. Lung function (FEV_1) was recorded before and after bronchoscopy and all participants received nebulised 0.5mg Salbutamol and 500ug Ipratropium bromide prior to the procedure. The Keymed BF260 bronchoscope (Olympus, UK) was used.

2.2.2 Bronchial biopsies

Six endobronchial biopsies were taken from the left segmental and subsegmental carinae from different sites with fenestrated forceps (FB-211D). Two biopsies were snap frozen on dry ice prior to transfer to liquid nitrogen storage. The other 4 biopsies were fixed in 4% paraformaldehyde and transferred to the department of lung pathology at the Brompton hospital for further processing for histology and immunohistochemistry.

2.2.3 Bronchial brushing collection

Six bronchial brushings were taken with 5mm sheathed endobronchial brushes (Olympus BC-202D-5010) under direct vision by inserting the bronchoscope into a bronchial sub segment and brushing 5–10 strokes against bronchial wall. Sheathed brushes were removed from the bronchoscope and shaken into warmed BEGM medium for primary bronchial epithelial cells in a single 20mL sterile tube. Brushes and sheaths were detached into the medium and this was immediately transported to the laboratory for counting and culture. The team aimed to get the samples from the patient into the incubator within 30mins and samples were kept at 37ºC whilst in transport.

2.2.4 Bronchial brushing processing

BEGM complete medium containing detached bronchial brushes and sheaths was transported to the laboratory and processed within 15min of sampling. The sample was gently vortexed to detach cells from the brushes and brush sheaths were flushed with fresh medium to detach any cells adhering to them. Any remaining cells attached to the brushes were removed by passing the brush through a pipette tip. After cells had been detached the medium containing cells was centrifuged at 1150rpm for 6min, the supernatant discarded and then the cell pellet resuspended in fresh warmed medium and counted using a haemocytometer. The total sample was transferred to a pre-coated 25cm² flask that had been incubated at $37^{o}C/5\%$ CO_2 overnight. The sample was then left undisturbed for 48h to allow for maximum adherence of cells to the flask after which the medium was discarded and

replaced by fresh medium containing Ultroser G (100µl per 10mL BEGM). The medium was then removed and replaced every 48h. BEGM with supplemented Ultroser G was used for the first week of culture only and after this time only BEGM complete was used. Cells were cultured until 70–80% confluent and split and used to seed further flasks.

2.3 *In vitro* methods

2.3.1 Tissue and virus culture

All cell culture medium and media supplements used in this study are listed in Table 2.3. All tissue culture was performed in a designated "clean" (i.e. free of respiratory viruses and bacteria) category II tissue culture hood and all RV infections in a designated "virus" category II tissue culture hood.

2.3.1.1 Maintenance of cells

HeLa cells were cultured in D-MEM and BEAS2B cells were culture in RPMI medium. All media were supplemented with 10% heat inactivated foetal bovine serum, 2.5% HEPES buffer solution, and 1% sodium bicarbonate solution. HeLa and BEAS2B cells were cultured in 175cm^2 tissue culture flasks (Nunc, USA), in a humid 37°C incubator with 5% CO2, and cells passaged when approximately 90% confluent. HBECs were cultured in BEGM consisting of BEBM, supplemented with single-quot additives (Table 2.3). Medium with the addition of supplements will be referred to as complete BEGM throughout this study. BEGM was changed every 2-3 days during the culturing period. HBECs were cultured in 80cm^2 tissue culture flasks (Nunc), in a humid 37°C incubator with 5% CO_2, and cells passaged when approximately 90% confluent.

2.3.1.2 HeLa cell subculture

Cells were washed 2X with D-PBS, and 2mL trypsin-EDTA diluted in D-PBS to 1X solution was added and cells incubated at 37°C until cells were detached. Cells were resuspended in 10mL pre-warmed complete D-MEM to inactivate the trypsin and 10-20% of cells transferred to a new 175cm^2 tissue culture flask containing pre-warmed medium.

2.3.1.3 BEAS2B cell subculture

Cells were washed 2X with D-PBS, and 2 mL trypsin-EDTA diluted in D-PBS to 1X solution was added and cells incubated at 37°C until cells were detached. Cells were resuspended in 10mL pre-warmed complete RPMI to inactivate the trypsin and 10-20% of cells transferred to a new 175cm^2 tissue culture flask containing pre-warmed medium.

2.3.1.4 HBEC subculture

Cells were washed with 4mL HEPES buffered saline solution, 2mL trypsin-EDTA was added and cells incubated at 37°C until cells were detached. 4mL TNS was added, and cells centrifuged at 1500rpm for 6 min. Cells were resuspended in 10mL complete BEGM and one third transferred into a new 80cm^2 tissue culture flask containing pre-warmed medium.

2.3.1.5 Cell quantification

Resuspended cells (50μl) were removed from the cell suspension (section 2.2.1.2-2.2.1.4) and an equal volume of 0.1% trypan blue (in D-PBS) added. A Neubauer haemocytometer was filled with cell/trypan blue suspension (approx 5-10μl) and viewed under a light microscope. Viable cells were counted, and cells per mL calculated.

2.3.1.6 Resuscitation of frozen HeLa, BEAS2B cells and HBECs

Cells were thawed quickly in a 37°C water bath and transferred to an 80cm^2 flask (Nunc) containing pre-warmed D-MEM (HeLa), RPMI (BEAS2B) or complete BEGM (HBECs). The cells were placed in a humid 37°C incubator with 5% CO2 (Table 2.8) overnight. The medium was changed the following day, and cells cultured as above.

2.3.1.7 RV culture

RV1B and RV16 were cultured using confluent HeLa cell monolayers in 175cm^2 flasks. HeLa Ohio cells were used for RV cultured for *in vitro* use and HeLa H1 were used for virus culture designated for *in vivo* use. Cells were washed with 2X 10mL HeLa cell infection medium and approximately 2mL of virus stock added to cells with 18mL of infection medium. Flasks were gently agitated for 1h at room temperature, and incubated overnight at 37°C with 5% CO_2 or until approximately 90% cytopathic effect was observed. Cells were lysed by freeze/thawing 3X and centrifuged at 4,000rpm for 15min to pellet cell debris. For *in vitro* use of RV, the resulting RV inoculum was decanted and filtered through a 0.2μm filter (Pall Life Sciences, UK) and then stored in 1.5mL aliquots at -80°C.

2.3.1.8 RV purification for in vivo use

The cell debris obtained after centrifugation was used to obtain purified RV for use *in vivo.* Cells were washed twice in D-PBS, spun down at 1,800rpm for 10min after each wash and resuspended in approximately 36mL of D-PBS. Cells were freeze thawed twice, spun down at 4,000rpm for 15min and supernatant was filtered through a 0.2μM syringe filter. 4mL of NaCl and 2.8 g of PEG6000 was added, mixed on a roller for 5min and incubated on ice for 1h. This was then spun at 4,500rpm for 1h and the obtained cell pellet was dissolved in 15mL of D-PBS, mixed on roller for 10min and spun down at 4,000rpm for 15min. Supernatant was transferred into a new 50mL falcon tube, filtered through a 0.2μM syringe filter into a centrifuge filter device (Millipore) and spun at 4,000rpm until approximately 2.8mL of purified virus was maintained in the filter. Virus was then tested using a virus titration assay (see below, 2.2.1.9).

2.3.1.9 RV titration in HeLa cells

HeLa cells were split, resuspended in HeLa cell medium, counted and further diluted in infection medium to give 0.8×10^5 cells/mL. 150µL of cells were placed in each well of a 96-well plate (Nunc). Undiluted RV inoculum or 10-fold dilutions of the RV inoculum were added to each well, and the plate incubated in a humid 37°C incubator with 5% CO_2 for 4 days. Viral cytopathic effect (CPE) was determined using light microscopy and infected wells were counted at day 4. Tissue culture infective dose 50% (TCID50)/mL value was determined by scoring the sum of positive wells and using the Spearman Karber formula (M= xk +d[0.5-(1/n)(r)], wherein xk= dose of highest dilution, r = sum of positive responses, d = spacing between dilutions and n = wells per dilution).

2.3.1.10 RV infection of BEAS2B, HBECs and BAL cells

BEAS2B cells and HBECs were grown in 6 or 12 well tissue culture plates (Nunc) until 80-90% confluent and were then starved by culturing in respectively RPMI infection medium or BEBM overnight before incubating with RV inoculum (200µl/well for a 12-well plate; 500µL/well for a 6-well plate) for 1h with agitation at room temperature. BAL cells were rested for 24h in a 96 well tissue culture plate (Nunc) and stimulated with RV1B inoculum (40µL) for 1h. This corresponds to a multiplicity of infection (MOI) of 1. Inoculum was removed and replaced with respectively RPMI infection medium, BEBM or BAL culture medium (1mL/well for a 12 well plate; 2mL/well for a 6 well plate; 200µL for a 96 well plate) and incubated at 37°C with 5% CO_2, before harvesting cell supernatants for ELISA and cells were lysed for protein and/or RNA analysis at required time points (0-48h). In some experiments, where RV replication was to be measured, after 1h agitation at room temperature the RV inoculum was removed, and the cell monolayer washed 3X in 0.5mL PBS, to remove non-adhered RV prior to incubation at 37˚C for required time points.

2.3.1.11 PolyIC treatment of HBECs

HBEC were grown in 6 well tissue culture plates (Nunc) until 80-90% confluent and were then starved by culturing in BEBM overnight before incubating with PolyIC (Sigma Aldrich, USA) at 10µg/mL (200µl/well for a 12-well plate; 500µL/well for a 6-well plate) for 1h with agitation at room temperature. PolyIC was removed and replaced with BEBM (2mL per well for a 6-well plate) and incubated at 37°C with 5% CO_2, before harvesting cells for protein and/or RNA analysis at required time points (0-48h).

2.3.1.12 Stimulation of HBECs and BEAS2Bs with exogenous recombinant proteins

BEAS2Bs and HBEC were grown in 6 well tissue culture plates (Nunc) until 80-90% confluent and were then starved by culturing in respectively RPMI infection medium or BEBM (Table 2.3) overnight before incubating with different proteins (Table 2.8) and incubated at 37°C with 5% CO_2, before harvesting cells for protein and/or RNA analysis at required time points (0-48h).

2.3.2 Protein analysis techniques

All buffers and general reagents used in this study are listed in Table 2.1. A list of commercially purchased kits used is provided in Table 2.2. The antibodies used for western blot are listed in Table 2.5.

2.3.2.1 Firefly luciferase reporter gene assay

The Dual Luciferase Assay Kit (Promega) was used to assess luciferase (reporter) gene expression, according to manufacturers' instructions. Supernatants were removed, and cells were washed with D-PBS and lysed in 100μL reporter lysis buffer (diluted 1/5 with distilled water). Extracts were collected and centrifuged at 12,000rpm for 2min to pellet cell debris and nuclei. Each sample (20μL) was used to quantify both firefly and Renilla luciferase, using a luminometer (Berthold technologies, Germany). Samples were measured for a 10sec time interval, and expressed as firefly/Renilla luciferase ratios of relative light units (RLU).

2.3.2.2 Sample preparation for Polyacrylamide gel electrophoresis (PAGE)

Supernatants were removed and cells washed with D-PBS. Protein sample buffer (Tris-glycine SDS sample buffer) was added to the cells (100μL/well), and scraped with a 1mL pipette tip. Samples were sonicated and 10% v/v β-mercaptoethanol added, and samples heated for 5min at 95°C.

2.3.2.3 PAGE

1L 1X running buffer was prepared and samples separated using a pre-cast 16% tris-glycine gel (NuPage, Invitrogen) in a novex mini gel tank. The outer chamber consisted of 800mL running buffer and the inner chamber contained 200mL running buffer with 0.5mL NuPAGE anti-oxidant (Table 2.1). Samples (15μL) were run alongside molecular weight markers (SeeBlue Plus2 pre-stained standard, Invitrogen) at 120V.

2.3.2.4 Protein transfer to nitrocellulose membrane

Following PAGE, the gel was submerged for approximately 5min in transfer buffer and then laid flat on nitrocellulose membrane (Invitrogen) and equilibrated with transfer buffer. This gel-transfer membrane assembly was both under-laid and overlaid with a pre-wetted filter pad (Biorad, USA), and the filter pad- filter paper -gel – membrane – filter paper - filter pad combination placed in a mini trans-blot electrophoretic transfer cell (Biorad, USA), and run at 100V for 1.5h. Following transfer of proteins onto the membrane, the membrane was washed with western blot washing solution for 15min and then incubated at room temperature with western blot blocking solution with shaking for 2h.

2.3.2.5 Western blotting

Blocking solution was removed and replaced with appropriate primary antibody (diluted in western blot blocking solution), and incubated at 4°C with shaking overnight. The membrane was washed with western blot washing solution 3X (5min washes each) before adding a secondary antibody-HRP conjugate, diluted in western blot blocking solution and incubated at room temperature for 1h. The membrane was washed with western blot washing solution as above, and then detected using an ECL kit, with X-ray film (GE Health care, UK) and an X-ray film developer.

2.3.2.6 Stripping nitrocellulose membranes

After detection the membrane was thoroughly washed in western blot washing solution, prior to incubation with stripping buffer at 50°C for 30min, to remove primary and secondary antibodies. The stripped gel was then washed for 15min, and blocked as in section 2.2.2.4 and re-probed with another primary antibody.

2.3.2.7 ELISA

ELISAs to quantify cytokines (CCL5/RANTES, CXCL1/KC, CXCL2/MIP-2, CXCL10/IP-10 and CCL11/Eotaxin, CCL22/MDC, CXCL5/LIX, CCL17/TARC) and IFN-λ2/3/IL-28AB production in BAL from mice were performed using commercially available paired antibodies and recombinant protein standards (R&D Systems, Table 2.2). 96-well microtitre plates (Nunc) were coated with 100μL capture antibody, and incubated overnight at room temperature. The plate was washed 3X with 250μL ELISA wash buffer, and blocked by adding 250μL ELISA blocking solution and incubated at room temperature for 2h. Recombinant protein standard was diluted, and a standard curve prepared by performing doubling dilutions. The plate was washed 3X, and 100μL of sample supernatant, standards, and ELISA diluent buffer controls (blanks) were added to the plate in

duplicate and incubated for 1h at room temperature. The plate was washed 3X, 100μL of biotinylated detection antibody added and the plate incubated for 1h at room temperature. The plate was washed 3X, 100μL strepavidin-HRP (diluted 1:5000 in ELISA diluent buffer) added and the plate incubated for 15min at room temperature with shaking. The plate was washed 3X, 100μL TMB solution added and the reaction allowed to develop in the dark for approximately 5min (or until background colour begins to appear in blank wells) before stopping the reaction with the addition of 50μL 2M H_2SO_4. Absorbance was measured at 450nm, on a microplate reader. Absorbance readings of the experimental samples were compared to the standard curve, to enable quantification of each sample in pg/mL. The sensitivities of each assay were approximately: CCL5/RANTES 30pg/mL, CXCL1/KC 30pg/mL, CXCL2/MIP-2 65pg/mL, CXCL10/IP-10 65pg/mL and IFN-λ2/3/ IL-28AB 100pg/mL, CCL11/Eotaxin 25/pg/mL, CCL22/MDC 30pg/mL, CXCL5/LIX 30pg/mL, CCL17/TARC 25pg/mL.

IFN-β and IFN-α were determined in the same samples using a kit containing a precoated plate (R&D systems, sensitivity for both approximately 25 pg/mL).

2.3.3 Molecular Biology Techniques

2.3.3.1 RNA extraction and cDNA synthesis

Cells were washed with PBS, 350μL buffer RLT (Qiagen) (containing 1% β-mercaptoethanol) added/well and the lysate pipetted until the viscosity of the lysate was reduced. Lysate was collected into an eppendorf tube and stored at -80°C until RNA extraction. Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to manufacturers' instructions. RNA was eluted using 39μL nuclease-free water, giving ~10ug RNA/sample extraction. 39μL of RNA was used to prepare cDNA using an Omniscript Reverse Transcription (RT) kit (Qiagen). The components of the reaction were assembled as shown in Table 2.10.

Table 2.10: Components of cDNA synthesis reaction.

Component	Volume/reaction	Final Concentration
Omniscript RT	3 μL	10 units/50 μL reaction
10× Buffer RT	6 μL	1X
dNTP mix (5mM each dNTP)	6 μL	0.5mM each dNTP
Random primers (Promega)	3 μL	
RNase free water	3 μL	
Template RNA	39 μL	
Total Volume	60 μL	

The reaction was incubated in a 37°C water bath for 1h, and stored at -80°C.

2.3.3.2 Realtime RT-PCR primer and probe design

Realtime RT-PCR primer and probes were designed for all of the genes measured in this study (Table 2.7), with exception of 18s and RV that were already established within the lab. If possible, primer and probe combinations were designed to hybridise to DNA over an exon-exon boundary to avoid specific amplification of the target genomic DNA. Genomic and mRNA sequences of the target gene were found using NCBI PubMed and compared to find the exon-intron boundaries within the genomic DNA sequence. Primers and probe, overlapping an exon-exon boundary in the mRNA, were selected using Primer Express v3.0. To optimise forward and reverse primer concentrations probe concentration was kept constant at 175nM per reaction and each forward/reverse primer combination varied using 50nM, 300nM and 900nM concentrations. The primer pair combination which gave the lowest threshold value (CT) and the highest normalised reporter dye signal (ΔRn), with no primer dimer formation or background amplification were chosen for use in future taqman RT-PCR analysis. No template controls (NTC) were used to detect background amplification and primer-dimers.

2.3.3.3 Realtime PCR set-up

mRNA for a number of genes were measured using TaqMan™ PCR (Applied Biosystems Fast 7500 machine) and specific primer and probe sequences. On each fast 96-well optical PCR plate (Applied

Biosystems), each gene of interest and 18S rRNA was analysed to assess cDNA loading. Each reaction consisted of 6.25μL 2X QuantiTect probe PCR Master Mix, sense and anti-sense primers (at optimised nM ratio, Table 2.6) and 175nM probe and1μL cDNA. For 18S rRNA measurements, the cDNA was diluted 1/100 in nuclease-free water (Promega). The 96-well optical PCR plate was sealed using a microamp optical adhesive cover (Applied Biosystems) and the reactions analysed using an applied biosystems 7500 fast real-time PCR systems. The amplification cycle consisted of 50°C for 2 min, 94°C for 10 min, and 45 cycles of 94°C for 15 s, 60°C for 15 s. Data was analysed using version 1.4 ABI Prism 7500 SDS Software. Unknown samples were compared to a standard curve produced from running dsDNA standards in 10 fold dilutions from 10^7 copies per reaction, to 1 copy per reaction. Quantitative PCR data was normalised for 18S rRNA and presented as normalised copy number per well.

2.3.3.4 PCR product purification

Standard curves were constructed by cloning a specific PCR amplicon in a plasmid. PCR product obtained from RT-PCR reaction were purified using QIAquick gel extraction kit (Qiagen) following manufacturers' instruction for the Qiaquick PCR purification microcentrifuge protocol. Briefly, 5 volumes of buffer PB1 were added to 1 volume of PCR reaction, this mixture was added to a Qiaquick column and spun for 30sec at 10,000rpm. The column was washed once with 0.75 mL buffer PE and spun for 30sec. To elute DNA 30 μL of Buffer EB was added, leaving the column for 1 min and spun for 1 min at 10,000rpm.

2.3.3.5 Ligation using pCR 2.1-TOPO

The pCR 2.1-TOPO vector (Invitrogen, Table 2.2) was used for cloning PCR products. Each ligation reaction consisted of 4μL gel extracted PCR product (15-100ng) or water (negative control ligation), 1μL salt solution and 1μL TOPO vector. The reaction was mixed by gentle pipetting and incubated at room temperature for 15min before placing reactions on ice.

2.3.3.6 Transformation of competent E. coli

Approximately 5-6μL of a ligation reaction or 1μL of stock plasmid DNA was added to 50μl of One shot competent *E. coli* (Invitrogen) and gently mixed with a pipette tip. The transformation reaction was incubated on ice for 30min, and transferred to a 42°C water bath for 45 sec, then placed on ice for 2min. LB broth (450μL, Table 2.3) was added to each transformation reaction and shaken for 1h at 37°C. 100μl of each transformation reaction was spread onto LB agar plates containing 100μg/mL ampicillin, and incubated at 37°C overnight.

2.3.3.7 Colony Screening and mini-prep analysis of plasmid DNA

Following competent cell transformation single colonies (5-10) were picked and used to inoculate 2mL LB broth with 100μg/mL Ampicillin. Cultures were grown at 37°C overnight and plasmid DNA isolated using a mini-prep method. Cells were pelleted, by spinning at 10,000rpm for 10sec, and the pellet re-suspended in 0.2mL solution 1 (Buffer P1), mixed, and 0.2mL solution 2 (Buffer P2) added. After gentle mixing, 0.2mL of solution 3 (Buffer P3) was added and the mixture was spun at 10,000rpm for 10min. The supernatant was then taken, and mixed with 1mL 100% ethanol. After incubating for 20min at room temperature, the plasmid DNA was precipitated by spinning at 10,000rpm for 20min. The plasmid DNA pellet was then washed by spinning at 10,000rpm for 2min in 70% ethanol and air-dried before re-suspension in 30μL nuclease free water. Mini-prep isolated plasmids were used to confirm the presence of a desired PCR product with Taqman RT-PCR using primers and probes specific for the gene.

2.3.3.8 Maxi-prep isolation of plasmid DNA

Samples confirmed to have the desired PCR product in section 2.3.6 were transformed into competent *E. coli* (as in section 2.3.5). A single colony from section 2.3.5 was picked and grown at 37°C for 8h with shaking, and this starter culture was transferred into 100mL LB broth containing 100μg/mL ampicillin, shaken at 37°C overnight and plasmid DNA prepared using a Maxi-prep method according to the manufacturers' instructions. The purified plasmid DNA was precipitated in 100% isopropanol (VWR), and spinning at 6,000rpm for 1h, the supernatant discarded, and the DNA pellet washed with 2 mL 70% ethanol and spun at 6,000rpm for 30min. Finally the pellet was air dried for 10min, the isolated DNA re-suspended in1300μL sterile TE buffer (pH 8.0, Table 2.1), and stored at -80°C. Plasmids isolated by this method were used as standards in TaqMan RT-PCR.

2.3.3.9 Quantification of plasmid DNA

Maxi-prep DNA weight per volume was measured using a nanodrop at 260nM. The quantity of DNA determined by assuming an absorbance of A260nm of 1.0 is equal to 50μg/mL DNA. Plasmids were diluted in distilled water to 1×10^{10} plasmid copies/μL (using the formula below) and stored at -80°C.

A.

$$\text{Moles} = \frac{\text{plasmid DNA yield (g)}}{\text{plasmid molecular weight}}$$

B.

$$\frac{\left(\text{moles x } 6.02\times10^{23}\right)}{\text{plasmid volume } (\mu\text{L})} = \text{molecules (copies) / } \mu\text{L}$$

2.3.3.10 TaqMan RT-PCR standard curve generation

To create a TaqMan standard curve for each gene (by equating cycle threshold (ct) values to copy number), each plasmid was diluted to 10^8 copies/2μL in distilled water and further serial 10-fold dilutions performed to give plasmid copy numbers down to 10 copies/2μL. Serially diluted plasmids (2μL) were combined in a TaqMan PCR reaction with specific gene targeting forward and reverse primers/probe, QuantiTect probe PCR Master Mix and distilled water. TaqMan RT-PCR standards were made for CISH, SOCS1-7. Standards for RV, mIFN-β, mIFN-λ2/3/IL-28AB, mOAS and mViperin were already established within the lab.

2.3.4 Transfection of human epithelial cells with plasmid DNA

All plasmids used in this study are listed in Table 2.9.

2.3.4.1 Transfection of BEAS2Bs

Table 2.11: Transient BEAS2B cell plasmid transfections performed in this study.

1	650ng SOCS1/ SOCS3/pORF + 250ng promoter + 100 ng Renilla
2	350ng expression vector/ constitutively active vector/control + 350ng SOCS1/ SOCS3/pORF + 200ng promoter + 100 ng Renilla

All stock plasmids were at 1μg/μL. Confluent monolayers of BEAS2B cells in 12-well plates (Nunc) were transiently transfected with plasmid DNA. A mastermix of plasmid DNA was diluted in serum free RPMI medium and a volume of 74μL per well to be transfected, was prepared and vortexed to mix. 3μL of Superfect was added per μg of DNA used, vortexed and incubated for 15 min at room temperature to allow lipid-plasmid complexes to form. 0.4mL of serum free RPMI was added per well. 475μL solution was added to each well accordingly. Transfections were incubated at 37°C (5% CO2), removed by washing 1X with D-PBS, and cells placed in 2% FCS RPMI medium and incubated for 24 h at 37°C (5% CO2). Cells transfected as in Table 2.11 point 1 were then treated as in section 2.2.1.10, 2.2.1.11 and 2.2.1.12 and harvested for luciferase assay (section 2.2.2.1). Cells transfected as in Table 2.11 point 2 were harvested for luciferase assay.

2.3.4.2 Transfection of HBECs

All stock plasmids were at 1μg/μL. Confluent monolayer cells in 12-well plates were transiently transfected with plasmid DNA. A master mix of plasmid DNA diluted in Optimem-1 medium, and a

volume of 100μL per well to be transfected, was prepared and vortexed to mix. LP2000 (1mg/mL) was diluted in Optimem-1 medium to 20μg/mL, and a volume of 100μL per well to be transfected prepared, mixed by vortexing and incubated at room temperature for 5min. Diluted LP2000 and diluted DNA were combined, vortexed to mix and incubated at room temperature for 20min to allow lipid-DNA complexes to form. Medium was removed from cells and replaced with 800μL BEGM complete medium. 200μL of complexes were added to each well and rotated to mix. Transfections were incubated for 5h at 37°C (5% CO_2). Cells were treated 24h post transfection as in section 2.2.1.10, 2.2.1.11 and 2.2.1.12 and harvested for luciferase assay (section 2.2.2.1).

2.4 Animal models/methods

2.4.1 Animals

6-8 week old, female BALB/c and C57/Bl6 mice were purchased from Charles River UK and IFN-$\gamma^{-/-}$Bl6 mice and SOCS1$^{-/-}$, IFN-$\gamma^{-/-}$Bl6 mice were bread and housed in specific pathogen free conditions within the CBS facility at Imperial College London, St Mary's Campus. All animal work was authorized by the appropriate licenses from the Home Office, UK.

2.4.2 Anesthesia

For intranasal challenges (i.n.), mice were lightly anaesthetized with vaporized isoflurane (Table 2.2). Terminal anesthesia was achieved by intraperitoneal injection (i.p.) of 250µL pentobarbitone solution (Table 2.2).

2.4.3 HRV1B infection model

Mice were challenged i.n. with 50µL approximately 1x10^6 TCID50 RV1B or UV-inactivated RV1B (UV-RV1B) on day (d) 0. Mice were culled at various time points post infection by terminal anaesthesia.

2.4.4 IL-13 treatment model

Mice were treated i.n. with 0.5µg/50µL recombinant mouse IL-13 (R&D, USA) or mock treated with PBS on d0. Mice were culled at various time points post infection by terminal anesthesia.

2.4.5 IL-13 pretreatment and HRV1B infection model

Mice were treated i.n. with 0.5µg/50µL recombinant mouse IL-13 (R&D, USA) on -8h. Mice were challenged i.n. with 50µL approximately 1x10^6 TCID50 RV1B, UV-inactivated RV1B (UV-RV1B) on d0. Mice were culled at various time points post infection by terminal anesthesia.

2.4.6 Sample recovery and processing

2.4.6.1 BAL

Lungs were lavaged via the trachea with 1.5mL of BAL buffer. Cells and supernatants from recovered fluid were spun down at 13,000rpm for 30s. BAL supernatants were snap frozen in liquid nitrogen and stored at -80˚C until use. Cell pellet was resuspended in ACK lysis buffer to remove red blood cells and immediately neutralized with medium. Cell suspension was spun down and resuspended to a total volume of 1mL. Cells were counted as in paragraph 2.2.1.5.

2.4.6.2 Cytospin assay

100uL of BAL cell suspension was added to a cytospin funnel and spun down onto Shandon Cytoslides at 500rpm for 5min using the Cytospin3 system (Shandon, USA). Slides were air-dried for at least 3h and stained using REASTAIN Quick-Diff Kit (Table 2.2). After staining, slides were again air-dried and at least 300 cells per slide were differentially counted blind to experimental conditions.

2.4.6.3 Lung tissue for RNA extraction

The apical lobe was harvested and stored in RNAlater at -80˚C. Upon defrosting the lobe was homogenized in 600µL RLT buffer + 1% β-ME. Suspension was then spun down for 3min at 13.000 RPM and liquid was transferred to an eppendorf with 600µL 70% ethanol. The same protocol was then used as in 2.2.3.1

2.4.6.4 Lung tissue for protein extraction

Protein was extracted from lung tissue using Cellytic NuCLEAR extraction kit (Sigma-Aldrich) using the protocol for nuclear and cytoplasmic extraction from 100mg of tissue (according to manufacturers protocol). Protein was then measured using protocols described in 2.2.2.3-2.2.2.6.

2.4.6.5 Ex vivo culture of BAL cells

BAL was obtained from approximately 15 IFN-$\gamma^{-/-}$Bl6 mice and SOCS1$^{-/-}$, IFN-$\gamma^{-/-}$Bl6 mice per experiment. Cells were spun down at 1500rpm for 15min and resuspended in 300µL ACK lysis (Table 2.1) buffer and neutralized in 5mL BAL cell culture medium (Table 2.3). Cells were spun down at 1500rpm for 15min resuspended in 2mL BAL cell culture medium and counted according to section 2.3.1.5. Cells were plated in a 96 well tissue culture plate (nunc) at a concentration of $4x10^5$ cells/mL and rested for 24h. Cell were then infected with RV1B or treated with PolyIC (section 2.3.1.10 and section 2.3.1.11) and harvested for RNA and supernatants at indicated time points.

2.5 Statistics

Data are presented as means ±SEM (standard error of the mean). Three to six experiments were performed for *in vitro* experiments. A minimum of two experiments were performed for *in vivo* experiments using four mice per group. Time course data in HBECs and mice were analyzed using two-way analysis of variance (ANOVA), at a 95% confidence interval with Bonferroni's multiple comparison test. Luciferase and siRNA data were analyzed using one-way ANOVA, at a 95% confidence interval with Bonferroni's multiple comparison test. Clinical data was analyzed using Kruskal-Wallis test, at a 95% confidence interval with Dunn's multiple comparison test. Correlations in clinical data were analyzed using Spearman's test. A p value of $p<0.05$ was considered statistically different.

3 Regulation and function of SOCS in HBECs

3.1 Introduction

Atopic asthma is an allergic inflammatory disease. The majority of asthma exacerbations are caused by respiratory viruses of which RVs account for two thirds of all exacerbations [18]. The major site of RV infection in the airways is the bronchial epithelium. HBECs also respond to inflammatory cytokines including IL-1β and TNF-α releasing a vast array of pro-inflammatory cytokines and chemokines [184]. HBECs also have receptors for IL-4 and IL-13 and contribute to asthma pathogenesis by producing pro-Th2 mediators, notably the Th2 chemokine TARC [184]. Further studies also suggest that lung epithelial cells including HBECs produce TSLP and perhaps IL-25; underlying the importance of HBECs in responding to and generating proinflammatory and Th2 signals.

Upon RV infection, HBECs also produce anti-viral cytokines, including type I and III IFNs. Cohort studies have shown that asthmatics have an increased susceptibility to RV infection, and experience greater reductions in lung function and longer duration and severity of lower respiratory symptoms compared to healthy subjects [15, 29]. A deficiency in RV induced type I IFN-β and type III IFN-λ in cultured HBECs and type III IFNs in cultured BAL cells has been described recently [47, 49], suggesting an important role of IFN deficiency in RV induced asthma exacerbations. The mechanism(s) behind this deficiency in IFN induction is unknown.

IFNs and cytokines (including Th2 cytokines) signal through the JAK/STAT signalling pathway. SOCS act by inhibiting JAK/STAT. SOCS1 and SOCS3 play a role in the Th1/Th2 balance and have been implicated in asthma [162, 170] and both SOCS1 and SOCS3 can negatively regulate type I and III IFN signalling [171, 173]. Several investigators have shown a role for SOCS in inhibiting other signalling pathways [175, 180, 181, 185]. SOCS1 and SOCS3 are expressed in HBECs and all SOCS genes are expressed in the bronchial epithelial cell line BEAS2B [175]. These observations indicate that SOCS1 and SOCS3 might play a role in RV induced asthma exacerbations. This is the first study investigating this role of SOCS in RV infection. The experiments in this chapter investigate the role of SOCS in HBECs.

The first aim of this chapter was to investigate the role of different mediators associated with asthma in the induction of SOCS in HBECs. It was hypothesized that SOCS1 and SOCS3 mRNA and protein are induced upon RV, type I and III IFNs, dsRNA PolyIC, inflammatory mediators and Th2 cytokines in HBECs. An *in vitro* model of primary HBECs was used where cells were infected with major (RV16) and minor (RV1B) group RV or were treated with type I (IFN-β) and type III IFNs (IFN-λ1). A TLR3 ligand (PolyIC) was used to mimic other RNA respiratory viruses. Inflammatory mediators (IL-1β and TNF-α) and Th2 cytokines (IL-4 and IL-13) were also incorporated as they are believed to be important in (allergic) asthma pathogenesis [186-189]. SOCS mRNA was measured by taqman RT-PCR

and protein using western blotting. SOCS1 and SOCS3 appeared to be the main induced SOCS family members in HBECs.

The second aim of this chapter was to investigate the role of SOCS1 and SOCS3 in the regulation of the downstream signalling of the above mentioned cytokines and RV. Firstly, it was hypothesized that SOCS1 and SOCS3 modulation *in vitro* affects RV induced IFN-β and IFN-λ promoter activation and type I IFN induced IFN-β and IFN-λ promoter activation. Secondly it was hypothesized that SOCS3 can inhibit Th2 induced STAT6 promoter activation and SOCS1 has no effect according to what has previously been observed in human cells [168]. An *in vitro* transfection model was used in which SOCS1 and SOCS3 were overexpressed using plasmids, and promoter activation was measured by a luciferase assay. To confirm these luciferase assays, the SOCS overexpression vectors were used to investigate their effect on RV induced RV replication and release and IFN mRNA and siRNA specific to SOCS1 and SOCS3 was used to investigate the effects of knockdown of SOCS1 and SOCS3 on IFN induced ISG mRNA induction and RV induced IFN mRNA induction. It was found that SOCS1 and SOCS3 play a clear role in negatively regulating IFN-β and RV1B induced IFN promoter activation and IL-4 and IL-13 induced STAT6 promoter activation.

3.2 Results

3.3 SOCS expression and induction in HBECs

3.3.1 SOCS mRNA was constitutively expressed in HBECs

To assess if SOCS mRNA was constitutively expressed in HBECs, taqman RT-PCR was used to determine the presence, absence and abundance of each SOCS mRNA. Cells were grown in 6 wells plate and lysed for mRNA upon confluency. It was not possible to obtain SOCS4 and SOCS6 standard curves, so absolute quantification was not performed. However both were clearly expressed (Figure 3.1B). Taqman RT-PCR curves had a threshold cycle (Ct) value of approximately 30 for SOCS4 and 31 for SOCS6. mRNA from all SOCS family members were expressed in HBECs (Figure 3.1A), with SOCS5 being the highest expressed.

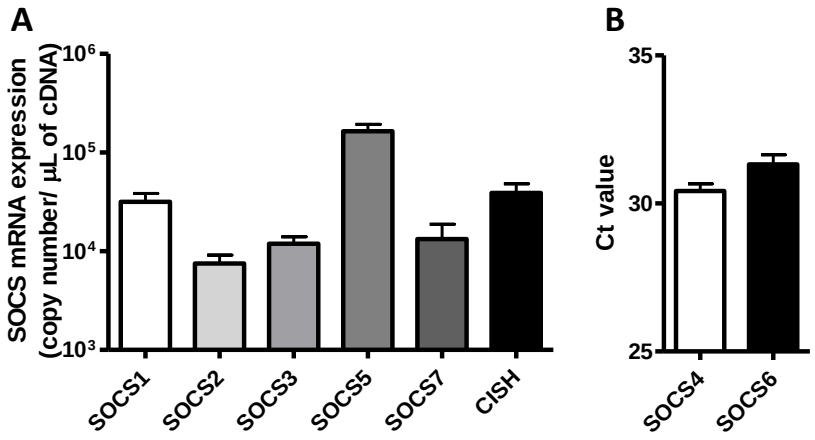

Figure 3.1: SOCS mRNA expression in HBECs. mRNA of all SOCS members were expressed in HBECs.

SOCS4 and SOCS6 were constitutively expressed, although no standard curve was obtained for conversion to copy numbers (n=5).

3.3.2 Major and minor group RV induced SOCS1 and SOCS3 mRNA and protein in HBECs

As discussed in Chapter 1, RV is the major cause associated with asthma exacerbations. SOCS family members have been associated with asthma and negatively regulating IFN signalling. However they have not yet been studied in RV infection. After determining expression of mRNA of SOCS in HBECs it was investigated whether minor group (RV1B) and major group RV (RV16) could induce both SOCS mRNA and protein expression. Cells were grown in 6 well plates until confluent. Confluent monolayers were infected with RV1B or RV16 and lysed for mRNA or protein at 0, 4, 8, 12, 18, 24 and 48h post infection, mRNA levels were measured using taqman RT-PCR and protein levels were measured using western blotting.

SOCS1 mRNA and protein was induced upon both RV1B and RV16 ($p<0.05$, Figure 3.2). RV1B increased SOCS1 mRNA in a time dependent manner showing an increase in mRNA from 12h, and RV1B significantly increased SOCS1 mRNA at 48h post infection compared to medium treated cells ($p<0.05$, Figure 3.2A). Similar results were observed for RV1B induced SOCS1 protein. SOCS1 protein was detectable at 12h and increased until 48h in a time dependent manner (Figure 3.2C).

RV16 induced SOCS1 mRNA was also increased in a time dependent matter. Increased RV16 induced SOCS1 mRNA was present at 24h and was statistically significant at 48h compared to medium treated cells ($p<0.05$, Figure 3.2B). RV16 induced SOCS1 protein was detectable from 8h and increased until 24h (Figure 3.2D).

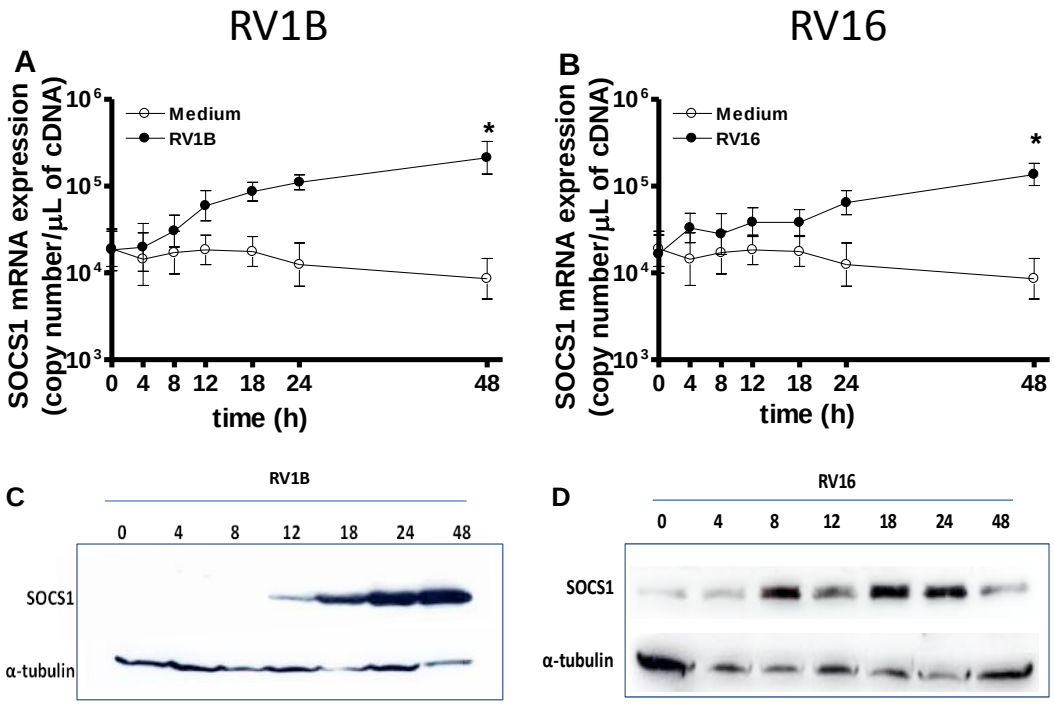

Figure 3.2: RV induced SOCS1 mRNA and protein.

SOCS1 mRNA was significantly induced by both RV1B (A) and RV16 (B) at 48h ($p<0.05$). A time dependent increase of RV1B and RV16 induced SOCS1 protein was seen (C, D) (n=5 for mRNA experiments, western blot experiments representative of 3 experiments).

SOCS3 mRNA and protein was induced upon both RV1B and RV16 (Figure 3.2). SOCS3 mRNA was increased by RV1B in a time dependent manner, with increased mRNA observed at 12h, which was statistical significant at 48h compared to medium treated cells ($p<0.01$, Figure 3.3A). SOCS3 protein was detectable at 4h post RV1B infection and increased until 24h in a time dependent manner (Figure 3.3C).

SOCS3 mRNA was also increased by RV16 in a time dependent manner, with increased mRNA observed at 12h, which reached statistical significance at 48h versus SOCS3 mRNA in medium treated cells ($p<0.01$, Figure 3.3B). SOCS3 protein was detectable at 8h post RV16 infection and increased until 48h in a time dependent manner (Figure 3.3D).

RV1B and RV16 induced SOCS1 and SOCS3 mRNA and protein in HBECs. Neither RV1B nor RV16 induced increased mRNA expression of any other SOCS family members (data not shown).

