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Therapeutic manipulation of inflammation in exacerbated lung disease

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Statement of originality

No work presented in this thesis has previously been submitted for a degree in any university and contains no material previously published or written by another person except where due acknowledgment is made. All work contained within the thesis was performed by myself or in collaboration with members of Leukocyte Biology or industrial collaborators. Experimental work was carried out at the National Heart and Lung Institute.

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Abstract

It is well known that existing and prior inflammatory events in the lung can adversely affect subsequent inflammatory events even though the inciting antigen might be different. For example, viral infections can enhance secondary bacterial infections and also the symptoms of asthma causing increased morbidity and mortality. Understanding changes in the lung micro-environment that lead to altered lung responsiveness is important given the increasing incidence in allergic diseases and the emergence of pandemic influenza strains. Elucidating the molecular mechanisms of these prolonged alterations could lead to new therapeutic strategies.

Susceptibility to bacterial super-infections has been attributed to reduced toll-like receptor (TLR) responses in the inflammation-experienced lung. The triggering receptor expressed on myeloid cells (TREM) family of innate immune receptors fine-tune TLR responses, but have yet to be studied in the context of influenza and secondary bacterial infection. Targeting specific immune signalling receptors or signalling kinases involved in the inflammatory process in the lung by use of inhaled drugs could provide specific treatment of inflammatory lung diseases minimising effects in other organs or tissues. The aim of this thesis was to investigate the role of TREMs during influenza and secondary bacterial infection and to determine the effects of inhaled signalling kinase inhibitors in models of viral exacerbations of allergic inflammation.

TREM-2 expression was shown to be upregulated during the peak of influenza-induced inflammation. Furthermore, soluble (s)TREM-1 in bronchoalveolar lavage (BAL), lung and serum coincide with heightened susceptibility to bacterial infection post-influenza virus. These novel findings suggest that targeted manipulation of TREM-1 (or prevention of its cleavage into the soluble form) and TREM-2 has the potential to restore the inflammatory tone of the airspaces to prevent subsequent infectious complications.

Narrow-spectrum signalling kinase inhibitor TC2 reduced BAL eosinophils and total and allergen-specific serum IgE production in house dust mite (HDM)-induced allergic inflammation. Furthermore, a new model of viral infection during HDM-induced allergic inflammation was developed. Surprisingly, exposure to HDM prior to respiratory syncytial virus (RSV) infection led to reduced RSV-induced weight loss, damage and proinflammatory mediator release, which correlated with increased TREM-2 expression in the early stages of infection. Treatment with the kinase inhibitor TC2 during viral infection in allergen-exposed mice, however, still reduced airway hyperresponsiveness. Thus, whether severe asthma is characterised by additive inflammatory conditions or exacerbation of an underlying problem by infection, kinase inhibitors still hold promise in the clinic.

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Abbreviations

AEC	Airway epithelial cell
AHR	Airway hyperresponsiveness
Arg	Arginase
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
C	Compliance
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CFU	Colony forming units
COPD	Chronic obstructive pulmonary disease
CPE	Cytopathic effect
DALY	Disability-adjusted life year
DAP	DNAX activation protein
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
E	Elastance
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
FoxP3	Forkhead box P3
FSC	Forward scatter
G	Tissue damping
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H	Tissue elastance
H&E	Haematoxylin and eosin
HA	Haemagglutinin
HBSS	Hanks' balanced salt solution
HDM	House dust mite

HMGB1	High-mobility group box 1
IFI	Interferon γ-inducible protein
IFN	Interferon
IFTIM	Interferon-induced transmembrane protein
Ig	Immunoglobulin
IGF	Insulin-like growth factor 1
IL	Interleukin
ILC	Innate lymphoid cells
IQR	Interquartile range
IRF	interferon regulatory factor
ISG	Interferon-stimulated gene
ITAM	Immunoreceptor-tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M2	Alternatively-activated macrophages
mAb	Monoclonal antibody
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
Muc5ac	Mucin 5AC
NA	Neuraminidase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHBEC	Normal human bronchial epithelial cells
NK	Natural killer
NLR	NOD-like receptor
NO	Nitric oxide
Nos	Nitric oxide synthase
NSKI	Narrow-spectrum kinase inhibitor
OAS	2'-5'-oligoadenylate synthetase

OD	Optical density
OVA	Ovalbumin
P(I:C)	Polyinosinic:polycytidylic acid, poly(I:C)
p38 MAPK	p38 mitogen-activated protein kinase
PAMP	Pathogen-associated molecular pattern
PAS	Periodic acid–Schiff
PBS	Phosphate buffered saline
PFU	Plaque forming units
PLC-γ	Phospholipase-C-γ
PR8	Influenza A virus Puerto Rico/8/34 H1N1
PSI	Protein synthesis inhibitors
qPCR	Quantitative polymerase chain reaction
R	Resistance
RANTES	Regulated on activation, normal T cell expressed and secreted
Relm-α	Resistin-like molecule-α
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNP	Neuraminidase
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
RV	Rhinovirus
SEM	Standard error of the mean
SIRPα	Signal-regulatory protein-α
SIT	Specific immunotherapy
Sp	Surfactant protein
SSC	Side scatter
sTREM	Soluble triggering receptor expressed on myeloid cells
Syk	Spleen tyrosine kinase
TAM	Tyro-3, Axl, and Mer
TARC	Thymus and activation regulated chemokine
TCR	T-cell receptor
TGF-β	Transforming growth factor beta-β

TLR	Toll-like receptor
TLT	TREM-like transcript
Treg	Regulatory T cell
TREM	Triggering receptor expressed on myeloid cells
TSLP	Thymic stromal lymphopoietin

Chapter 1: Introduction

Chapter 1: Introduction

1.1 Global burden of respiratory disease

It is estimated that lower respiratory infections account for one of the highest global burden of disease, as measured by disability-adjusted life years (DALYs). This is expressed as the number of years lost due to illness, disability or premature death, as summarised in Table 1 (WHO, 2014). While asthma burden is not as high (25,202,418 DALYs), asthma is associated with lower respiratory tract infections and high risk of early death in asthma sufferers from low- and middle-income countries and remains an important respiratory disease (Beran et al., 2015).