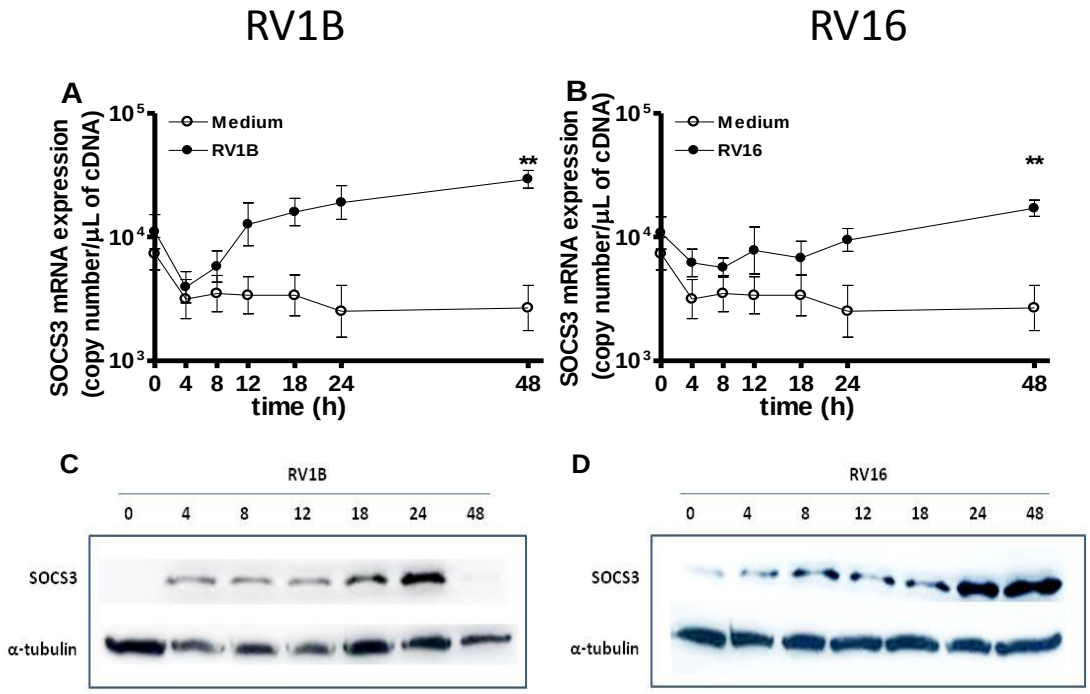

Figure 3.3: RV induced SOCS3 mRNA and protein.

SOCS3 mRNA was significantly induced by both RV1B (A) and RV16 (B) at 48h ($p<0.01$). A time dependent increase of RV1B and RV16 induced SOCS3 protein was seen (C, D) (n=5 for mRNA experiments, western blot experiments representative of 3 experiments).

3.3.3 IFN-β induced SOCS1 mRNA and protein and SOCS3 protein in HBECs

Type I and III IFNs are the first line of defence against virus infection. SOCS1 and SOCS3 have been shown to be induced upon IFN-β treatment [190, 191], however this has not been shown in HBECs. To investigate whether IFN-β and IFN-λ1 induce SOCS family members, confluent cultured HBECs were treated with either 10ng/mL IFN-β or IFN-λ1 and lysed for mRNA or protein at 0, 4, 8, 12, 18, 24 and 48h post treatment. mRNA levels were measured using taqman RT-PCR and protein levels were measured using western blotting.

SOCS1 mRNA was significantly induced upon IFN-β treatment in HBECs, with mRNA levels peaking at 4h post treatment ($p<0.01$, Figure 3.4A) compared to medium treated cells and remaining elevated until 48h post treatment ($p<0.01$ at 8h and 24h, $p<0.05$ at 12h, 18h and 48h, Figure 3.4A). IFN-β induced SOCS1 protein in a time dependent manner (Figure 3.4C), with protein detectable at 8h post treatment which increased until 48h post treatment. A trend was observed for IFN-λ1 induced SOCS1 mRNA although this was not statistically significant (Figure 3.4B).

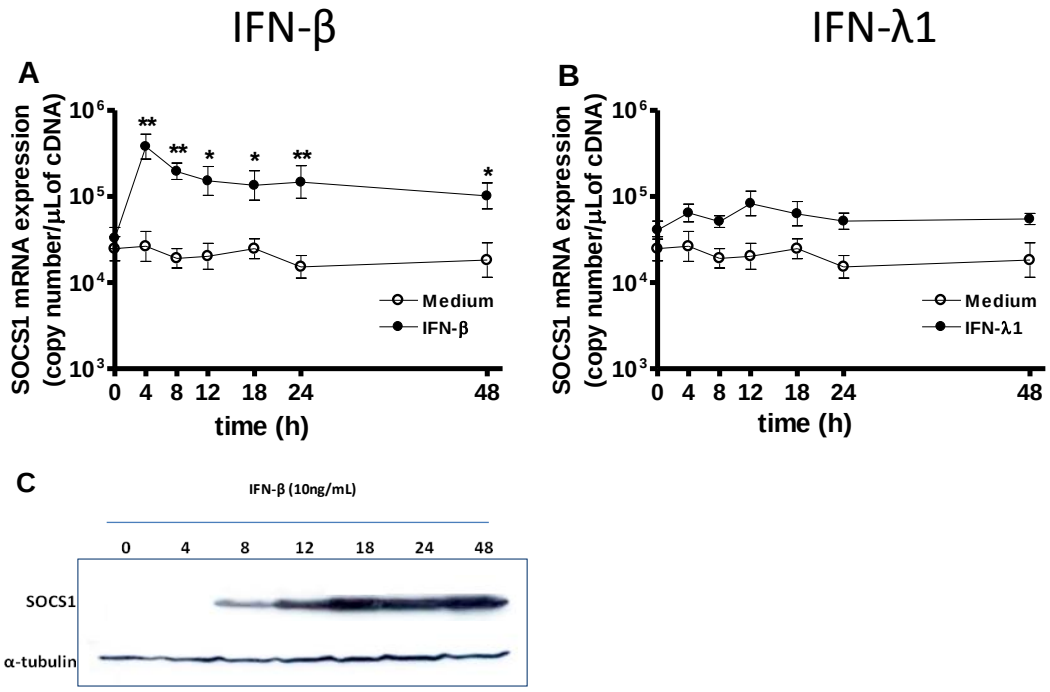

Figure 3.4: IFN-β and IFN-λ1 induced SOCS1 mRNA and IFN-β induced SOCS1 protein.

SOCS1 mRNA was significantly induced by IFN-β ($p<0.01$ at 4,8 and 24h and $p<0.05$ at 12, 18 and 48h, A). IFN-λ1 did not significantly induce SOCS1 mRNA (B). A time dependent increase of IFN-β induced SOCS1 protein was observed (C) (n=5 for mRNA experiments, western blot experiments representative of 3 experiments).

SOCS3 mRNA was not significantly induced upon IFN-β or IFN-λ1 protein treatment (Figure 3.5A and 3.5B), although a non-significant increase was observed for induction of SOCS3 mRNA and protein upon IFN-β treatment (Figure 3.5A and 3.5C).

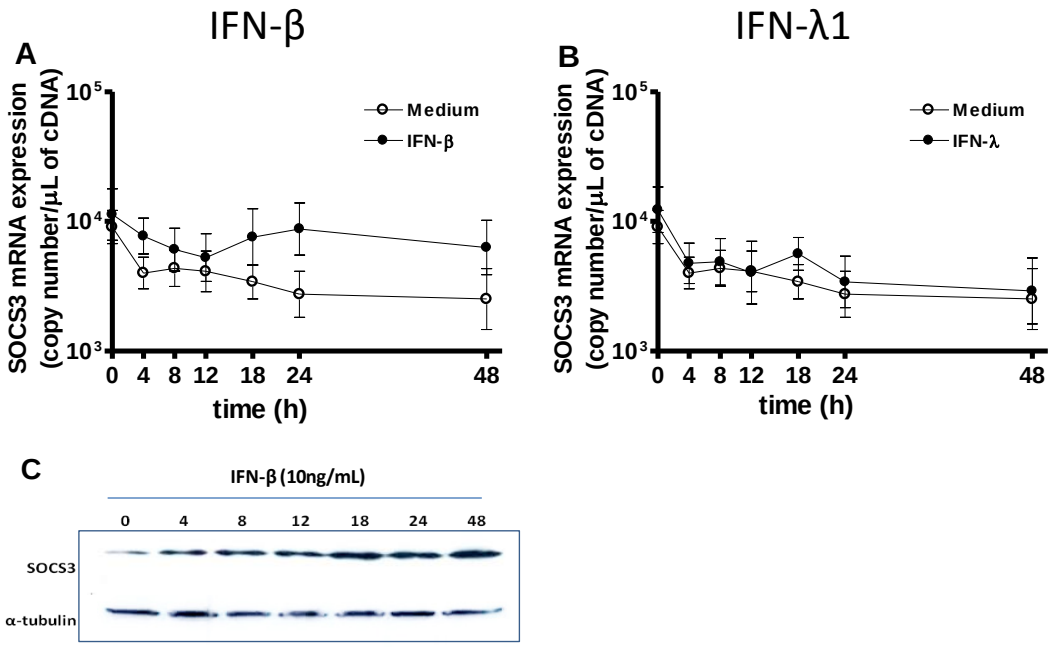

Figure 3.5: IFN-β and IFN-λ induced SOCS3 mRNA and IFN-β induced SOCS3 protein.

SOCS3 mRNA was not induced by IFN-β or IFN-λ1 (A, B). A time dependent increase of IFN-β induced SOCS3 protein was seen (C) (n=5 for mRNA experiments, western blot experiments representative of 3 experiments).

IFN-β induced SOCS1 mRNA and protein. A trend was observed towards IFN-λ1 induced SOCS1 mRNA. IFN-β and IFN-λ1 protein did not induce increased mRNA expression of any of the other SOCS family members (data not shown).

3.3.4 PolyIC induced SOCS1 and SOCS3 mRNA and protein in HBECs

PolyIC is a dsRNA activating signalling through the TLR3 pathway, triggering antiviral immune responses. RV has been shown to signal through both TLR3 and RIG-I/MDA-5 and SOCS1 has been shown to be induced upon PolyIC treatment [192], however this has not yet been shown in HBECs. To asses if SOCS mRNA and protein can be induced by PolyIC, the effect of PolyIC on induction of SOCS family members was determined. Confluent cultured HBECs were treated with either 10µg/mL PolyIC and lysed for mRNA or protein at 0, 4, 8, 12, 18, 24 and 48h post treatment. mRNA levels were measured using taqman RT-PCR and protein levels were measured using western blotting.

PolyIC significantly induced SOCS1 mRNA peaking at 4h post treatment ($p<0.05$, Figure 3.6A) compared to medium treated cells and mRNA levels remained elevated until 48h post treatment. Induction was statistically significant at 8h, 12h and 18h post treatment ($p<0.05$, Figure 3.6A). SOCS1 protein was induced by PolyIC in a time dependent manner (Figure 3.6B), with protein detectable at 12h post infection, peaking at 18h and remaining detectable until 48h post treatment.

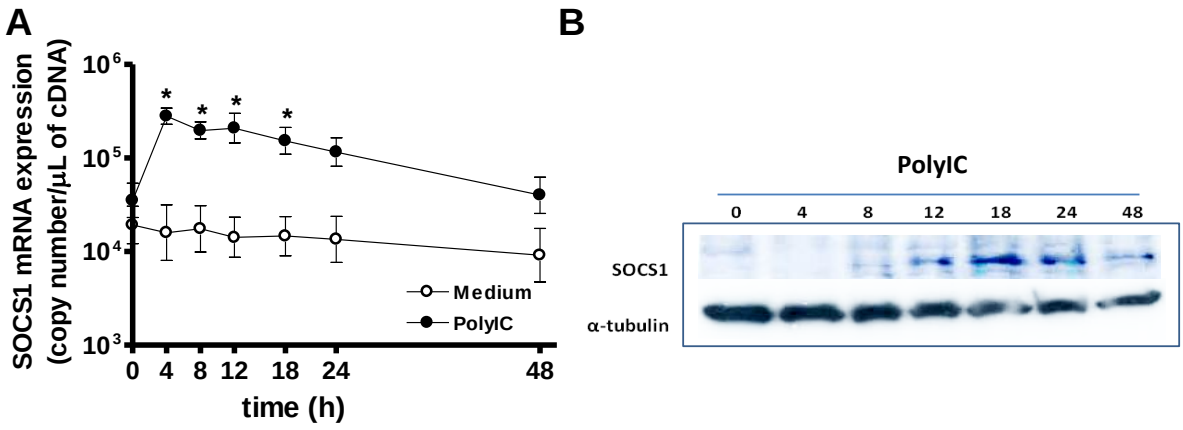

Figure 3.6: PolyIC induced SOCS1 mRNA and protein.

(A) SOCS1 mRNA was significantly induced by PolyIC ($p<0.05$ at 4, 8, 12 and 18h). (B) A time dependent increase of PolyIC induced SOCS1 protein was observed (n=5 for mRNA experiments, western blot experiments representative of 3 experiments).

PolyIC induced SOCS3 mRNA, showing a statistically significant peak at 4h post treatment ($p<0.01$, Figure 3.7A) and remaining elevated until 48h. SOCS3 protein was also induced by PolyIC in a time dependent manner with protein detectable at 8h post treatment and increasing until 48h post treatment (Figure 3.7B).

PolyIC induced SOCS1 and SOCS3 mRNA and protein. PolyIC did not induce increased mRNA expression of any other SOCS family member (data not shown).

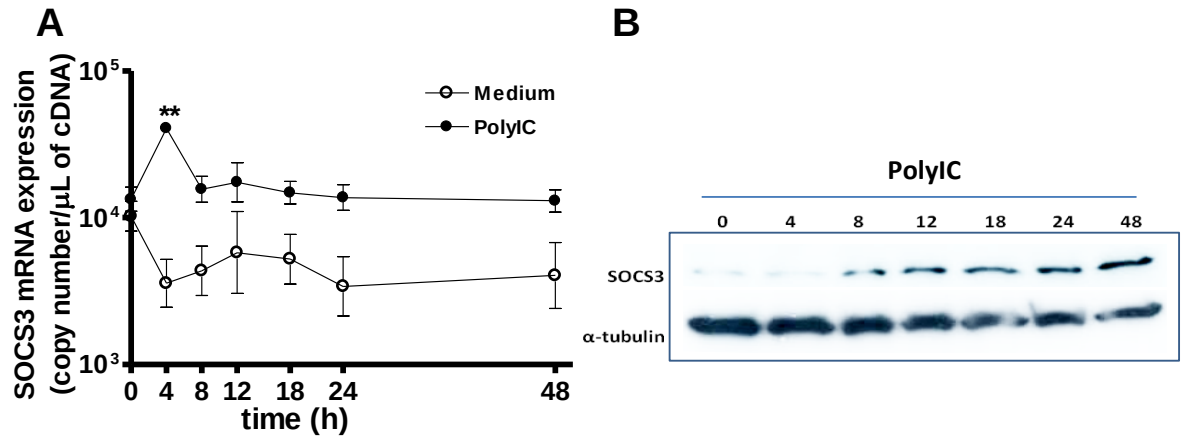

Figure 3.7: PolyIC induced SOCS3 mRNA and protein.

SOCS3 mRNA was significantly induced by PolyIC ($p<0.01$ at 4h, A) SOCS3 protein was induced upon PolyIC treatment (B) (n=5 for mRNA experiments, western blot experiments representative of 3 experiments).

3.3.5 The inflammatory mediators TNF-α and IL-1β induced SOCS1 mRNA and protein in HBECs.

To assess whether SOCS can be induced by the inflammatory mediators, IL-1β and TNF-α which are important in the pathogenesis of asthma [187, 188], the effect of IL-1β and TNF-α on SOCS family member expression was determined. Confluent cultured HBECs were treated with either 10ng/mL IL-1β or TNF-α and lysed for mRNA or protein at 0, 4, 8, 12, 18, 24 and 48h post treatment. mRNA levels were measured using taqman RT-PCR and protein levels were measured using western blotting.

IL-1β induced SOCS1 mRNA, showing a statistically significant peak at 4h post treatment ($p<0.05$, Figure 3.8A) with levels remaining elevated until 48h. SOCS1 protein was induced by IL-1β in a time dependent manner (Figure 3.8C), with protein detectable at 4h post infection, peaking at 24h and remaining detectable until 48h post treatment.

TNF-α increased SOCS1 mRNA at 4h post treatment, with levels remaining elevated until 48h, which was statistically significant at 18h, 24h and 48h post treatment compared to medium treated cells (Figure 3.8B, 18h and 24h $p<0.05$ and 48h $p<0.01$ respectively]. SOCS1 protein was induced by TNF-α in a time dependent manner (Figure 3.8D) with protein detectable at 8h post infection, peaking at 18h and remaining detectable until 24h post treatment.

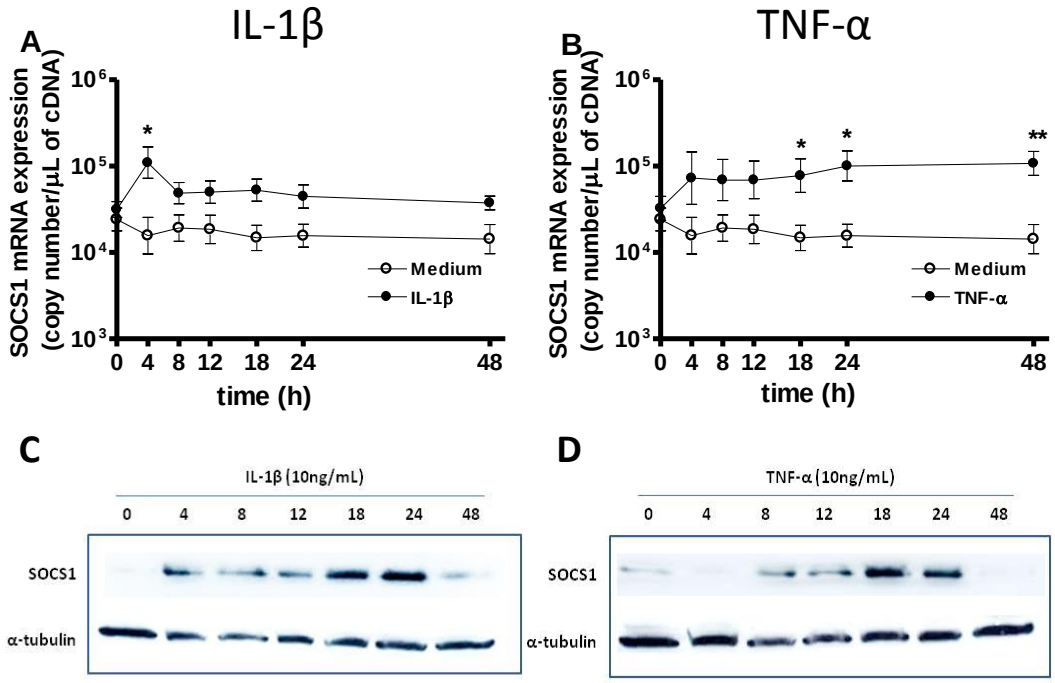

Figure 3.8: IL-1β and TNF-α induced SOCS1 mRNA.

SOCS1 mRNA was significantly induced by IL-1β ($p<0.05$ at 4h [A]) and TNF-α ($p<0.05$ at 18h and 24h, $p<0.01$ at 48h [B]). IL-1β and TNF-α induce SOCS1 protein ([C] and [D] respectively) (n=5 for mRNA experiments, western blot experiments representative of 3 experiments).

IL-1β and TNF-α significantly induced SOCS1 mRNA and protein. Both IL-1β and TNF-α induced SOCS3 mRNA however this was not statistically significant (Figure 3.9). IL-1β and TNF-α did not induce increased mRNA expression of any other SOCS family member (data not shown).

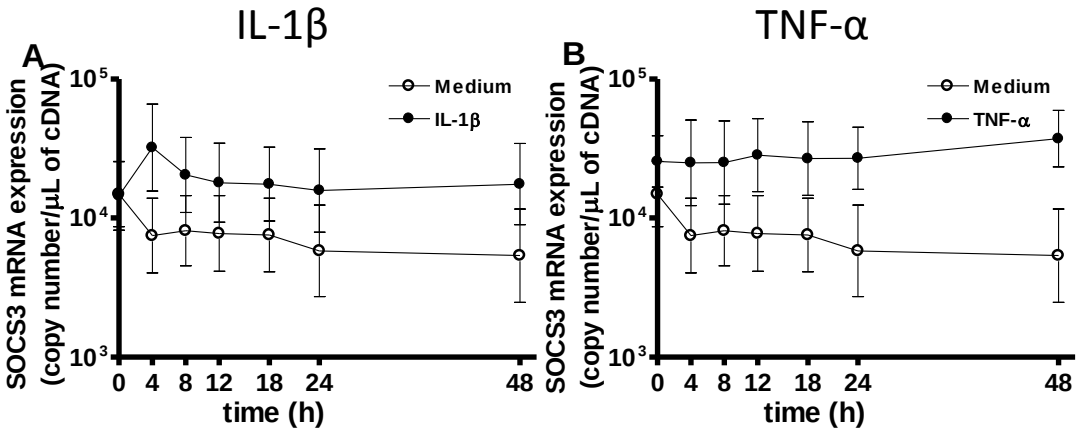

Figure 3.9: IL-1β and TNF-α induced SOCS3 mRNA.

SOCS3 mRNA was not significantly induced by IL-1β (A) and TNF-α (B) (n=5).

3.3.6 The Th2 cytokines IL-4 and IL-13 induced SOCS1 and CISH mRNA and protein in HBECs

IL-4 and IL-13 have been shown to upregulate SOCS1 and signal through JAK/STAT. Furthermore IL-4 and IL-13 have a role in the pathogenesis of atopic asthma .The effects of these Th2 cytokines on SOCS induction was investigated. Confluent cultured HBECs were treated with either 50ng/mL IL-4 or IL-13 and lysed for mRNA or protein at 0, 4, 8, 12, 18, 24 and 48h post treatment. mRNA levels were measured using taqman RT-PCR and protein levels were measured using western blotting.

IL-4 induced SOCS1 mRNA, peaking at 4h post treatment ($p<0.05$, Figure 3.10A) and levels remained elevated until 48h ($p<0.05$ at 18h and 24h). SOCS1 protein was induced by IL-4 in a time dependent manner (Figure 3.10C), with protein detectable at 4h post infection, peaking at 24h and remaining detectable until 48h post treatment.

IL-13 induced SOCS1 mRNA, peaking at 4h post treatment ($p<0.05$, Figure 3.10B) and levels remained elevated until 48h [$p<0.05$ at 24h and $p<0.01$ at 48h]. SOCS1 protein was induced by IL-13 in a time dependent manner (Figure 3.10D), with protein increased from baseline at 4h post infection and remained elevated until 48h post treatment .

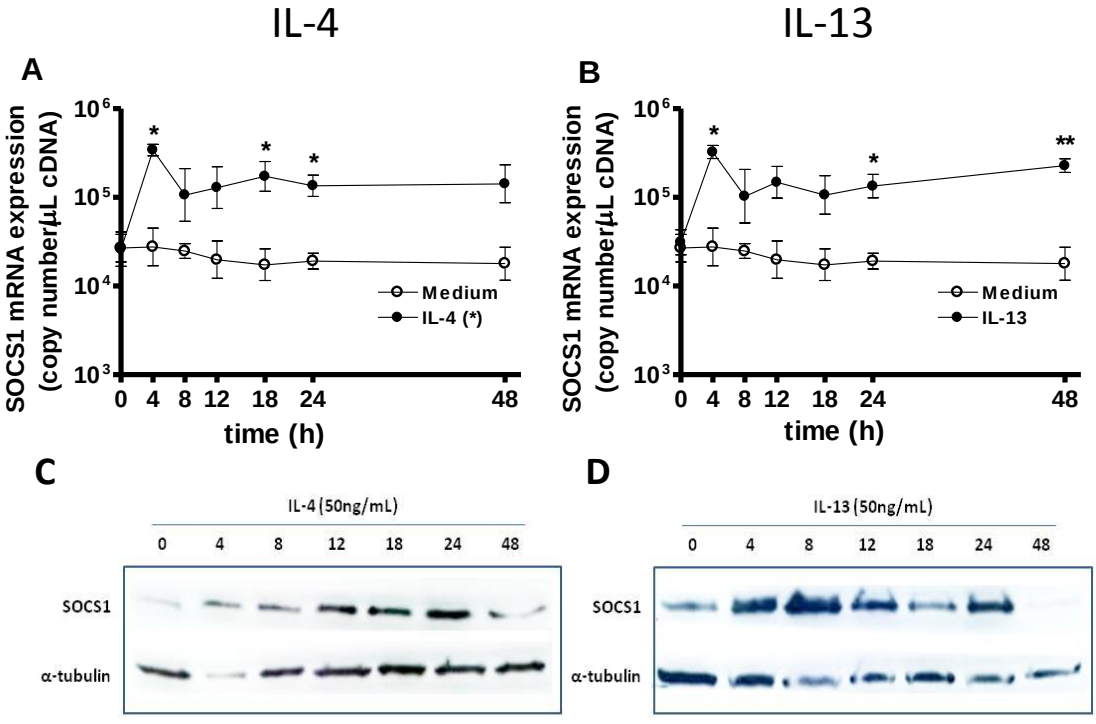

Figure 3.10: IL-4 and IL-13 induced SOCS1 mRNA and protein.

SOCS1 mRNA was significantly induced by IL-4 ($p<0.05$ at 4h, 18h and 24h A) and IL-13 ($p<0.05$ at 4h and 24h, $p<0.01$ at 48h B). IL-4 and IL-13 induced SOCS1 protein (C, D) (n=5 for mRNA experiments, western blot experiments representative of 3 experiments).

IL-4 increased CISH mRNA was statistically significant at 4h post treatment ($p<0.01$, Figure 3.11A) compared to medium treated cells and mRNA levels remain elevated until 48h [$p<0.05$ at 8h, and 18h, $p<0.01$ at 24h and 48h]. CISH protein was induced by IL-4 in a time dependent manner (Figure 3.11C), with protein detectable at 4h post infection, peaking at 18h and remaining detectable until 48h post treatment.

IL-13 increased CISH mRNA was statistically significant at 4h post treatment ($p<0.05$, Figure 3.11B) compared to medium treated cells and mRNA levels remain elevated until 48h ($p<0.05$ at 8h, 12h, 18h and 48h, $p<0.01$ at 24h). CISH protein was induced by IL-4 in a time dependent manner (Figure 3.11D), with protein detectable at 4h post infection, peaking at 18h and remaining detectable until 48h post treatment.

IL-4 and IL-13 induced SOCS1 and CISH mRNA and protein. IL-4 and IL-13 did not induce increased mRNA expression of any other SOCS family member (data not shown).

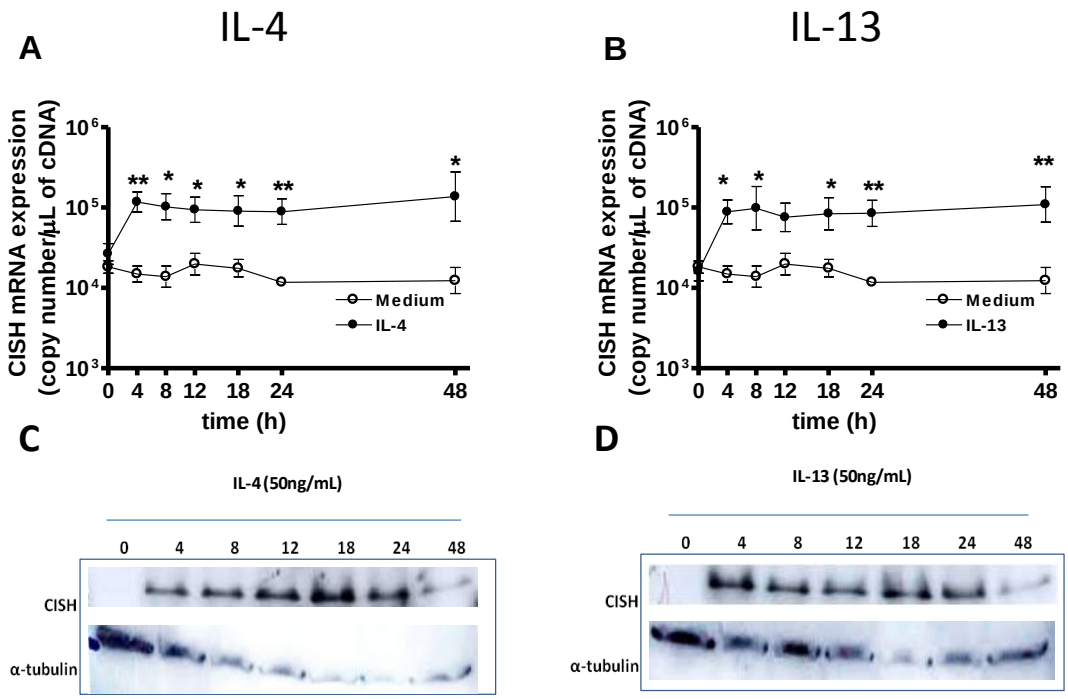

Figure 3.11: IL-4 and IL-13 induced CISH mRNA.

CISH mRNA was significantly induced by IL-4 ($p<0.05$ at 8h, 12h, 18h and 48h, $p<0.01$ at 4h and 24h, A) and IL-13 ($p<0.05$ at 4h, 8h, and 18h, $p<0.01$ at 24 and 48h, B). CISH protein was induced by IL-4 (C) and IL-13 (D) (n=5 for mRNA experiments, western blot experiments representative of 3 experiments).

3.3.7 Summary of induction of SOCS by different mediators

Table 3.1: Summary of induction of SOCS mRNA and protein by different mediators.

↑ means significantly induced at least one time point post treatment or post infection. – means no significant induction at any time point post treatment or post infection.

	SOCS1	SOCS2	SOCS3	SOCS4	SOCS5	SOCS6	SOCS7	CISH
RV1B	↑	-	↑	-	-	-	-	-
RV16	↑	-	↑	-	-	-	-	-
IFN-β	↑	-	-	-	-	-	-	-
IFN-λ	-	-	-	-	-	-	-	-
PolyIC	↑	-	↑	-	-	-	-	-
IL-1β	↑	-	-	-	-	-	-	-
TNF-α	↑	-	-	-	-	-	-	-
IL-4	↑	-	-	-	-	-	-	↑
IL-13	↑	-	-	-	-	-	-	↑

3.4 Function of SOCS1 and SOCS3 in HBECs

Having shown that SOCS1 and SOCS3 were induced upon RV, IFN-β, PolyIC, inflammatory mediators and Th2 cytokines in HBECs, it was aimed next to investigate the effects of SOCS1 and SOCS3 on anti-viral IFN-λ1 and IFN-β promoter activation, proinflammatory IL-6 and CXCL8/IL-8 promoter activation and Th2 induced STAT6 promoter activation.

3.4.1 SOCS1 and SOCS3 vector transfection leads to protein overexpression in HBECs

To confirm that transfection of HBECs with SOCS1 and SOCS3 vectors leads to overexpression of the proteins in these cells, protein expression of SOCS1 and SOCS3 was measured using western blotting. SOCS1 overexpression resulted in increased SOCS1 protein expression in HBECs (Figure 3.12A) compared to SOCS3 and control (pORF) vector transfection and untransfected cells. SOCS3 over-expression resulted in increased SOCS3 protein expression in HBECs (Figure 3.12A) compared to SOCS1 and control (pORF) transfection and untransfected cells. Overexpression of SOCS1 and SOCS3 led to increased expression of their respective proteins.

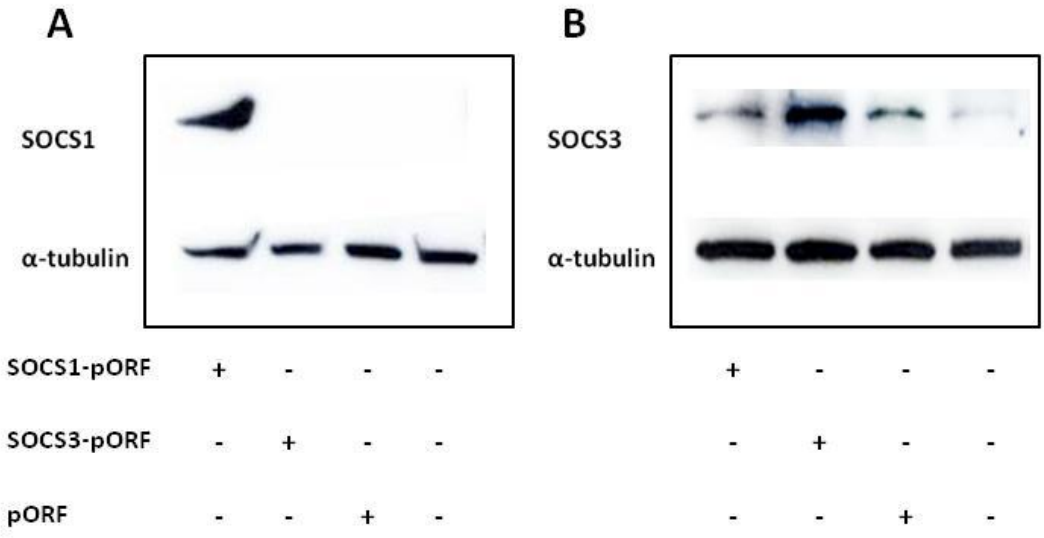

Figure 3.12: SOCS1 and SOCS3 vector transfection leads to protein over-expression in HBECs.

HBECs were tranfected with SOCS1, SOCS3 or control vector (pORF) and lysed 24h post transfection for western blotting. Data representative of 4 experiments.

3.4.2 Overexpression of SOCS1 and SOCS3 inhibit IFN-β induced IFN-β and IFN-λ1 promoter activation

Previous studies have shown that SOCS1 and SOCS3 can negatively regulate IFN-β induced innate immune responses [173, 193], here it was aimed to confirm this in HBECs. To assess whether SOCS1 and SOCS3 inhibited IFN-β induced IFN-λ1 and IFN-β promoter activation in HBECs, HBECs were transfected with SOCS1, SOCS3 or pORF control vectors and IFN-λ1 or IFN-β promoter and promoter activation was measured by luciferase assay.

IFN-β significantly induced IFN-λ1 and IFN-β promoter activity in HBECs ($p<0.01$, Figure 3.13 A, B). This effect was completely blocked by overexpression of SOCS1 and SOCS3 ($p<0.001$).

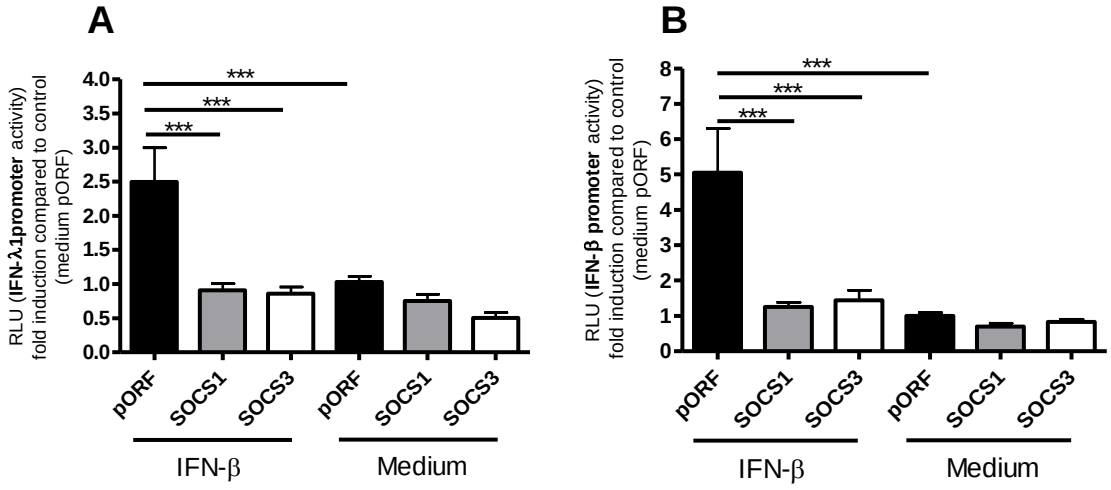

Figure 3.13: IFN-β induced IFN-λ1 and IFN-β promoter activation is reduced by SOCS1 and SOCS3.

HBECs were transfected with IFN-λ1 and IFN-β promoter and SOCS1, SOCS3 or control (pORF) vector, 24h post transfection cells were treated for 24h with IFN-β (10ng/mL) or medium. Data is expressed as relative light units (luciferase RLU, normalised to Renilla) fold induction compared to medium treated control vector transfected cells (n=4),

3.4.3 Overexpression of SOCS1 and SOCS3 inhibited RV1B induced IFN-β and IFN-λ1 promoter activation

Having shown that RV can induce SOCS1 and SOCS3 mRNA and protein, it was aimed to investigate whether these proteins play a role in negatively regulating RV induced signalling. It was assessed whether SOCS1 and SOCS3 had an effect on RV induced IFN-λ1 and IFN-β promoter activation in HBECs. HBECs were transfected with SOCS1, SOCS3 or pORF control vectors and IFN-λ1 or IFN-β promoter and promoter activation was measured by luciferase assay.

RV1B significantly induced IFN-λ1 and IFN-β promoter activity in HBECs ($p<0.001$ IFN-λ1 promoter, Figure 3.14A; $p<0.01$ IFN- β promoter, Figure 3.14B). This effect was completely blocked by SOCS1

and SOCS3 overexpression ($p<0.001$, IFN-λ1 promoter Figure 3.14A; IFN -β promoter $p<0.01$, Figure 3.14B).

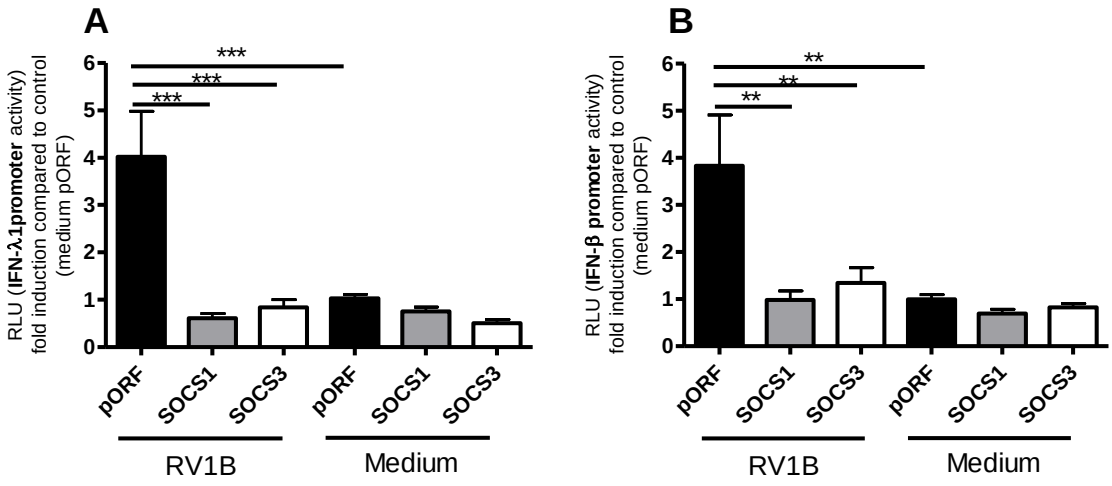

Figure 3.14: RV1B induced IFN-λ1 and IFN-β promoter activation is reduced by SOCS1 and SOCS3 in HBECs.

HBECs were transfected with IFN-λ1 or IFN-β promoter and SOCS1, SOCS3 or control (pORF) vectors, 24h post transfection cells were infected with RV1B or treated with media and lysed 24h post infection. Data is expressed as RLU (RLU luciferase normalised to Renilla) fold induction compared to medium treated control vector transfected cells (n=4)

RV16 did not significantly induce IFN-λ1 and IFN-β promoter activity in HBECs or BEASE2Bs. The effect of SOCS1 and SOCS3 on RV16 induced IFN-λ1 and IFN-β promoter activation was therefore not determined. SOCS1 and SOCS3 completely blocked RV1B induced IFN-β and IFN-λ1 promoter activation.

3.4.4 Overexpression of SOCS1 and SOCS3 had no effect on constitutively active ΔRIG-I and ΔTRIF induced IFN-λ1 promoter activation

In the previous paragraph it was shown that SOCS1 and SOCS3 could negatively regulate RV1B induced IFN promoter activation. Here we investigated if this was related to RNA helicase or TLR3 pathway induction of the IFN-λ1 promoter. To determine the effects of SOCS1 and SOCS3 on the RIG-I and TLR3 pathways inducing IFN promoters, constitutively active ΔRIG-I and ΔTRIF was used to induce the IFN-λ1 promoter. ΔRIG-I and ΔTRIF significantly induced the IFN-λ1 promoter in BEAS2Bs (ΔRIG-I $p<0.05$, Figure 3.15A, ΔTRIF $p<0.01$, Figure 3.15B). Although ΔRIG-I and ΔTRIF induced IFN-λ1 promoter activation was reduced by SOCS1 and ΔTRIF induced IFN-λ1 promoter activation was reduced by SOCS3, this was not statistically significant. SOCS1 and SOCS3 overexpression did not have an effect on ΔRIG-I and ΔTRIF induced IFN-λ1 promoter activation, similar results were observed with the IFN-β promoter (data not shown).

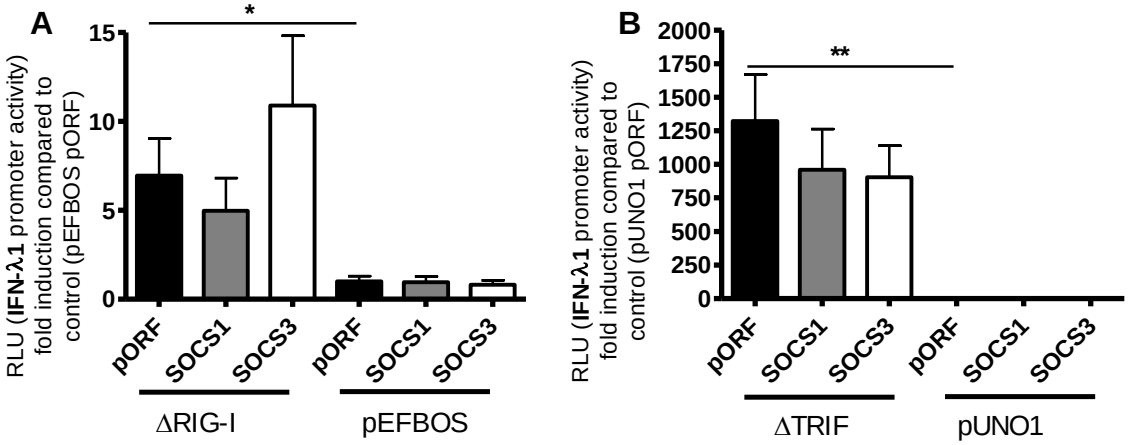

Figure 3.15: ΔRIG-I and ΔTRIF induced IFN-λ1 promoter activation is not inhibited by SOCS1 and SOCS3 in BEAS2Bs.

BEAS2Bs were transfected with the IFN-λ1 promoter, SOCS1, SOCS3 or control vector (pORF) and ΔRIG-I (A), ΔTRIF (B) or control vector (pEFBOS for ΔRIG-I and pUNO1 for ΔTRIF) and lysed 16h post transfection. Data is expressed as RLU (RLU Luciferase normalised to RLU Renilla) fold induction compared to control vector transfected cells (n=3).

3.4.5 Overexpression of SOCS1 and SOCS3 had no effect on TBK-1, IKKβ and IKKε induced IFN-λ1 promoter activation

In the previous paragraph it was shown that SOCS1 and SOCS3 did not negatively regulate ΔRIG-I and ΔTRIF induced IFN promoter activation. It was aimed to determine whether SOCS1 and SOCS3 could negatively regulate induction of the IFN-λ1 promoter by signalling intermediates downstream in the RIG-I and TLR3 pathways. To determine the effects of SOCS1 and SOCS3 downstream on the RIG-I and TLR3 pathway, TBK-1, IKKβ and IKKε were used to induce the IFN-λ1 promoter. All kinases significantly induced the IFN-λ1 promoter in BEAS2Bs (TBK-1 and IKKβ $p<0.001$, Figure 10A and Figure 10B respectively, IKKε $p<0.05$, Figure 10C). A reduction of TBK-1 and IKKβ promoter activation by SOCS1 and IKKε promoter activation by SOCS3 was observed, however this was not significant, similar results were observed with the IFN-β promoter (data not shown).

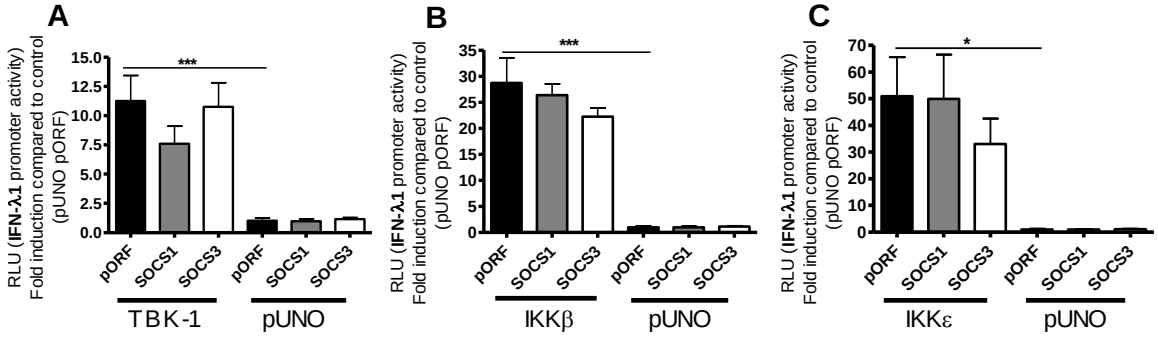

Figure 3.16: TBK-1, IKKβ and IKKε induced IFN-λ1 promoter activation is not inhibited by SOCS1 and SOCS3 in BEAS2Bs.

BEAS2Bs were transfected with the IFN-λ1 promoter, SOCS1, SOCS3 or control vector (PORF) and TBK-1 (A), IKKβ (B), IKKε (C) or control vector (pUNO) and lysed 24h post transfection. Data is expressed as RLU (RLU Luciferase normalised to RLU Renilla) fold induction compared to control vector transfected cells (n=3).

3.4.6 Overexpression of SOCS1 and SOCS3 did not decrease IRF3 induced IFN-λ1 and IFN-β promoter activation

IRF3 is a crucial transcription factor required for IFN-λ1 and IFN-β expression. To determine the effects of SOCS1 and SOCS3 on IRF3 induced IFN-λ1 and IFN-β promoter an IRF-3 overexpression vector was used. IRF3 overexpression significantly induced the IFN-λ1 and IFN-β promoter in BEAS2Bs (IFN-λ1 $p<0.01$, Figure 3.17A and IFN-β $p<0.05$, Figure 3.17B). SOCS3 had no effect on IRF3 induced IFN-λ1 and IFN-β promoter activation. SOCS1 had no effect on IRF3 induced IFN-λ1 promoter activity, but significantly increased IFN-β promoter activation (Figure 3.17B, $p<0.05$).

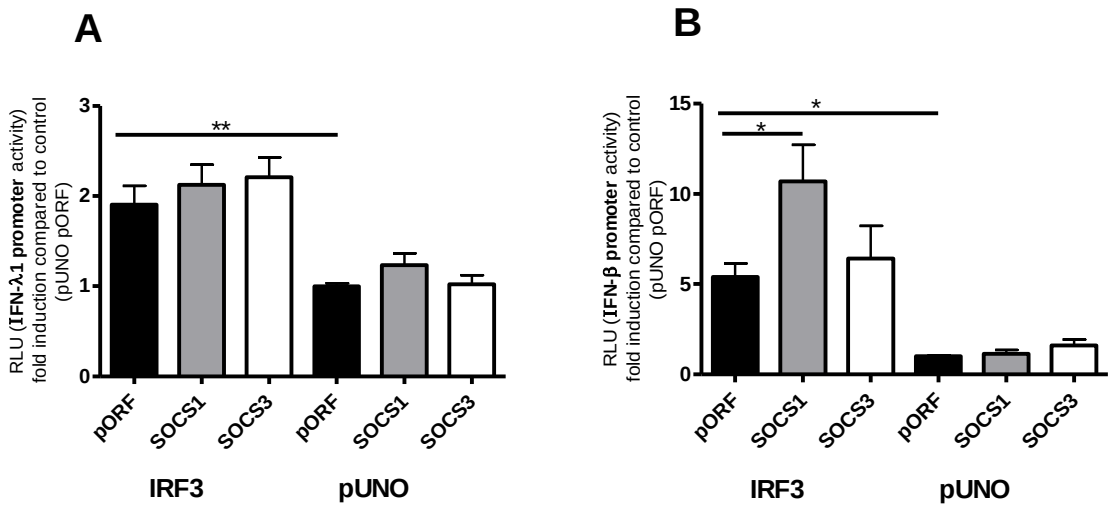

Figure 3.17: IRF3 induced IFN-λ1 and IFN-β promoter activation was not inhibited by SOCS1 and SOCS3 in BEAS2Bs.

SOCS1 significantly increased IRF3 induced IFN-β promoter activation (B). BEAS2Bs were transfected with the IFN-λ1 (A) and IFN-β (B) promoter, SOCS1, SOCS3 or control vector (pORF) and IRF3 or control vector (pUNO) and lysed 24h post transfection. Data is expressed as RLU (RLU Luciferase normalised to RLU Renilla) fold induction compared to control vector transfected cells (n=3).

3.4.7 Overexpression of SOCS1 and SOCS3 inhibited IL-4 and IL-13 induced STAT6 promoter activation

IL-4 and IL-13 play a role in atopic asthma and signal through JAK/STAT. SOCS1/3 have been shown to negative regulate IL-4 and IL-13 induced signalling, however this has not yet been shown in BECs [185, 194, 195]. To determine the effect of SOCS1 and SOCS3 on IL-4 and IL-13 induced signalling, IL-4 and IL-13 were used to induce STAT6 minimal promoter activation.

IL-4 and IL-13 significantly induced STAT6 minimal promoter activation in BEAS2Bs ($p<0.001$, Figure 3.18). Over-expression of SOCS1 and SOCS3 significantly reduced IL-4 ($p<0.001$, Figure 3.18A) and IL-13 ($p<0.001$, Figure 3.18B) induced STAT6 minimal promoter activation.

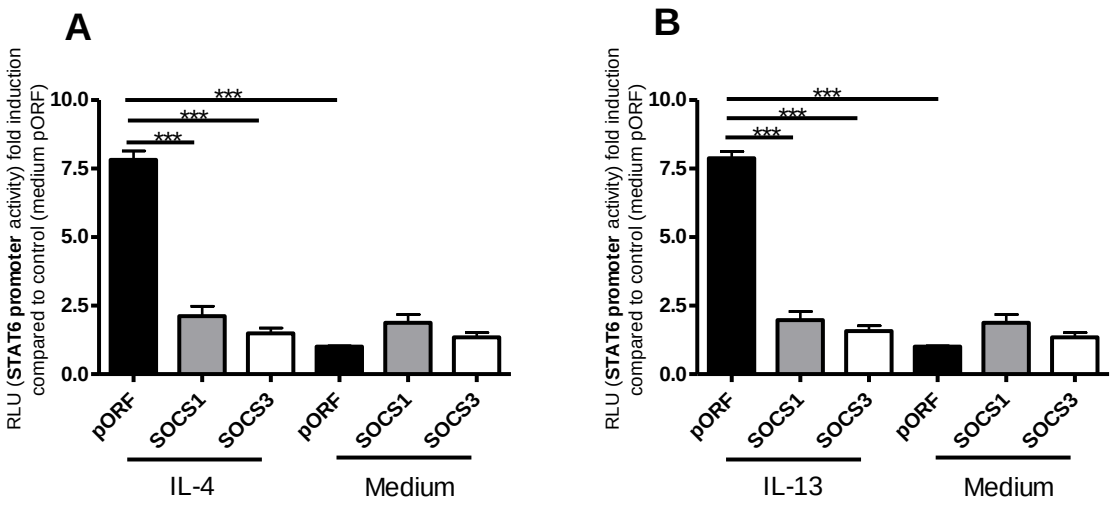

Figure 3.18: IL-4 and IL-13 induced STAT6 minimal promoter is significantly inhibited by both SOCS1 and SOCS3.

BEAS2Bs were transfected with STAT6 minimal promoter, SOCS1, SOCS3 or control vector (pORF) and treated with 50ng/mL of IL-4 (A) or IL-13 (B) or medium and lysed 24h post treatment. Data is expressed as RLU (RLU Luciferase normalised to RLU Renilla) fold induction compared to control vector transfected cells (n=3).

3.4.8 Overexpression of SOCS1 and SOCS3 increased IL-1β and TNF-α induced CXCL8/IL-8 promoter activation, reduced IL-1β and TNF-α induced IL-6 promoter activation and had no effect on IL-1β and TNF-α induced NF-κB promoter activation

One study indicates that SOCS1 might be able to negatively regulate induction of NF-κB dependant genes [181]. SOCS1 was significantly induced by IL-1β and TNF-α, and a trend was observed for a similar pattern of SOCS3 induction (Figure 3.8 and Figure 3.9). To determine whether the induction of SOCS1 and SOCS3 could relate to a role of these proteins in negatively regulating IL-1β and TNF-α induced promoter activation, reporter assays were used to determine the effect of overexpression of SOCS1 and SOCS3 on IL-1β and TNF-α induced IL-6, CXCL8/IL-8 and a minimal promoter for NF-κB promoter activation. IL-1β and TNF-α induce the promoters of pro-inflammatory cytokines (e.g. IL-6 and CXCL8/IL-8) through NF-κB [61, 62].

Both IL-1β and TNF-α significantly induced IL-6 promoter activation ($p<0.001$, Figure 3.19A and Figure 3.19B). SOCS1, and to a lesser extent SOCS3 overexpression, inhibited IL-1β (Figure 3.19A, respectively $p<0.01$ and $p<0.05$) and TNF-α (Figure 3.19B, $p<0.001$) induced IL-6 promoter activation.

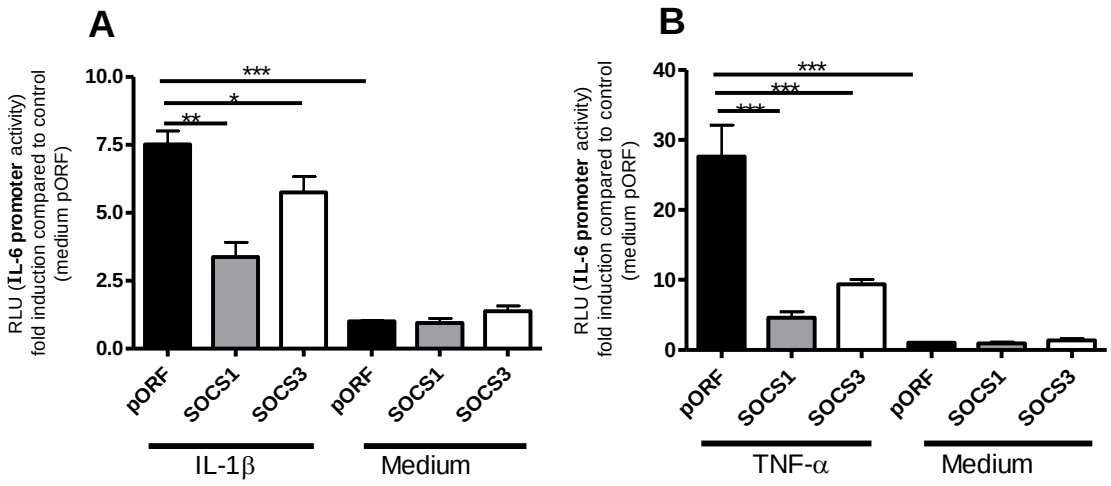

Figure 3.19: IL-1β and TNF-α induced IL-6 promoter activation is inhibited by SOCS1 and SOCS3 in BEAS2Bs.

BEAS2Bs were transfected with the IL-6 promoter, SOCS1, SOCS3 or control vector (pORF) and treated with IL-1β (A) and TNF-α (B) and lysed 24h post treatment. Data is expressed as RLU (RLU Luciferase normalised to RLU Renilla) fold induction compared to control vector transfected cells (n=4).

IL-1β and TNF-α also activated the IL-8 promoter (Figure 3.20A and Figure 3.20B, $p<0.05$). In contrast to the results observed with the IL-6 promoter, SOCS1 and SOCS3 overexpression significantly induced IL-1β (Figure 3.20A, respectively $p<0.001$) and TNF-α (Figure 3.20B, $p<0.001$) induced CXCL8/IL-8 promoter activity.

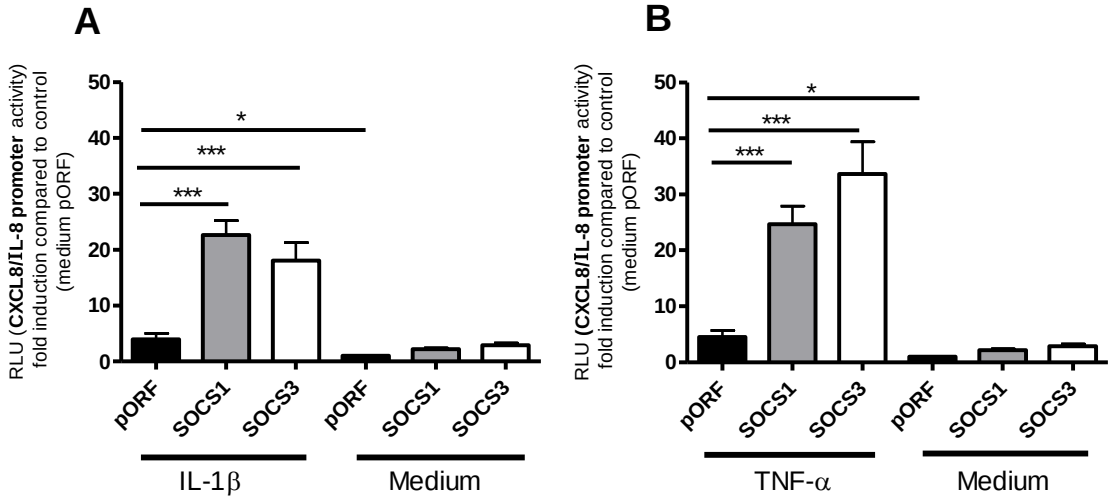

Figure 3.20: IL-1β and TNF-α induced CXCL8/IL-8 promoter activation is inhibited by SOCS1 and SOCS3 in BEAS2Bs.

BEAS2Bs were transfected with the CXCL8/IL-8 promoter, SOCS1, SOCS3 or control vector (pORF), treated with IL-1β (A) and TNF-α (B) and lysed 24h post treatment. Data is expressed as RLU (RLU Luciferase normalised to RLU Renilla) fold induction compared to control vector transfected cells (n=4).

Finally an NF-κB minimal promoter was used. NF-κB is involved in IL-1β and TNF-α induced IL-6 and CXCL8/IL-8 transcriptional regulation, with their promoters containing NF-κB binding sites necessary for their activation[61, 62, 64]. IL-1β and TNF-α significantly induced the NF-κB minimal promoter (Figure 3.21, p<0.001). However SOCS1 and SOCS3 had no effect on this activation (Figure 3.21).

The role of SOCS1 and SOCS3 in negatively regulation IL-1β and TNF-α induced NF-κB dependant promoters was observed to be unclear. SOCS1 and SOCS3 increased IL-1β and TNF-α induced CXCL8/IL-8 promoter activation, reduced IL-1β and TNF-α induced IL-6 promoter activation and had no effect on IL-1β and TNF-α induced NF-κB promoter activation.

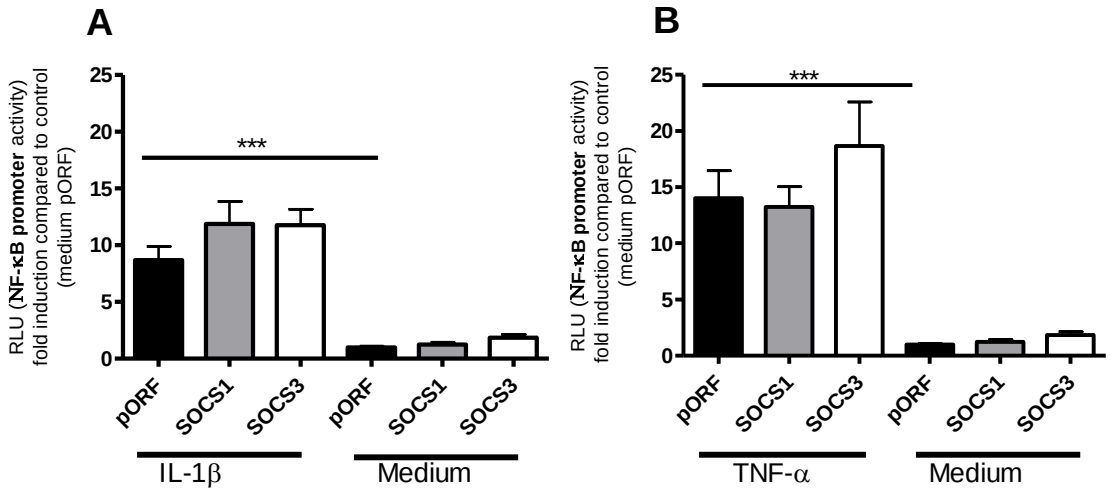

Figure 3.21: IL-1β and TNF-α induced NF-κB minimal promoter activation is inhibited by SOCS1 and SOCS3 in BEAS2Bs.

BEAS2Bs were transfected with the NF-κB promoter, SOCS1, SOCS3 or control vector (pORF) and treated with IL-1β (A) and TNF-α (B) and lysed 24h post treatment. Data is expressed as RLU (RLU Luciferase normalised to RLU Renilla) fold induction compared to control vector transfected cells (n=5).

3.4.9 Overexpression of SOCS1 and SOCS3 inhibited RV1B induced IL-6 and CXCL10/IP-10 promoter activation, SOCS1 increased RV1B induced CXCL8/IL-8 promoter activation and SOCS3 had no effect on RV1B induced CXCL8/IL-8 promoter activation

To determine whether the induction of SOCS1 and SOCS3 could have a direct effect on RV1B induced proinflammatory cytokine promoter activation, reporter assays were used to determine the effect of overexpression of SOCS1 and SOCS3 on RV1B induced IL-6, CXCL8/IL-8, CXCL10/IP-10 and NF-κB minimal promoter activation.

RV1B significantly induced IL-6 and CXCL8/IL-8 promoter activation in BEAS2Bs (p<0.001 IL-6 promoter, Figure 3.22A; p<0.01 CXCL8/IL-8 promoter, Figure 3.22B). Over-expression of SOCS1 and SOCS3 significantly reduced IL-6 promoter activation (p<0.001, Figure 3.22A). RV1B induced

CXCL8/IL-8 promoter activation was increased with SOCS1 over-expression ($p<0.001$, Figure 3.22B) and no effects were observed with SOCS3. RV1B did not significantly induce a minimal promoter for NF-κB (data not shown).

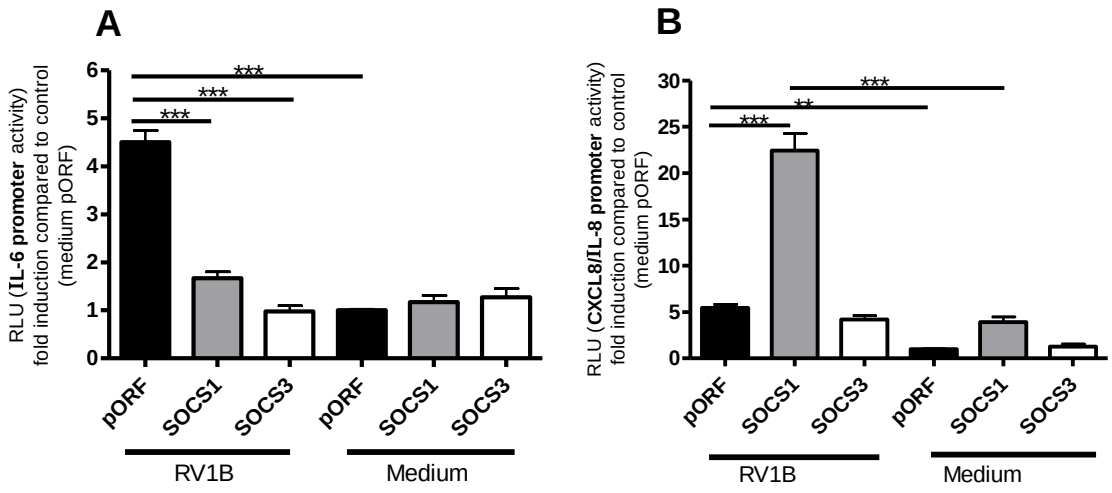

Figure 3.22: Effect of SOCS1 and SOCS3 on RV1B induced IL-6 and CXCL8/IL-8 promoter activation.

BEAS2Bs were transfected with the IL-6 (A) or CXCL8/IL-8 (B) promoter, SOCS1, SOCS3 or control vector (pORF) and infected with RV1B and lysed 24h post infection. RV1B induced CXCL8/IL-8 promoter activation is inhibited by SOCS1 and SOCS3 in BEAS2Bs (A, $p<0.001$). RV1B induced CXCL8/IL-8 promoter activation is increased by SOCS1 in BEAS2Bs (B, $p<0.001$). Data is expressed as RLU (RLU Luciferase normalised to RLU Renilla) fold induction compared to control vector transfected cells (n=4).

The effects of SOCS1 and SOCS3 on RV1B induced IL-6 and CXCL8/IL-8 promoter activation were expected to be similar, because RV1B induced signalling leading to the activation of these promoters are very similar. Therefore it was aimed to investigate whether the inhibitory effects of SOCS1 and SOCS3 on the IL-6 promoter were representative of RV1B induced NF-κB dependant proinflammatory promoter induction or the IL-8/CXCL8 results. Hence COX2, CXCL-5/ENA-78 and CXCL10/IP-10 were used as other NF-κB inducible promoters. BEAS2Bs were transfected with these promoters and infected with RV1B, the CXCL10/IP-10 promoter was the only promoter induced by RV1B ($p<0.001$, Figure 3.23). SOCS1 and SOCS3 overexpression both significantly reduced this induction ($p<0.001$).

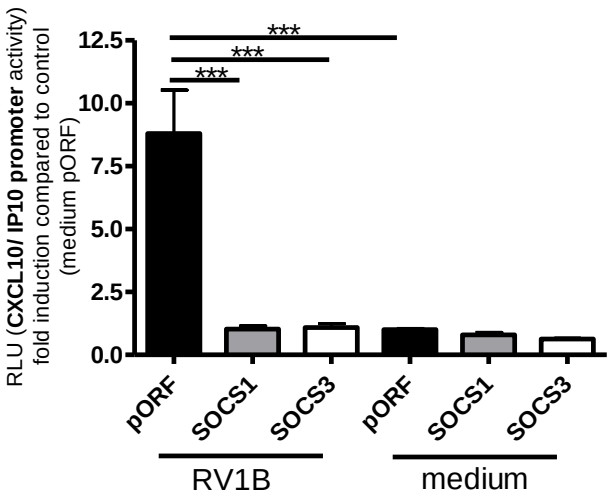

Figure 3.23: Effect of SOCS1 and SOCS3 on RV1B induced CXCL10/ IP-10 promoter activation.

BEAS2Bs were transfected with the CXCL10/IP-10 promoter promoter, SOCS1, SOCS3 or control vector (pORF) and infected with RV1B and lysed 24h post infection. RV1B induced CXCL10/ IP-10 promoter activation is inhibited by SOCS1 and SOCS3 in BEAS2Bs ($p<0.001$). Data is expressed as (RLU Luciferase normalised to RLU Renilla) fold induction compared to control vector transfected cells (n=5).

The role of SOCS1 and SOCS3 in negatively regulation RV1B induced NF-κB dependant promoters was observed to be controversial. Overexpression of SOCS1 and SOCS3 inhibited RV1B induced IL-6 and CXCL10/IP-10 promoter activation, SOCS1 increased RV1B induced CXCL8/IL-8 promoter activation and SOCS3 had no effect on RV1B induced CXCL8/IL-8 promoter activation

3.4.10 SOCS1 and SOCS3 knockdown had no effect on IFN induced ISGs mRNA and RV induced IFN mRNA expression

As a comparison to the overexpression data, siRNA was used to assess the effect of SOCS1 and SOCS3 knockdown on type I IFN induced ISG mRNA and RV induced type I and III IFN mRNA expression.

First knockdown of SOCS1 and SOCS3 mRNA was determined by their individual siRNAs. HBECS were transfected with SOCS1, SOCS3 or control siRNA, left for 24h and treated with IFN-β (10ng/mL) for 24h and cells were lysed for mRNA. The results of individual siRNA used and their effect on the knockdown of their target are summarised in table 3.2 (SOCS1) and table 3.3 (SOCS3).

Table 3.2: SOCS1 siRNAs used and their knockdown effect on SOCS1 mRNA.

siRNA=short interfering RNA, shRNA=short hairpin RNA.

SOCS1			
Company	**RNAi method**	**Product code**	**Knockdown**
Dharmacon	siRNA	10	0
Dharmacon	siRNA	11	0
Dharmacon	siRNA	12	<25%
Dharmacon	siRNA	13	<15%
Dharmacon	siRNA	Pool	<10%
Invivogen	shRNA	-	0
Santa Cruz	siRNA	-	~95%

Table 3.3: SOCS3 siRNAs used and their knockdown effect on SOCS3 mRNA.

SOCS3			
Company	**RNAi method**	**Product code**	**Knockdown**
Dharmacon	siRNA	9	<15%
Dharmacon	siRNA	10	0
Dharmacon	siRNA	11	0
Dharmacon	siRNA	12	<30%
Dharmacon	siRNA	Pool	0
Santa Cruz	siRNA	-	<15%
Qiagen	siRNA	1	0
Qiagen	siRNA	4	0
Qiagen	siRNA	6	0
Qiagen	siRNA	7	<20%

siRNA knockdown was more difficult to achieve than expected, with SOCS1 mRNA expression only significantly reduced by the Santa Cruz siRNA, and virtually no knockdown by the other siRNAs (Table 3.2. IFN-β (1ng/mL) significantly increased SOCS1 mRNA expression in HBECs transfected with control siRNA ($p<0.05$, Figure 3.24). The Santa Cruz siRNA targeting SOCS1 (Table 3.2) significantly reduced IFN-β induced SOCS1 mRNA in HBECs ($p<0.01$, Figure 3,24). There was no significant knockdown observed of SOCS3 mRNA expression by any of the SOCS3 siRNAs used (Table 3.3, Figure 3.25). IFN-β (1ng/mL) did not increase SOCS3 mRNA expression in HBECs transfected with control siRNA (Figure 3.25). The Dharmacon siRNA targeting SOCS3 (product code 12, Table 3.3) showed a trend towards reduction of reduced SOCS3 mRNA in HBECs, this however was not significant (Figure 3.25).

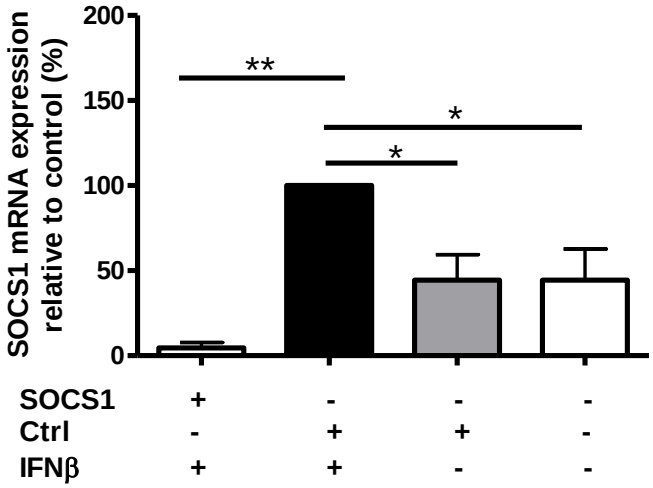

Figure 3.24: Knockdown by SOCS1 siRNA of its target SOCS1 mRNA in HBECs.

IFN-β (10ng/mL) treated HBECs significantly induced SOCS1 mRNA at 24h post treatment ($p<0.05$). SOCS1 siRNA transfected and IFN-β treated cells had a significant reduction of SOCS1 mRNA compared to IFN-β treated and control transfected HBECs ($p<0.01$, n=4).

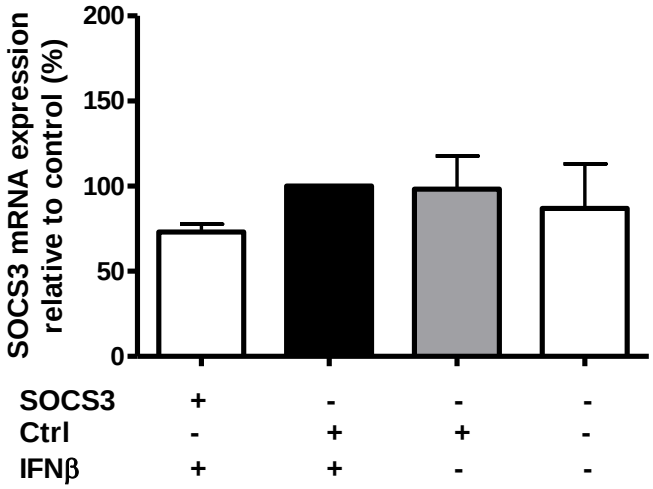

Figure 3.25: Knockdown by SOCS3 siRNA of its target SOCS3 mRNA in HBECs.

IFN-β (10ng/mL) treated HBECs did not induce SOCS3 mRNA at 24h post treatment. SOCS3 siRNA transfected and IFN-β treated cells had no significant reduction of SOCS3 mRNA compared to IFN-β treated and control transfected HBECs (n=4).

Previous studies have shown a clear role for SOCS1 in negatively regulating IFN-β induced signalling [173, 193] and in paragraph 3.4.2 it was shown that overexpression of SOCS1 inhibited RV1B and IFN-β induced IFN-β and IFN-λ1 promoter activation in HBECs. Here it was aimed to confirm the effect of SOCS1 on RV1B and IFN-β induced signalling by using siRNA targeting SOCS1. HBECS were transfected with SOCS1 siRNA (Santa Cruz) and treated with IFN-β (10ng/mL) as previously described and ISGs Viperin, MxA, OAS and RIG-I mRNA levels were measured (Figure 3.26). No significant

increase was observed between control siRNA transfected HBECs and SOCS1 siRNA transfected cells for all ISGs measured, however Viperin and MxA exhibited a trend for increased expression after treatment with SOCS1 specific siRNA. SOCS1 siRNA did not affect RV1B induced IFN mRNA expression (data not shown).

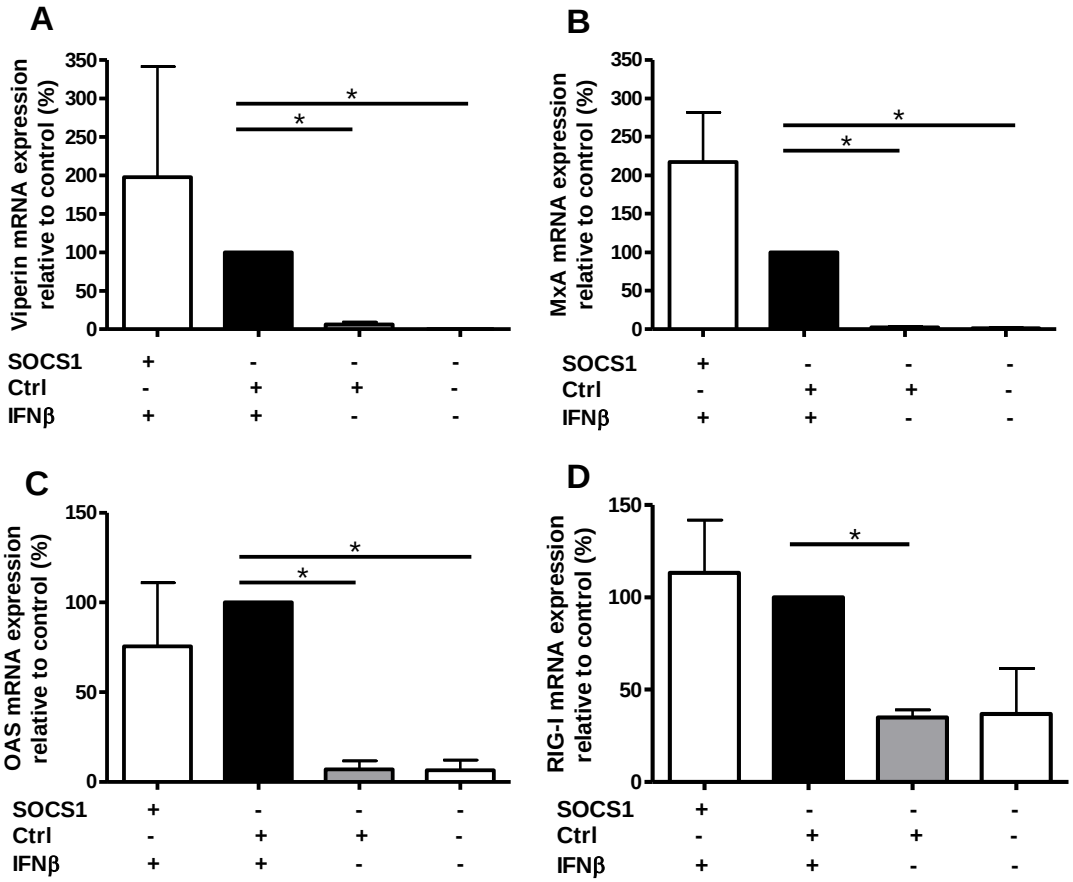

Figure 3.26: Effect of SOCS1 siRNA on IFN-β induced ISG mRNA in HBECs.

IFN-β (10ng/mL) treated and control siRNA transfected HBECs significantly induced Viperin, MxA, OAS and RIG-I mRNA compared to control siRNA transfected and untreated cells ($p<0.05$). SOCS1 siRNA transfected and IFN-β treated cells had no significant different induction of SOCS1 mRNA compared to IFN-β treated and control transfected HBECs (n=3).

SOCS1 knockdown by siRNA had no effect on IFN induced ISGs mRNA and RV induced IFN mRNA expression. There was no significant knockdown observed by any of the SOCS3 targeting siRNAs used in this study.

3.4.11 Overexpression of SOCS1 and SOCS3 had no effect on RV viral RNA expression and release and RV induced IFN mRNA induction

Previous studies have shown a clear role for SOCS1 and SOCS3 in negatively regulating IFN-β induced signalling [173, 193] and in paragraph 3.4.2 it was shown that overexpression of SOCS1 and SOCS3 inhibited RV1B induced IFN-β and IFN-λ1 promoter activation in HBECs. Here it was aimed to confirm the effect of SOCS1 and SOCS3 on RV1B induced promoter activation by determining the effect of SOCS1 and SOCS3 overexpression on RV viral RNA (vRNA) expression and release and RV1B induced IFN mRNA expression. No differences were observed between control vector (pORF) transfected cells and SOCS1 or SOCS3 transfected cells for RV1B vRNA expression (Figure 3.27A), RV release into supernatants (Figure 3.27B) or RV1B induced IFN mRNA expression (data not shown). Although SOCS1 and SOCS3 overexpression could inhibit RV1B induced IFN-β and IFN-λ1 promoter activation, this had no effect on RV1B vRNA and release and RV induced IFN mRNA.

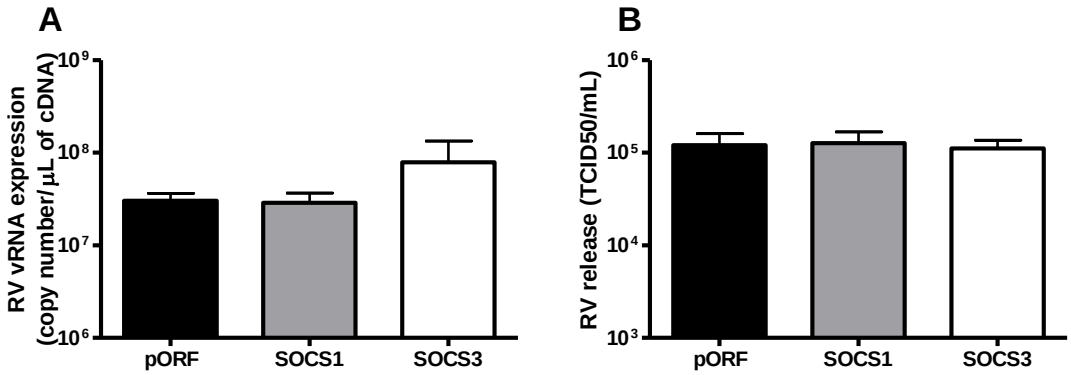

Figure 3.27: Effect of SOCS1 and SOCS3 overexpression on RV vRNA and RV release upon RV1B infection in BEAS2Bs.

SOCS1 and SOCS3 overexpression had no significant effect on RV vRNA expression (A) and RV release (B) compared to control vector (pORF, n=5).

3.5 Discussion

In this chapter it was investigated whether SOCS family members were induced in HBECs upon RV infection, or on stimulation with type I and type III IFNs, Th2 cytokines, inflammatory mediators and on dsRNA treatment. The role of SOCS family members in RV induced asthma exacerbations was unknown and all these mediators play a role in asthma or RV induced asthma exacerbations. SOCS1 was induced by RV, IFN-β, Th2 cytokines, PolyIC (a TLR3 ligand) and inflammatory mediators. SOCS3 was induced by RV and PolyIC and trends were observed for induction by IFN-β and inflammatory mediators.

Secondly, it was investigated whether SOCS1 and SOCS3 could modulate RV, IFN, pro-inflammatory cytokine and Th2 induced promoter activation, which would indicate a role of SOCS1 and SOCS3 in inhibiting the RV, IFN-β, Th2 cytokine signalling pathways. SOCS1 and SOCS3 negatively inhibited RV and IFN induced IFN-β and IFN-λ1 promoter activation and Th2 induced STAT6 promoter activation. Contradictory effects of SOCS1 and SOCS3 were seen on inflammatory mediator induced NF-κB dependant promoter activation, with SOCS1 and SOCS3 negatively regulating TNF-α, IL-1β and RV1B induced IL-6 promoter activity, increasing CXCL8/IL-8 promoter activity and having no effect on a NF-κB minimal promoter activity.

3.5.1 SOCS1 and SOCS3 induction in HBECs

The potential role of SOCS proteins in RV induced asthma exacerbations was unknown. These proteins had not been studied in HBECs in relation to RV infection and could play a role in RV induced asthma exacerbations. It is known that SOCS family members are constitutively expressed in a bronchial epithelial cell line [175] and that they are induced by other respiratory viruses [175-177, 179] in bronchial epithelium and other cell types. To determine whether RVs were able to induce SOCS primary HBECS were stimulated with major and minor group RV (respectively RV16 and RV1B) and SOCS family mRNA and protein induction was measured. Both RV1B and RV16 induced SOCS1 and SOCS3 mRNA in a time dependent manner, which was significantly increased at 48h, with increased mRNA observed from 12h post infection. SOCS1 and SOCS3 protein were also induced upon RV1B and RV16 with increases in protein observed between 4 and 12h. This is the first report of RV inducing SOCS1 and SOCS3. RV1B induced type I and III IFN mRNA were increased at 4h, peaks at 24h and stays elevated at 48h post infection [196]. RV induced SOCS1 and SOCS3 were induced around the same time compared with the time course of RV induced IFN induction and increase during the same time course of infection. This relatively quick induction of SOCS1 and SOCS3 occurs at a time when RV induced IFN-β and IFN-λ are still increasing [196] suggesting that if SOCS proteins act as negative

regulators upon expression that this negative inhibition of IFN signalling by SOCS1 and SOCS3 proteins is a slow effect rather than a rapid shut down of signalling after expression.

PolyIC, a dsRNA activating TLR3 signalling, was shown to induce both SOCS1 and SOCS3 mRNA and protein in HBECs, with mRNA peaking at 4h and remaining elevated for both SOCS1 and SOCS3 and protein visible at respectively 12h and 8h and increasing in a time dependent manner. Induction of SOCS1 by PolyIC had not been studied in HBECs but had been shown in keratinocytes [192]. The innate immune response to RV infection in HBECs had been shown to be coordinated between TLR3 and RNA helicase signalling, with TLR3 recognition of RV being the initial response followed by RNA helicase signalling [196]. IFN-β was also shown to increase SOCS1 mRNA peaking at 4h and remaining elevated until 48h post treatment, with protein detectable 8h post treatment and increasing in a time dependent manner [173]. Although a trend was seen for type III IFN-λ induced SOCS1 mRNA and IFN-β induced SOCS3 mRNA no statistical significant increase was seen. The quick induction of SOCS1 and SOCS3 by PolyIC and SOCS1 by IFN-β supports the statement in the previous paragraph that SOCS1 and SOCS3 more likely act slowly in their inhibition of IFN signalling after their initial expression.

TNF-α and IL-1β both play an important role in inflammatory responses in asthma and have been shown to be increased in the airways of asthmatics compared to healthy subjects [187-189]. TNF- α causes increased airway neutrophila [189] and airway hyperresponsiveness. IL-1β plays both an indirect and direct role in mucus hypersecretion [197]. Their signalling pathways activate NF-κB, but are however distinct from the JAK/STAT pathway. Several studies have shown inhibition of NF-κB dependent signalling by both SOCS1 and SOCS3 [82, 180, 198, 199]. Previous studies have shown induction of SOCS1, SOCS2 and SOCS3 in mice liver cells [200] and overexpression of SOCS1 protects hepatocytes from TNF induced damage [200], strongly suggesting that SOCS1 Is a natural negative regulator of TNF-α induced signalling. Here we showed induction of SOCS1 mRNA and protein upon TNF-α and IL-1β treatment, and a trend of SOCS3 mRNA induction in HBECs by these mediators. The increased levels of TNF-α and IL-1β in asthmatics might cause increased levels of SOCS1 present in the airways. Upon RV infection this could contribute to reduced type I and III IFNs, via IL-1β or TNF-α. IFN deficiency has been shown in pro-inflammatory, non allergic disease lung diseases, such as chronic obstructive pulmonary disease (COPD) [201] and cystic fibrosis (CF) (M.Varielle, manuscript submitted). This indicates that increased SOCS1 and SOCS3 levels in asthmatics could relate to both inflammatory mediators and Th2 cytokines, causing the IFN deficiency and inflammatory mediator cause increased SOCS1 and SOCS3 levels which might be related to IFN deficiency in COPD and CF.