Table 1. DALYs of disorders of greatest burden worldwide.
Data taken from (WHO, 2014)

Rank	Disorder	DALYs (x 10^3)
1	Ischaemic heart disease	165,717
2	Lower respiratory infections	146,864
3	Stroke	141,348
4	Pre-term birth complications	107,210
5	Diarrhoeal diseases	99,728
|	|	|
..	Asthma	25,202

The WHO estimates that respiratory infections cause more than 5% of the global burden of disease and the highest burden is found in low-income countries (WHO, 2014). More recently, the rapid emergence of new respiratory viral strains (e.g. H1N1 'swine flu', and H5N1 'avian flu') have caused global epidemics (Trombetta et al., 2015). Furthermore, the prevalence of asthma in both low-income and high-income countries and access to effective treatment is poor in low-income countries, and the association of viral-induced development and exacerbation of asthma is well-recognised, but effective treatments are lacking for specific patient populations (WHO, 2013). Therefore, understanding of the mechanisms of

airway diseases and drug development to reduce disease pathogenesis is clearly an important area of medical research. This begins with understanding the basic biology of the respiratory tract and its function under normal conditions.

1.2 Mucosal immunology of the respiratory tract

The respiratory tract is the second largest mucosal surface in the body. It serves vital functions in gas exchange and is also an important site of immunity, with a variety of immune-cell populations, which serve to provide a balance between homeostasis and protective response against pathogens.

The human respiratory tract consists of consists of two main parts, the upper respiratory tract (nasal cavity, and pharynx) and the lower respiratory tract (trachea, bronchi, bronchioles, and lungs). Although historically thought of as sterile, the respiratory tract is now known to be colonised with commensal microbes in healthy individuals (Beck et al, 2012). The upper and lower respiratory tract becomes colonised with resident and opportunistic microbes, e.g. *Streptococcus pneumoniae*, early on in life, which do not pose a risk in healthy individuals (Murphy et al., 2009). However, the respiratory tract must also contend with pathogenic microorganisms, such as influenza and respiratory syncytial virus (RSV), and foreign material, e.g. particulate matter present in pollution and allergens such as dust or pollen. The airway is constantly exposed to harmless antigens through breathing and therefore, must avoid mounting an inappropriate inflammatory response, which is achieved by active regulation of immune-cell activation to maintain homeostasis. The cell populations and soluble mediators involved in the maintenance of pulmonary homeostasis will be discussed in the following section.

1.3 The lung at homeostasis

1.3.1 Epithelial-derived mediators of homeostasis

In addition to acting as an initial physical barrier to incoming foreign antigens, the airway epithelium is also the source of a variety of soluble mediators responsible for maintaining homeostasis in the lung.

At homeostasis, the lung epithelium secretes a variety of soluble factors that regulate the function of leukocytes in the airway (Figure 1.1). For example, surfactant proteins (Sp) A and D are released by type II alveolar epithelial cells and are abundant in the lower airway lumen (Khoor et al., 1993, Snelgrove et al., 2011). Both SpA and SpD are able to suppress alveolar macrophage phagocytosis by 1) inhibiting complement C1 complex formation—crucial for opsonisation of antigens, 2) blocking the binding of pathogen-associated molecular patterns (PAMPs) to pathogen recognition receptors (PRRs), e.g. toll-like receptor (TLR)2, TLR4, and their co-receptors, e.g. MD2 and CD14, and 3) binding to the macrophage negative regulator protein signal regulatory protein alpha (SIRPα) (Yamada et al., 2006, Sano et al., 2006, Janssen et al., 2008).

Epithelial-derived nitric oxide (NO), transforming growth factor (TGF)-β and interleukin (IL)10 can also suppress proinflammatory cytokine production by alveolar macrophages, and reduce the responsiveness of dendritic cells (DCs) leading to the differentiation of regulatory T (Treg) cells and the inhibition of effector T cells (Wissinger et al., 2009, Strickland et al., 2006). Production of the growth factor GM-CSF by epithelial cells induces the differentiation of recruited monocytes into alveolar macrophages, rather than differentiation into antigen-presenting cells (Ward et al., 2009), thereby minimising the initiation of inflammatory responses during homeostasis.

In the absence of proinflammatory signals, conventional and plasmacytoid DCs facilitate tolerance to harmless antigens by promoting the development of Treg cells (Akbari et al., 2001). Alveolar macrophages actively inhibit the antigen-presenting function of DCs, and subsequently prevent effector T-cell function (Holt et al., 1993, Thepen et al., 1991). Alveolar macrophages can also directly suppress IL-2-mediated T-cell activation by the production of immunoregulatory mediators TGF-β and prostaglandins (Roth and Golub, 1993).

1.3.2 The role of macrophages in homeostasis

Alveolar macrophages are a unique population of macrophages that are phenotypically distinct from interstitial macrophages residing between the epithelium and endothelium.

Alveolar macrophages represent over 95% of all leukocytes in the alveolar space and have a low turnover rate of 40% in 1 year (Wissinger et al., 2009, Maus et al., 2006). In order to prevent unnecessary activation by innocuous antigens, alveolar macrophage activation is controlled by soluble factors such as TGF-β, NO and surfactant proteins. Direct cell-cell interactions also play a role in regulating the activity of macrophages. Several of these regulatory pathways have been characterised in mice, for example, the interaction of CD200R (expressed on macrophages) with CD200 on the surface of type II epithelial cells.

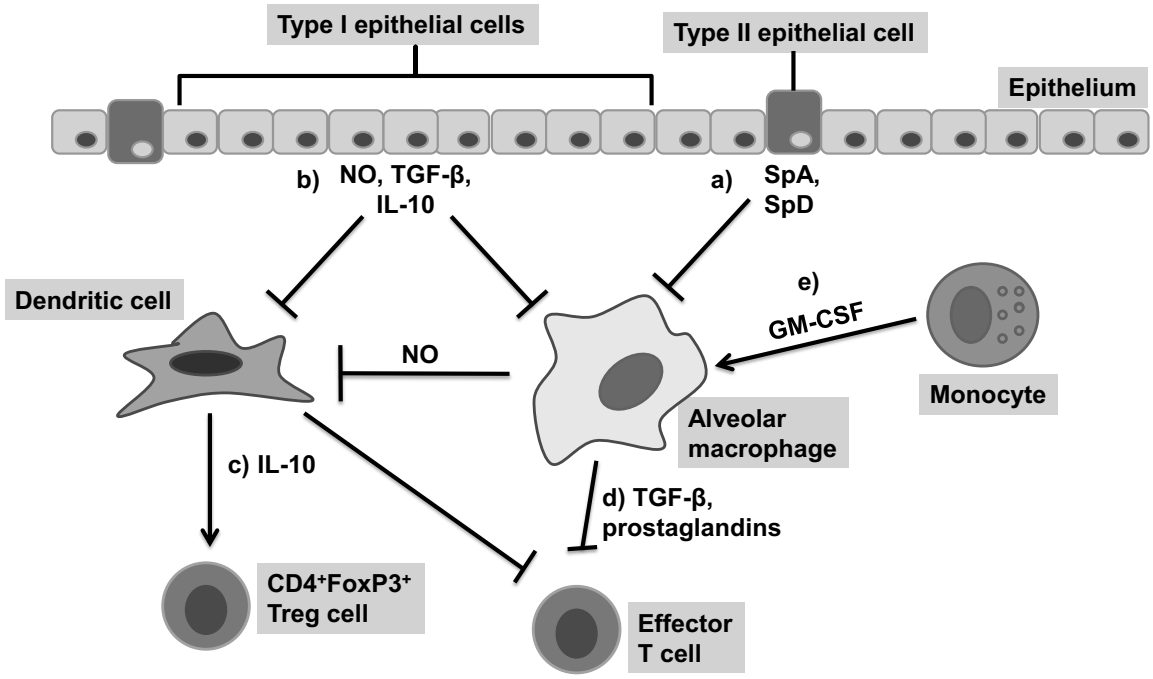

Figure 1.1 Selected mechanisms of maintaining homeostasis in the lower airway lumen.

a) Surfactant proteins SpA and SpD secreted by type II epithelial cells line the airway lumen and suppress alveolar macrophage phagocytosis. b) NO, TGF-β and IL-10 secreted by epithelial cells inhibit DC and alveolar macrophage activation. c) IL-10-producing DCs promote $CD4^+$ T-cell differentiation into forkhead box P3 $(FoxP3)^+$ Tregs and effector T cell function is inhibited by IL-10 (secreted by DCs) and d) TGF-β and prostaglandins (secreted by alveolar macrophages). e) Granulocyte-macrophage colony-stimulating factor (GM-CSF) skews the differentiation of monocytes towards alveolar macrophages, and away from antigen-presenting DCs.

CD200R is present on most leukocytes, but is particularly highly expressed on alveolar macrophages (Snelgrove et al., 2008). Ligation of CD200R by CD200 results in the inhibition of myeloid cell and T-cell interaction through the dampening of extracellular-

signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (MAPK) signalling (Mihrshahi et al., 2009). Another example of a negative regulator of alveolar macrophages is mannose receptor. Recognition of unopsonised bacteria, e.g. commensal bacteria, results in the inhibition of proinflammatory cytokine secretion by macrophages (Zhang et al., 2005). Triggering receptor expressed on myeloid cells (TREM)-2—an innate immune cell receptor—is also thought to suppress proinflammatory responses to lipopolysaccharide (LPS) in macrophages (Turnbull et al., 2006), and will be discussed in more detail in a later chapter.

1.4 Lung immune response to microorganisms and allergens

1.4.1 Influenza

Influenza A is an airborne virus that causes an acute respiratory disease, which remains one of the most important infectious diseases worldwide. Not only highly prevalent during seasonal epidemics, affecting 5-10% of the adult population each season (WHO, 2010), influenza also causes pandemic infections. Occurring in 2009, the most recent "swine flu" H1N1 influenza pandemic was rapidly transmitted globally and infected approximately 60 million people in the USA alone by the following April 2010 (Shrestha et al., 2011). While seasonal influenza infection is usually self-limiting, a subset of the population is a high risk of suffering from severe illness or complications as a result of influenza infection, i.e. infants, those over the age of 65, pregnant women and those with existing health problems. Furthermore, in the case of pandemics when completely novel strains are the cause, disease progression cannot always be predicted, as an example, there was a higher frequency of hospitalisation of patients aged between 5 and 54 during the 2009 pandemic compared to an average seasonal epidemic (Myles et al., 2012).