Induction of SOCS1 and SOCS3 by Th1 and Th2 cytokines and in allergic asthma and the role of SOCS1 in regulating the Th1/Th2 balance is controversial. SOCS1 is mainly expressed in Th1 cells [202] and induced by Th2 cytokines and IFN-γ in different cell types [194, 195, 203-205] with IL-13 inducing SOCS1 in ASMCs [170]. The lethality of SOCS1 mice can be prevented by either knockout of IFN-γ or STAT6, indicating a role for SOCS1 in inhibiting both Th1 and Th2 signalling [165, 166]. Consistent with this, T cell-specific deletion of SOCS1 leads to enhanced production of IFN-γ and IL-4 and enhanced differentiation into Th1 and Th2 cells [167]. However, recent studies found a correlation between elevated SOCS1 expression and asthma [168], and suggest that SOCS1 may inhibit IFN-γ-dependent Th1 differentiation, thereby enhancing Th2-mediated pathology [202]. However, this is not supported by the phenotype of SOCS1 $^{-/-}$ IFN-γ $^{-/-}$ C57/Bl6 mice, which show increased eosinophilia in the SOCS1 knockouts compared to IFN-γ$^{-/-}$ C57/Bl6 mice, indicating increased Th2 differentiation of SOCS$^{-/-}$ IFN-γ $^{-/-}$ C57/Bl6 mice [169]. SOCS3 is preferentially expressed in Th2 cells [202] and induced by Th2 cytokines in keratinocytes but not in airway smooth muscle cells [203]. SOCS3 mRNA levels were higher in asthmatics and atopic dermatitis patients [162]. These values correlated with IgE levels for both diseases and disease severity in the asthmatics. In the same study, it was shown that SOCS3 is expressed in a Th2 environment in humans and mice. Furthermore, SOCS3 transgenic mice have an enhanced Th2 response [162]. High levels of CISH are expressed in Th1 and Th2 cells [206]. Transgenic CISH mice have increased differentiation of Th cells into Th2 cells [207]. In HBECs it was shown that both IL-4 and IL-13 induced SOCS1 and CISH mRNA and protein. There was no induction of SOCS3 mRNA by these cytokines. Previous studies and these results suggest that induction of SOCS family members by regulators of the Th1 and Th2 balance are cell type specific, with most studies done in T cells. This might indicate that the role of SOCS1 and SOCS3 in inhibiting these pathways could also be cell type specific.

3.5.2 Effects of SOCS1 and SOCS3 modulation on IFN expression in BECs

It was investigated whether SOCS plays a role in inhibiting IFN-β signalling and production as a negative feedback mechanism to its induction. It was determined whether SOCS1 and SOCS3 overexpression plasmids could inhibit IFN signalling by measuring IFN-β induced IFN promoter activation, which we confirmed. Following that we measured RV induced IFN promoter activation which was also inhibited by SOCS1 and SOCS3. This could be an indication that SOCS1 and SOCS3 can inhibit IFN signalling and RV induced IFN production. The role of SOCS1 and SOCS3 in negatively regulating IFN signalling is well established [173] however its role in regulating virus induced IFN is less clear. Our data confirms that SOCS1 and SOCS3 are negative regulators of RV induced IFNs in *in vitro* models.

The role of SOCS1 and SOCS3 in inhibiting RV1B induced IFN production is more suggestive than conclusive in this study. Previous studies have shown that RV induced IFN-β and IFN-λ are detectable at 4h post infection, rise at 24h and plateau at 48h [196]. At early time points post RV infection, RV induced IFN production occurs by downstream PRR signalling leading IFN induction in an IRF3 dependent manner. Using siRNA specific for IRF3, it was also show that IRF3 siRNA reduced approximately 75% of the IFN mRNA levels at 24h post infection [196] suggesting that the majority of IFN at 24h post RV infection is virus rather than IFN induced. At later time points, IFNs are produced both directly through RV signalling via IRF3 and IFN induced IFN production through JAK/STAT. It is however, unclear at what time point IFN induced IFN via JAK/STAT signalling becomes more important than virus induced IFN. The complete inhibition of RV induced IFN promoter activation by SOCS1 and SOCS3 does suggest that SOCS1/3 can inhibit RV as well as IFN induced IFN, assuming that IFN promoter activation at 24h may be a combination of both processes. Hence this data is strongly suggestive, but not definitive.

The role of SOCS1 and SOCS3 in inhibiting IFN production is barely studied with only one study showing a role for SOCS1 and SOCS3 in IFN production [175]. In this study it was determined that SOCS1 inhibits Influenza induced IFN production by reducing IRF3 minimal promoter activation and SOCS3 by reducing NF-κB minimal promoter activity [175]. This observation shows an effect of SOCS1 and SOCS3 on virus induced IFN production distinct form JAK/STAT signalling. One study shows an inhibitory effect of SOCS3 on cJun phosphorylation [185]. Although IFN production was not investigated, ATF-2/c-Jun are implicated in type I IFN production which could be an explanation of SOCS3 reduced IFN production [59, 182].

Next it was attempted to investigate the effects of SOCS1 and SOCS3 directly on virus induced IFN by examining the effects of SOCS over-expression on PRR driven IFN promoter activation. TLR3 pathway inducers (constitutively active TRIF) and RIG-I pathway inducers (constitutively active RIG-I) were used. Furthermore the effects of SOCS1 and SOCS3 on kinase induced IFN promoter activation were investigated. Although some inhibitory effects of SOCS1 and SOCS3 on promoter activation were observed by these stimuli, there was no statistical significant reduction. Finally, we investigated whether IRF3 induced IFN promoter activity was reduced and no differences were observed. It is unclear whether the results suggest that the induction of these promoters by over-expressing signalling intermediates is too strong for the SOCS plasmids to inhibit, or whether SOCS1 and SOCS3 did not inhibit these pathways. As a partial effect of SOCS1 and SOCS3 with some of these stimuli was observed this may suggest that SOCS1 and SOCS3 could cause reduced promoter activation by

inhibiting some of these signalling intermediates. Further experiments are required to ascertain what exactly, SOCS1 and SOCS3 do inhibit to reduced RV1B induced promoter activation.

Evidence that SOCS1 and SOCS3 are involved in other signalling pathways then JAK/STAT is accumulating which could indicate towards mechanism(s) by which SOCS1 and SOCS3 might be able to inhibit RV induced IFN production. SOCS1 has been shown to directly associate with TLR signalling intermediates, Mal [180] and IL-1 receptor activated kinase (IRAK) 1 [208] via its SH_2 domain. SOCS1 causes Mal poly-ubiquitination and degradation to prevent TLR2 dependent NF-κB signalling. The role of SOCS as a cytoplasm inhibitor of JAK/STAT signalling is well established, recently, SOCS1 was found to be able to translocate into the nucleus via a nuclear localisation sequence (NLS) in the structure. In the nucleus SOCS1 regulated nuclear p65 stability and limiting TLR4 induced NF-κB dependent gene induction [209, 210]. The ability of SOCS1 and SOCS3 to inhibit IRF3 activation has only been studied by reporter gene activation, it would be interesting to study the ability of SOCs1/3 to directly associate with IRF3, given that IRF3 is essential for RV induced IFN gene expression.

As a method to confirm our findings in the experiments using overexpression of SOCS1 and SOCS3 we intended to perform siRNA knockdown experiments. siRNA (Dharmacon, Santa Cruz) and shRNA (Invitrogen) against SOCS1 and SOCS3 were used and cells stimulated with IFN-β or RV to show differences in IFN and ISG mRNA and protein production compared to control samples. Although SOCS siRNA inhibited SOCS1 mRNA, a non-significant increase was observed for increased IFN-β induced ISGs. The current literature suggests that knockdown of SOCS1 should increase IFN signalling and because we did not observe any increases in RV1B induced IFN mRNA (data not shown) this method was aborted. SOCS3 siRNA reagents gave no significant knockdown of their target gene. We further explore the involvement of SOCS1 in IFN signalling in the next chapter using $SOCS1^{-/-}$ IFN-$\gamma^{-/-}$ C57/BL6 mice to study IFN responses upon RV infection *in vivo* and *ex vivo*. Differences observed in the effectiveness of siRNA knockdown between manufacturers could be due to secondary structure, SOCS being a difficult target, or the regulation of SOCS in the cell and the turnover of SOCS RNA. This could indicate why SOCS1 and SOCS3 were difficult to suppress by siRNA in our system, this does not explain why, when suppression is obtained for SOCS1, no biological effect is observed. In physiological conditions where a robust induction of IFNs is observed it might be difficult to see an effect by knocking back negative regulators such as SOCS1 and SOCS3. Overexpression of these molecules might be an experimental method giving a better indication of the biological effect. In the promoter studies a biological effect is measured by abundance of luciferase protein induced by a synthetic promoter-reporter gene; compared to native gene expression in the siRNA experiments. Measuring mRNA expression, although indicative does not

inform on what the effect is on protein production, cellular localisation and biological effect. To confirm the effect of SOCS1 and SOCS3 overexpression on IFN induction and signalling, STAT and IRF DNA binding could be used, as determined by electromobility shift assay or chromatin immunoprecipitation. Further studies are required to understanding exactly why, no effect on IFN mRNA was observed by RNA interference.

3.5.3 Effect of SOCS1 and SOCS3 on Th2 cytokine signalling in BECs

Several studies have shown a role for SOCS1 and SOCS3 in controlling the Th1/Th2 balance. SOCS1 has been shown to be a negative regulator of both Th1 and Th2 signalling while SOCS3 has been shown to only inhibit Th1 signalling (paragraph 3.4.1, [162, 165-168, 202, 206]). In this study we overexpressed both SOCS1 and SOCS3 to measure the effect on Th2 cytokine signalling using STAT6 promoter activation as an outcome. Contrary to what was expected, a reduction of STAT6 promoter activation with both SOCS1 and SOCS3 was observed where only SOCS1 was originally expected to have an effect. An explanation of this could be experimental settings; by overexpression of these proteins we might induce such increased SOCS protein that non-specific binding is obtained to inhibit the signalling. The induction by Th1 and Th2 cytokines and the negative regulatory effect of SOCS1 on both Th1 and Th2 signalling seems to be cell type specific, and this could also explain our results. SOCS1 is induced by Th2 cytokines and IFN-γ in airway smooth muscle cells, by IFN-γ in murine embryonic fibroblasts [195] and by Th2 cytokines in macrophages, keratinocytes and cancer cells [194, 203-205]. The induction of SOCS3 is less well studied and induction by Th2 cytokines is not identical in all cell types [202, 203]. This could indicate that not only the induction but also the effects of SOCS1 and SOCS3 on regulating the Th1/ Th2 balance are cell type dependent. Also, the cell type specific importance of SOCS is worth reflection. SOCS1 and SOCS3 are well studied in regulating T cell differentiation which is probably their main site of effect for regulating the Th1/Th2 balance. The suppression of STAT6 in HBECs could be significantly less important with regards to Th1/Th2 differentiation, and may be a fine tuning mechanism to reduce or terminate bronchial epithelial derived Th2 chemokines such as CCL17/TARC or CCL22/MDC.

3.5.4 The effects of SOCS1 and SOCS3 on inflammatory signalling in BECs

Last in this chapter the role of SOCS1 and SOCS3 in IL-1β and TNF-α mediated signalling was investigated. To start, reporter assays with different promoters were used; a minimal NF-κB promoter, and an IL-6 and CXCL8/IL-8 promoter. Surprisingly the effects of SOCS1 and SOCS3 on these promoters were different from each other. SOCS1 and SOCS3 inhibited IL-1β and TNF-α induced IL-6 promoter activation, increased IL-8 promoter activation, but had no effect on the NF-κB

minimal promoter. SOCS1 and SOCS3 showed similar results upon RV1B induced IL-6 and CXCL8/IL-8 promoter activation.

A possible explanation for this could be that SOCS1 and SOCS3 auto activates the IL-8 promoter. SOCS1 and SOCS3 have been shown to inhibit inflammatory signalling through distinct pathways than the JAK/STAT pathway. Several papers have shown a role for SOCS1 in negatively regulating IL-1 receptor and TLR induced signalling through degradation of MAL [180], binding to and upstream kinase to JNK and p38 [199], or direct binding to p65 [82]. SOCS3 can inhibit the association between TRAF6 and TAK1, which consequently causes inhibition of NF-κB transcription [198]. These studies would also suggest that the activation of the used promoters would be similar. The inhibition of SOCS1 and SOCS3 on RV1B induced CXCL10/IP10 promoter activation could be by inhibition of IFN-α/β signalling as well as inflammatory signalling [211].

Both the IL-6 and IL-8 promoter contain AP-1, NF-IL-6 (c/EBP-β) and NF-κB binding sites [212-214]. It has been shown that different stimuli can activate different transcription factors e.g TNF-α activates all three transcription factors which bind to the CXCL8/IL-8 promoter [212], this could explain differences in inhibition of SOCS1 and SOCS3 upon the same stimuli. However, in RV infection in BEAS2Bs it has been shown that AP-1 and NF-IL-6 are not necessary for both IL-6 [62] and CXCL8/IL-8 [61] promoter activation; both are driven exclusively by NF-κB.

Although previous studies have clearly shown an inhibitory role for SOCS3 in inhibiting TNF-α and to a lesser extent IL-1β signalling [215, 216], the results in this study are inconclusive as to SOCS1 and SOCS3 can inhibit IL-1β and TNF-α signalling. If SOCS1 and SOCS3 are investigated as a target to increase RV induced IFN responses, preferably no effect would be observed on RV induced proinflammatory responses. However, current results have not definitively shown whether SOCS1 and SOCS3 negatively regulate these signalling pathways, or whether subgroups of proinflammatory cytokines could be inhibited by SOCS1 and/or SOCS3 based on an undetermined mechanism. Promoter activation experiments, while useful are artificial and rely on NF-κB driving a exogenous gene from a plasmid, rather than actual chromatin. Other experiments that could be used to determine the effect of SOCS1 and SOCS3 on TNF-α, IL-1β and RV induced NF-κB signalling would be gel shift, chromatin immuneprecipitation (CHIP) or CHIP sequencing. Gel shift, although still artificial because of transcription factor binding to synthetic oligos outside of the cell, could show absolute binding to the specific promoter. CHIP would show specific quantative binding to the actual cellular chromatin on specific promoters, and CHIP-sequencing could give a full view on NF-κB binding to every promoter in the DNA [217]. Such a global view of the effects of SOCS1 and/or SOCS3 on NF-κB binding

would be necessary to ascertain if SOCS are indeed negative regulators of NF-κB induced inflammation.

3.6 Conclusion

The results in this study have shown a role for SOCS proteins in RV infection in BECs. SOCS1 and SOCS3 are induced upon RV infection and several other stimuli, by inhibiting IFN induced signalling SOCS1 and SOCS3 could reduce innate immunity upon RV infection. Whether SOCS can play a role in inhibiting IFN production is not yet conclusively known and will be studied further. Both SOCS1 and SOCS3 have an inhibitory role on Th2 cytokine signalling in HBECs.

4 Analysis of SOCS1 and SOCS3 expression in HBECs from asthmatic and non-asthmatic subjects

4.1 Introduction

RV is the major cause of asthma exacerbations [18], with bronchial epithelium being the major site of RV infection in the lower airway. Cultured HBECs from atopic asthmatic subjects (AA) showed exaggerated RV replication compared to non-atopic non asthmatics subjects (NA) [47], with both major group virus (RV16), and minor group virus (RV1B). The same study demonstrated deficient RV induced IFN-β production in AA HBECs compared to NA [47]. The deficient RV induced IFN-β response was negatively correlated to increased RV vRNA, however signalling pathways downstream of the IFN-β response remained intact [47]. A deficiency in RV induced IFN-λ has also been observed, deficient RV induced IFN-λ production in AA HBECs was inversely correlated to viral replication *ex vivo* and defective RV induced type III IFNs was also shown in AA BAL cells. IFN-λ production by AA BAL cells *ex vivo* was also inversely correlated with clinical outcomes related to asthma exacerbation severity in the same study [49]. The above data suggests that IFN-β and IFN-λ production, and their downstream anti-viral responses are important in the pathogenesis of RV induced asthma exacerbations.

Other investigators have failed to confirm defective RV induced IFN production in asthma [50, 51] and experimental RV infection studies did not show clear differences in RV shedding between AA and NA [29, 52, 53]. Many factors could explain the differences in the above results, such as severity of asthma or IFN deficiency associated with a subgroup of AA rather than all AA. Why IFNs are impaired in some AA is complex and more studies are needed to understand these observations.

Type I and III IFNs are the first line of defence against viruses and are rapidly induced upon RV infection [196]. IFNs signal downstream to induce more IFNs and ISGs contributing to antiviral immunity. IFNs interact with specific transmembrane receptor units of the hemopoietin receptor family which induces dimerization of these subunits leading to intracellular activation of the JAK/STAT pathway to induce these genes. The SOCS complete a negative feedback loop in the JAK/STAT pathway (Figure 1.4). Activated STATs stimulate the transcription of SOCS genes [118]. Several studies have shown induction of SOCS1 and SOCS3 by IFNs in macrophages [190] and Th2 cytokines in keratinocytes and cancer cells [203, 205], work in this thesis has shown that in HBECs SOCS1 can be induced by Th2 cytokines and IFN-β (Chapter 3). Furthermore, SOCS1 and SOCS3 have been shown to be induced by Influenza virus in BEAS2Bs [175]and RV in HBECs (Chapter 3). SOCS1 and SOCS3 are associated with negative regulation of both IFNs and cytokines in several cell types, including ASMCs [170], PBMCs [162], hepatic cells [171] and in an *in vivo* model [173], playing a role in the Th1/Th2 balance. Besides negative regulation of JAK/STAT, SOCS have been shown to negatively regulate other signalling pathways [175, 180, 181, 185]; including one study showing that SOCS1 can inhibit influenza

induced IFN production in BEAS2Bs [175]. The mechanism behind RV induced IFN deficiency is currently unknown. Increased levels of negative regulators of RV induced signalling could play a role in IFN deficiency in AA. Several negative regulators of IFN induction have been described. SOCS are well described negative regulators of IFN induced JAK/STAT signalling [190].

The first aim was to investigate whether RV induced SOCS1 and SOCS3 levels in HBECs of AA and NA were associated with impaired IFN expression in two separate studies. In the first study, HBECs were grown from well controlled mild adult AA and NA. In the second study, HBECs were taken from children with badly controlled severe therapy resistant AA and NA. Secondly SOCS1 and SOCS3 expression data was correlated to several study outcomes, including RV replication and release, IFN induction, lung function, AHR and other study outcomes. It was hypothesised that SOCS1 and SOCS3 were increased in AA HBECs that exhibit IFN deficiency and SOCS1 and SOCS3 levels positively correlate with RV replication and negatively correlate with type I and III IFN induction.

4.2 Results

4.3 Outline of study using AA and NA adult subjects as a source of bronchial biopsies and cultured HBECs for the study of SOCS expression

Bronchial biopsies and HBECs were obtained by bronchoscopy from a recent clinical study performed in our laboratory which compared AA to NA (performed by A.Sykes). Inclusion criteria for AA were age between 18-65, clinical diagnosis of asthma, symptom questionnaires positive for asthma [218], skin prick test positive to at least one allergen, PC_{20} <8mg/mL, no other chronic respiratory or systemic disease, intermittent or mild persistent asthma according to the BTS guidelines of asthma (correlates with on GINA guidelines) and were non smokers (<1 pack year) and if ex smokers <5 pack year history. Patients were not recruited based on whether they wheeze upon RV infection, or experiences a worsening of asthma symptoms upon virus infections.

Inclusion criteria for NA were age between 18-65, symptom questionnaires negative for asthma, skin prick test negative, PC_{20}>16mg/mL, no other chronic respiratory or systemic disease, IgE <80IU/mL and current non smokers (>1 year) and if ex smokers <5 pack year history. Exclusion criteria for all subjects were a respiratory tract infection in the last 6 weeks and significant other abnormalities in lung function i.e. restrictive defect.

Group sizes were based on power calculations showing that n=11 can confirm that 3 fold changes between groups are achievable in 90% of the analytes tested by qPCR. Research in adults has been approved by St Mary's NHS Trust (08/H0712/39 & 07/Q0403/20, Prof S.L. Johnston). Each Research Ethics Committee is informed on anonymisation of data, data storage, complications and adverse effects, and insurance. All subjects gave written informed consent.

4.3.1 Clinical characteristics of subjects from whom bronchial biopsies were obtained

Biopsies were obtained from 17 AA and 14 NA. Clinical characteristics of subjects are summarised in table 4.1.

Table 4.1: Clinical characteristics of subjects from whom bronchial biopsies were obtained.

Values are presented as mean (±SEM) except for sex. ACQ=asthma control questionnaire, ICS=inhaled corticosteroids, LABA=long acting beta agonists, FVC=forced expiratory vital capacity, SPT=number of positive skin prick test results. (ICS and ICS+LABA; 0 was defined as non-treated subjects, 1 was defined as treated subjects), NA n=14 and AA n=17 (A.Sykes, unpublished data).

Clinical characteristic	**AA**	**NA**	**p value**
Sex	76% M 24% F	43% M 57% F	
Age	35.4 (± 1.903)	37.9 (± 2.623)	0.6196
ACQ	0.799 (±0.110)	0 (± 0)	<0.0001
ICS	0.53 (± 0.125)	0 (± 0)	0.0020
ICS and LABA	0.18 (± 0.098)	0 (± 0)	0.1250
Exacerbations/year	1.4 (± 0.2)	0 (± 0)	<0.0001
Oral steroids/year	0.47 (± 0.124)	0 (± 0)	0.0039
PEF % predicted	93.4 (± 3.76)	99.57 (± 3.01)	0.1901
FEV_1 % predicted	91.8 (± 4.39)	94.0 (± 2.21)	0.4625
FEV_1/FVC ratio	77.28 (± 1.57)	87.35 (± 1.124)	0.0002
PC_{20} (mg/mL)	1.75 (± 0.416)	>16	0.0002
IgE (units/mL)	193.42 (± 56.74)	18.63 (± 4.87)	<0.0001
SPT (number)	3.6 (± 0.34)	0 (± 0)	0.0001

There were no significant differences between the subjects for age, PEF% predicted and FEV1% predicted. Expected significant differences were observed for ACQ ($p<0.0001$), ICS treatment ($p<0.01$), number of exacerbations in the last year ($p<0.0001$), number of oral steroids required in the last year ($p<0.05$), airway obstruction (FEV_1/FVC) ($p<0.001$), AHR (PC_{20}) ($p<0.001$), total IgE ($p<0.0001$) and number of positive SPT ($p<0.0001$). There was no significant difference in combination treatment (ICS and LABA) ($p=0.1250$).

The subjects studied were mild and well controlled AA as evidenced by normal lung function, low frequency of exacerbations in the last year and low number of subjects requiring ICS and LABA treatment. Furthermore, these AA have an ACQ of 0.799; an ACQ lower than 3 indicates well controlled asthma [218].

4.3.2 SOCS1 protein levels in bronchial biopsies from AA were elevated compared to NA

It has previously been shown that a SOCS1 promoter polymorphism associated with asthma can increase SOCS1 levels in A549 cells [168]. Furthermore IL-4 and IL-13 induced SOCS1 in HBECs (Section 3.2.7) which could indicate that AA have higher levels of SOCS1 at baseline. Here it was investigated whether SOCS1 protein epithelial staining scores were higher in bronchial biopsies from AA compared to NA (Staining of bronchial biopsies was performed by Dr. Jie Zhu).

Bronchial biopsies from AA had significantly higher levels of SOCS1 protein in epithelium compared to NA ($p<0.05$, Figure 4.1 and Figure 4.2).

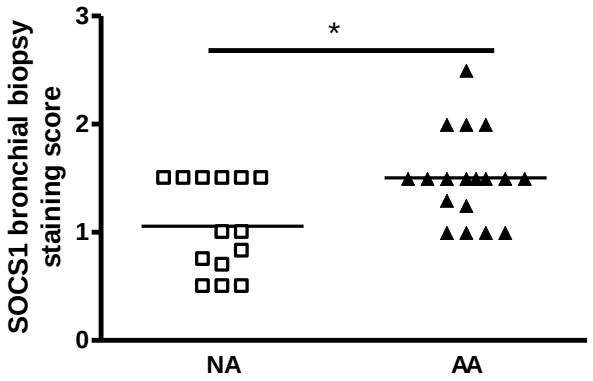

Figure 4.1: SOCS1 bronchial biopsy staining intensity score from NA compared to AA.

SOCS1 protein levels were significantly higher in bronchial biopsies from AA compared to NA ($p<0.05$). The staining intensity scores represent the immunostaining signals from negative to strong: 0= negative; 1= weak, 2= moderate and 3= strong. Squares denote NA, n=14; triangles denote AA, n=17

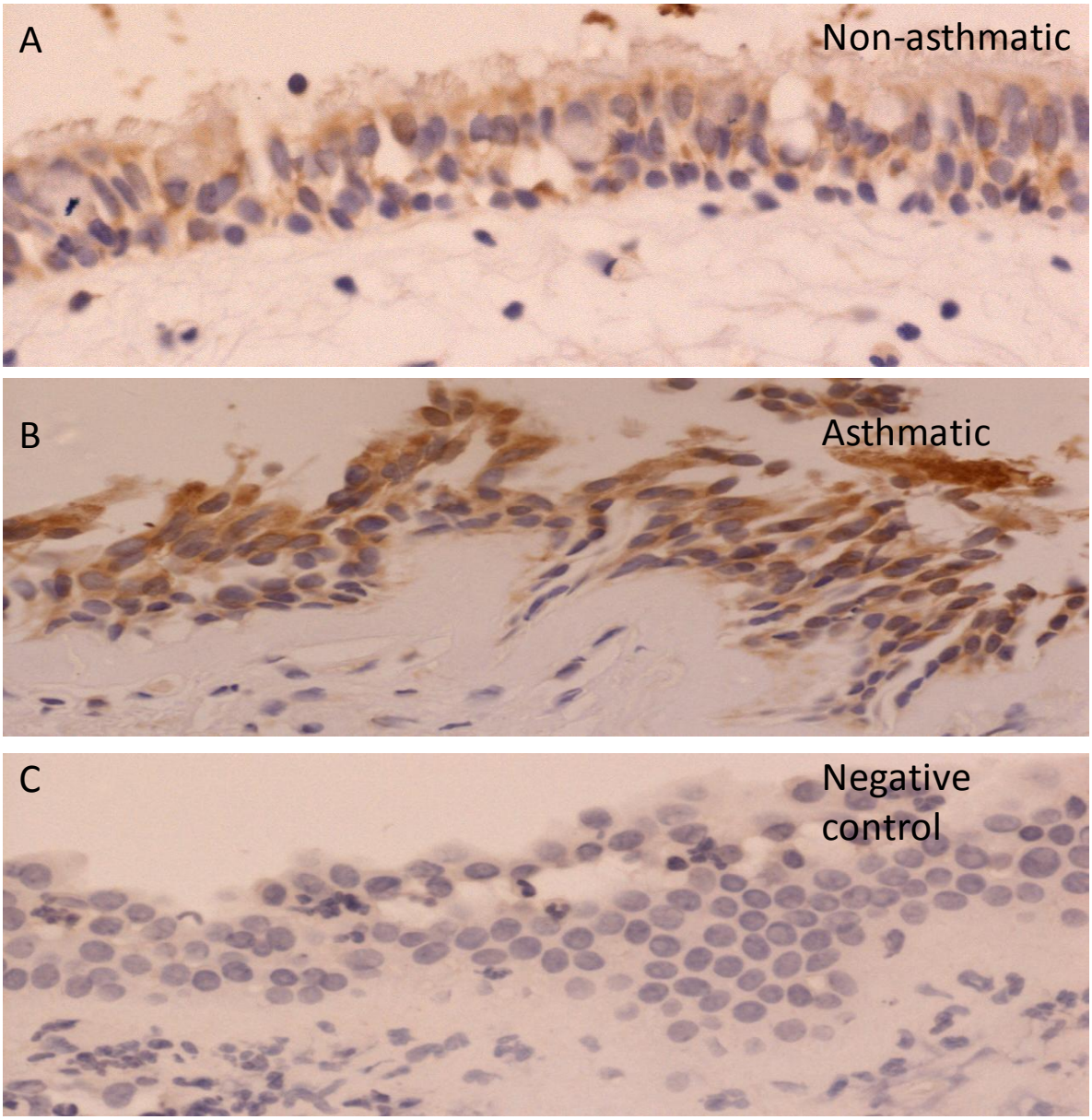

Figure 4.2: SOCS1 protein expression in bronchial biopsies from NA compared to AA.

SOCS1 protein staining is brown, nuclear staining is purple. (A) NA, (B) AA, (C) negative control (2nd Ab only).

4.3.3 SOCS3 protein levels in bronchial biopsies from AA were not different compared to NA

It has previously been shown that SOCS3 mRNA expression is higher in PBMCs from AA compared to NA [173]. It was demonstrated in Chapter 3 that IL-4 and IL-13 did not increase SOCS3 in HBECs. Here it was investigated whether SOCS3 protein staining was higher in bronchial biopsies from AA compared to NA. Bronchial biopsies from AA did not have increased levels of SOCS3 protein in epithelium compared to NA (Figure 4.3 and Figure 4.4, staining of bronchial biopsies was performed by Dr. Jie Zhu).

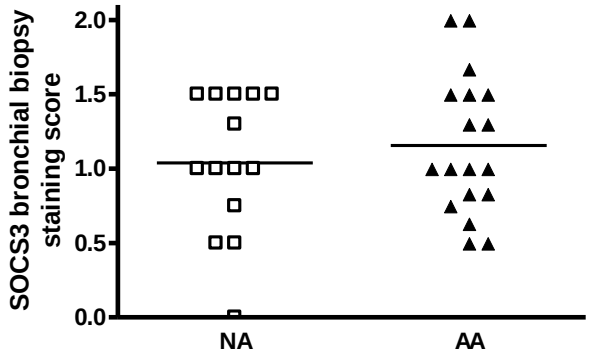

Figure 4.3: SOCS3 bronchial biopsy staining intensity score from NA compared to AA.

SOCS3 protein levels were not different in bronchial biopsies from AA and NA ($p<0.05$). The staining intensity scores represent the immunostaining signals from negative to strong: 0= negative; 1= weak, 2= moderate and 3= strong. Squares denote NA, n=14; triangles denote AA, n=17

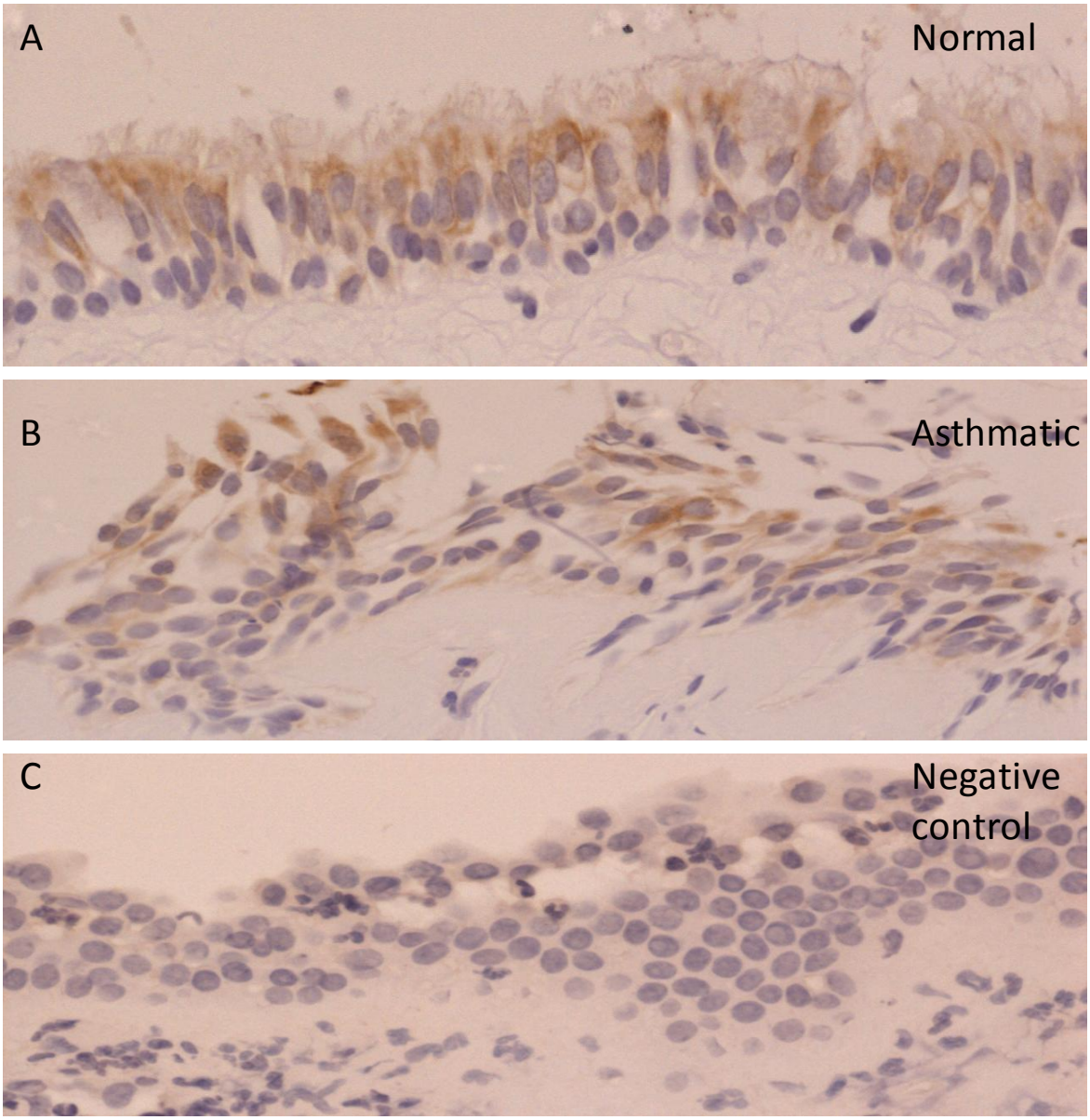

Figure 4.4: SOCS3 protein expression in bronchial biopsies from NA compared to AA.

SOCS3 protein staining is brown, nuclear staining is purple. (A) NA, (B) AA, (C) negative control (2nd Ab only).

4.4 RV1B and RV16 induced SOCS1 and SOCS3 mRNA expression in HBECs from AA and NA

4.4.1 Clinical characteristics of subjects from whom HBECS were successfully cultured

HBECs were successfully cultured from 10 AA and 10 NA (A.Sykes unpublished data). Bronchial biopsy samples obtained and HBECs cultured from 4 NA and 8 AA were from the same subjects. Clinical characteristics of subjects are summarised in Table 4.2.

Table 4.2: Clinical characteristics of subjects from whom HBECs were obtained and successfully cultured.

Values are presented as mean (±SEM) except for sex. NA n=10 and AA n=10 (A.Sykes, unpublished data).

Clinical characteristic	AA	NA	p value
Sex	80% M 20% F	50% M 50% F	
Age	34.2 (± 2.678)	38.4 (± 3.121)	0.3527
ACQ	0.599 (±0.139)	0 (± 0)	0.0039
ICS	0.5 (± 0.166)	0 (± 0)	0.0313
ICS and LABA	0.3 (± 0.153)	0 (± 0)	0.1250
Exacerbations/year	1.1 (± 0.1)	0 (± 0)	0.0010
Oral steroids/year	0.2 (± 0.133)	0 (± 0)	0.2500
PEF % predicted	95.1 (± 4.20)	105.8 (± 2.75)	0.0089
FEV_1 % predicted	90.4 (± 5.1)	96.8 (± 2.66)	0.2799
FEV_1/FVC ratio	77.26 (± 1.99)	86.04 (± 2.23)	0.0068
PC_{20} (mg/mL)	1.38 (± 0.500)	>16	0.0010
IgE (units/mL)	154.6 (± 37.97)	12.20 (± 3.65)	<0.0001
SPT (number)	4.1 (± 0.48)	0 (± 0)	0.0010

There were no significant differences between the subjects for age and FEV1% predicted. Expected significant differences were seen for ACQ ($p<0.01$), ICS treatment ($p<0.05$), number of exacerbations in the last year ($p<0.001$), PEF% predicted ($p<0.05$), airway obstruction (FEV_1/FVC) ($p<0.01$), AHR (PC_{20}) ($p<0.001$), total IgE ($p<0.0001$) and number of positive SPT ($p<0.001$). There was no significant difference in combination treatment (ICS and LABA) ($p=0.125$) and number of oral steroids required in the last year ($p=0.25$).

The subjects studied were mild and well controlled AA as evidenced by normal lung function, low frequency of exacerbations in the last year and low number of subjects requiring ICS and LABA treatment. Furthermore, these AA have an ACQ of 0.799; ACQ lower than 3 indicates well controlled asthma [218].

4.4.2 Summary of RV1B and RV16 induced IFN protein and mRNA induction in HBECs from AA and NA

To establish whether the previously reported defective RV induced IFN-β and IFN-λ in AA was also present in this group of AA, RV1B and RV16 induced type I and III IFN mRNA and protein were measured in HBECs in this study (A.Sykes, unpublished data). In summary, RV1B significantly induced IFN-β, IFN-λ1, IFN-λ2/3 mRNA at 8h, 24h and 48h post infection. RV16 significantly induced IFN-β at 48h post infection and RV16 significantly induced IFN-λ1, IFN λ2/3 mRNA at 8h, 24h and 48h post infection. RV1B significantly induced IFN-β and IFN-λ protein 48h post infection. RV16 did not significantly induced type I or type III IFN protein at any time points. No significant differences in RV induced type I and III IFNs were observed between AA and NA (A.Sykes, unpublished data).

4.4.3 RV1B and RV16 induced SOCS1 mRNA expression in HBECs from AA was not different compared to NA

The mechanism behind RV induced IFN deficiency is currently unknown. Increased levels of negative regulators, such as SOCS1, of RV induced signalling could play a role in IFN deficiency in AA. To assess whether baseline, RV1B or RV16 induced SOCS1 mRNA expression was different in AA compared to NA, SOCS1 mRNA levels were measured in medium treated cells and RV1B and RV16 infected cells at 8h, 24h and 48h post infection using taqman RT-PCR.

SOCS1 mRNA was not induced by RV1B at any time point by either AA or NA, although a trend was observed for RV1B induced SOCS1 mRNA in AA at 24h and 48h post infection (Figure 4.5). SOCS1 mRNA was not significantly induced by RV16 at any time point by either AA or NA, although a trend was observed for increased RV16 induced SOCS1 mRNA in both AA and NA at 48h post infection (Figure 4.6). No differences were observed between SOCS1 mRNA levels between AA and NA (Figure 4.5 and Figure 4.6).

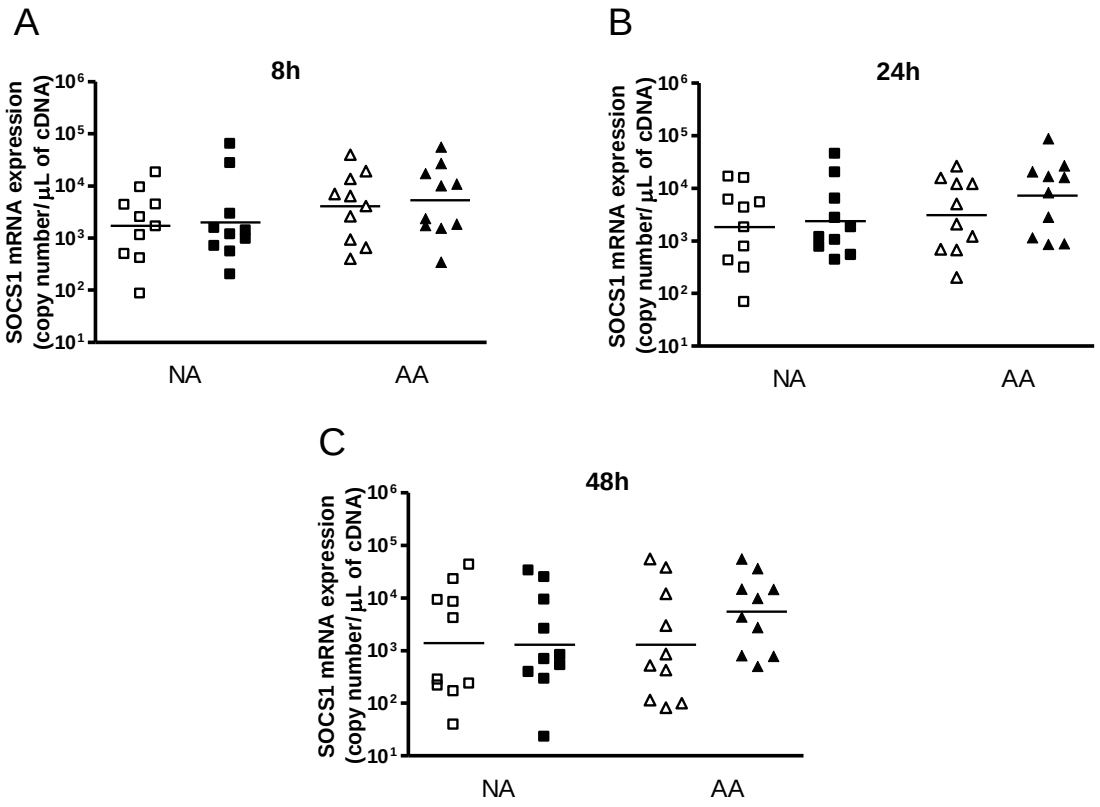

Figure 4.5: SOCS1 mRNA levels post RV1B infection in HBECs from AA and NA.

SOCS1 mRNA at 8h (A), 24h (B), 48h (C) post RV1B infection. Squares denote NA, n=10; triangles denote AA, n=10; open symbols denote medium treated cells; black symbols denote RV1B infected cells.

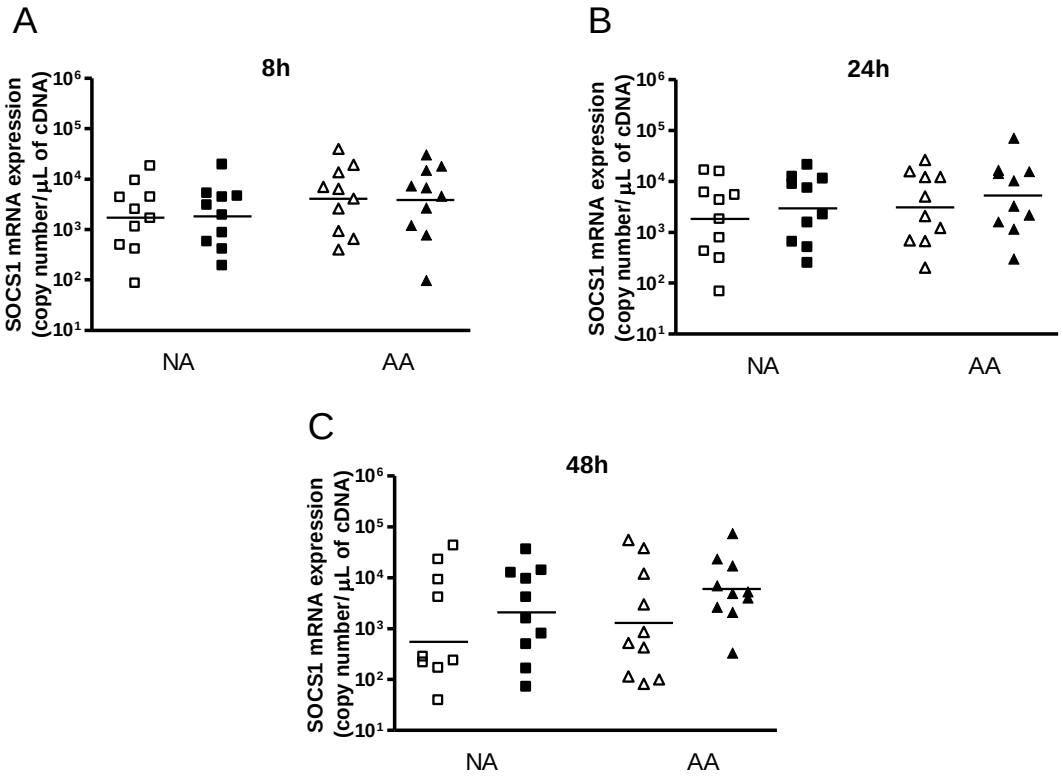

Figure 4.6: SOCS1 mRNA levels post RV16 infection in HBECs from AA and NA.