1.4.1.1 Classification

Influenza viruses belong to the *Orthomyxoviridae* family, and are single-stranded negative sense RNA viruses. Three types of influenza virus exist, A, B and C, influenza types A and B are important for seasonal epidemics, while C is responsible for sporadic infections and are not well characterised (Stephenson and Zambon, 2002). Type A influenza can be categorised based on the glycoproteins haemagglutinin (HA) and neuraminidase (NA) that are present on

the virion surface. HA is essential for binding and fusion of the virus particle to the host cell surface via sialic acid residues. NA cleaves sialic acid residues from viral proteins and the surfaces of infected cells, thus preventing viral aggregation and promoting viral release. There are currently 18 HA and 11 NA subtypes present in influenza viruses, and antigen variation in these surface glycoproteins contribute to viral evasion of host immunity (Tong et al., 2013). Variability is achieved by: 1) antigenic drift (gradual mutations) and 2) antigenic shift (mutations that lead to the development of novel viral strains). Although less frequent, antigenic shift can result in larger epidemics or pandemics. While commonly spread via human-to-human contact, influenza is also a zoonotic infection, with pigs and wild birds acting as important reservoirs of the virus (Medina and Garcia-Sastre, 2011).

1.4.1.2 Influenza virus replication cycle

Transmission of influenza virus occurs via large particle aerosols that contain virus and the point of viral entry in humans is the mucosal surface of the upper respiratory tract. The influenza virus attaches to the host cell surface via HA binding to α-2,6- or α-2,3-linked sialic acid. The virus then enters the cell via endocytosis. HA is cleaved by host cell proteases, exposing the HA peptide which enables fusion of the viral envelope and endosomal membrane. The viral M2 ion channel facilitates acidification and subsequent uncoating of the viral genome-containing ribonucleoprotein (RNP) complexes. The RNP translocates to the nucleus where RNA transcription and replication takes place. Viral RNAs are then translated in the cytoplasm, and newly formed RNPs are packaged. Essential viral proteins HA, NA and M2 are transferred via the Golgi apparatus to the plasma membrane and the virus particle is formed. NA enables the release of budding viruses by cleaving sialic acid via which the virion is attached to the host cell surface, reviewed in (Iwasaki and Pillai, 2014).

1.4.1.3 Immune response to influenza

Influenza can be recognised by three classes of PRRs: TLRs, Rig-like receptors (RLRs), such as RIG-I, and Nod-like receptors (NLRs), such as NLRP3. TLR3 detects viral RNA that has been engulfed by phagocytes such as macrophages. TLR7/8 detect viral RNA present in the

endosomes of infected cells and RIG-I and NLRP3 detect virus that is present in the cytosol of infected cells (Kawai and Akira, 2007, Kato et al., 2005, Allen et al., 2009).

Upon detection of influenza virus or viral RNA, the innate immune response is triggered to limit viral replication and pathogenesis associated with cell-death at the site of infection. Intracellular signalling pathways initiated by engagement of RLRs, TLRs and NLRs lead to the activation of both anti-viral and proinflammatory responses (Julkunen et al., 2000). Activation of the transcription factors interferon (IFN)-regulatory factor (IRF) 3 and 7 result in the transcription of type I and III IFN genes in plasmacytoid DCs (Mordstein et al., 2008). Type I and III IFNs, which include IFN-α, IFN-β and IFN-λ, prevent viral replication by inducing the transcription of IFN-stimulated genes (ISGs), IFN-α also contributes to reducing immunopathology (Arimori et al., 2013). ISGs are a range of genes encoding proteins that play various roles in restricting influenza infection in all stages of the influenza replication cycle. For example, interferon-induced transmembrane (IFTIM) proteins prevent fusion of the virus with the host membrane following viral attachment, 2'-5'-oligoadenylate synthetase (OAS) activates RNase L which cleaves viral RNA, ISG15 targets newly translated viral proteins for modification, and viperin blocks the release of new virus particles by altering lipid raft formation at the endoplasmic reticulum (Everitt et al., 2012, Silverman, 2007, Lenschow et al., 2007, Wang et al., 2007).

Secretion of IL-1β by infected DCs and macrophages causes the upregulation of chemokine receptor CC-chemokine receptor 7 (CCR7) on bystander DCs, which then migrate to the draining lymph nodes to activate T cells (Heer et al., 2008). Chemokines, such as regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), monocyte chemotactic protein (MCP) 1/CCL2 and CXCL8, are released by infected epithelial cells and macrophages, which lead to the recruitment of peripheral blood mononuclear cells to the infection site (Matsukura et al., 1996). Neutrophils are the predominant leukocyte population recruited in response to influenza infection that, together with macrophages, promote clearance of virus-infected cells by phagocytosis (Hashimoto et al., 2007). High dose influenza infection is associated with an increased number of neutrophils infiltrating the lung, however, neutrophils have also been shown to reduce tissue injury following influenza infection (Bradley et al., 2012, Tate et al., 2009). IFN-γ secretion by T cells and natural killer

(NK) cells activates APCs and induces granzyme-B-mediated lysis of infected cells (McElhaney et al., 2009). Cytotoxic $CD8^+$ T cells and NK cells also kill infected cells by inducing lysis or apoptosis (Gazit et al., 2006, Cerwenka et al., 1999). B cells secrete antibodies specific to viral antigens such as HA, which prevent virus infectivity and release of new virus particles, reviewed in (Waffarn and Baumgarth, 2011).