SOCS1 mRNA at 8h (A), 24h (B), 48h (C) post RV16 infection. Squares denote NA, n=10; triangles denote AA, n=10; open symbols denote medium treated cells; black symbols denote RV16 infected cells.

4.4.4 RV1B and RV16 induced SOCS3 mRNA expression in HBECs from AA was not different compared to NA

SOCS3 could also play a role in RV induced IFN deficiency in AA. To assess whether baseline or RV1B and RV16 induced SOCS1 mRNA expression was different in AA compared to NA, SOCS1 mRNA levels were measured in medium treated cells and Rv1B and RV16 infected cells at 8h, 24h and 48h post infection.

SOCS3 mRNA was not induced by RV1B at any time point by either AA or NA, although a trend was observed for increased RV1B induced SOCS3 mRNA in both AA and NA at 24h and 48h post infection (Figure 4.7). SOCS3 mRNA was not induced by RV16 at any time point by either AA or NA, although a trend was observed for increased RV16 induced SOCS3 mRNA in both AA and NA at 48h post infection (Figure 4.8). No differences were observed between SOCS3 mRNA levels between AA and NA (Figure 4.7 and Figure 4.8).

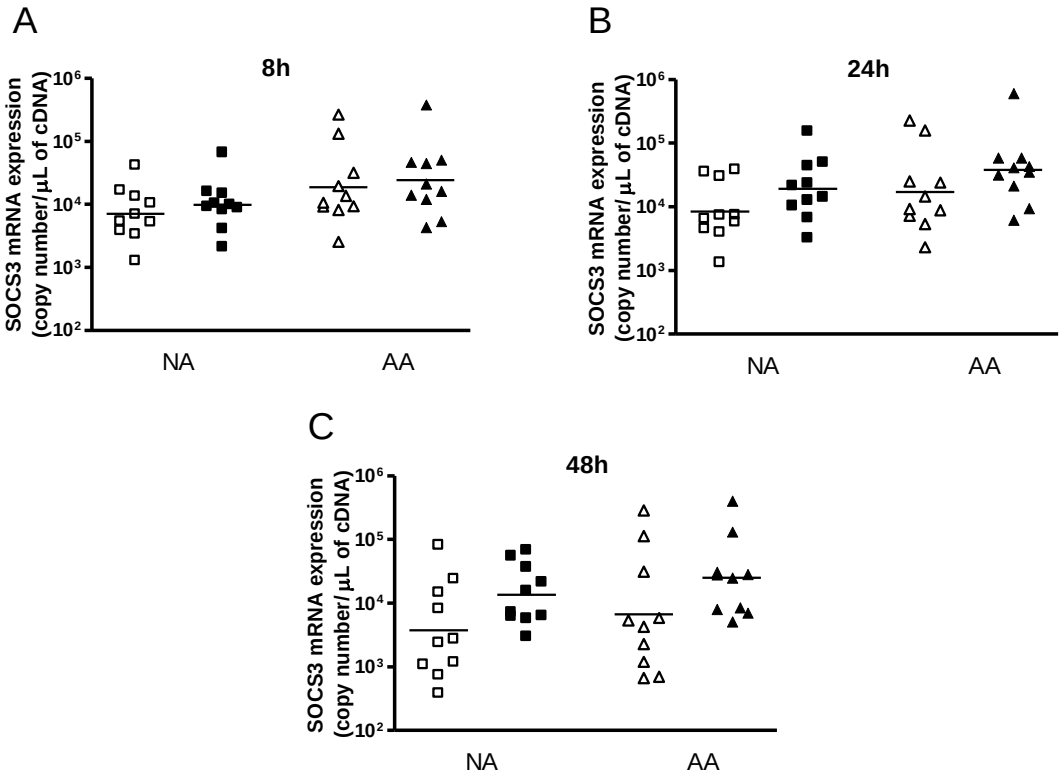

Figure 4.7: SOCS3 mRNA levels post RV1B infection in HBECs from AA and NA.

SOCS3 mRNA at 8h (A), 24h (B), 48h (C) post RV1B infection. Squares denote NA, n=10; triangles denote AA, n=10; open symbols denote medium treated cells; black symbols denote RV1B infected cells.

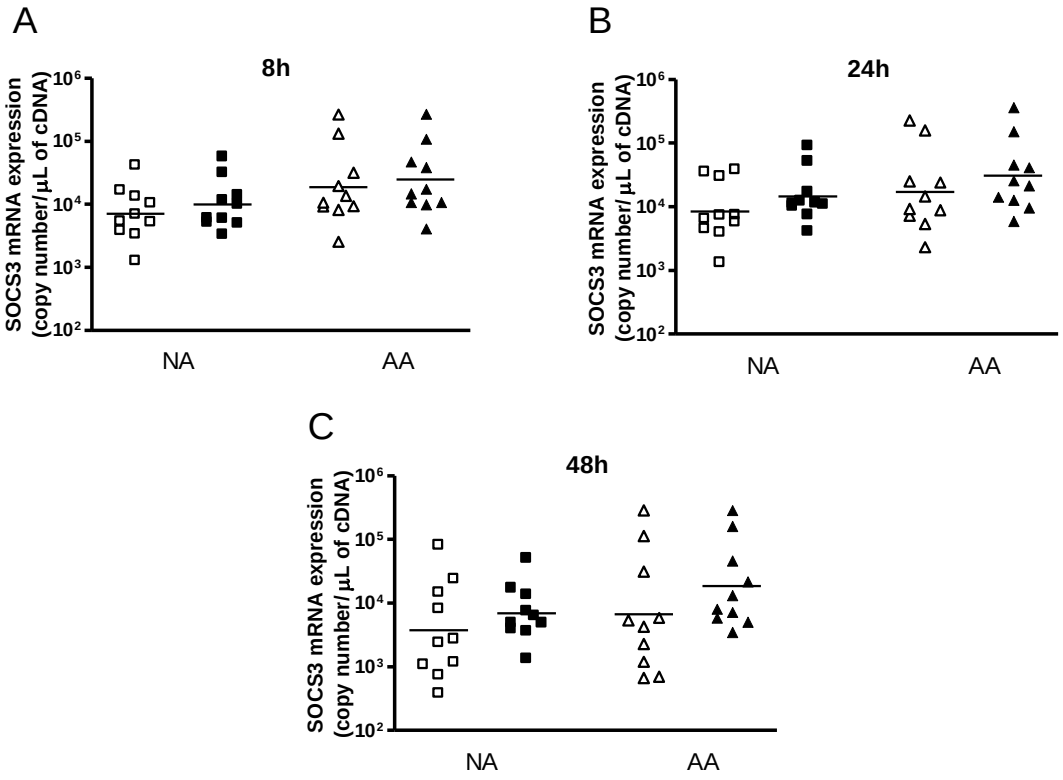

Figure 4.8: SOCS3 mRNA levels post RV16 infection in HBECs from AA and NA.

SOCS3 mRNA at 8h (A), 24h (B), 48h (C) post RV16 infection. Squares denote NA, n=10; triangles denote AA, n=10; open symbols denote medium treated cells; black symbols denote RV16 infected cells.

4.5 Correlation between SOCS1 and SOCS3 protein and mRNA expression in HBECs from AA and NA and lung function, AHR and atopy related clinical outcomes

4.5.1 SOCS1 protein levels in biopsies correlated with allergic and AHR clinical outcomes, SOCS3 protein levels did not correlate with any clinical study outcomes

To asses if the higher SOCS1 protein levels observed in the biopsies of AA (Section 4.3.2) were related to atopy, AHR and lung function, SOCS1 protein staining scores in bronchial biopsies were correlated with measurements of lung function (PEF% predicted, FEV_1% predicted and FEV_1/FVC ratio), AHR (PC_{20}) and atopy related clinical measurements (IgE and SPT) (Table 4.3, Figure 4.9). Relationships between SOCS3 protein staining scores (Section 4.3.3) and these clinical measurements were also determined (Table 4.3).

Table 4.3: Correlations between SOCS1 and SOCS3 protein expression and clinical study outcomes.

Data is expressed as p value and spearman r. NA n=14 and AA n=17.

		PEF % predicted	FEV_1 % predicted	FEV_1/FVC ratio	PC_{20}	IgE	SPT
SOCS1	**Spearman r**	-0.0461	0.0699	-0.2378	***-0.5254***	0.3443	***0.4728***
	p value	0.8055	0.7085	0.1977	***0.0024***	0.0578	***0.0072***
SOCS3	**Spearman r**	0.0584	0.3100	-0.0981	-0.1523	-0.0286	0.0169
	p value	0.7550	0.0896	0.5997	0.4135	0.8788	0.9281

There was no significant correlation between SOCS1 protein levels in bronchial biopsies and lung function (Table 4.3), however there was a significant positive correlation between number of positive SPT and SOCS1 ($R=0.4728$, $p<0.01$, Figure 4.9A) and a significant negative correlation between PC_{20} and SOCS1 ($R=-0.5254$, $p<0.01$, Figure 4.9B). Increased SOCS1 was related to increased number of positive skin prick tests to allergens (SPT) and increased AHR (reduced PC_{20}). A trend for a relationship between increased levels of total serum IgE levels and increased SOCS1 protein expression in bronchial biopsies was also observed ($R=0.3443$, $p=0.0578$, Figure 4.9C). Notably, Figure 4.9A,B shows data from AA only, criteria for this study were that SPT is zero and PC_{20} >16mg/mL for all NA (Section 4.3.1). AA had higher levels of SOCS1 which related to more reactivity to allergens (number of positive SPT), increased AHR (reduced PC_{20}) and higher total serum IgE levels (Figure 4.9). However, despite correlations being observed between atopic disease outcome and SOCS1 levels these did not appear to be related to asthmatics specifically (Table 4.4).

Table 4.4: Correlations between SOCS1 and SOCS3 protein expression and clinical study outcomes

Data is expressed as p value and spearman r. NA n=14 and AA n=17.

		PC_{20}	IgE		SPT
		AA	**NA**	**AA**	**AA**
SOCS1	**Spearman r**	-0.3842	0.1233	-0.02732	0.1818
	p value	0.1279	0.6746	0.9171	0.6746

SOCS3 protein levels did not correlate with atopy related clinical outcomes (IgE and number of SPT), AHR (PC_{20}) and lung function related outcomes (PEF% predicted, FEV_1% predicted and FEV_1/FVC ratio, Table 4.3).

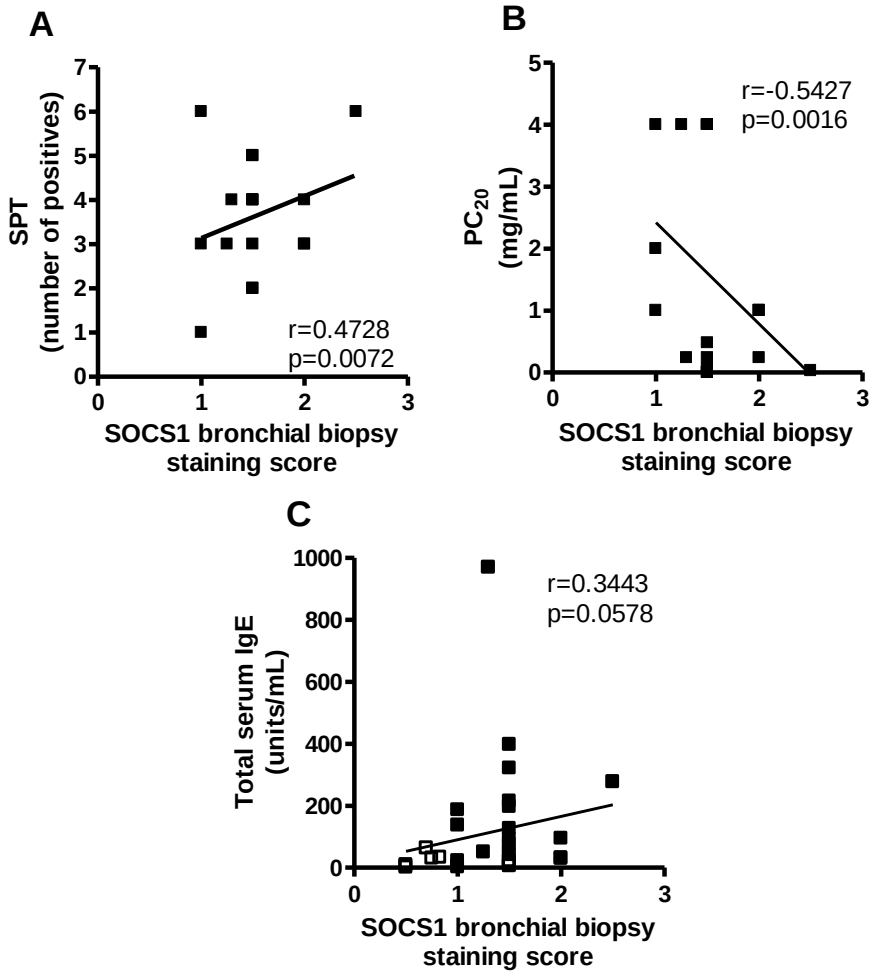

Figure 4.9: Correlations between SOCS1 protein levels in bronchial biopsies and allergic related clinical study outcomes.

SOCS1 protein positively correlated with number of positive SPT in AA [A], SOCS1 negatively correlated with PC_{20} in AA [B] and a trend was observed for a positive correlation between total serum IgE and SOCS1 protein in NA and AA [C]. Open squares denote NA, n=14; closed squares denote AA, n=17.

4.5.2 SOCS1 and SOCS3 protein expression in biopsies did not correlate with RV induced IFN mRNA expression, production and RV vRNA and release in HBECs

To asses if the higher SOCS1 protein levels observed in the biopsies of AA (Section 4.3.1) were related to RV induced IFN mRNA levels and protein levels and RV replication and release in HBECs cultured from these subjects, SOCS1 protein levels in bronchial biopsies were correlated with RV1B and RV16 induced type I and III IFN mRNA and protein levels 8, 24 and 48h post infection and RV1B and RV16 replication 8, 24 and 48h post infection. Relationship between SOCS3 protein levels in bronchial biopsies (Section 4.3.2) and these study outcomes were also determined.

SOCS1 and SOCS3 protein levels did not correlate with RV1B and RV16 induced type I and III IFN mRNA and protein levels 8, 24 and 48h post infection and RV1B and RV16 replication 8h, 24h and 48h post infection. Correlations for RV1B induced IFN-β, IFN-λ1 and IFN-λ2/3 mRNA 24h post infection and RV1B replication and release 48h post infection for both SOCS1 and SOCS3 are shown in Table 4.5 which was representative for all treatments and time points.

Table 4.5: Correlation outcomes between SOCS1 and SOCS3 protein expression and RV1B induced IFN mRNA 24h post infection and RV1B replication and release 48h post infection in HBECs.

Data is expressed as p value and spearman r. NA n=4 and AA n=8 (subjects who had both biopsies and successfully cultured HBECs).

RV1B induced						
		IFN-β mRNA	**IFN-λ1 mRNA**	**IFN-λ2/3 mRNA**	**RV1B vRNA**	**RV1B release**
SOCS1	**Spearman r**	0.3851	0.0828	0.0267	0.2617	-0.0488
	p value	0.1563	0.7692	0.9246	0.3460	0.8628
SOCS3	**Spearman r**	0.0018	0.3676	0.1676	0.2451	-0.0460
	p value	0.9949	0.1777	0.5505	0.3787	0.8707

4.5.3 SOCS1 and SOCS3 mRNA expression in HBECs did not correlate with clinical study outcomes

To asses if the SOCS1 and SOCS3 mRNA levels in HBECs (Section 4.4.3 to Section 4.4.6) were related to markers of atopy and lung function, SOCS1 and SOCS3 mRNA levels in medium treated cells, RV1B and RV16 infected cells 8h, 24h and 48h post infections were correlated with measurements of lung function (PEF% predicted, FEV_1% predicted and FEV_1/FVC ratio), AHR (PC_{20}) and markers of atopy (IgE and SPT).

SOCS1 and SOCS3 mRNA levels in HBECs did not correlate with markers of atopy (IgE and number of positive SPT), AHR (PC_{20}) or lung function (PEF% predicted, FEV_1% predicted and FEV_1/FVC ratio). Table 4.6 shows that SOCS1 and SOCS3 mRNA levels 8h post medium treatment in HBECs did not correlate with lung function and markers of atopy. This was representative for all treatment and time points (8h, 24h, 48h; RV1B, RV16 infection and medium treatment).

Table 4.6: Correlations between SOCS1 and SOCS3 mRNA levels 8h post medium treatment in HBECs and clinical study outcomes.

Data is expressed as p value and spearman r. NA n=10 and AA n=10.

		PEF % predicted	FEV_1 % predicted	FEV_1/FVC ratio	PC_{20}	IgE	SPT
SOCS1 mRNA	**Spearman r**	0.1860	-0.0083	0.1331	-0.0829	0.2332	0.3661
	p value	0.4324	0.9723	0.5758	0.7284	0.3225	0.1124
SOCS3 mRNA	**Spearman r**	-0.1039	0.1990	-0.2806	-0.2896	0.3069	0.3079
	p value	0.6628	0.4002	0.2309	0.2156	0.1881	0.1866

4.5.4 SOCS1 and SOCS3 mRNA expression did not correlate with RV induced IFN mRNA expression, production and RV replication and release in HBECs

In this paragraph it was aimed to assess whether SOCS1 and SOCS3 mRNA levels in HBECs (Section 4.4.3 to Section 4.4.6) were related to RV induced IFN mRNA levels, IFN protein levels, RV replication or release in HBECs cultured from these subjects. SOCS1 and SOCS3 mRNA levels in medium treated, RV1B and RV16 infected HBECs 8h, 24h and 48h post infection were correlated with RV1B and RV16 induced type I and III IFN mRNA and protein levels 8h, 24h and 48h post infection and RV1B and RV16 replication 8h, 24h and 48h post infection.

SOCS1 and SOCS3 mRNA levels did not correlate with RV1B and RV16 induced IFN-β and IFN-λ1 mRNA and protein levels at 8h, 24h and 48h post infection or RV1B and RV16 replication and release 8h, 24h and 48h post infection. Table 4.7 shows that SOCS1 and SOCS3 mRNA levels 8h post medium treatment in HBECs did not correlate with RV1B induced IFN-β, IFN-λ1 and IFN-λ2/3 mRNA 24h post infection or RV1B replication and release 48h post infection for both SOCS1 and SOCS3 which were representative for all time points and treatments.

Table 4.7: Correlations between SOCS1 and SOCS3 mRNA levels 8h post medium treatment with and RV1B induced IFN mRNA 24h post infection and RV1B replication and release 48h post infection in HBECs.

Data is expressed as p value and spearman r. NA n=10 and AA n=10.

RV1B induced						
		IFN-β mRNA	**IFN-λ1 mRNA**	**IFN-λ2/3 mRNA**	**RV replication**	**RV release**
SOCS1	**Spearman r**	0.4180	-0.0481	***0.4857***	-0.1203	-0.2625
	p value	0.0666	0.8403	***0.0299***	0.6134	0.2635
SOCS3	**Spearman r**	0.2301	-0.1940	0.3158	0.3489	-0.3528
	p value	0.3291	0.4125	0.1750	0.1317	0.1271

RV1B and RV16 induced SOCS1 mRNA levels positively correlated with RV1B and RV16 induced IFN-λ2/3 mRNA levels at several time points post infection (Table 4.7 and Table 4.8), indicating that increased levels of SOCS1 mRNA were related to increased levels of IFN-λ2/3 mRNA in HBECs. No clear subdivision was seen between AA and NA, e.g. AA having low levels of both IFN- λ2/3 and SOCS1 mRNA and NA having high levels of IFN- λ2/3 and SOCS1 mRNA or vice versa.

Table 4.8: Correlations between RV16 and RV1B induced SOCS1 mRNA levels and IFN-λ2/3 mRNA in HBECs.

Data is expressed as p value and spearman r. Highlighted values denote significant correlation between SOCS1 mRNA and IFN-λ2/3 mRNA levels in HBECs. NA n=10 and AA n=10.

	IFN-λ2/3		**8h**		**24h**		**48h**	
SOCS1			**RV16**	**RV1B**	**RV16**	**RV1B**	**RV16**	**RV1B**
8h	**RV16**	**Spearman r**	0.2677	***0.4586***	0.2917	***0.4752***	0.0361	0.0466
		p value	0.2539	***0.0420***	0.2120	***0.0342***	0.8799	0.8453
	RV1B	**Spearman r**	0.4120	***0.7158***	***0.4526***	***0.6617***	0.2511	0.3474
		p value	0.0710	***0.0004***	***0.0451***	***0.0015***	0.2855	0.1334
24h	**RV16**	**Spearman r**	0.1654	0.4376	0.2992	***0.5158***	0.1925	0.1489
		p value	0.4858	0.0537	0.1999	***0.0199***	0.4162	0.5310
	RV1B	**Spearman r**	0.2857	***0.5850***	0.4105	***0.6436***	0.1128	0.2376
		p value	0.2220	***0.0067***	0.0722	***0.0022***	0.6359	0.3131
48h	**RV16**	**Spearman r**	0.1970	0.2827	0.1519	0.2842	0.1143	-0.1414
		p value	0.4052	0.2272	0.5227	0.2246	0.6314	0.5522
	RV1B	**Spearman r**	0.3579	***0.5233***	0.3820	***0.6556***	0.2692	0.3398
		p value	0.1213	***0.0179***	0.0965	***0.0017***	0.2511	0.1426

4.6 RV1B and RV16 induced SOCS1 and SOCS3 mRNA and SOCS1 protein expression in HBECs from severe AA and NA

HBECs were obtained by bronchoscopies performed on nine paediatric patients with severe therapy resistant asthma (STRA). STRA was defined as persistent (most days, for at least 3 months) chronic symptoms (requiring a rescue bronchodilator on at least three days per week) of airway obstruction, despite treatment with high dose inhaled corticosteroids (at least 800 μg/day of beclometasone equivalent) and trials of add on drugs (LABA, leukotriene receptor antagonists and oral theophylline in a low, anti-inflammatory dose) and/or recurrent severe asthma exacerbations**.** Patients were not recruited based on whether they wheeze upon RV infection, or had worse asthma symptoms upon experiencing a cold. None of the subjects were prescribed antibiotics at the time of the study and all were considered to be clinically free of infections. Non-atopic NA controls (NA) were recruited through the Royal Brompton Hospital and Children's Hospital in Bern. NA had no history of asthma and no asthma in family history and no record of food allergy, rhinitis or eczema. HBECS were cultured and infected with RV1B, RV16 or treated with the dsRNA PolyIC (M.R.Edwards and N.Regamey, unpublished data).

Group sizes were based on power calculations showing that n=11 can confirm that 3 fold changes between groups are achievable in 90% of the analytes tested by qPCR (study still in progress). The use of bronchial brushings from severe asthmatic and control children have been approved by both the Royal Brompton NHS Trust (02/302, Prof A. Bush) and the Ethic Committee for the canton of Bern, for University Children's Hospital Bern, Switzerland (77/09, A/Prof N. Regamey). Each Research Ethics Committee is informed on anonymisation of data, data storage, complications and adverse effects, and insurance. Written informed consent from parents and age-appropriate assent from children was obtained in each case.

4.6.1 Clinical characteristics

Table 4.9: Clinical characteristics of AA and NA.

Values are presented as mean (±SEM) except for sex. ACT=asthma control test, BDR=bronchodilator responsiveness, FeNO,50= fractional exhaled nitric oxide in 50mL, n/a = not available. NA n=11, AA n=9 (M.R.Edwards and N.Regamey, unpublished data).

Clinical characteristic	AA	NA
Sex	67% M 33% F	70% M 30% F
Age	11.6 (± 0.8)	7.3 (2-15)
ACT	12.2 (±1.5)	n/a
FEV_1 % predicted	70.1 (± 8.52)	n/a
IgE (units/mL)	981.8 (± 463.8)	n/a
RAST (sum of positive total RAST data)	78.9 (± 18.5)	n/a
BDR (%)	15.4 (± 2.6)	n/a
FeNO,50 (ppb)	46.5 (± 7.4)	n/a

BDR is measured by (FEV_1% predicted post inhalation of 400µg of salbutamol- FEV_1% predicted baseline)/baseline FEV_1% predicted [219]. A reversibility of >12% is an indication of active asthma. FeNO is a non invasive marker of eosinophilic airway inflammation and steroid responsiveness; ≥45 ppb indicates significant eosinophilic airway inflammation [220]. An ACT score <17 indicates uncontrolled asthma [221]. The ACT and FeNO,50 data showed that this group of patients has severe asthma.

4.6.2 Summary of RV1B and RV16 induced IFN protein and mRNA induction and RV release in HBECs from severe AA and NA

To establish whether the previously reported defective RV induced IFN-β and IFN-λ in AA was also present in HBECs cultured from severe therapy resistant asthmatic (STRA) children, RV1B, RV16 and PolyIC induced type I and III IFN mRNA and protein was measured in this study (M.R. Edwards, unpublished data). In summary, in NA controls RV16 significantly induced IFN-β ($p<0.05$), IFN-λ1 ($p<0.01$) and IFN-λ2/3 ($p<0.01$) versus medium at 24h post infection; RV1B also significantly induced IFN-β ($p<0.001$), IFN-λ1 ($p<0.001$) and IFN-λ2/3 ($p<0.001$) versus medium at 24h post infection, PolyIC did not significantly induced IFN-β, but did significantly induce IFN-λ1 ($p<0.05$) and IFN-λ2/3 ($p<0.05$) versus medium at 8h post treatment. In severe AA, RV16 did not significantly induce IFN-β, IFN-λ1 and IFN-λ2/3 compared to medium at 24h post infection; and RV1B significantly induced IFN-λ1 ($p<0.05$) but did not significantly induced IFN-β and IFN-λ2/3 versus medium at 24h post

infection. There was a significant difference between AA and NA for each virus tested. For RV16, the mRNA of IFN-β ($p<0.05$), IFN-λ1 ($p<0.01$) and IFN-λ2/3 ($p<0.01$) was significantly lower than for NA at 24h post infection. For RV1B, the mRNA of IFN-β ($p<0.01$), IFN-λ1 ($p<0.05$) and IFN-λ2/3 ($p<0.05$) was significantly lower than for NA at 24h post infection. For PolyIC, the mRNA of IFN-λ1 ($p<0.01$) and IFN-λ2/3 ($p<0.01$) was significantly lower than for NA at 8h post treatment.

4.6.3 RV1B and RV16 induced and medium treated SOCS1 mRNA levels in HBECs from severe AA and NA showed increased baseline SOCS1 mRNA levels

To assess whether baseline, RV1B or RV16 induced SOCS1 mRNA expression was different in severe AA compared to NA, SOCS1 mRNA levels were measured in medium treated and RV infected cells at 8h and 24h post infection.

SOCS1 mRNA was not induced by RV1B at either time point by either AA or NA in HBECs (Figure 4.10). Although a trend was observed for increased RV1B induced SOCS1 mRNA at 24h post infection in NA HBECs (Figure 4.10B). SOCS1 mRNA levels were significantly higher in medium treated HBECs of AA compared to NA at 8h (Figure 4.10A and Figure 4.11A, $p<0.05$). This trend was also observed for RV1B induced SOCS1 mRNA levels at 8h (Figure 4.10A).

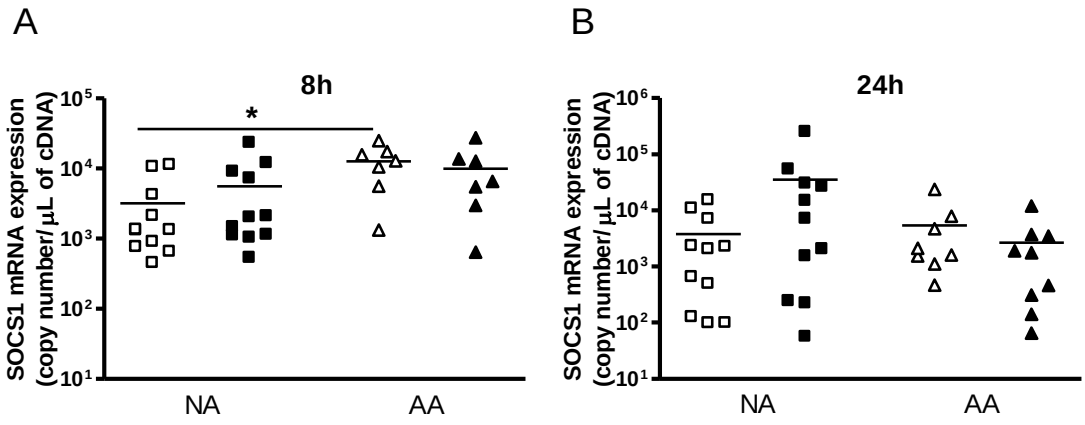

Figure 4.10: RV1B induced SOCS1 mRNA expression in HBECs from AA and NA, 8h (A) and 24h (B) post infection.

Squares denote NA, n=11; triangles denote AA, n=7 (8h, A) and n=9 (24h, B); open symbols denote medium treated cells; black symbols denote RV1B infected cells.

RV16 induced SOCS1 mRNA was significantly higher 24h post infection in NA (Figure 4.11B, $p<0.05$). A trend was observed for increased RV16 induced SOCS1 mRNA 8h post infection in NA HBECs (Figure 4.11A) and 24h post infection in AA HBECs (Figure 4.11B). RV16 induced SOCS1 mRNA levels at 8h showed a trend towards increased levels in AA compared to NA (Figure 4.11A).

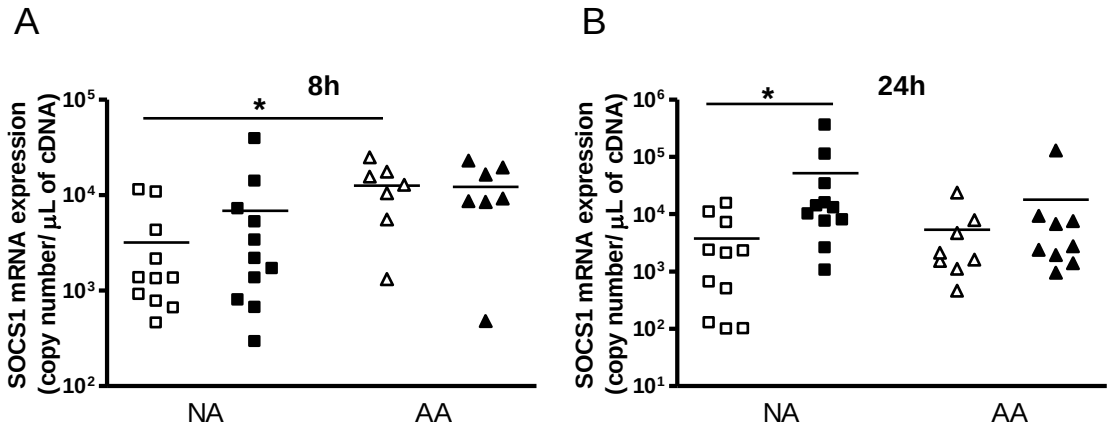

Figure 4.11: RV16 induced SOCS1 mRNA expression in HBECs from AA and NA, 8h (A) and 24h (B) post infection.

Squares denote NA, n=11; triangles denote AA, n=7 (8h, A) and n=9 (24h, B); open symbols denote medium treated cells; black symbols denote RV16 infected cells.

4.6.4 PolyIC induced SOCS1 mRNA expression in HBECs from severe AA was not different compared to NA

In this study IFN deficiency was observed in AA HBECs upon PolyIC as well as RV (Section 4.6.2). To assess whether PolyIC induced SOCS1 mRNA expression was different in AA compared to NA, PolyIC induced and medium treated SOCS1 mRNA levels were measured at 8h post infection. Samples from PolyIC treated HBECs were obtained from a smaller amount of subjects compared to RV infected HBECs, due to the amount of cells obtained for each subject.

SOCS1 mRNA was significantly induced by PolyIC in NA HBECs ($p<0.05$, Figure 4.12). A trend was observed for increased PolyIC induced SOCS1 mRNA in the AA HBECs (Figure 4.12). In this subset the same increase in SOCS1 mRNA levels in medium treated HBECs from AA was observed compared to NA, although this was not significant, probably due to that samples from PolyIC treated HBECs were obtained from a smaller number of subjects (Figure 4.12).

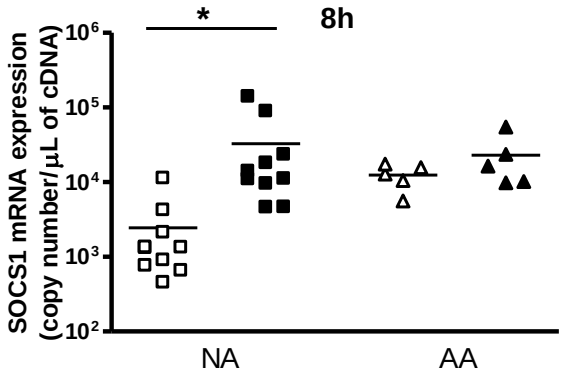

Figure 4.12: PolyIC induced SOCS1 mRNA expression in HBECs from AA and NA, 8h (A) and 24h (B) post infection.

Squares denote NA, n=10; triangles denote AA, n=5; open symbols denote medium treated cells; black symbols denote PolyIC treated cells.

4.6.5 RV1B and RV16 did not induce SOCS3 mRNA expression in HBECs from severe AA and NA

To assess whether RV1B and RV16 induced SOCS3 mRNA expression was different in AA compared to NA, SOCS3 mRNA levels were measured at 8h and 24h post infection. SOCS3 mRNA was not induced by RV1B (Figure 4.13) and RV16 (Figure 4.14) at either time point in AA or NA HBECs and no differences were observed between SOCS3 mRNA levels in AA and NA HBECs.

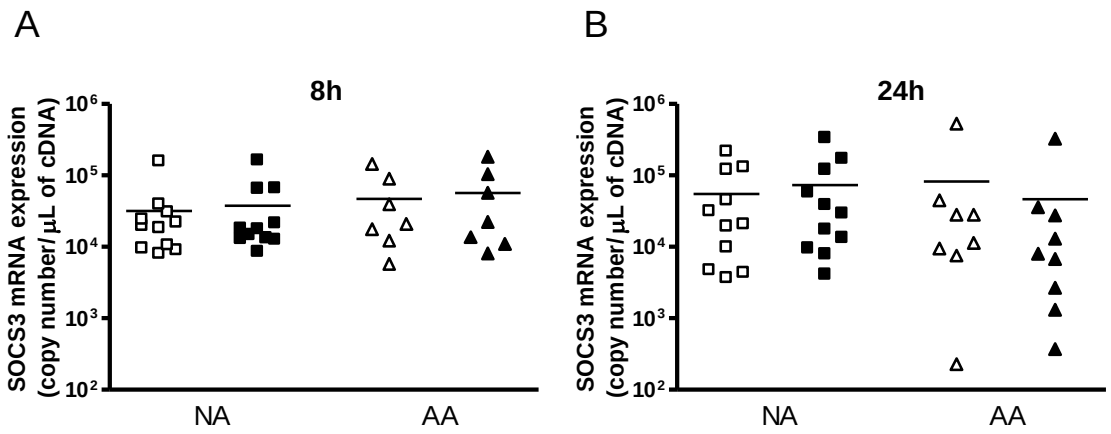

Figure 4.13: RV1B induced SOCS3 mRNA expression in HBECs from AA and NA, 8h (A) and 24h (B) post infection.

Squares denote NA, n=11; triangles denote AA, n=7 (8h, A) and n=9 (24h, B); open symbols denote medium treated cells; black symbols denote RV1B infected cells.

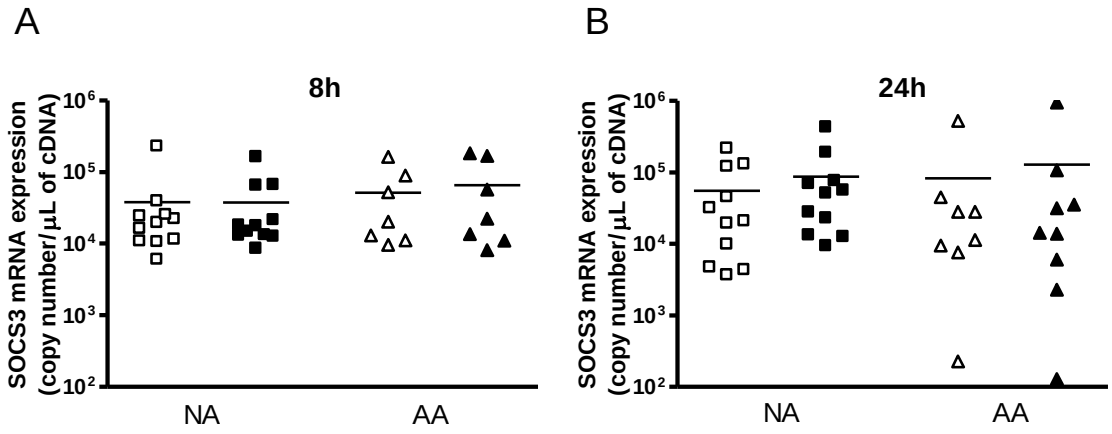

Figure 4.14: RV16 induced SOCS3 mRNA expression in HBECs from AA and NA, 8h [A] and 24h [B] post infection.

Squares denote NA, n=11, triangles denote AA, n=7 (8h, A) and n=9 (24h, B); open symbols denote medium treated cells, black symbols denote RV16 infected cells.

4.6.6 PolyIC did not induce SOCS3 mRNA expression in HBECs from severe AA and NA

To assess whether PolyIC induced SOCS3 mRNA expression was different in AA compared to NA, SOCS1 mRNA levels were measured at 8h post PolyIC treatment. SOCS3 mRNA was not induced by PolyIC at either time point in AA or NA HBECs (Figure 4.13) and no differences were observed between SOCS3 mRNA levels in AA and NA HBECs.

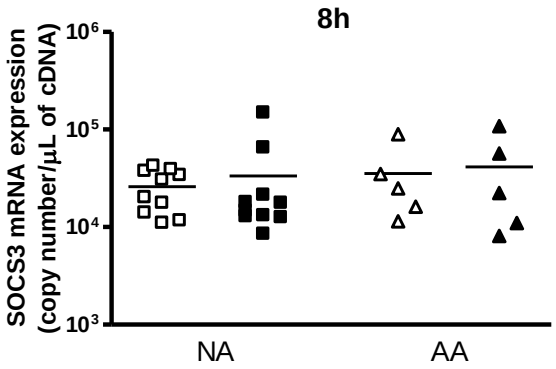

Figure 4.15: PolyIC induced SOCS3 mRNA expression in HBECs from AA and NA, 8h (A) and 24h (B) post infection.

Squares denote NA, n=10; triangles denote AA, n=5; open symbols denote medium treated cells, black symbols denote PolyIC treated cells.

4.6.7 SOCS1 protein in medium treated, RV1B and RV16 infected HBECs from severe AA was not different compared to NA

To assess whether the increased SOCS1 mRNA levels at baseline in AA HBECs compared to NA correlated with SOCS1 protein levels, SOCS1 protein levels were measured 24h post RV1B and RV16 infection and medium treatment using western blotting. SOCS1 protein levels were not different between AA and NA (Figure 4.16). Figure 4.17 is a representation of the SOCS1 protein levels observed for the different treatments.

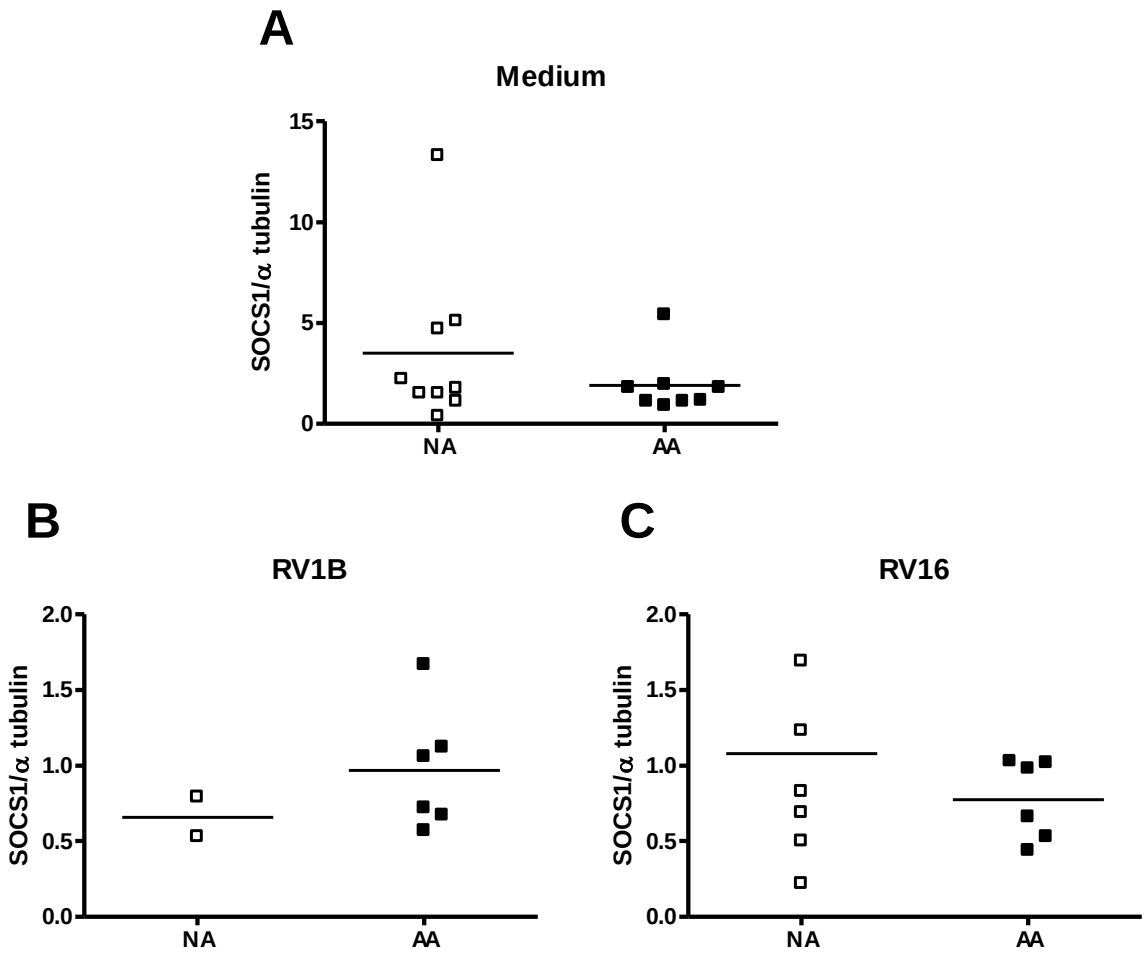

Figure 4.16: SOCS1 protein levels in medium treated (A), RV1B (B) or RV16 (C) infected HBECs.

Data is presented as ratio of SOCS1 protein over α-tubulin. Open squares denote NA, closed squares denote AA. Medium NA n=10, AA=8; RV1B NA n=2, AA=6; RV16 NA n=7, AA n=6.

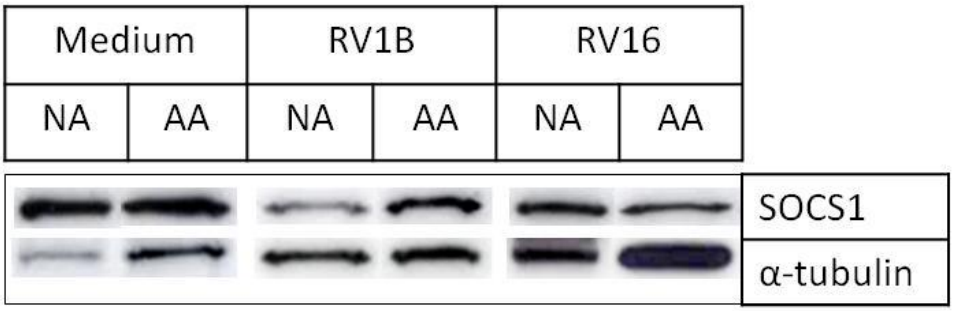

Figure 4.17: SOCS1 protein levels in medium treated, RV1B or RV16 infected HBECs from a NA and AA, representative of all samples.

4.7 Correlation between SOCS1 mRNA expression in HBECs 8h post medium treatment from severe AA and NA and markers of atopy, asthma or lung function

4.7.1 SOCS1 mRNA expression 8h post medium treatment in HBECs did not correlate with clinical study outcomes

To asses if the increased SOCS1 mRNA levels 8h post medium treatment in HBECs from AA (Section 4.6.3 and 4.6.4) were related to markers of atopy, asthma or lung function. SOCS1 mRNA levels 8h post medium treatment in HBECs were correlated with clinical measurements of FEV_1% predicted, BDR, ACT, FeNO,50, total serum IgE levels and RAST. None of these clinical parameters were obtained from the NA.

Table 4.10: Correlation outcomes between SOCS1 mRNA levels 8h post medium treatment and clinical study outcomes.

Data is expressed as p value and spearman r. AA n=7.

		FEV_1 % predicted	BDR (%)	ACT	FeNO,50	IgE	RAST
SOCS1 mRNA	**Spearman r**	-0.1622	0.3591	0.0180	-0.2143	-0.2500	-0.6429
	p value	0.7131	0.5167	0.9635	0.6615	0.5948	0.1389

Table 4.10 demonstrates that SOCS1 mRNA levels 8h post medium treatment did not correlate with lung function, asthma and atopy related clinical outcomes in AA. No correlation were observed between SOCS3 mRNA levels in HBECs, at any time point or treatment, and lung function, asthma and atopy related clinical outcomes (data not shown).

4.7.2 Correlation between SOCS1 mRNA expression 8h post medium treatment and RV1B and RV16 induced IFN mRNA expression and RV release in HBECs

To assess whether the increased SOCS1 mRNA levels 8h post medium treatment in HBECs (Section 4.6.3 and 4.6.4) in AA compared to NA correlated with deficient RV1B and RV16 induced IFN mRNA levels and protein levels and RV release in HBECs (Section 4.2.4.2). SOCS1 mRNA levels 8h post medium treatment in HBECs were correlated with RV1B and RV16 induced type I and III IFN mRNA levels 24h post infection and RV1B and RV16 release 48h post infection in HBECs.

RV1B induced type I and III IFN mRNA levels 24h post treatment did not correlate with SOCS1 mRNA levels 8h post medium treatment in HBECs (Figure 4.18), although a trend was observed towards a negative correlation of SOCS1 mRNA levels with IFN-λ1 and IFN-λ2/3 mRNA levels (p=0.0931 and p=0.0672 respectively). This could indicate an association between greater SOCS1 mRNA and lower

IFN-λ1 and IFN-λ2/3 mRNA levels. Notably, data from HBECs from AA and NA were separated, with AA having greater SOCS1 mRNA levels and lower IFN-λ1 and IFN-λ2/3 mRNA production (Figure 4.18B,C).

SOCS1 mRNA levels 8h post medium treatment positively correlated with RV1B release 48h post infection in HBECs ($p<0.05$, Figure 4.20A), indicating a relation between higher SOCS1 mRNA and higher RV1B protein levels.

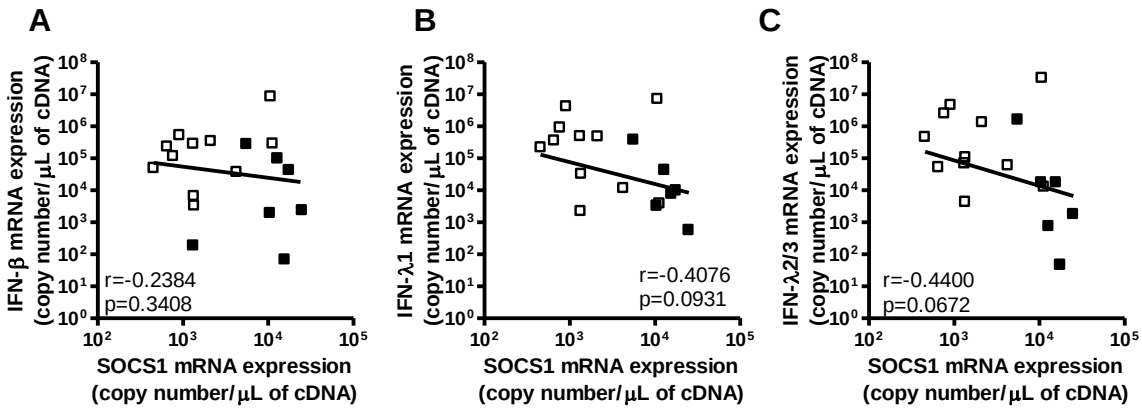

Figure 4.18: Correlations between SOCS1 mRNA levels 8h post medium treatment and RV1B induced type I and III IFN mRNA production 24h post infection in HBECs.

SOCS1 mRNA levels did not correlate with RV1B induced IFN-β mRNA (A), IFN-λ1 mRNA (B) and IFN-λ2/3 mRNA (C). Open squares denote NA, n=11; closed squares denote AA, n=7.

RV16 induced type I and III IFN mRNA levels 24h post infection negatively correlated with SOCS1 mRNA levels 8h post medium treatment in HBECs (IFN-β $p<0.05$, IFN-λ1 $p<0.001$, IFN-λ2/3 $p<0.01$; Figure 4.19), indicating a relation between greater SOCS1 mRNA and lower IFN-β, IFN-λ1 and IFN-λ2/3 mRNA levels, however SOCS1 mRNA levels 8h post medium treatment did not correlate with RV16 release 48h post infection in HBECs (Figure 4.20B).

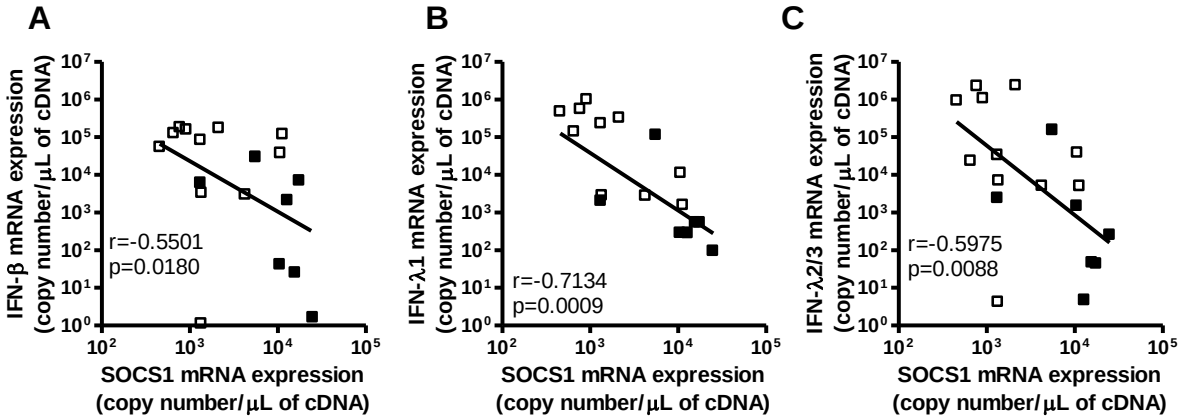

Figure 4.19: Correlations between SOCS1 mRNA levels 8h post medium treatment and RV16 induced type I and III IFN mRNA production 24h post infection in HBECs.

SOCS1 mRNA levels negatively correlated with RV16 induced IFN-β mRNA (A), IFN-λ1 mRNA (B) and IFN-λ2/3 mRNA. Open squares denote NA, n=11; closed squares denote AA, n=7.

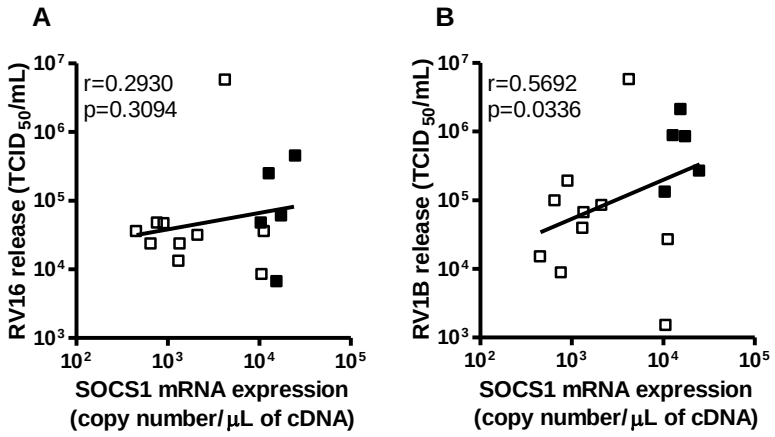

Figure 4.20: Correlations between SOCS1 mRNA levels 8h post medium treatment and RV1B and RV16 release in HBECs 48h post infection.

SOCS1 mRNA levels positively correlated with RV1B release (A). SOCS1 mRNA levels did not correlate with RV16 release (B). Open squares denote NA, n=9; closed squares denote AA, n=5.

However, despite correlations being observed between RV-induced IFNs and SOCS1 mRNA levels these did not appear to be related to specifically to asthma (Table 4.11 and Table 4.12).

Table 4.11: Correlation outcomes between SOCS1 mRNA levels 8h post medium treatment and RV1B-induced type I and III IFN mRNA production 24h post infection in HBECs

Data is expressed as p value and spearman r. NA=11 and AA n=7.

	RV1B induced				
SOCS1		**IFN-β mRNA**	**IFN-λ1 mRNA**	**IFN-λ2/3 mRNA**	**RV release**
NA	**Spearman r**	0.4060	-0.198	0.0285	0.1197
	p value	0.2154	0.5595	0.9337	0.7418
AA	**Spearman r**	0.1920	0.5831	0.4529	0.3528
	p value	0.6800	0.1694	0.3075	0.5603

Table 4.12: Correlation outcomes between SOCS1 mRNA levels 8h post medium treatment and RV16-induced type I and III IFN mRNA production 24h post infection in HBECs.

Data is expressed as p value and spearman r. NA=11 and AA n=7.

	RV16 induced				
SOCS1		**IFN-β mRNA**	**IFN-λ1 mRNA**	**IFN-λ2/3 mRNA**	**RV release**
NA	**Spearman r**	-0.01296	-0.3337	-0.2697	-0.0758
	p value	0.9698	0.3159	0.4224	0.8352
AA	**Spearman r**	-0.5712	-0.5385	-0.5347	0.1307
	p value	0.1804	0.2124	0.2163	0.8341

No correlation were observed between SOCS3 mRNA levels in HBECs, at any time point or treatment, and RV1B and RV16 induced type I and III IFN mRNA or RV1B and RV16 release (data not shown).

4.7.3 Correlation between SOCS1 mRNA expression 8h post medium treatment and PolyIC treatment in HBECs

To asses if the increased SOCS1 mRNA levels 8h post medium treatment in HBECs (Section 4.2.4.3 and 4.2.4.4) in AA compared to NA correlated with deficient PolyIC induced IFN mRNA levels and protein levels and RV release in HBECs (Section 4.2.4.2). SOCS1 mRNA levels 8h post medium treatment in HBECs were correlated with PolyIC induced type I and III IFN mRNA levels 8h post treatment in HBECs.

PolyIC induced type III IFN mRNA levels 8h post treatment negatively correlated with SOCS1 mRNA levels 8h post medium treatment in HBECs ($p<0.05$, Figure 4.21B,C), indicating an association between high SOCS1 mRNA and low IFN-λ1 and IFN-λ2/3 mRNA levels. Notably, data from HBECs

from AA and NA were clearly separated, with AA having greater SOCS1 mRNA levels and lower IFN-λ1 and IFN-λ2/3 mRNA production (Figure 4.21B,C). PolyIC induced IFN-β mRNA did not correlate with SOCS1 mRNA levels in HBECs (Figure 4.21A).

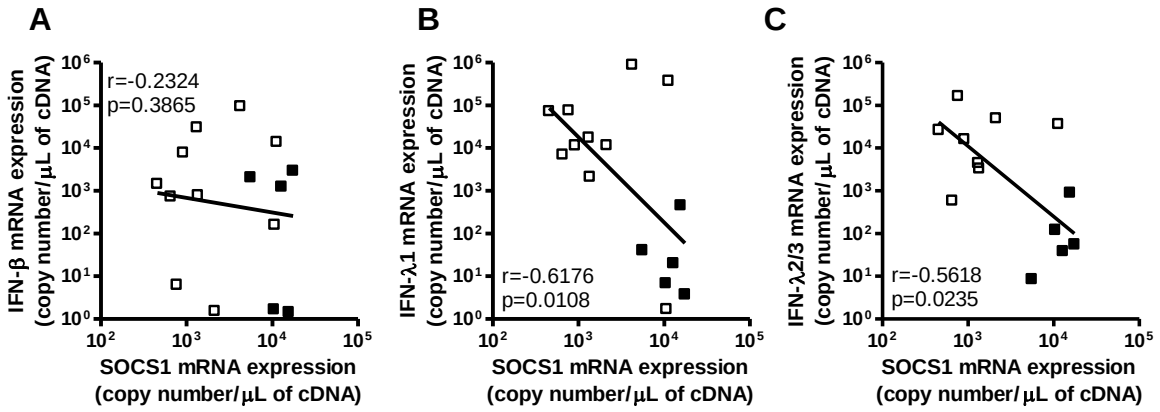

Figure 4.21: Correlations between SOCS1 mRNA levels 8h post medium treatment and PolyIC induced type I and III IFN mRNA production 8h post treatment in HBECs.

SOCS1 mRNA levels did not correlate with PolyIC induced IFN-β mRNA (A). SOCS1 mRNA levels negatively correlated with PolyIC induced IFN-λ1 mRNA (B) and IFN-λ2/3 mRNA. Open squares denote NA, n=10; closed squares denote AA, n=5.

However, despite correlations being observed between PolyIC-induced IFNs and SOCS1 mRNA levels these did not appear to be related to asthma specifically (Table 4.13).

Table 4.13: Correlation outcomes between SOCS1 mRNA levels 8h post medium treatment and RV1B-induced type I and III IFN mRNA production 24h post infection in HBECs

Data is expressed as p value and spearman r. NA=11 and AA n=7.

	PolyIC induced			
SOCS1		**IFN-β mRNA**	**IFN-λ1 mRNA**	**IFN-λ2/3 mRNA**
NA	**Spearman r**	0.1696	-0.2389	-0.3039
	p value	0.6396	0.5063	0.3933
AA	**Spearman r**	-0.2015	-0.0474	0.6971
	p value	0.7451	0.9397	0.2073

No correlation were observed between SOCS3 mRNA levels in HBECs, at any time point or treatment, and PolyIC induced type I and III IFN mRNA levels (data not shown).

4.8 Discussion

In this chapter it was aimed to investigate whether SOCS1 and SOCS3 mRNA in HBECs and protein in bronchial biopsies and HBECs were increased in AA and whether they play a role in RV induced IFN deficiency previously observed in AA, the mechanism of which is currently unknown. Increased levels of negative regulators of RV induced signalling could potentially play a role in IFN deficiency in AA and of the several negative regulators that have been described SOCS are a potential candidate as they are involved in negatively regulating IFNs via JAK/STAT and possibly virus induce IFN production [175, 190].

SOCS1 and SOCS3 levels were determined in two separate studies. In the first study, SOCS1 and SOCS3 protein levels were determined in bronchial biopsies from mild AA and SOCS1 and SOCS3 mRNA in *ex vivo* cultured HBECs from the same study group which were infected with RV1B and RV16. SOCS1 protein was increased in AA compared to non-atopic NA in bronchial biopsies; no differences were observed in SOCS3 protein levels in biopsies or SOCS1 and SOCS3 mRNA in HBECs between the two groups. The previously described IFN deficiency in AA was not observed in this study [47, 49] (A. Sykes PhD thesis & unpublished observations). The increased SOCS1 protein levels correlated with clinical study outcomes for AHR and allergies, and showed a trend towards a correlation with IgE levels. This indicates that higher SOCS1 protein in lung biopsies is related to increased IgE, increased AHR and increased number of allergies, although this did not appear to be disease specific.

In the second study SOCS1 and SOCS3 mRNA levels and SOCS1 protein levels were determined in *ex vivo* cultured and RV1B, RV16 infected and PolyIC treated HBECs from severe AA children and NA. SOCS1 mRNA was increased in HBECs without treatment in AA compared to NA, no differences were observed in RV1B, RV16 and PolyIC induced SOCS1 and SOCS3 mRNA between the two study groups. No differences were observed in SOCS1 protein between the two study groups. IFN deficiency in AA was observed in this study. The increased SOCS1 mRNA levels negatively correlated with RV induced IFN mRNA and positively correlated with RV release. This indicates that increased SOCS1 mRNA levels were related to decreased RV induced IFNs and increased RV release in HBECs, although this did not appear to be disease specific.

4.8.1 SOCS1 and SOCS3 in asthma

Previous studies have investigated SOCS1 and SOCS3 in clinical asthma. SOCS3 mRNA has been shown to be increased in CD3+ T cells isolated from whole blood in AA compared to healthy subjects [162]. More severe AA had higher levels of SOCS3 mRNA than mild and moderate AA. In this study a

positive correlation was observed between concentration of serum IgE and SOCS3 mRNA in T cells in the AA [162]. This indicates that SOCS3 expression in T cells might play a role in regulating allergic asthma, however in the present study, no increase in SOCS3 protein was observed in lung biopsies and SOCS3 mRNA in untreated HBECs between mild AA and NA. Furthermore, no increase in SOCS3 mRNA or protein from severe AA compared to NA was observed. The failure of IL-4 and IL-13 to induce SOCS3 in HBECs (Chapter 3) suggests that there was no role for SOCS3 in atopy in this cell type. The results suggest that atopy may increase SOCS3 but this is cell type specific, and not present in HBECs, but is a feature of T cells. The results shown in this chapter did not indicate that SOCS3 expression was important in RV infection of HBECs and was not associated with IFN deficiency observed in AA.

Clinical studies of SOCS1 in AA are contradictory. A polymorphism has been found in the SOCS1 promoter in AA. In A549 cells this promoter variant leads to increased SOCS1 protein expression and reduced IFN-β induced STAT1 phosphorylation [168]. This suggests that SOCS1 levels are higher in AA and that SOCS1 might be involved in asthma, however, untreated ASMCs showed no differences in SOCS1 mRNA between AA and NA [162] and a failure of induction of SOCS1 mRNA by IL-13 in AA. In the present study it was observed that mild AA had an increase of SOCS1 protein in bronchial biopsies compared to NA. The increased SOCS1 protein levels correlated with clinical outcomes of atopy and AHR suggesting a relationship between SOCS1 and atopic asthma severity; however no increase was observed in SOCS1 mRNA in untreated cultured HBECs from mild AA. Bronchial biopsies are processed immediately while cultured HBECs are passaged a few times before experiments are completed on these, which might be an explanation for the differences observed between these samples. In untreated HBECs from severe AA however, a significant increase in SOCS1 mRNA levels was observed compared to NA. No bronchial biopsies were obtained in this study. The present study and previous literature are controversial, however this could, as for SOCS3, be a cell type specific difference. The results in this study showed increased SOCS1 in HBECs in severe asthma, suggesting that increased SOCS1 expression in HBECs may be related to asthma severity, as HBECs are the main site of infection of RVs, this indicates that SOCS1 might be associated with impaired IFN production and therefore might play a role in RV induced asthma exacerbations.

4.8.2 Induction of SOCS1 and SOCS3 mRNA in HBECs grown from bronchial brushings compared to commercially obtained HBECs

In Chapter 3 it was observed that both RV1B and RV16 significantly induce SOCS1 and SOCS3 mRNA and protein in HBECs that were obtained from a commercial source. In this chapter HBECs were cultured from bronchial brushings obtained from living volunteers by bronchoscopies performed in

house. In the severe asthmatic children study, samples were only taken 24h post RV infection in HBECs. RV16 significantly induced SOCS1 mRNA at 24h and similar trends were observed for RV1B induced SOCS1 mRNA, but not for SOCS3 mRNA. In the mild asthmatic adult study no significant increase was observed of SOCS1 and SOCS3 mRNA post RV16 and RV1B infection up to 48h in HBECs. Furthermore in the severe asthmatic children study an increase was observed in SOCS1 mRNA, but not SOCS3 mRNA. This contradicts what was shown in chapter 3. The *ex vivo* cultured HBECs were cultured according to exactly the same protocol. Differences in SOCS expression due to handling was unlikely to be the explanation. Different batches of RV were used however, although all tested for RV type specificity strength, this might be an explanation of different responsiveness. Another explanation could be that due to HBECs from different donors (9-11 per subgroup in each study) in the present study, more variability was observed in baseline levels of SOCS1 and SOCS3 mRNA, causing difficulty to show significant increased RV induced SOCS1 and SOCS3 mRNA over all subjects.

4.8.3 SOCS1 mRNA baseline levels are increased in severe AA and correlate with RV and IFN study outcomes in severe AA but not mild AA

Several studies have investigated IFN deficiency in AA with contradicting results including the two present studies [47, 49-52]. In the present studies an IFN deficiency was observed in HBECs from severe AA, but not in mild AA. It appears that IFN deficiency may be related to severity of disease; RV induced IFN deficiency was observed in HBECs from severe AA and it was not observed in HBECs from mild AA.

The mechanism behind RV induced IFN deficiency is currently unknown. Increased levels of negative regulators of RV induced signalling could play a role in IFN deficiency in AA. SOCS are well described negative regulators of IFN induced JAK/STAT signalling [190]. Th2 cytokines, such as IL-4 and IL-13, have been shown to induce SOCS1 in HBECs and macrophages [194] (Chapter 3). AA have increased SOCS1 [168] and SOCS3 [162], although differences in SOCS levels have not yet been studied in HBECs from AA compared to NA. SOCS1 and SOCS3 were also induced by RV in HBECs and SOCS1 and SOCS3 negatively regulated RV induced IFN signalling and possibly IFN production in HBECs (Chapter 3). This could indicate a role for SOCS1 and SOCS 3 in IFN deficiency in AA, which was investigated in this chapter. As mentioned in section 4.3.1 no increase was observed in SOCS3 in HBECs or lung biopsies, SOCS1 however was increased in biopsies from mild AA and untreated HBECs in severe AA. When no IFN deficiency was observed (in the mild atopic asthmatic study) no correlation was observed with increased SOCS1 protein levels in AA. As mentioned previously, in this study no increase of SOCS1 in untreated HBECs was observed. In AA HBECs that do not have RV induced IFN deficiency no differences are observed in SOCS1 and SOCS3 mRNA levels in HBECs. SOCS1 was increased in lung

biopsies from AA compared to NA suggesting a role for SOCS1 in atopic asthma. HBECs were cultured for several passages before experiments were completed on these, while bronchial biopsies are processed immediately. Increased inflammatory mediators and Th2 cytokines are present in the lung of AA [187, 188] which could contribute to increased induction of SOCS1 protein in the bronchium. Due to culturing HBECs away from the lung and these mediators this might not be observed .

It is noteworthy that SOCS1 mRNA levels in severe AA were elevated compared to NA, suggesting that severe AA have a higher baseline of SOCS1 mRNA and therefore a greater potential for SOCS1 protein production and SOCS1 mediated inhibition of IFN production. In the severe AA an IFN deficiency was observed which correlates with RV release in HBECs. Here a negative correlation was observed between the increased SOCS1 mRNA in AA and RV1B induced type I and III IFNs and a positive correlation with RV16 release in HBECs. Trends were observed for similar correlations with RV16 induced type I and III IFNs and RV1B release. These results suggested that the increased baseline levels of SOCS1 in HBECs were involved in regulating RV induced IFN deficiency in AA and consequently increased RV release. Although a trend was observed towards increased RV1B and RV16 induced SOCS1 mRNA at 8h at 24h no differences were observed. Furthermore, no differences were observed in SOCS1 protein levels between NA and AA in HBECs. The RV induced SOCS1 mRNA levels might be explained by a maximum amount of SOCS1 mRNA being produced and with the increased levels already present they could not be increased to a higher level. This would not explain SOCS1 protein levels observed, although western blotting as a method was complex due to large differences in amount of protein in samples, shown by α-tubulin. Adjustments were made for loading but it is a semi-quantitative measurement at best. The recent discovery that SOCS1 can act as nuclear protein inhibiting the function of NF-kB p65 suggests that the cellular distribution of SOCS1 may be important in SOCS1 function [181]. Further studies are required to investigate if AA have enhanced nuclear translocation of SOCS1, this may account for the impaired IFN production in AA without necessarily having differences in overall abundance of SOCS1 protein as detectable by western blot.

Other negative regulators of this pathway could be involved and would be interesting to investigate, such as LGP2, A20 and Pin1 (section 1.4.3). SOCS1 is however the first protein to be associated with RV induced IFN deficiency in asthma. Overall, the data showed elevated SOCS1 mRNA levels in bronchial epithelial cells of AA and elevated SOCS1 protein in bronchial biopsies, and that elevated levels of SOCS1 mRNA correlated with diminished innate immunity to RV infection. Furthermore, the correlations of SOCS1 levels and asthma disease severity suggest that SOCS1 induction is related to

asthma severity. This is the first report of an association between SOCS1 expression with innate immunity to RV infection.

4.9 Conclusion

SOCS1 but not SOCS3 was increased in AA in HBECs and lung biopsies. RV induced IFN deficiency in HBECs from AA appears to be related to asthma severity. Increased SOCS1 mRNA in HBECs from severe AA was increased at baseline and was related to decrease RV induced IFNs and increased RV release in HBECs. This however appeared to be unrelated to asthma alone. This is the first report associating increased SOCS1 expression with reduced IFN production and RV release.

5 Role of SOCS1 in *in vivo* RV1B infection

5.1 Introduction

In this thesis it has been shown that SOCS1 is induced upon RV infection and plays a role in negatively regulating RV induction of type I and III IFNs in HBECs. SOCS1 mRNA levels negatively correlate with defective RV induced IFN mRNA expression in HBECs from AA and NA. Although this data suggests an important role for SOCS1 in regulating RV induced IFN expression in asthma, this data is based on studies *in vitro* and associations between SOCS1, IFN and RV load *ex vivo*. In this chapter, the role of SOCS1 in a mouse model of RV1B infection was investigated. There were several rationales for moving to an *in vivo* model. In Chapter 3 it was sought to determine whether knockdown of SOCS1 by siRNA would show increased RV induced IFN responses showing the opposite of overexpression of SOCS1 on RV1B induced IFNs shown in Chapter 3, however SOCS1 siRNA did not show increased RV induced IFN mRNA. Here it was aimed to determine whether SOCS1 deficient mice or their cells would show increased RV induced IFN mRNA and protein therefore confirming the functional importance of SOCS1 as a negative regulator of IFN expression.

Previous studies have shown that mice lacking the SOCS1 gene die within weeks due to IFN-γ induced inflammation [146, 222]. SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice survive however and have been studied in models of atopy and allergic airway inflammation. Upon saline treatment, SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice have higher levels of total cells and eosinophilia in the BAL compared to IFN-γ$^{-/-}$ C57/Bl6 mice [169] indicating that in the absence of treatment, SOCS1$^{-/-}$ mice are biased towards a Th2 response suggesting that SOCS1 in mice may regulate both Th1 and Th2 responses. Upon OVA treatment, the differences in total cells and eosinophilia in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 were even greater [169] and other Th2 markers were increased, such as CD4+ T cell derived Th2 cytokines and serum IgE compared to IFN-γ$^{-/-}$ C57/Bl6. Intratracheal IL-13 treatment also showed increased levels of eosinophilia and the Th2 chemokine Eotaxin/CCL11 in BAL from SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 compared to IFN-γ$^{-/-}$ C57/Bl6 [170]. The above data show that in the mouse, SOCS1 is a negative regulator of Th2 immunity with knockouts having increased Th2 responses prior to and during OVA or IL-13 treatment. These experiments suggest that SOCS1 might be a less ideal target for increased IFN responses in the context of virus induced allergic airway inflammation. The role of SOCS1 *in vivo* during viral infections has not been studied however.

Recently RV1B infection models have been described in Balb/c and C57/Bl6 mice [223, 224]. In these models, successful RV1B infection is shown by increases in inflammatory cells in BAL. An early influx of neutrophils into the lung peaking at d1 and a delayed influx of lymphocytes peaking at d4 was observed upon RV1B infection compared to UV-RV1B treated mice. Type I and III IFNs and chemokines were induced upon RV1B infection. In addition, a model of RV induced exacerbation of

OVA induced allergic airway disease has been described [223, 225]. In this model RV and OVA treated mice show increased levels of neutrophils and lymphocytes compare to RV and OVA alone control mice, increased mucins and Th2 cytokines were also shown.

In this chapter IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice were used to investigate RV1B infection of *ex vivo* cultured BAL macrophages of these mice and an RV1B infection model *in vivo*. In the RV1B infection model responses to infection between IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice were compared, investigating type I and III IFN responses, differences in inflammatory cell influx into BAL, RV vRNA and pro-inflammatory chemokine production. The role of SOCS1 in RV induced exacerbation of allergic airway disease was also planned to investigate. Due to amount of SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 and IFN-γ$^{-/-}$ C57/Bl6 mice bred and increased allergic airway inflammation in these mice previously shown[169, 170], this was not investigated. Furthermore, in the RV/OVA induced exacerbation model increased RV replication and type I and III IFN was previously observed in the RV/OVA group compared to RV controls (Nicholas Glanville, PhD thesis), with the increased IFN likely due to increased repluication at Day 1 and Day 2 post infection. The role of SOCS1 in the RV/OVA model and role in moculating IFN and hence RV replication would be of interest to the present study. Instead, IL-13 pretreatment and RV1B infection was investigated with IL-13 providing a means to induce SOCS1 prior to RV1B infection in an attempt to mimic increased SOCS1 in allergic airway disease, without increased IFN responses in the control groups. Finally, the results obtained in this chapter could indicate whether SOCS1 could be realistic target *in vivo*, allowing insights into RV responses in the airways including IFN, RV vRNA and inflammation in the absence of SOCS1.

The main hypothesis was that RV1B infected and IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice would have higher levels of RV1B induced type I and III IFN responses and type I and III IFNs are upregulated for an increased amount of time compared to IFN-γ$^{-/-}$ C57/Bl6 control mice. It was also hypothesised that upon RV1B infection in C57/Bl6 mice SOCS1 mRNA and protein was induced and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice would have similar levels of chemokines upon RV1B infection compared to IFN-γ$^{-/-}$ C57/Bl6 mice, confirming that SOCS1 is a negative regulator of RV1B induced IFN but not of RV1B induced inflammation.

5.2 Results

5.3 RV1B infection in C57/Bl6 mice

5.3.1 RV1B infection in C57/Bl6 mice induced inflammatory cells in BAL, type I and III IFN mRNA in the lung and type I and III IFN and chemokines in BAL fluid

To confirm whether RV1B successfully infected C57/Bl6 mice after intranasal (i.n.) challenge it was determined whether RV1B induced inflammatory cells in BAL as previously shown in BALB/c mice and C57/Bl6 mice [223, 224], it was determined whether RV1B infection induced neutrophils, lymphocytes, macrophages and total cells in C57/Bl6 mice compared to UV-RV1B in C57/Bl6 mice.

A significant increase in total cells in BAL of RV1B infected C57/Bl6 mice was observed at d4 and d7 compared to UV-RV1B infected C57/Bl6 mice, macrophages at d7 and neutrophils at d1. A prolonged trend to RV1B induced increased lymphocytes was observed, detectable at d2 and increasing d7 (data not shown).

To further determine whether RV1B infection in mice showed similar outcomes as previously shown [223, 224], IFN-β and IFN-λ (IL-28) mRNA were determined in the lung and IFN-α, IFN-β, IFN-λ, the neutrophil attracting chemokines KC/CXCL1, MIP-2/CXCL2, and the T cell attracting chemokines IP-10/CXCL10, RANTES/CCL5 protein were determined in BAL fluid of RV1B infected and UV-RV1B treated C57/Bl6 mice.

RV1B significantly induced IFN-β and IFN-λ mRNA at 8h post infection in the lung of C57/Bl6 mice compared to UV-RV1B treated C57/Bl6 mice RV1B significantly induced IFN-α and IFN-λ protein at d1 post infection in BAL fluid of C57/Bl6 mice compared to UV-RV1B treated C57/Bl6 mice RV1B did not induce IFN-β protein in BAL fluid of C57/Bl6 mice compared to UV-RV1B treated C57/Bl6 mice (data not shown).

RV1B significantly induced KC/CXCL1 protein levels at 8h and d1, MIP-2/CXCL2 at 8h, IP-10/CXCL10 at d1 and RANTES/CCL5 at 8h and d2 in BAL from C57/Bl6 mice compared to UV-RV1B treated C57/Bl6 mice (data not shown).

5.3.2 RV1B infection in C57/Bl6 mice induced SOCS1 and SOCS3 mRNA and SOCS1 protein in the lung

To assess whether RV1B infection in C57/Bl6 mice induced SOCS1 and SOCS3 mRNA and SOCS1 protein in the lung compared to UV-RV1B treated mice, SOCS1 and SOCS3 mRNA and SOCS1 protein were determined in the lung.

RV1B significantly induced SOCS1 and SOCS3 mRNA at 8h ($p<0.01$, SOCS1; $p<0.001$, SOCS3) and d1 ($p<0.05$, SOCS1; $p<0.01$, SOCS3) post infection in the lung of C57/Bl6 mice compared to UV-RV1B treated C57/Bl6 mice (Figure 5.1A and Figure 5.1B). SOCS1 protein was induced in the lung of RV1B infected C57/Bl6 mice at 8h, d1, d4 and d7 post infection compared to UV-RV1B treated C57/Bl6 mice (Figure 5.1C).

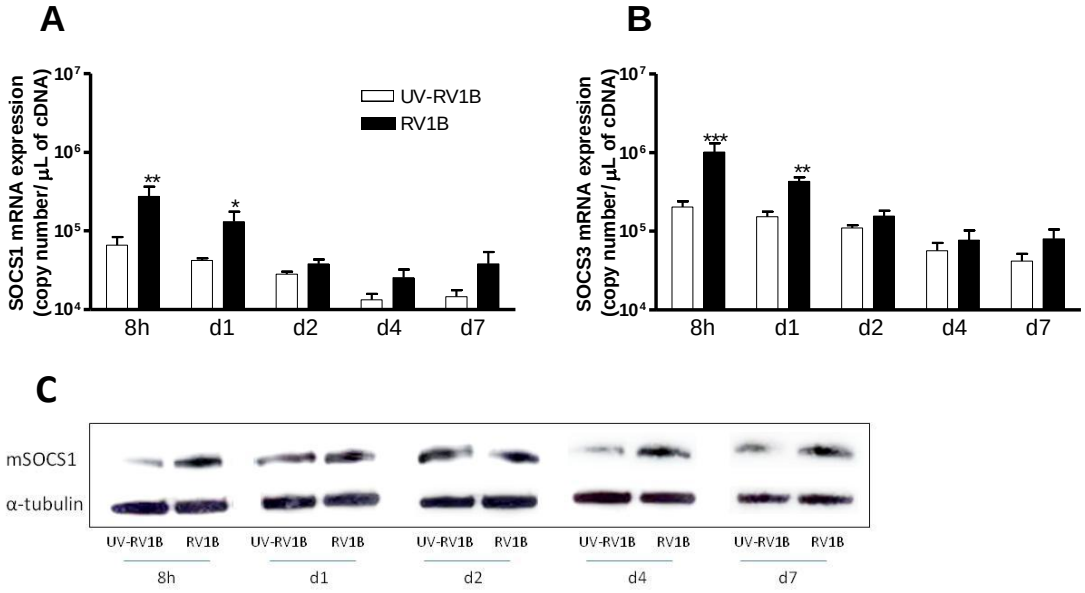

Figure 5.1: RV1B induced SOCS1 and SOCS3 mRNA and SOCS1 protein in lung from C57/Bl6 mice.

RV1B induced SOCS1 mRNA (A) and SOCS3 mRNA (B) and SOCS1 protein (C) in lung from C57/Bl6 mice compared to UV-RV1B treated C57/Bl6 mice. Open bars denote UV-RV1B, black bars denote RV1B. mRNA data represent mean (±SEM) of 2 experiments with each experiment having 4 mice per group, western blot data is presented from protein lysate from 1 mouse, representative of all samples.

5.4 RV1B infection in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 compared to IFN-γ$^{-/-}$ C57/Bl6 mice and wild type C57/Bl6 mice

To investigate the role of SOCS1 in RV1B induced type I and III IFNs and chemokines and inflammatory cells *in vivo*, SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6, IFN-γ$^{-/-}$ C57/Bl6 and wild type (wt) C57/Bl6 mice were infected with RV1B and outcomes previously described were investigated.

5.4.1 Confirmation of knockout of SOCS1 and IFN-γ$^{-/-}$ in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 and IFN-γ$^{-/-}$ in IFN-γ$^{-/-}$ C57/Bl6 mice

To ensure adequate knockdown of SOCS1 and IFN-γ genes in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 and IFN-γ gene in IFN-γ$^{-/-}$ C57/Bl6 mice, SOCS1 and IFN-γ mRNA was determined in the lung of SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6, IFN-γ$^{-/-}$ C57/Bl6 and C57/Bl6 mice. SOCS1 mRNA expression was almost completely knocked back in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 (Figure 5.2A) and IFN-γ mRNA was completely knocked back in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 and IFN-γ$^{-/-}$ C57/Bl6 (Figure 5.2B).

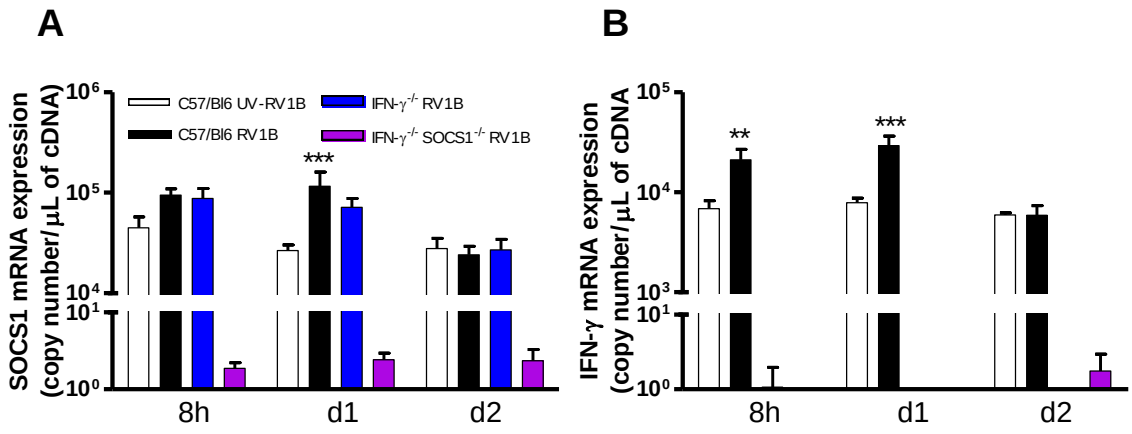

Figure 5.2: RV1B induced SOCS1 and IFN-γ mRNA in lung from C57/Bl6 mice, IFN-γ$^{-/-}$ C57/Bl6 mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice.

RV1B induced SOCS1 mRNA (A) and IFN-γ mRNA (B) in lung from C57/Bl6 mice, IFN-γ$^{-/-}$ C57/Bl6 mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice compared to UV-RV1B treated C57/Bl6 mice. Open bars denote UV-RV1B in C57/Bl6 mice, black bars denote RV1B in C57/Bl6 mice, blue bars denote RV1B in IFN-γ$^{-/-}$ C57/Bl6 mice. Data represent mean (±SEM) of 2 experiments with each experiment having 4 mice per group.

5.4.2 RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice had increased lymphocytes and eosinophils compared to RV1B infected IFN-γ$^{-/-}$ C57/Bl6 mice or wild type C57/Bl6 mice in BAL

To assess whether inflammatory cell influx in the lung of in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice upon RV1B infection compared to IFN-γ$^{-/-}$ and wt mice was different; total BAL cells, BAL macrophages, lymphocytes, neutrophils and eosinophils were determined.

Total cells were significantly increased in BAL at d2 in RV1B infected wt mice ($p<0.05$) compared to UV-RV1B treated wt mice (Figure 5.3A). No differences in total cell counts were observed between RV1B infected IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice (Figure 5.3A).

Macrophages were significantly increased in BAL at d2 in RV1B infected wt mice compared to UV-RV1B treated wt mice (Figure 5.3B; $p<0.05$). No differences in BAL macrophages were observed between RV1B infected IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice (Figure 5.3B).