Although proinflammatory cytokines such as IL-1β and TNF-α play a vital role in controlling infection, prolonged cytokine release can lead to pathology at the site of infection. Negative regulators of inflammatory cells and cytokines, e.g. IL-10 and CD200, promote the resolution of inflammation by suppressing inflammation, while tissue repair is induced, e.g. by TGF-β. The balance of inflammation versus resolution determines disease severity and can also influence subsequent inflammatory events in the lung, such as secondary bacterial infection (Hussell and Cavanagh, 2009).

1.4.2 RSV

RSV is a virus that causes respiratory tract infections in adults and lower respiratory tract illnesses, e.g. bronchiolitis, in infants and children (Iwane et al., 2004). Infection is common, and severe disease can occur in infants younger than 6 months and infants with other medical concerns. High-risk groups for RSV infection include infants born prematurely and those with other disorders, such as chronic lung disease, heart disease, and immunodeficiency. RSV is a successful virus and individuals infected do not develop long-term immunity to the virus, and reinfection can occur in infants and in the eldery (Simoes, 1999). There is currently no safe and effective vaccine against RSV. The monoclonal antibody palivizumab (which recognises an epitope on the F protein) is used as a prophylactic in infants at high risk of complications following infection, however, its high cost prohibits its widespread use (Shadman and Wald, 2011). An anti-viral drug, ribavirin, is able to stop RSV replication, however, it has not proved cost-effective, so is also limited to use in high-risk infants (Ventre and Randolph, 2007).

1.4.2.1 Classification

RSV belongs to the *Paramyxoviridae* family, and is a negative sense, single-stranded RNA virus. The genome consists of ten genes that code for 11 viral proteins. There are two main antigenic groups of RSV, A and B, which were originally determined based on their reactivity to monoclonal antibodies, based on the expression of surface glycoproteins F and G (Anderson et al., 1985). Two main laboratory strains of RSV, A2 and line 19, have been used to model disease parameters, but produce different disease parameters, which could be the result of differential viral protein expression (Lukacs et al., 2006).

1.4.2.2 RSV virus replication cycle

RSV primarily infects airway epithelial cells, but can also persist and replicate in alveolar macrophages (Sarmiento et al., 2002, Hegele et al., 1994). RSV enters the cell via the binding of F and G proteins to host cell surface nucleolin and glycosaminoglycans, e.g. heparan sulphate (Tayyari et al., 2011, Lay et al., 2013). A study in normal human bronchial epithelial cells (NHBECs) also showed that RSV utilises lipid rafts in order to gain entry in the host cell (San-Juan-Vergara et al., 2012).

Following hemifusion (partial fusion) of the viral membrane with the host cell membrane, the viral RNP complex is released into the cytoplasm. This process requires phosphorylation of the viral P protein, which dissociates RNP binding to the matrix protein (Asenjo et al., 2005). Actin and profilin are thought to play a role in promoting the transcription of RSV genes, which takes place in specific sites in the cytoplasm called inclusion bodies (Jeffree et al., 2007). Following viral protein translation at the host ribosome, F protein mediates filament formation at the cell surface, and other viral proteins aggregate at the cell surface ready for viral assembly. Host cell actin and heat shock protein 90 are utilised in this process of viral assembly. Viral budding then occurs at the apical surface of the host cell. Infected-cell surface F protein mediates the merging of adjacent cells, i.e. formation of characteristic syncytia, which are evident in human disease and *in vitro* models (Johnson et al., 2006, Villenave et al., 2012).

1.4.2.3 Immune response to RSV

Host cells can recognise RSV via RLRs, TLRs and NLRs. While RIG-I and Nod2 recognise viral RNA in the cytoplasm, TLRs can recognise viral PAMPs on the cell surface and present in endosomes. TLRs 2, 3, 4, 6 and 7 are involved in the detection of RSV virus. Recognition of viral proteins by TLR2 and TLR6 on the host cell surface activates innate immune responses to RSV (Murawski et al., 2009). TLR3 and TLR7 detect viral dsRNA and ssRNA in the endosomes respectively, and also contribute to the proinflammatory response through the activation of transcription factors NF-κB and IRFs (Bermejo-Martin et al., 2007, Lukacs et al., 2010). TLR4 has been shown to recognise RSV F protein via its co-receptor CD14, which leads to NF-κB activation and inflammation (Kurt-Jones et al., 2000, Haeberle et al., 2002). As well as mediating inflammatory responses through direct intracellular signalling, TLR expression is also markedly altered upon RSV infection of airway epithelial cells, in order to increase the magnitude of the response (Xie et al., 2009).

Activation of IRF3 and IRF7 leads to type I IFN production. Type I IFNs promote the cytotoxic killing of infected cells by NK and $CD8^+$ T cells, and promote the maturation of DCs. IFN production also induces the control of viral replication by ISGs such as OAS2 and interferon-induced protein (IFI) 44 (Behera et al., 2002, Fjaerli et al., 2006). Upon nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation following PRR recognition of viral proteins, airway epithelial cells (AECs) produce inflammatory mediators such as IL-1, IL-6 and IFN-γ. Macrophages are also known to produce proinflammatory cytokines such as IL-6 and also IFN-α in response to RSV infection (Pribul et al., 2008). While proinflammatory cytokines promote leukocyte activation in the lung tissue and ISGs and IFNs promote anti-viral activity, a range of chemokines are secreted that induce leukocyte infiltration into the lung. The production of CCL5, CCL2, CXCL8 and interferon gamma-induced protein (IP-10/CXCL10) by AECs and also macrophages, result in the recruitment of monocytes/macrophages, NK cells, T cells, eosinophils and neutrophils into the lung (Bueno et al., 2011). These cells play similar roles in response to RSV as in influenza virus infection as described earlier.

Recently, other mediators have been shown to contribute to the immune response to RSV. For example, cathelicidin was found to mediate anti-viral activity in human bronchial epithelial cells (Currie et al., 2013). The expression of thymic stromal lymphopoietin (TSLP)—associated with the development of allergic responses—is also upregulated upon RSV infection and promotes T helper cell (Th)2 responses in AECs (Lee et al., 2012). The expression of programmed death-ligand-2 and OX40L on DCs is important for the restoration of cytokine balance following recall responses to RSV in mice (Wythe et al., 2012). $CD4^+CD25^+FoxP3^+$ Tregs are upregulated following RSV infection and contribute to recovery of infected mice (Lee et al., 2010). While studies have indicated the importance of proinflammatory and anti-viral responses to RSV, the contribution of the humoral response is not well studied. The antibody response to RSV is poor and, as a result, re-infection with the virus is common (Singleton et al., 2003).

In addition to its importance as a primary infection, particularly in infants, RSV infection is also, albeit controversially, associated with the development of allergic disease later in life, i.e. allergic asthma, which will be discussed in the following section.

1.4.3 Allergic Asthma

Asthma is a heterogeneous, respiratory disease characterised by chronic inflammation and airway hyperreactivity (AHR), which lead to symptoms such as shortness of breath, wheeze and cough (GINA, 2015). Asthma affects approximately 300 million people worldwide, while in the UK 18% of the population have been diagnosed as asthma sufferers (GINA, 2015, To et al., 2012). The complex and heterogeneous nature of the disease means that there is no cure. Current therapies aim to control specific aspects of the disease, i.e. controlling muscle contraction or reducing inflammation. Glucocorticoids, potent inhibitors of inflammation, are widely used in asthma therapy and are effective in controlling symptoms in the majority of patients. However, a small group of patients (5-10%) respond poorly to glucocorticoid therapy and account for a large proportion of the clinical and economic burden of asthma (Masoli et al., 2004). There is clearly an urgent need for the development of novel therapeutic drugs for this disorder, and many groups have focussed on targeting inflammatory processes involved in the disease development.

1.4.3.1 Immune response to allergens

Inhalation of allergens into the airway triggers an adaptive immune response via the activation of resident DCs, which process antigen and present antigen peptides in the context of major histocompatibility complex (MHC) II to T-cell receptors (TCRs) present on the surface of naive $CD4^+$ T cells (Figure 1.2). T cells that encounter peptide via MHC II become activated and differentiate into a Th2 phenotype (reviewed in (Lambrecht and Hammad, 2015)). The cytokines typically released by Th2 cells are IL-4, IL-5 and IL-13. Type 2 innate lymphoid cells (ILC2s) are also an important source of Th2 cytokines in the lung and produce substantial amounts of IL-5 and IL-13 in allergic inflammation (Halim et al., 2012). The Th2 cytokine milieu results in B-cell class-switching to immunoglobulin (Ig)E, activation of mast cells via IgE, activation of eosinophils and the release of inflammatory mediators, such as histamines, leukotrienes and prostaglandins. Physiological changes in the airway, e.g. contraction of airway smooth muscle (ASM) and increased permeability of capillaries, resulting in AHR, lead to damage to the airway epithelium and initiates the process of repair. Dysregulated repair, subepithelial fibrosis, increased mucus production and increased smooth muscle mass contribute to increased AHR and the classical symptoms of asthma; shortness of breath, wheeze and cough (reviewed in (Lambrecht and Hammad, 2015)).

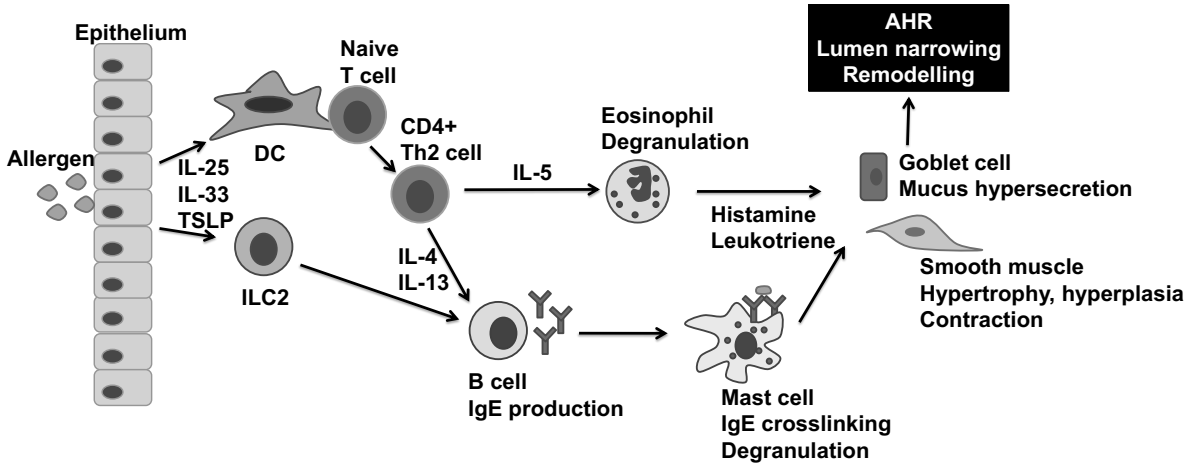

Figure 1.2 Inflammatory cascade in allergic inflammation.