Lymphocytes were significantly increased in BAL at d2 in RV1B infected wt mice compared to UV-RV1B treated wt mice (Figure 5.3C; $p<0.05$). RV1B infected SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice had significantly more BAL lymphocytes than RV1B infected IFN-$\gamma^{-/-}$ mice at d2 post infection (Figure 5.3C, $p<0.05$).

Neutrophils were significantly increased in BAL at d1 in RV1B infected wt mice (Figure 5.3D; $p<0.01$) compared to UV-RV1B treated wt mice. No differences in BAL neutrophils were observed between RV1B infected IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice (Figure 5.3D).

Eosinophils were not increased in BAL in RV1B infected wt mice compared to UV-RV1B treated wt mice (Figure 5.3E). RV1B infected SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice had significantly more BAL eosinophils than RV1B infected IFN-$\gamma^{-/-}$ mice at d1 post infection, the same trend was observed at d2 (Figure 5.3E, $p<0.001$).

RV1B infected IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice had a trend towards increased inflammatory cell influx into the lung compare to C57/Bl6 mice, which could be due to increased viral load. Results observed were consistent between experiments.

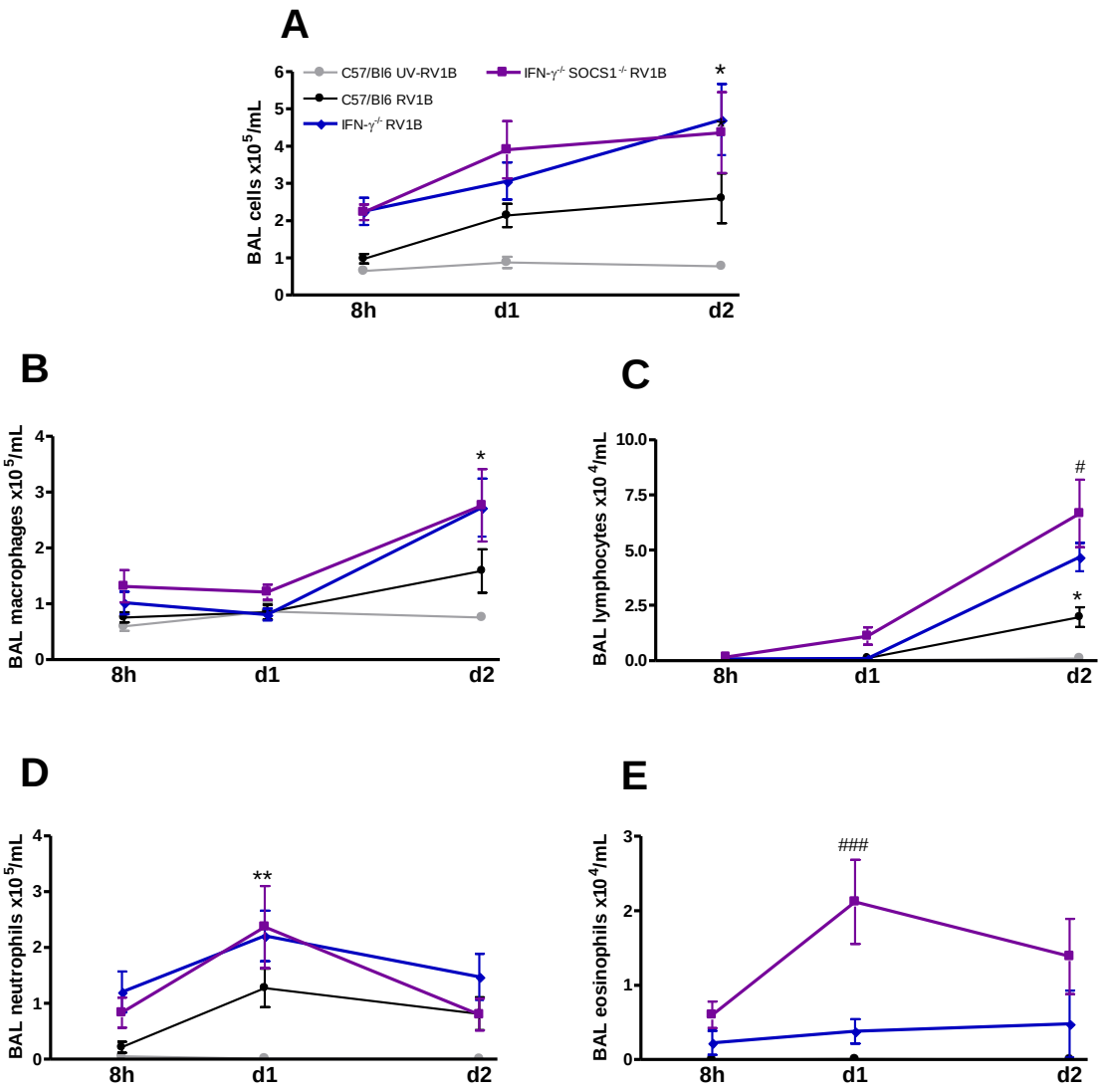

Figure 5.3: Differential and total cells in BAL from RV1B infected C57/Bl6, IFN-γ$^{-/-}$ C57/Bl6 and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice.

Grey line denotes UV-RV1B infected C57/Bl6 mice, black line denotes RV1B in C57/Bl6 mice, blue line denotes RV1B in IFN-γ$^{-/-}$ C57/Bl6 mice, purple line denotes RV1B in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6. Data represent mean (±SEM) of 2 experiments with each experiment having 4 mice per group. * denotes p<0.05 between UV-RV1B and RV1B infected wt mice, ** denotes p<0.01 between UV-RV1B and RV1B infected C57/Bl6 mice, # denotes p<0.05 between RV1B infected IFN-γ$^{-/-}$ mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice , ### denotes p<0.001 between RV1B infected IFN-γ$^{-/-}$ mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice.

5.4.3 RV1B infection in SOCS1$^{-/-}$ IFNγ$^{-/-}$ C57/Bl6 mice showed no increase in type I and III IFN mRNA and protein production compared to IFNγ$^{-/-}$ C57/Bl6 mice or wild type C57/Bl6 mice

To assess whether knockout of SOCS1 increased RV1B induced type I and III IFN, we determined RV1B induced type I and III IFN mRNA and protein production in wt, IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice.

RV1B significantly induced IFN-β and IFN-λ mRNA in wt mice compared to UV-RV1B treated mice as previously shown (Figure 5.4; IFN-β 8h, $p<0.05$; IFN-λ d1 $p<0.001$). SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice showed a trend towards higher levels of IFN-β mRNA at d2 compared to IFN-γ$^{-/-}$ mice, no differences were observed between these groups at 8h and d1 (Figure 5.4A). IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ showed significantly lower levels of RV1B induced IFN-β mRNA compared to wt mice at d1 post infection (Figure 5.4A, $p<0.05$).

SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice showed decreased IFN-λ mRNA at 8h compared to IFN-γ$^{-/-}$ and wt mice ($p<0.05$, $p<0.001$ respectively), the same trend was observed at d1 and d2 (Figure 5.4B).

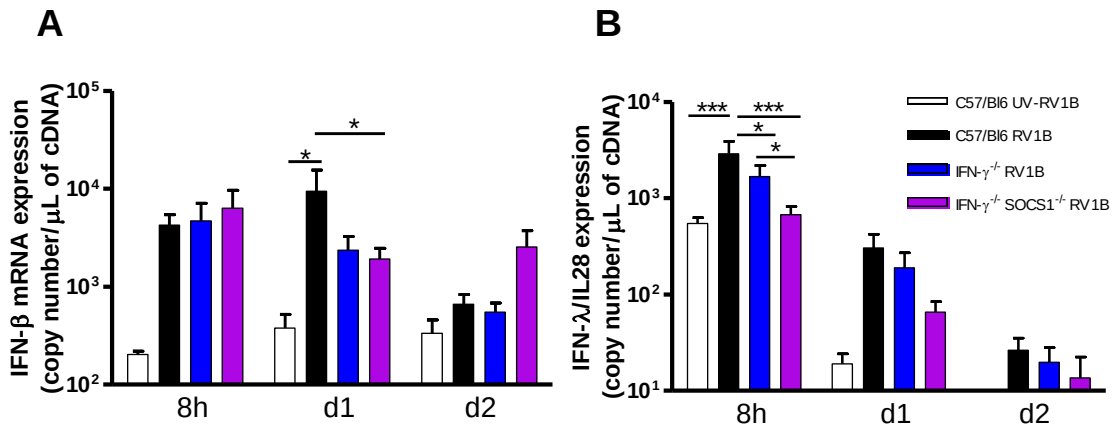

Figure 5.4: RV1B induced IFN-β (A) and IFN-λ mRNA (B) in the lung from RV1B infected C57/Bl6, IFN-γ$^{-/-}$ C57/Bl6 and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice.

Open bars denote UV-RV1B infected C57/Bl6 mice, black bars denote RV1B in C57/Bl6 mice, blue bars denote RV1B in IFN-γ$^{-/-}$ C57/Bl6 mice, purple bars denote RV1B in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6. Data represent mean (±SEM) of 2 experiments with each experiment having 4 mice per group.

RV1B significantly induced IFN-α and IFN-λ protein in BAL from wt mice compared to UV-RV1B treated wt mice as previously shown (Figure 5.5; d1 $p<0.01$, d2 $p<0.001$), IFN-β protein was not induced. Wt mice had significantly increased levels of IFN-α compared to IFN-γ$^{-/-}$ and IFN-γ$^{-/-}$ SOCS1$^{-/-}$ mice at d2 post RV1B infection ($p<0.001$), the same trend was observed at d1 (Figure 5.5A). No differences were observed between RV1B induced type I and III IFNs in IFN-γ$^{-/-}$ mice and IFN-γ$^{-/-}$ SOCS1$^{-/-}$ (Figure 5.5).

No differences were observed between RV1B induced type I and III IFNs in IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice, however C57/Bl6 mice had higher levels of RV1B induced type I and III IFNs compared to RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice. In Section 5.4.2 it was discussed that increased inflammatory cell influx in RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice could be due to increased viral load compared to C57/Bl6 mice. Increased viral load in RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ^{-}

$^{-/-}$ mice compared to C57/Bl6 mice could also be related to a reduced induction of IFNs by RV1B which was observed here. Results observed were consistent between experiments.

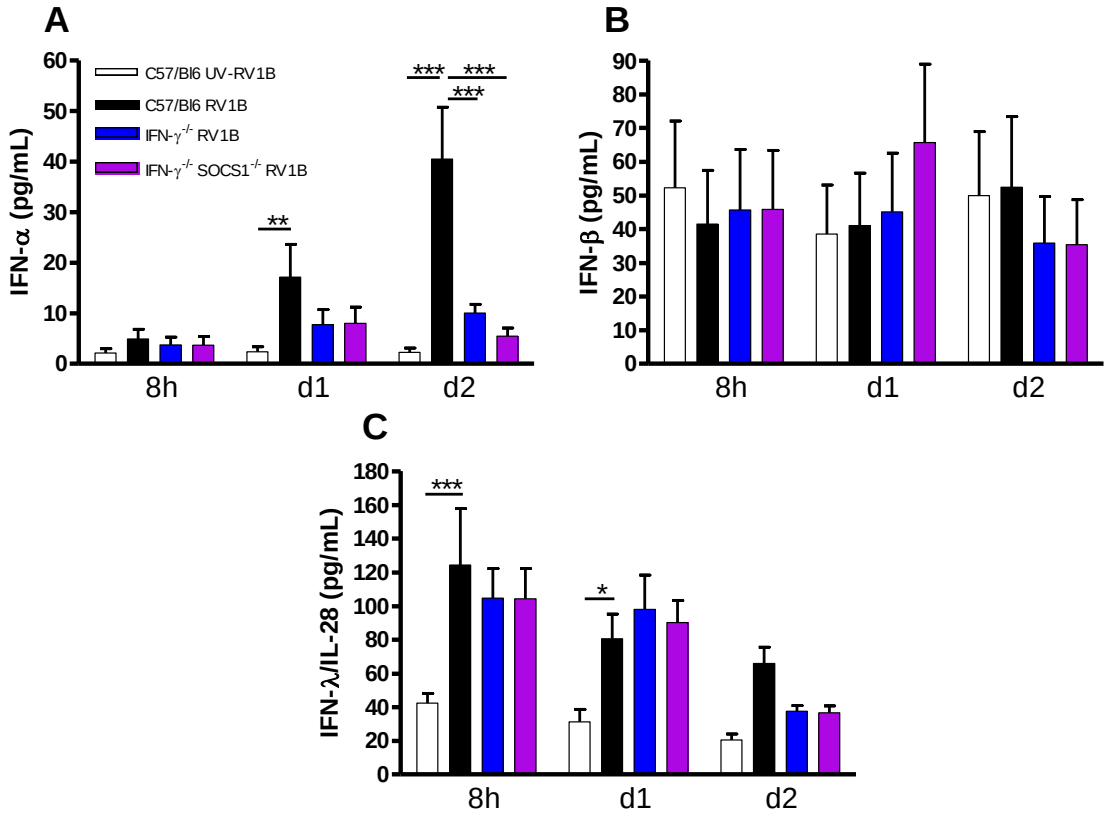

Figure 5.5: RV1B induced IFN-α (A), IFN-β (B) and IFN-λ (C) protein in BAL from C57/Bl6 mice, IFN-γ$^{-/-}$ C57/Bl6 mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 infected mice.

Open bars denote UV-RV1B infected C57/Bl6 mice, black bars denote RV1B in C57/Bl6 mice, blue bars denote RV1B in IFN-γ$^{-/-}$ C57/Bl6 mice, purple bars denote RV1B in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6. Data represent mean (±SEM) of 2 experiments with each experiment having 4 mice per group.

5.4.4 RV mRNA was not different between SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6, IFN-γ$^{-/-}$ C57/Bl6 mice and wt C57/Bl6 mice in the lung after i.n infection with RV1B

To investigate whether knockout of SOCS1 had an effect on RV replication upon RV1B infection *in vivo,* RV vRNA levels were determined in the lung of RV1B infected wt, IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice.

A trend towards an increase in RV mRNA was observed in IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to wt mice 8h post infection and a trend towards decreased RV vRNA in IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice at d2 post infection compared to wt mice (Figure 5.6). No differences were observed between RV vRNA from RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice (Figure 5.6).

As discussed in Section 5.4.2 and 5.4.3 the trend towards increased RV vRNA could explain increased RV1B induced inflammatory cell influx and reduced RV1B induced type I and III IFNs in RV1B infected IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice compared to C57/Bl6 mice. This might have impaired our ability in these experiments to observe difference in type I and III IFN induction between RV1B infected IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice. Results observed were consistent between experiments.

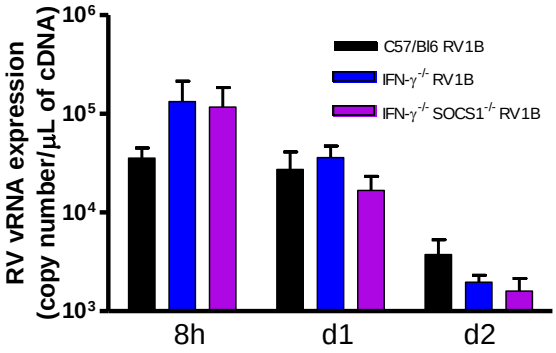

Figure 5.6: RV1B vRNA in lung from C57/Bl6 mice, IFN-$\gamma^{-/-}$ C57/Bl6 mice and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ C57/Bl6 infected mice.

Black bars denote RV1B in C57/Bl6 mice, blue bars denote RV1B in IFN-$\gamma^{-/-}$ C57/Bl6 mice, purple bars denote RV1B in SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ C57/Bl6. Data represent mean (±SEM) of 2 experiments with each experiment having 4 mice per group.

5.4.5 RV1B infected SOCS1$^{-/-}$ IFNγ$^{-/-}$ C57/Bl6 mice showed no increase in chemokines compared to RV1B infected IFNγ$^{-/-}$ C57/Bl6 mice or wt C57/Bl6 mice

To assess whether SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice produced higher levels of chemokines upon RV1B infection compared to wt and IFN-$\gamma^{-/-}$ mice, KC/CXCL1 and RANTES/CCL5 protein were determined in RV1B infected wt, IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice.

RV1B infection significantly increased KC/CXCL1 and RANTES/CCL5 protein in BAL from wt mice compared to UV-RV1B treated wt mice (Figure 5.7; KC/CXCL1 8h, $p<0.001$; RANTES/CCL5 d1, $p<0.01$). RV1B infected IFN-$\gamma^{-/-}$ mice had significantly higher levels of KC/CXCL1 protein in BAL compared to wt and SOCS1$^{-/-}$IFN-$\gamma^{-/-}$ mice 8h post infection (Figure 5.7A, $p<0.001$), no differences were observed between the groups at d1 and d2.

RV1B induced RANTES/CCL5 protein was significantly lower in SOCS1$^{-/-}$IFN-$\gamma^{-/-}$ mice compared to wt mice at 8h post infection in BAL ($p<0.05$), no differences were observed between IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$IFN- $\gamma^{-/-}$ mice at any time points (Figure 5.7B).

Increased levels of KC/CXCL1 in RV1B infected IFN-$\gamma^{-/-}$ mice compared to C57/Bl6 mice could again be explained by the trend towards increased vRNA in IFN-$\gamma^{-/-}$ mice described in Section 5.4.4, however the reduced levels of KC/CXCL1 in SOCS1$^{-/-}$IFN-$\gamma^{-/-}$ mice compare to IFN-$\gamma^{-/-}$ mice indicate that SOCS1

did not negatively regulate this chemokine and most likely SOCS1 plays a role in causing increased KC/CXCL1 induced inflammation.

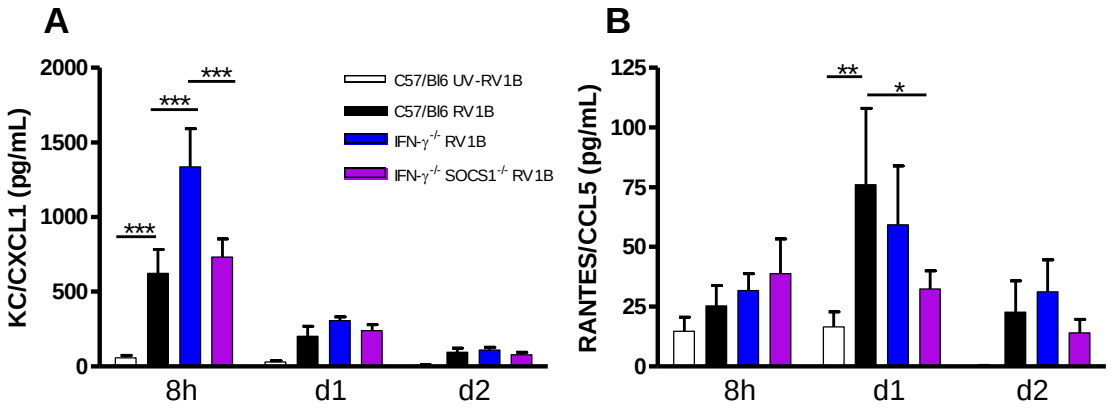

Figure 5.7: RV1B induced KC/CXCL1 (A) and RANTES/CCL5 (B) protein in BAL from C57/Bl6 mice, IFN-γ$^{-/-}$ C57/Bl6 mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 infected mice.

Open bars denote UV-RV1B infected C57/Bl6 mice, black bars denote RV1B in C57/Bl6 mice, blue bars denote RV1B in IFN-γ$^{-/-}$ C57/Bl6 mice, purple bars denote RV1B in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6. Data represent mean (±SEM) of 2 experiments with each experiment having 4 mice per group.

5.5 IL-13 treatment in IFN-γ$^{-/-}$ C57/Bl6 mice

The IL-13 treatment model was designed to determine the effects of increased SOCS1 protein on RV induced IFNs. IFN-γ$^{-/-}$ mice were first used in pilot studies, with the overall aim to eventually perform IL-13 pretreatment in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice. By performing these studies in SOCS1$^{-/-}$ mice and controls, it could be assessed if IL-13 induced SOCS1 could suppress RV induced IFN responses *in vivo*.

5.5.1 IL-13 treatment in IFNγ$^{-/-}$ C57/Bl6 mice induced Th2 chemokines in BAL fluid, but not eosinophilia

Previous studies have used intratracheal IL-13 treatment [170] in mice to induce Th2 chemokines and eosinophils in the lung. Here it was assessed whether the same amount of IL-13 (0.5µg) administered i.n. could induce eosinophilia and Th2 chemokines in the lung.

IL-13 treatment did not induce increased BAL cell influx (Figure 5.8A). IL-13 treatment did not significantly induce any inflammatory cell type (Figure 5.8B) in IFN-γ$^{-/-}$ mice compared to PBS treated mice (>98% macrophages, data not shown).

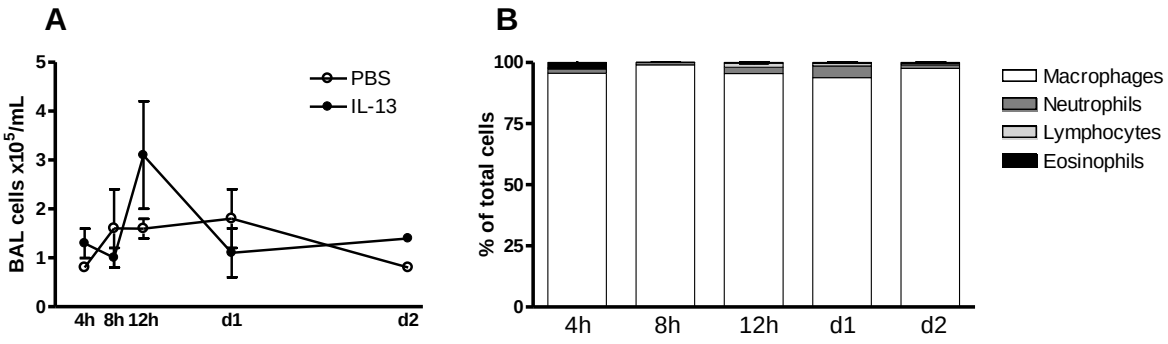

Figure 5.8: IL-13 induced total BAL cells and differential inflammatory cell counts in IFN-γ$^{-/-}$ mice.

(A) Open circles denote PBS treated mice and closed circles denote IL-13 treated mice. (B) Differential inflammatory cell counts (% total cells) in IL-13 treated mice. Data represent mean (±SEM) of 1 experiment, 2 mice per group.

IL-13 treatment in IFN-γ$^{-/-}$ mice induced Th2 chemokines compared to PBS treated mice. IL-13 significantly induced MDC/CCL2 d2 post IL-13 treatment (Figure 5.9A, $p<0.01$), Eotaxin/CCL11 12h post IL-13 treatment (Figure 5.9B, $p<0.01$) and TARC/CCL17 4h, d1 and d2 post treatment (Figure 5.9C; $p<0.05$, $p<0.001$, $p<0.001$ respectively).

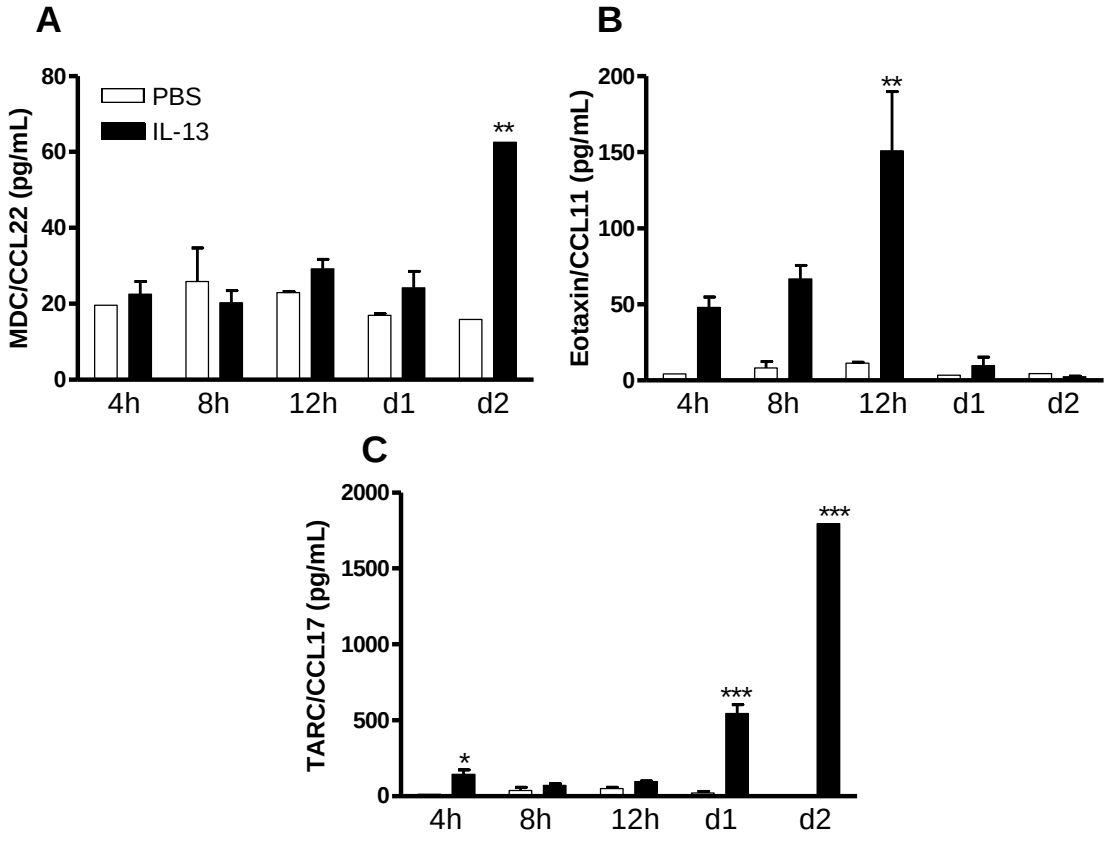

Figure 5.9: IL-13 induced Th2 chemokines in IFN-γ$^{-/-}$ mice, (A) MDC/CCL22, (B) Eotaxin/CCL11, (C) TARC/CCL17.

Open bars denote PBS treated mice and black bars denote IL-13 treated mice. Data represent mean (±SEM) of 1 experiment, 2 mice per group.

5.5.2 IL-13 treatment in IFN γ $^{-/-}$ C57/Bl6 mice induced SOCS1 mRNA in the lung

To assess whether IL-13 treatment could induce SOCS1, SOCS1 mRNA was determined in the lung of IFN-γ$^{-/-}$ mice. IL-13 significantly induced SOCS1 mRNA in the lung of IFN-γ$^{-/-}$ mice compared to PBS treated at 4h, 8h and 12h post treatment (Figure 5.10; $p<0.05$, $p<0.01$, $p<0.01$ respectively).

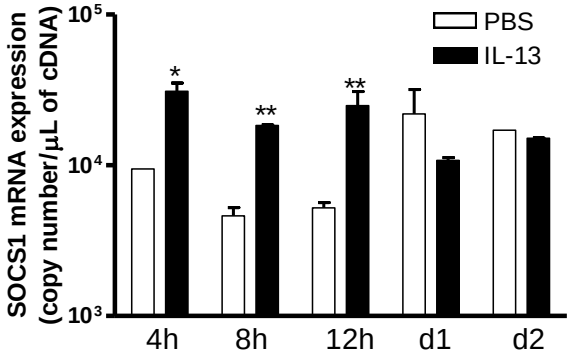

Figure 5.10: IL-13 induced SOCS1 mRNA in IFN- $\gamma^{-/-}$ mice.

Open bars denote PBS treated mice and closed bars denote IL-13 treated mice. Data represent mean (±SEM) of 1 experiment, 2 mice per group.

5.6 IL-13 pretreatment and RV1B infection in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice compared to IFN-γ$^{-/-}$ C57/Bl6 mice or wild type C57/Bl6 mice

IL-13 treatment i.n. increased SOCS1 mRNA levels as shown in Section 5.5.2. It was hypothesised that pretreatment with IL-13 would reduce RV induced IFN in control mice (IFN-γ$^{-/-}$ mice) due to induction of SOCS1, showing lower levels of RV induced IFNs in IFN-γ$^{-/-}$ mice compared to SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice.

5.6.1 IL-13 pretreatment and RV1B infection in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice showed significantly increased total cells, lymphocytes and eosinophils in BAL fluid compared to IFN-γ$^{-/-}$ C57/Bl6 mice

To assess whether SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice had differences in inflammatory cell influx in the lung upon IL-13 treatment and RV1B infection compared to IFN-γ$^{-/-}$ mice; total BAL cells, BAL macrophages, lymphocytes, neutrophils and eosinophils were determined. Control mice used for UV-RV1B treatment and UV-RV1B and IL-13 treatment were SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice.

Total cells were significantly increased in BAL at d1 and d2 in response to virus infection in IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ and IFN-γ$^{-/-}$ mice (Figure 5.11A; d1 $p<0.001$; d2 $p<0.001$) compared to IL-13 and UV-RV1B treated control mice. RV1B infected IL-13 treated SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice had significantly more total BAL cells at d2 post infection compared to IL-13 treated IFN-γ$^{-/-}$ control mice (Figure 5.11A, $p<0.05$).

No differences were observed between macrophages in BAL from IL-13 treated and RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice and IL-13 pretreated UV-RV1B treated control mice (Figure 5.11B).

Lymphocytes were significantly increased in BAL in responses to virus infection at d1 and d2 in IL-13 pretreated RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice and at d2 in IFN-γ$^{-/-}$ mice compared to IL-13 pretreated and UV-RV1B treated mice (Figure 5.11C; $p<0.05$, $p<0.001$, $p<0.001$ respectively). IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice had significantly more lymphocytes compared to IFN-γ$^{-/-}$ mice at d1 and d2 post infection (Figure 5.11C; $p<0.05$, $p<0.01$ respectively).

Neutrophils were significantly increased in response to virus infection in BAL at d1 and d2 in IL-13 pretreated and RV1B infected IFN-γ$^{-/-}$ mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice (Figure 5.11D; $p<0.001$) compared to IL-13 pretreated UV-RV1B treated control mice. No differences in BAL neutrophils were observed between the IL-13 pretreated RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice (Figure 5.11D).

Eosinophils were significantly increased in BAL in response to virus infection at d1 and d2 in IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice and d2 in IL-13 pretreated and RV1B infected IFN-γ$^{-/-}$ mice compared to IL-13 pretreated and UV-RV1B treated wt mice (Figure 5.11E; $p<0.001$ SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice; $p<0.05$ IFN-γ$^{-/-}$ mice). IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice had markedly and significantly more BAL eosinophils than IL-13 pretreated RV1B infected IFN-γ$^{-/-}$ mice at d1 and d2 post infection (Figure 5.11E, $p<0.001$).

Increased levels of total inflammatory cell influx in response to RV1B infection in IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compare to IL-13 pretreated and RV1B infected IFN-γ$^{-/-}$ mice was probably due to increased lymphocytes and eosinophils in the IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice. Increased eosinophils in response to RV1B infection in IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compare to IL-13 pretreated and RV1B infected IFN-γ$^{-/-}$ mice indicates that in the absence of SOCS1 mice are biased towards a Th2 response as previously shown [169, 170]. In IFNAR knockout mice it has been shown that lymphocytes are not induced upon RV1B infection, indicating that lymphocytes induction is dependent on type I IFN signalling (Bartlett et all, manuscript in preparation). Increased lymphocytes in response to RV1B infection in IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IL-13 pretreated and RV1B infected IFN-γ$^{-/-}$ mice could indicate increased protection from virus infection due to increased RV1B induced type I IFNs.

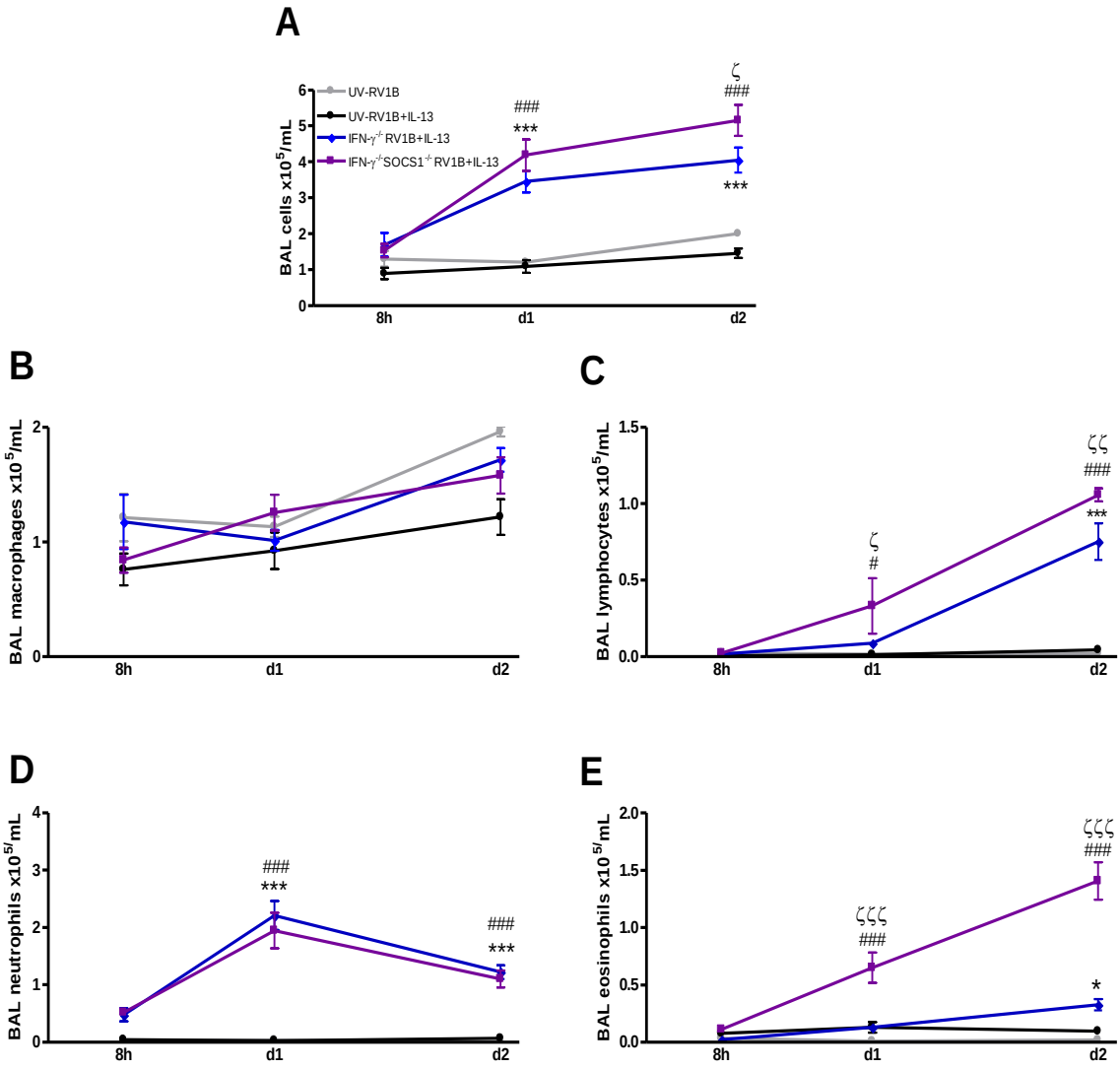

Figure 5.11: Differential and total cells in BAL from IL-13 pretreated RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice.

Grey line denotes UV-RV1B control mice, black line denotes IL-13/UV-RV1B control mice, blue line denotes IL-13/RV1B in IFN-γ$^{-/-}$ C57/Bl6 mice, purple line denotes IL-13/RV1B in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6. Data represent mean (±SEM) of 3 experiments with each experiment having 4-7 mice per group. * denotes p<0.05 between IL-13/UV-RV1B mice and IL-13/RV1B IFN-γ$^{-/-}$ mice, *** denotes p<0.001 between IL-13/UV-RV1B mice and IL-13/RV1B IFN-γ$^{-/-}$ mice, # denotes p<0.05 between IL-13/UV-RV1B mice and IL-13/RV1B SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice, ### denotes p<0.001 between IL-13/UV-RV1B mice and IL-13/RV1B SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice, ζ denotes p<0.05 between IL-13/ RV1B IFN-γ$^{-/-}$ mice and IL-13/RV1B SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice, ζζ denotes p<0.01 between IL-13/ RV1B IFN-γ$^{-/-}$ mice and IL-13/RV1B SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice, ζζζ denotes p<0.001 between IL-13/ RV1B IFN-γ$^{-/-}$ mice and IL-13/RV1B SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice.

5.6.2 IL-13 pretreatment and RV1B infection in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice showed increased Eotaxin/CCL11 in BAL fluid compared to IFN-γ$^{-/-}$ C57/Bl6 mice

To confirm whether IL-13 pretreatment induced Th2 mediated inflammation, it was determined whether IL-13 induced eotaxin/CCL11. IL-13 pretreatment significantly increased eotaxin/CCL11 in BAL from UV-RV1B treated control mice at 8h (Figure 5.12, p<0.001) and RV1B infected IFN-γ$^{-/-}$ and

SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice at 8h and d1 compared to UV-RV1B treated control mice (Figure 5.12, p<0.001). BAL from IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice had significantly higher levels of eotaxin/CCL11 compared to IL-13 pretreated UV-RV1B treated mice and RV1B infected IFN-γ$^{-/-}$ mice at 8h (Figure 5.12; p<0.001) and d1 (p<0.001 and p<0.01 respectively).

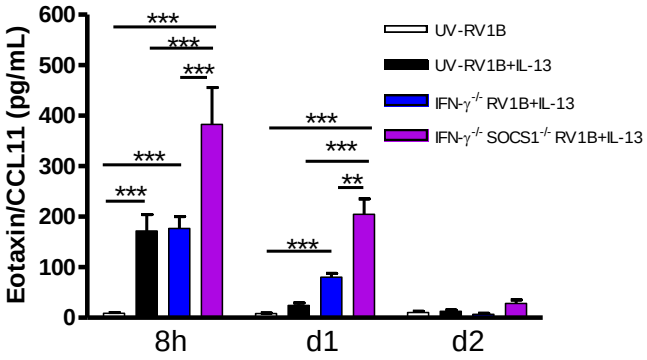

Figure 5.12: Eotaxin/CCL11 induction in IL-13 pretreated UV-RV1B treated control mice and RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice.

Open bars denote UV-RV1B mice, black bars denote UV-RV1B/IL-13 mice, blue bars denote IL-13/RV1B in IFN-γ$^{-/-}$ C57/Bl6 mice, purple bars denote IL-13/RV1B in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6. Data represent mean (±SEM) of 3 experiments with each experiment having 4-7 mice per group.

5.6.3 IL-13 pretreatment and RV1B infection in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice showed increased type I and III IFN production in BAL fluid compared to IFN-γ$^{-/-}$ C57/Bl6 mice

To assess whether knockout of SOCS1 increased RV1B induced type I and III IFN, we determined RV1B induced type I and III IFN mRNA and protein production in IL-13 pretreated and RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice.

RV1B significantly induced IFN-β and IFN-λ mRNA in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IL-13 pretreated UV-RV1B treated mice 8h and d1 post infection (Figure 5.13; IFN-β, 8h p<0.001, d1 p<0.05; IFN-λ, 8h p<0.01, d1 p<0.05) and IFN-γ$^{-/-}$ mice at 8h (Figure 5.13; p<0.001), the same trend was observed at d1 and d2 post infection. No differences were observed between RV1B induced IFN-β and IFN-λ mRNA levels between IL-13 treated and RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice (Figure 5.13).

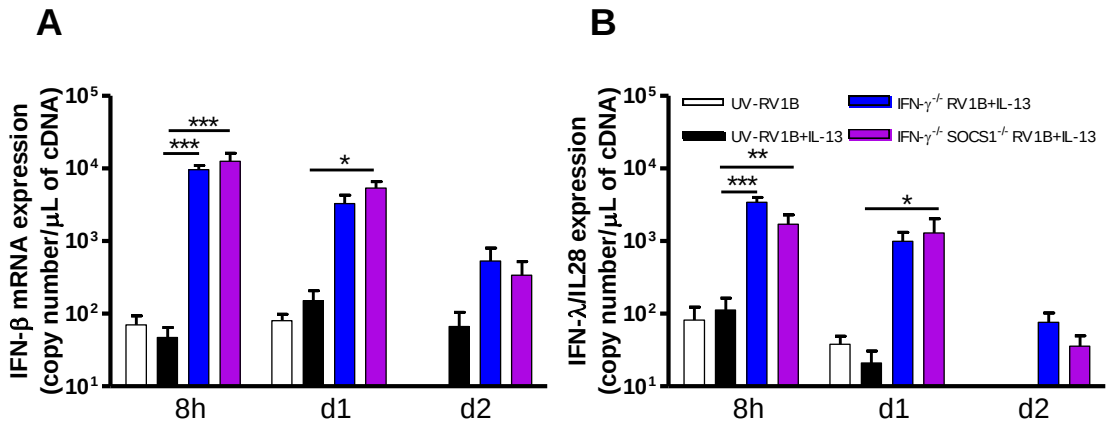

Figure 5.13: RV1B induced IFN-β (A) and IFN-λ mRNA (B) in the lung from IL-13 pretreated RV1B infected IFN-γ$^{-/-}$ C57/Bl6 and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice.

Open bars denote UV-RV1B mice, black bars denote UV-RV1B/IL-13 mice, blue bars denote IL-13/RV1B in IFN-γ$^{-/-}$ C57/Bl6 mice, purple bars denote IL-13/RV1B in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6. Data represent mean (±SEM) of 3 experiments with each experiment having 4-7 mice per group.

RV1B significantly induced IFN-α protein in BAL from IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice at 8h ($p<0.01$ and $p<0.001$ respectively) and d1 ($p<0.001$) compared to IL-13 pretreated UV-RV1B treated mice (Figure 5.14A). IFN-β protein was not significantly induced by RV1B in either group (Figure 5.18B). RV1B significantly induced IFN-λ in BAL from both IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice at 8h ($p<0.001$) and d1 ($p<0.001$) compared to IL-13 pretreated UV-RV1B treated mice (Figure 5.14C). IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice had significantly increased levels of IFN-α compared to IFN-γ$^{-/-}$ mice 8h post RV1B infection ($p<0.001$, Figure 5.14A) and IFN-λ at d1 ($p<0.05$, Figure 5.14C). The same trend was observed at 8h for IFN-β (Figure 5.14B) and IFN-λ (Figure 5.14C) and at d1 for IFN-α (Figure 5.14A).

No differences were observed between RV1B induced type I and III IFN mRNA levels in IL-13 pretreated and RV1B infected IFN-γ$^{-/-}$ mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice, however SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice had increased levels of IFN-α and IFN-λ protein compared to IFN-γ$^{-/-}$ mice. This indicates that in the absence of SOCS1 mice have increased RV1B induced type I and III IFN production.