Cells and mediators involved in allergic inflammation from initial antigen encounter at the epithelium eventually leading to AHR, and airway remodelling. After allergen recognition in the airway lumen, epithelial cells secrete mediators such as IL-25, IL-33 and TSLP, which activate DCs and ILCs. These cells, in turn, activate Th2 cells and B cells, which produce IL-5, IL-4 and IL-13 and antibodies,

respectively. Activation of granulocytes such as eosinophils and mast cells leads to the production of histamines and leukotrienes, which cause goblet cell activation and airway smooth muscle cell hypertrophy, hyperplasia, and contraction, leading to AHR and airway narrowing.

1.4.4 Animal models of inflammatory lung disease

In order to understand the basic biology of disease for the development of potential treatments for diseases such as viral infection and asthma, animals have been widely used in respiratory science research.

Animal models of influenza infection play an important part in the understanding of viral pathogenicity and allow the testing of new vaccine candidate and therapeutic drugs. Animal models of influenza infection used in research include mice, ferrets, guinea pigs, swine, non-human primates, and zebrafish (Margine and Krammer, 2014; Gabor et al., 2014). Mice are particularly widely used for influenza research. Although mice are not natural hosts for influenza viruses, laboratory strains have been developed that can be infected with certain influenza viruses. While caveats remain, and other models are better models for comparison with human disease (e.g. swine models), mice are the most widely used animal model for influenza research, as they have advantages over other species (i.e. their size, low cost, and the availability of transgenic strains and availability of research reagents such as antibodies; Margine and Krammer, 2014). The investigations in this thesis used mouse models of influenza infection and secondary bacterial infection following influenza infection.

For the same reasons as in influenza infection, mouse models have been widely used in research into other RSV infection. The BALB/c laboratory mouse strain is particularly popular for experimental modelling of human RSV infection. Models of intranasal inoculation of RSV in mice result in measurable disease parameters such as weight loss, viral replication in the lung and Th2 responses upon reinfection (Jafri et al, 2014). However, development of a robust mouse model of RSV infection has proved to be challenging compared with influenza infection, as a result of the relative resistance of animals to RSV; RSV is well adapted to infecting human hosts, no animal reservoir of RSV exists and RSV is unstable in the environment (Sacco et al., 2015). In this thesis, a new model of RSV infection

following allergen-induced allergic airway disease was explored. The allergen used to induce airway disease was HDM.

The study of allergic responses in mice requires the experimental induction of asthma with an allergen, since mice do not spontaneously develop asthma nor usually encounter allergens in their specific pathogen free environments. Early studies used OVA as an allergen and, which required intraperitoneal sensitisation of mice with OVA followed by inhalation of OVA to induce Th2 responses (Lloyd et al., 2001). More clinically relevant allergens, such as HDM, have since been developed, which not only induce Th2 responses, but also induce AHR, mucus production and airway remodelling (Johnson et al., 2004, Gregory et al., 2009). In this study, the HDM model of allergic airway disease was used to test the effects of signalling kinase inhibitors and to develop a new model of viral infection after allergic airway disease.

1.5 Secondary complications of inflammatory lung disease

While an encounter with an infectious agent or allergen will induce an immune response and the appearance of symptoms in the individual, often this primary response to a stimulus can affect immune responses to subsequent infection. Viral infections can be succeeded by secondary bacterial infections, while those with allergic asthma may experience exacerbation of asthma symptoms as a result of viral infections, such as RSV. Such secondary inflammatory events can result in pathology that surpasses that of the primary inflammatory episode, particularly in those with existing health concerns. Therefore, understanding the mechanisms that lead to susceptibility to further disease is of importance and can identify new targets for drug development and testing.

1.5.1 Secondary bacterial infection following influenza infection

As discussed in the previous sections, influenza infection alone can activate the immune response, leading to the production of proinflammatory cytokines and chemokines and subsequent inflammation of the upper respiratory tract. However, bacterial infection following influenza infection can result in greater pathological consequences. For example, during the 1918 influenza pandemic, over 50 million people died as a result of secondary

bacterial infections post-influenza infection (Morens et al., 2008). While bacterial species, such as *Staphylococcus aureus,* are becoming more prominent, *Streptococcus pneumoniae* is one of the most common causes of bacterial super-infection (McCullers, 2014).

1.5.1.1 *Streptococcus pneumoniae*

S. pneumoniae is an opportunistic and commensal bacteria that usually resides in the nasal mucosa in humans (Lynch and Zhanel, 2009). It is a gram-positive bacterial species, which utilises its polysaccharide capsule to evade the host immune response (Nelson et al., 2007). It is usually harmless, but can cause mild symptoms such as otitis media and sinusitis, and less commonly, septicaemia and pneumonia. Those that are susceptible to severe disease are young children, the elderly and the immunocompromised. Also vulnerable are those recently infected with a virus, such as influenza. Secondary bacterial infection results in worse outcomes following influenza infection (Rice et al., 2012, Palacios et al., 2009).

1.5.2 Asthma and microbes

Microbial infections are also important in non-infectious diseases such as asthma. The role of infectious agents and asthma can either be in the development of asthma or in influencing asthma pathogenesis. The hygiene hypothesis suggests that a reduction in exposure to infections such as parasitic infections, as a result in improved sanitation and personal hygiene, has led to an increase in allergies. This is due to impaired maturation of immunity leading to inappropriate immune responses to harmless stimuli such as house dust mite (Okada et al., 2010, Ege et al., 2011). Also, early exposure to viral infections, such as rhinovirus (RV) or RSV, are thought to predispose individuals to the development of allergic asthma later in life (Gern, 2015; Sigurs et al., 2010). Moreover, infection in those who already have established asthma can exacerbate symptoms. Viral and bacterial infections can contribute to the exacerbation of asthma (Bisgaard et al., 2007, Johnston et al., 1995). Studies in asthmatics have revealed that patients are more susceptible to bacterial infections such as *S. pneumoniae* (Klemets et al., 2010). Other bacteria such as *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* have also been linked to exacerbation of asthma (Johnston et al., 2006b). Increases in fungal spores in the environment have been correlated with increased

hospital admissions in children with asthma (Atkinson et al., 2006), and *Alternaria alternata* fungal infection has been shown to increase IL-33 production and Th2 inflammation in both paediatric patients with asthma and in a mouse model of HDM allergic airway disease (Castanhinha et al., 2015, Snelgrove et al., 2014).