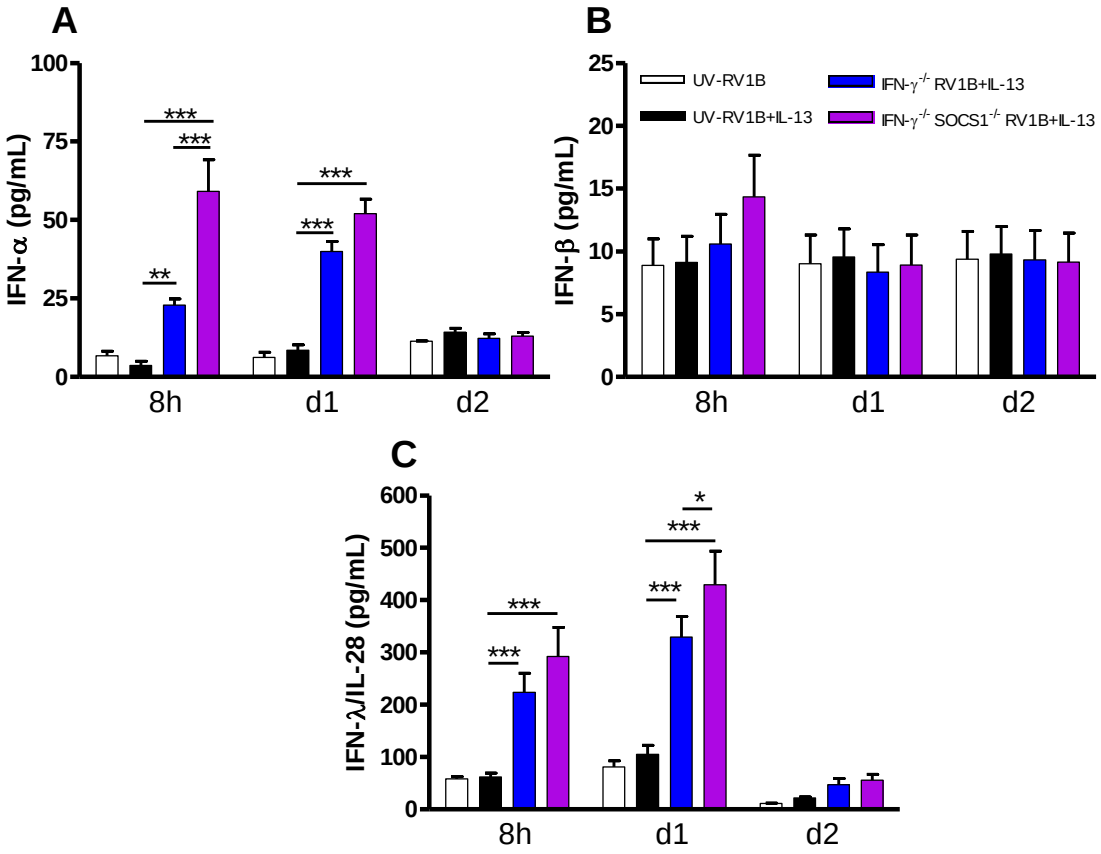

Figure 5.14: RV1B induced IFN-α (A), IFN-β (B) and IFN-λ (C) protein in BAL from IL-13 pretreated and RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ $^{-/-}$ C57/Bl6 mice.

Open bars denote UV-RV1B mice, black bars denote UV-RV1B/IL-13 mice, blue bars denote IL-13/RV1B in IFN-γ$^{-/-}$ C57/Bl6 mice, purple bars denote IL-13/RV1B in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6. Data represent mean (±SEM) of 3 experiments with each experiment having 4-7 mice per group.

5.6.4 IL-13 pretreatment and RV1B infection in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice showed no differences in RV mRNA in the lung compared to IFN-γ$^{-/-}$ C57/Bl6 mice

To investigate whether knockout of SOCS1 had an effect on RV replication with IL-13 pretreatment and RV1B infection *in vivo,* RV vRNA levels were determined in the lung of IL-13 pretreated and RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice.

No significant differences were observed between RV vRNA from IL-13 pretreated and RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice, however a trend towards an decrease in RV vRNA was observed in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IFN-γ$^{-/-}$ mice d2 post infection (Figure 5.15).

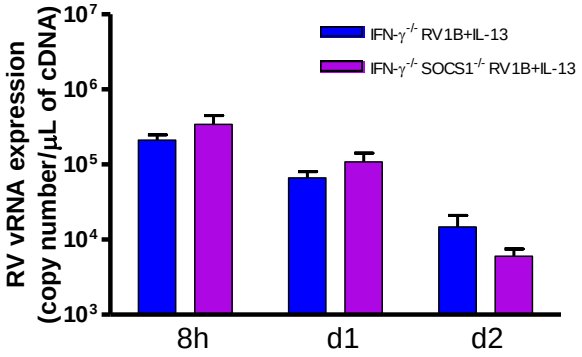

Figure 5.15: RV1B vRNA in lung from C57/Bl6 mice, IFN-γ$^{-/-}$ C57/Bl6 mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 infected mice.

Blue bars denote IL-13/RV1B in IFN-γ$^{-/-}$ C57/Bl6 mice, purple bars denote IL-13/RV1B in SOCS1$^{-/-}$ IFN- γ$^{-/-}$ C57/Bl6. Data represent mean (±SEM) of 3 experiments with each experiment having 4-7 mice per group.

5.6.5 IL-13 pretreatment and RV1B infection in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice showed no differences in KC/CXCL1, decreased LIX/CXCL5 and increased RANTES/CCL5 in BAL fluid compared to IFN-γ$^{-/-}$ C57/Bl6 mice

To assess whether SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice produced higher levels of chemokines upon IL-13 pretreatment and RV1B infection compared to IFN-γ$^{-/-}$ mice, KC/CXCL1, LIX/CXCL5 and RANTES/CCL5 protein were determined in IL-13 pretreated RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice.

RV1B infection significantly increased KC/CXCL1 at 8h (Figure 5.16A; $p<0.001$) and LIX/CXCL5 (Figure 5.16B; 8h, IFN-γ$^{-/-}$ $p<0.05$, SOCS1$^{-/-}$ IFN-γ$^{-/-}$ $p<0.001$; d1 $p<0.001$) and RANTES/CCL5 (Figure 5.20C; $p<0.001$) protein at 8h and d1 post infection in BAL from IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IL-13 and UV-RV1B treated wt mice. RV1B infected IFN-γ$^{-/-}$ mice had significantly higher levels of LIX/CXCL5 protein in BAL compared to SOCS1$^{-/-}$IFN-γ$^{-/-}$ mice d1 post infection (Figure 5.20B, $p<0.001$), a similar trend was observed for KC/CXCL1 at 8h and d1 post infection (Figure 5.20A). RV1B induced RANTES/CCL5 protein was significantly higher in SOCS1$^{-/-}$IFN-γ$^{-/-}$ mice compared to IFN-γ$^{-/-}$ mice at 8h post infection in BAL (Figure 5.16C; $p<0.05$).

IL-13 pretreatment and RV1B infection significantly induced KC/CXCL1, LIX/CXCL5 and RANTES/CCL5 protein in BAL from both IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice. KC/CXCL1 and LIX/CXCL5 are induced less in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IFN-γ$^{-/-}$ mice. RANTES/CCL5 has been shown to be regulated by type I IFN (Bartlett manuscript in preparation) and was increased in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared IFN-γ$^{-/-}$ mice.

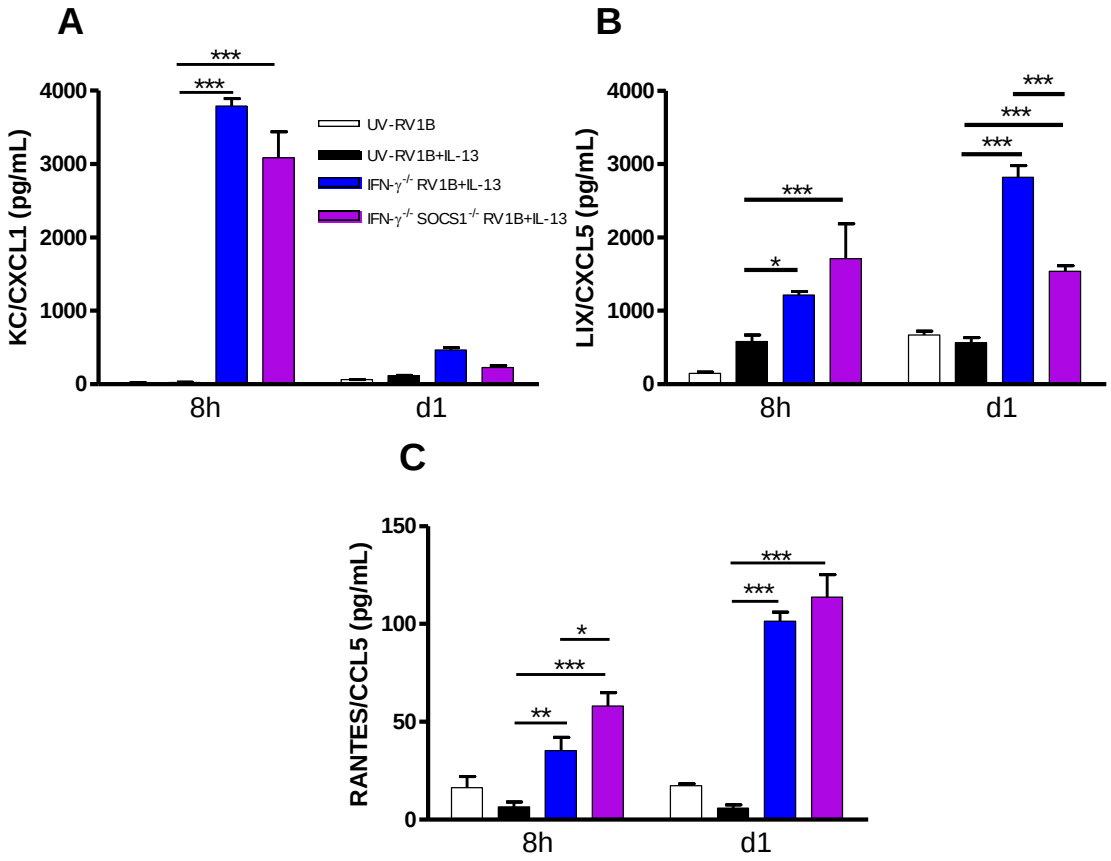

Figure 5.16: RV1B induced KC/CXCL1 (A), LIX/CXCL5 (B) and RANTES/CCL5 (C) protein in BAL from IL-13 pretreated and RV1B infected IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice.

Open bars denote UV-RV1B mice, black bars denote UV-RV1B/IL-13 mice, blue bars denote IL-13/RV1B in IFN-γ$^{-/-}$ C57/Bl6 mice, purple bars denote IL-13/RV1B in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6. Data represent mean (±SEM) of 3 experiments with each experiment having 4-7 mice per group.

5.7 RV1B infection in *ex vivo* cultured BAL macrophages from SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice compared to IFN-γ$^{-/-}$ C57/Bl6 mice

To determine whether SOCS1 knockout could increase RV1B induced type I and III IFN responses in BAL macrophages as a comparison to *in vivo* studies previously described, these cells from IFN-γ$^{-/-}$ mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice were cultured *ex vivo* and infected with RV1B for the indicated time points.

5.7.1 Adherent BAL cells from IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice consist mainly of macrophages

Firstly it was determined whether BAL cells were mainly macrophages in BAL from both IFN-γ$^{-/-}$ mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice before culture and after cell adherence. A small fraction of lymphocytes was observed in BAL cells from both IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice (Figure 5.17), although these were non-adherent cells. In BAL from SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice a fraction of cells were eosinophils (approximately 6%, Figure 5.17) and these cells did adhere.

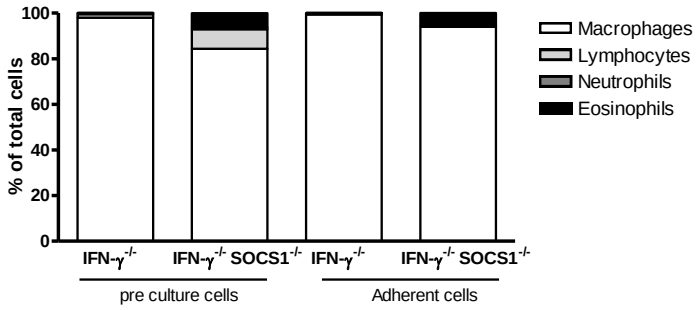

Figure 5.17: Type of cells in BAL from IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice before and after adherence to cell culture plates.

Macrophages were 100% of adherent cells in IFN-γ$^{-/-}$ mice and 94% in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice (n=3).

5.7.2 RV1B infection in *ex vivo* cultured BAL macrophages from SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice showed increased type I IFN mRNA levels compared to IFN-γ$^{-/-}$ C57/Bl6 mice

RV mRNA levels were determined, showing no differences between RV1B infected BAL macrophages IFN-γ$^{-/-}$ mice and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice at any time point (Figure 5.18A). RV1B significantly induced SOCS1 mRNA in IFN-γ$^{-/-}$ mice (data nor shown). RV1B significantly induced IFN-β mRNA in BAL macrophages from both IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice at 4, 8 and 24h post infection (Figure 5.18B; $p<0.01$ 4h, 8h and 24h IFN-γ$^{-/-}$ mice and 4h SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice and $p<0.001$ 8h, 24h SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice), and at 48h in BAL macrophages from SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice (Figure 5.18B, $p<0.05$). RV1B induced IFN-β mRNA in BAL macrophages from SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice was increased at 4h and 8h post infection ($p<0.05$) compared to IFN-γ$^{-/-}$ mice, a similar trend was observed at 24h and 48h (Figure 5.18B).

IFN-λ mRNA was not detectable in RV1B infected BAL macrophages, as was IFN-λ and IFN-α protein in supernatants (data not shown). IFN-β protein was not significantly induced upon RV1B infection in supernatants from BAL macrophages (data not shown).

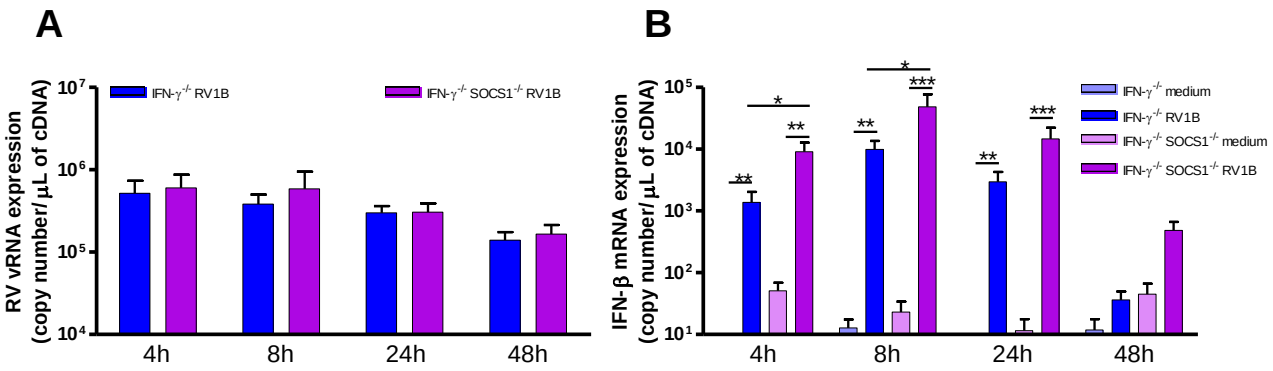

Figure 5.18: RV mRNA levels and RV1B induced IFN-β mRNA levels in BAL macrophages from IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice.

Blue bars denote IFN-γ$^{-/-}$ C57/Bl6 mice, purple bars denote SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 (n=6).

5.7.3 Upon RV1B infection in *ex vivo* cultured BAL macrophages from SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 mice and IFN-γ$^{-/-}$ C57/Bl6 mice chemokines were not detectable in BAL fluid

To determine whether SOCS1 knockout has an effect on RV1B induced chemokines, RANTES/CCL5, KC/CXCL1, LIX/CXCL5, MIP-2/CXCL8, IP-10/CXCL10 and GM-CSF were determined in supernatants from RV1B infected BAL macrophages from IFN-γ$^{-/-}$ and SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice. RANTES/CCL5, KC/CXCL1, LIX/CXCL5, MIP-2/CXCL8, IP-10/CXCL10 and GM-CSF were not detectable in supernatants from RV1B infected BAL macrophages (data not shown).

5.8 Discussion

In this chapter the effects of RV1B infection on SOCS1 deficient mice were explored. Previous data had supported a role for SOCS1 to be a negative regulator of IFN-β and IFN-λ production. The use of SOCS1 deficient mice offered the opportunity to investigate if these observed results could be confirmed *in vivo*. The use of an *in vivo* model also offered the opportunity to examine the effects of SOCS1 on RV induced inflammation in the lung, and therefore to examine if targeting SOCS1 would affect virus induced airway inflammation.

SOCS1 and SOCS3 mRNA levels were rapidly induced upon RV1B infection in C57/Bl6 mice. Effectiveness of RV1B infection was determined by neutrophil and lymphocyte influx into the BAL and induction of type I and III IFNs and chemokines as previously shown [223]. The rapid induction of SOCS1 and SOCS3 *in vivo* was comparable to what was observed in HBECs (Chapter 3). This again suggest that if SOCS1 and SOCS3 act as negative regulators of IFNs upon induction, this effect probably occurs as a slow effect rather than a rapid shut down of signalling after expression.

Secondly, IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice were infected with RV1B. RV1B infected SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice exhibited no increase in levels of type I and III IFNs compared to IFN-$\gamma^{-/-}$ mice and wt mice. A decrease in RV induced IFN-α were observed in both SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ and IFN-$\gamma^{-/-}$ mice compared to wt mice. This indicates a role for IFN-γ in regulating IFN-α production. Although type I IFNs have been shown to induce IFN-γ production through IL-15 mediated NK cell activation [226], IFN-γ has not been shown to induced IFN-α, and our data could represent a previously unknown regulatory mechanism of IFN-γ controlling IFN-α *in vivo*. This might however be explained by the fact that the control C57BL/6 mice where from a commercial supplier, while the IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ where littermates. Differences in the mice, such as microflora differences, may account for different magnitude of responses that are independent of our stimulus.

Next it was investigated whether IL-13 treatment could induce SOCS1 to use this as a method to upregulate SOCS1 in IFN-$\gamma^{-/-}$ mice before infection. IL-13 treatment induced SOCS1 mRNA, upon which a new model was designed to investigate whether SOCS1 knockout increased type I and III IFN mRNA. IL-13 pretreatment and RV1B infection in SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice increased IFN-α protein and IFN-λ in BAL and non-significant increased IFN-β protein was observed compared to IFN-$\gamma^{-/-}$ mice. This supports a role for SOCS1 induction in allergic airway inflammation regulating RV1B induced type I and possibly type III IFN responses *in vivo*.

5.8.1 Effects of SOCS1 on RV1B infection *in vivo* compared to SOCS1 overexpression in HBECs

In vitro it was shown that SOCS1 overexpression abolished RV1B induced IFN-β and IFN-λ1 promoter activation in HBECs. Assays using a SOCS1 overexpression vector measuring RV replication and release (Figure 3.27) and type I and III IFN mRNA did not confirm the promoter studies (data not shown) and using SOCS1 siRNA did not show increased RV induced IFN responses (data not shown), although trends were observed for IFN-β induced ISG responses (Figure 3.26). As a method to confirm the effects of overexpression of SOCS1 on RV induced IFNs the aim was to grow tracheal epithelial cells from IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice and investigate differences in RV induced IFN responses. Due to inability to optimise this culturing system BAL macrophages from IFN-$\gamma^{-/-}$ and SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice were investigated instead.

Using BAL macrophages from SOCS1 deficient mice, it was shown that RV1B induced IFN-β mRNA in from SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice was increased compared to controls. IFN-β protein was not detectable, IFN-λ mRNA and protein and IFN-α protein were not induced upon RV1B infection in BAL macrophages. IFN-β mRNA was however increased in the SOCS deficient cells at every time point. This supports the role of SOCS1 in negatively regulating RV induced IFN expression.

5.8.2 SOCS1 as a regulator of RV1B infection and RV1B induced IFN responses *in vivo*

SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ C57/Bl6 have been used to study Semlike forest virus infection in vivo [173], showing that SOCS1 deficiency augmented type I IFN antiviral actions independent of IFN-γ. Here SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice and IFN-$\gamma^{-/-}$ mice were used to compare type I and III IFN responses in RV1B infection in the absence of SOCS1. In IFNAR1 knockout mice, where type I IFN signalling is absent, lymphocytes are markedly decreased upon RV infection (Bartlett et al, manuscript in preparation), indicating a role for type I IFNs in inducing lymphocytes upon RV infection. Interestingly, lymphocytes were increased in RV infected SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice compared to IFN-$\gamma^{-/-}$ mice at d2 post infection, however IFN mRNA and proteins were not increased in these experiments. This indicates that SOCS1 did not play a significant role in RV1B induced IFN responses *in vivo*. In HBECs knockdown of SOCS1 by siRNA had no effect on RV induced IFN responses. This indicates that observing effects of a reduced negative regulator is difficult to obtain both *in vitro* and *in vivo*. An explanation for why overexpression of negative regulators show clear effects (Chapter 3) and that differences with siRNA experiments are difficult to observe is that a negative regulator needs to be induced or activated before it can have an effect. This would agree with the results observed in Chapter 4, where atopic asthmatics have increased SOCS1 before RV1B infection in bronchial biopsies or HBECs, which could be explained by increased Th2 cytokines or inflammatory mediators present in the airway. The increased SOCS1 mRNA in HBECs negatively correlated with reduced RV induced IFNs, suggesting

that SOCS1 might play a role in RV induced IFN deficiency in AA. In BAL macrophages from SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice however, higher levels of IFN-β mRNA compared to IFN-γ$^{-/-}$ mice showing were observed without induction of SOCS1 indicating that in some cell types the effects of SOCS1 depletion are more obvious and absence of SOCS1 is adequate to observe differences in RV1B induced type I IFN induction without prior SOCS1 induction in control cells.

To circumvent the lack of effect observed in the SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IFN-γ$^{-/-}$ mice and test the theory of induction of SOCS1 prior to infection, IL-13 was used to induce SOCS1 in the control group (IFN-γ$^{-/-}$ mice) prior to RV infection. Chapter 3 demonstrated that IL-13 was an excellent inducer of SOCS1 *in vitro*. The lack of SOCS1 induction prior to RV1B infection in the control group could be an explanation for the lack of difference in RV induced IFN production between SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice and IFN-γ$^{-/-}$ mice observed in the RV1B infection model. Furthermore, one study showed that IL-13 pretreatment and dsRNA treatment in airway epithelial cells reduced IFN-λ production compared to dsRNA alone [227], although not studied here this could be due to induction of SOCS1 in IL-13 treated cells. IFN-α protein was significantly more induced in IL-13 pretreatment and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IFN-γ$^{-/-}$ mice, similar trends were observed for IFN-β and IFN-λ protein. These results did show a role for SOCS1 in regulating RV induced IFN responses *in vivo*, though the effects were most prominent with IFN-α and IFN-λ rather than IFN-β. Differences in transcription factors that are important in their promoters could explain differences in activation and subsequent protein production. SOCS1 has been shown to target NF-κB [181] in the nucleus but might also target IRFs [175]. IRF-1, IRF-3 and IRF-7, but not NF-κB/p65 have been shown to be important for activation of the IFN-α promoter [228], while IRF-3 and NF-κB/p65 have been shown to be important for IFN-λ [228] and ATF2/cJun, IRF-3 and NF-κB/p65 for IFN-β [228]. If SOCS1 targets specific transcription factor this could explain difference in the effect of SOCS1 on protein production. Also, it is not known which cells produced the IFNs measured in BAL from IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice and IFN-γ$^{-/-}$ mice. DCs, epithelial cells and macrophages are probably the source of IFNs in this model. No differences were observed in RV induced IFN mRNA from the lung, however RV induced IFN-β mRNA was increased in *ex vivo* cultured BAL macrophages. RV1B infected BAL macrophages did not produce IFN-α and IFN-λ, but did produce IFN-β mRNA. IFN-α will probably be more prominently produced by DCs [229] while IFN-β might be produced more by lung epithelium or macrophages. More work is required to understand the relative contributions of each cell type to IFN production in the BAL, and how dependent these different cell types are to each IFN gene.

At early time points post RV infection, RV induced IFN production occurs by downstream TLR3, and later RIG-I and MDA5, induced signalling leading to IFN induction in an IRF3 dependent manner [196]. In HBECs it has been shown that the majority of IFN at 24h post RV infection is virus rather than IFN induced [196]. At later time points, IFNs are produced both directly through RV signalling via IRF3 and IFN induced IFN production through JAK/STAT. Although this has not been studied *in vivo*, the results describing increased RV1B induced IFN protein in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IFN-γ$^{-/-}$ mice were observed at 8h and d1 post RV1B infection, indicating that SOCS1 may negatively regulate both RV and IFN induced IFN production. Collectively with the reduction of RV1B induced IFN promoter activity by SOCS1 and SOCS3 overexpression in HBECs (results shown in Chapter 3) and increased RV1B induced IFN-β mRNA in BAL macrophages from SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IFN-γ$^{-/-}$ mice it supports our hypothesis that SOCS1 can negatively regulate both RV induced IFN production as well as IFN induced JAK/STAT signalling and subsequent IFN production.

Although the comparison between human and mice and *in vivo* versus a single cell type is difficult and complicated, the increased RV1B induced IFN protein levels shown *in vivo* shown in this chapter indicate that inhibition of SOCS1 could be a target to increase IFN responses in humans. This underscores the importance of the results observed in Chapter 4. SOCS1 levels were higher in AA HBECs compared to NA HBECs which negatively correlated with RV1B and RV16 induced IFN levels, which could be related to increased Th2 and inflammatory signalling in AA. When SOCS1 was targeted *in vivo* (SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice), RV1B induced IFN protein levels were higher compared to IFN-γ$^{-/-}$ mice when SOCS1 mRNA levels were induced by Th2 cytokine pretreatment before RV1B infection. The current data suggest however that although SOCS1 could be a target to increase IFN responses, this might not affect all IFNs and for that reason might give a partial solution to abolish the IFN deficiency observed in AA. In addition to that it would be interesting to investigate whether the increased levels of IFN were sufficient to improve lung function in these mice. This would be an indication whether reduction or knockback of SOCS1 could lead to improvement of exacerbations *in vivo.*

5.8.3 The role of SOCS1 in other mouse models

Several studies have investigated allergic airway inflammation in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice [169, 170]. Our observation that at baseline no increased eosinophilia in BAL was observed in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IFN-γ$^{-/-}$ mice is in contradiction with previous literature [169] where approximately 30% of BAL cells in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice were eosinophils at baseline. A previous study showed that upon OVA treatment the differences in total cells and eosinophilia in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 were even greater [169] and other Th2 markers were increased, such as Th2 cytokines in CD4+ T cells in the lung

and serum IgE compared to IFN-γ$^{-/-}$ C57/Bl6. One study has investigated the role of IL-13 in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 and IFN-γ$^{-/-}$ C57/Bl6 [170]. IL-13 was administered intratracheally 3 times and increased eosinophilia and eotaxin/CCL11 were observed in BAL from SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 compared to IFN-γ$^{-/-}$ C57/Bl6. In the present study IL-13 was administered i.n. before RV1B infection and IL-13 treated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 showed increased eosinophilia and eotaxin/CCL11 compared to IFN-γ$^{-/-}$ C57/Bl6. Interestingly, RV1B infection alone increased eosinophilia in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ C57/Bl6 compared to IFN-γ$^{-/-}$ C57/Bl6, however when RV1B infection alone is compared to IL-13 pretreatment and RV1B infection an exaggeration of eosinophilia seemed to occur in the latter. Overall SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice appeared to have a Th2 biased phenotype shown by eosinophilia and eotaxin/CCL11 compared to IFN-γ$^{-/-}$ mice. This Th2 phenotype however was less apparent in our study than in previous studies [169, 170].

Our results indicate that RV1B infection exaggerated an increased Th2 biased phenotype in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IFN-γ$^{-/-}$ mice. This would be a limitation in using SOCS1 as a target to increase IFN responses. If this were to relate to the effect of targeting SOCS1 in humans, especially AA, although increased IFN responses would be beneficial, increased Th2 would lead to consequent increased morbidity. In the OVA treatment study [169] the authors showed that the regulation of Th2 responses by SOCS1 is more specifically for hematopoietic cells and not airway epithelium. A functional polymorphism in the SOCS1 promoter has been associated with asthma in adults [168]. By selectively targeting SOCS1 in the airway epithelium of AA and not of the lung, could be a way of safeguarding against unwanted exaggerated Th2 responses, however how realistic that would be needs to be investigated further. Targeting SOCS1 could be achieved through a peptide or siRNA targeting [160, 230], which have been successfully applied in murine models [230-232].

5.9 Conclusion

RV1B infection induced SOCS1 mRNA and protein in C57/Bl6 mice. Upon induction of SOCS1 by IL-13 in IFN-$\gamma^{-/-}$ mice and subsequent RV1B infection, these mice have a reduced production of IFN-α and IFN-λ protein in BAL compared to SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice and a non-significant reduction of IFN-β compared to SOCS1 deficient mice. These increased IFN responses in SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice compared to IFN-$\gamma^{-/-}$ mice showed a role for SOCS1 in *in vivo* RV1B infection, however this effect was not observed in a single RV1B infection without IL-13 pretreatment. The increase in IFN was only partial and Th2 responses are increased in SOCS1$^{-/-}$ IFN-$\gamma^{-/-}$ mice compared to IFN-$\gamma^{-/-}$ mice. The data support the idea that SOCS1 is a negative regulator of IFN production and that targeting SOCS1 could reduce the suppression of virus induced IFN *in vivo*.

6 Discussion

6.1 Key study findings

6.1.1 RV induced SOCS1 and SOCS3 expression

This is the first report investigating the potential role of SOCS proteins in deficient innate IFN production and increased susceptibility to RV infection in asthmatics. Major (RV16) and minor (RV1B) group RV induced SOCS1 and SOCS3 mRNA and protein in HBECs, but not other SOCS family members. RV1B induced SOCS1 mRNA and protein and SOCS3 mRNA in lungs of C57/Bl6 mice, and RV1B significantly induced SOCS1 mRNA in *ex vivo* cultured BAL macrophages from IFN-$\gamma^{-/-}$ mice. This points to the potential role of SOCS1 and SOCS3, but not other SOCS family members, in modulating aspects of immunity to RV.

Effects of other mediators involved in atopic asthma and RV infection on SOCS expression were also studied. IFN-β, PolyIC, the inflammatory mediators TNF-α and IL-1β and the Th2 cytokines IL-4 and IL-13 induced SOCS1 mRNA and protein in HBECs. PolyIC induced SOCS3 mRNA and protein in HBECs. IL-4 and IL-13 induced CISH mRNA and protein in HBECs. IL-13 was also shown to induce SOCS1 mRNA *in vivo*. Although several of these mediators have been shown to induce SOCS1 and/or SOCS3 [190-192, 194], this had not yet been shown in HBECs. This would suggest that in the context of allergy and asthma, SOCS1, SOCS3 and CISH have the potential to modify ongoing immune responses in HBECs.

6.1.2 SOCS1 and SOCS3 regulated RV induced IFN responses, Th2 cytokine responses, but not RV induced proinflammatory responses

RV induced IFN promoter activation in HBECs at 24h post infection was completely blocked by overexpression of SOCS1 and SOCS3. *In vivo* IL-13 pretreatment and RV1B infection induced IFN production was increased in SOCS1$^{-/-}$ mice compared to control mice at 8h and d1. RV1B induced IFN-β mRNA was increased in *ex vivo* cultured BAL macrophages from SOCS1$^{-/-}$ mice compared to control mice as early as 4h post infection. This data suggests that SOCS1 is a negative regulator of RV induced IFN production. Although it could not be ruled out that SOCS1 was negatively regulating IFN induced IFN during RV infection. At early time points, 4h and 8h, most of the IFN produced was probably due to RV only, while at 24h both RV induced IFN production and IFN induced IFN production would take place. The effects of SOCS1 on RV induced IFN responses at these early time points indicates that SOCS most likely negatively regulated RV induced IFN production. The effects of SOCS1 on RV induced IFN versus IFN induced IFN could be further tested by blocking the IFNAR1 receptor, such as through a blocking antibody, or by the selective knockdown of STAT1. We would predict that SOCS1 would still reduce RV induced IFN in the presence of blocking antibody or in STAT1 deficient cells, showing that SOCS1 is effecting RV induced IFN directly.

A role for SOCS1 in negatively regulating Th2 cytokine and IFN-γ responses, in human studies using T cells, and studies in SOCS1$^{-/-}$ knockout mice have been reported [165-167]. In this thesis it was shown that IL-4 and IL-13 induced STAT6 promoter activation was completely blocked by overexpression of SOCS1 and SOCS3, and IL-13 treated SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice had increased Th2 chemokines compared to IFN-γ$^{-/-}$ mice. These results show a role for SOCS1 and SOCS3 in negatively regulating Th2 responses. The results shown in this report support findings from previous studies that SOCS1 negatively regulated Th2 responses both in HBECs and in mice. This would be a limitation in using SOCS1 as a target to increase IFN responses. If this were to relate to the effect of targeting SOCS1 in humans, especially AA, although increased IFN responses would be beneficial, increased Th2 responses could lead to consequent increased morbidity. Previously it has been shown that in mice the regulation of Th2 responses by SOCS1 was specific for hematopoietic cells and not airway epithelium [168]. Selectively targeting SOCS1 in the airway epithelium of AA, could be a way of safeguarding against unwanted exaggerated Th2 responses, however how realistic that would be needs to be investigated further.

The role of SOCS1 and SOCS3 on inflammatory mediator induced signalling has been less well studied. One report has indicated a role for SOCS1 in negatively regulating NF-κB mediated signalling [181]. This could indicate that RV induced NF-κB dependant chemokines might be negatively regulated by SOCS1. In HBECs, overexpression of SOCS1 and SOCS3 led to reduced RV induced IL-6 and IP-10/CXCL10 induced promoter activation, increased RV induced IL-8/CXCL8 promoter activation and had no effect on TNF-α and IL-1β induced NF-κB minimal promoter activation. *In vivo* in IL-13 pretreated and RV1B infected SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice, a decrease in the chemokines KC/CXCL1 and LIX/CXCL5 compared to IFN-γ$^{-/-}$ mice was observed. The chemokine RANTES/CCL5, which has been shown to be IFN induced (Bartlett N, et al; manuscript in preparation) was increased in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice compared to IFN-γ$^{-/-}$ mice. Overall, the role of SOCS1 and SOCS3 on RV induced inflammatory responses was less clear compared to RV induced IFN responses, although most data suggested that SOCS1 did not play a role in negatively regulating RV induced inflammatory responses.

6.1.3 SOCS1, but not SOCS3, was increased in HBECs from asthmatics compared to non-asthmatics, and SOCS1 levels negatively correlated with RV induced IFN responses

This is the first report investigating SOCS1 and SOCS3 expression in HBECs from asthmatics compared to non-asthmatics. SOCS1 and SOCS3 have been associated with asthma in other cell types. SOCS3 has previously been shown to be increased in T cells from atopic asthmatics compared to non-asthmatics [162] and a functional polymorphism in the SOCS1 promoter has been associated

with asthma in adults [168]. A promoter variant of this polymorphism enhanced SOCS1 mRNA expression levels in an alveolar lung cell line (A549) and reduced STAT1 phosphorylation after IFN-β stimulation, this might indicate that SOCS1 is increased in asthmatics [168]. Another study however showed that IL-13 could not induce SOCS1 in ASMCs from asthmatics compared to non-asthmatics [170]. The data in this thesis is generally consistent with the literature, however potential cell type specific expression levels of SOCS1 and SOCS3 in asthma should be kept in mind when considering the overall role SOCS1 and SOCS3 play in asthma.

Here it was demonstrated that SOCS3 mRNA in HBECs from severe AA children and mild AA adults and SOCS3 protein in bronchial biopsies from mild AA was not different from NA controls. SOCS1 mRNA was increased in HBECs from severe AA and SOCS1 protein was increased in bronchial biopsies from mild AA compared to NA controls, suggesting a role for SOCS1 in bronchial epithelium of atopic asthmatics.

The increased SOCS1 protein in bronchial biopsies positively correlated with atopic and AHR clinical study outcomes. This indicated that sensitisation to a larger number of allergens, increased serum IgE and increased AHR were related to increased levels of SOCS1. IL-4 and IL-13 induced SOCS1 mRNA and protein in HBECs supports this finding (Section 6.1.1), however the increased SOCS1 could also be due to increased inflammatory mediators such as TNF-α and IL-1β [187, 188, 201] which also induced SOCS1 in HBECs (Section 6.1.1). In mild AA no increased SOCS1 mRNA was observed in HBECs. In severe AA however there was an increase in SOCS1 mRNA in AA at baseline compared to NA. The results indicate that SOCS1 might play a role in atopic asthma and increased SOCS1 is probably related to disease severity.

RV induced IFN responses were deficient in HBECs from severe AA compared to NA, this was not observed in the mild AA study. This indicates that severity of asthma might relate to IFN deficiency in HBECs in AA. Furthermore increased SOCS1 mRNA levels in HBECs were only observed in severe AA. Increased SOCS1 mRNA levels in HBECs at baseline negatively correlated with RV induced IFN mRNA and positively correlated with RV release in HBECs. This indicates that SOCS1 might play a role in RV induced IFN although this was not related to asthmatics specifically. This agrees with our findings *in vitro* and *in vivo* that SOCS1 can negatively regulate RV induced IFN.

6.2 Future work

This work has identified several important questions that need to be addressed by future work. These future studies will be able to strengthen the argument that SOCS1 can inhibit RV induced IFN production as well as IFN induced IFN production. This would further strengthen the concept that SOCS1 is involved in impaired RV induced IFN production in asthmatics. Future work should be:

- To establish the relationship between increased SOCS1 mRNA and protein and reduced IFN expression. Our data show that SOCS1 mRNA was increased and is associated with impaired IFN in severe AA, but total SOCS1 protein was not different between AA and NA; however in biopsies from mild moderate AA SOCS1 was increased and related to clinical outcomes of asthma. In HBECs total cellular SOCS1 protein may not necessarily be associated with impaired IFN, these inconsistencies could be explained by an examination of the cellular distribution of SOCS1. Recently, SOCS1 has been shown to be a nuclear protein and has distinct roles from that when expressed in the cytoplasm [181]. Inhibition of IFN induced signalling by SOCS1 through JAK/STAT occurs in the cytoplasm. If SOCS1 inhibits RV induced IFN production, this could be a nuclear event with SOCS1 inhibiting a transcription factor such as IRF3. IRF3 has been identified as the only transcription factor to be required for both RV induced IFN-β and IFN-λ in HBECs (Slater, unpublished observations). SOCS1 may inhibit IRF3 in a similar to how it inhibits NF-κB subunit p65 in the nucleus in HEK293 cells [181].
- To investigate whether SOCS1 function on RV induced IFN promoter in HBECs requires nuclear translocation. The suppressive effects of SOCS1 on IFN would be lost by mutating the nuclear localisation sequence (NLS) in the SOCS1 overexpression vector. Recently a nuclear localisation sequence on SOCS1 protein has been identified [181]. If SOCS1 would have been shown to enter the nucleus, a role for SOCS1 as a negative regulator different than the JAK/STAT pathway, which occurs in the cytoplasm, could be demonstrated by using the NLS mutated SOCS1 vector to establish whether SOCS1 function occurs in the nucleus as well.
- To investigate whether SOCS1 and IRF3 bind, immunoprecipation assays could be performed. If the previously mentioned experiments have shown that SOCS1 enters the nucleus and mutation of the SOCS1 NLS causes los of function this would indicate a role for SOCS1 in negatively regulating IRF3. If SOCS1 would be able to negatively regulate IRF3, this could be through formation of a SOCS1-IRF3 complex effectively sequestering IRF3 from binding IRF binding sites with IFN-β and IFN-λ promoters.

- To investigate whether SOCS1 overexpression affects RV induced IRF3 nuclear translocation in HBECs, demonstrating that SOCS1 has an effect on RV induced IRF3 activation and subsequent IFN gene induction.
- To determine whether the increased RV1B-induced IFN levels in SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice improve disease outcomes, lung function should be measured in the models used in this thesis.

RV induced IFN deficiency in asthmatics has been shown in both HBECs and BAL macrophages. It has not yet been determined whether RV induced IFN deficiency is specific to atopic asthmatics or is present in non-atopic asthmatics as well. Furthermore, RV induced IFN deficiency has been shown in other respiratory diseases such as CF (Varielle M, et al, manuscript in preparation) and COPD [201]. Future work could be:

- To determine whether increased levels of SOCS1 in AA are cell type specific it should be established whether SOCS1 is also increased in BAL macrophages from AA who have RV induced IFN deficiency
- To determine whether the increased levels of SOCS1 in AA were due to atopy, it should be investigated whether non-atopic asthmatics have RV induced IFN deficiency in addition to increased levels of SOCS1. Experiments could be performed comparing SOCS1 levels and RV induced IFN responses in HBECs from non-atopic non-asthmatics, atopic non-asthmatics, non-atopic asthmatics and atopic asthmatics. This could indicate whether increased SOCS1 expression in asthmatics is due to atopy, possible induction by Th2 cytokines, or might be due to other factors such as increased inflammatory mediators in asthmatics, e.g. IL-1β and TNF-α [187, 188].
- To determine whether increased levels of SOCS1 observed in asthmatics are due to the previously described SOCS1 promoter polymorphism[168], experiments could be performed on HBECs from asthmatics selected with and without the polymorphism to determine increased levels of SOCS1 and IFN deficiency.
- To determine whether SOCS levels are increased in other respiratory diseases such as CF and COPD where RV induced IFN deficiency has been observed. If SOCS1 levels are increased in cells from these patients this would again be an indication that increased SOCS1 might be due to increased inflammatory mediators.
- To determine whether increased SOCS1 levels correlate with disease outcomes and in vivo RV infection study could be performed in AA compared to NA. SOCS1 should not only be correlated to IFN levels and RV replication but also with lung function, this would determine

whether SOCS1 has an effect on disease outcomes and whether it is worth considering as a target in RV-induced asthma exacerbations.

6.3 Conclusion

This is the first report investigating SOCS1 and SOCS3 in relation to RV induced IFN responses. RV induced SOCS1 and SOCS3 *in vitro, ex vivo* and *in vivo.* SOCS3 negatively regulated RV induced IFN responses *in vitro*. SOCS1 negatively regulated RV induced IFN responses *in vitro, ex vivo* and *in vivo.* SOCS1 protein, but not SOCS3, was increased in bronchial biopsies from AA compared to NA. SOCS1 mRNA levels were increased at baseline in HBECs from severe AA and this negatively correlated with RV induced IFN deficiency in AA. This is the first report finding an association between increased expression of a protein, SOCS1, with impaired IFN production and RV release in asthma.

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