Respiratory viruses, however, are the most common cause of asthma exacerbations, affecting over 50% of exacerbations in children and adults, with lower respiratory tract infections, such as RV and RSV most prevalent (Johnston et al., 2006a, Johnston et al., 1995). The most common viral infection to infect older children and adults is RV. A link between exacerbations and RV has been established for decades (Nicholson et al., 1993, Johnston et al., 1995), and infection led to increased inflammation in the airways of asthmatics in an experimental model of RV infection (Grünberg et al., 2001). Conversely, allergic asthma induced by the fungus *Aspergillus fumigatus* and ovalbumin (OVA) resulted in reduced susceptibility to infection (Ishikawa et al., 2012, Samarasinghe et al., 2014). Samarasinghe et al. suggest a role for insulin-like growth factor (IGF)-1, a proinflammatory hormone, in promoting anti-viral activity leading to a reduction in viral burden and subsequent exacerbation of influenza infection during chronic allergic inflammation.

1.5.2.1 RSV infection and allergic asthma

RSV is a major cause of asthma exacerbations in young children under the age of two, but is also important in adult disease (Westerly and Peebles, 2010). Early studies in mice into the effect of RSV on inducing sensitisation of asthma have shown that RSV can induce AHR itself but also increases sensitisation to OVA-induced allergic inflammation (Schwarze et al., 1997, Tourdot et al., 2008). Since then, further studies have pointed to a role for RSV in the development of asthma, reviewed in (Busse et al., 2010). Jackson and colleagues (2008) showed that RSV infection in the first 3 years of life led to a 2.6-fold increase in risk of wheeze by the age of six. Others argue that genetic factors are more important in predisposing children to asthma than environmental factors such as viral infections (Thomsen et al., 2009). In studying the infection in mice, another group showed that RSV infection-induced allergic airway inflammation to OVA, in mice previously tolerized to OVA (Krishnamoorthy et al., 2012). This was due to the effect of RSV skewing regulatory T cells

towards a Th2 phenotype, which increased susceptibility to allergic inflammation. A study in human monocyte-derived DCs suggested that *in vitro* RSV-induced enzyme indoleamine 2,3-dioxygenase, which promotes Th2 immune responses, could be a factor contributing to the development of allergic asthma following RSV infection (Ajamian et al., 2015). Regardless of the relative importance of RSV in the development of asthma in later life, it is clear that a link does exist in some patient cohorts studied, and investigations continue to focus on this aspect of RSV infection in allergic asthma.

In addition to its role in influencing the development of asthma, RSV is also an important respiratory virus in the exacerbation of existing asthma. Johnston et al. (1995) found that respiratory viruses were present in samples taken from asthmatic children during an symptomatic episode, and RSV was present in 4% of cases. RSV is also associated with asthma exacerbations in adults (Nicholson et al., 1993). *In vitro* studies of peripheral blood mononuclear cells isolated from asthmatic patients have previously indicated impaired IFN I responses to RSV infection compared to those cells isolated from healthy controls, an effect observed both in children and adults (Bufe et al., 2002, Gehlhar et al., 2006). Studies of IFN responses in bronchial epithelial cells isolated from asthmatic adults, however, showed similar responses to RSV in non-asthmatic and asthmatic subjects (Patel et al., 2014). Other features in the immune responses of asthmatic patients to RSV infection include: increased IL-10 production, increased IFN-γ and increased RSV-specific IgG_1 and IgG_2 (Grissell et al., 2005, Douville et al., 2007, Hancock et al., 2001). The differences in the response to RSV between asthmatics and non-asthmatics may contribute to the exacerbations observed in asthmatic patients. The various mechanisms by which co-pathogenesis of lung inflammation occurs will be discussed in the following section.

1.5.3 Mechanisms of co-pathogenesis

Following an initial infection or inflammatory event such as allergic airway disease, alterations to the airway environment cause the lung to respond differently to a subsequent infection. Several factors are likely to be involved in this phenomenon.

1.5.3.1 Disruption to the epithelium

After a viral infection, such as influenza infection, extensive cell infiltration and cytopathic activity leads to damage to the epithelium, which functions as a barrier to foreign material. Disruption of tight junctions, removal of surfactant layers and cell death lead to increased access to cells for opportunistic pathogens, such as *S. pneumoniae*, and increased nutrient availability promote bacterial growth in the area (Fukushi et al., 2011). Similarly, viral infection during allergic inflammation also leads to local cell death and sloughing (Bossios et al., 2005), which increases permeability of the epithelium and subsequently increase recognition of pathogens and allergens, contributing to increased pathogenesis and aggravation of symptoms.

1.5.3.2 Excessive inflammation

Viral infection can lead to a potent proinflammatory response, which is augmented by additional inflammatory mediators stimulated by a subsequent bacterial infection (Kuri et al., 2013). In fact, synergy between bacterial and influenza infections has been observed in the inflammatory effects of PB1-F2—an accessory protein expressed by influenza that promotes inflammation (McAuley et al., 2007). The activation of shared TLR pathways by viruses and bacteria can also lead to excessive cytokine production, e.g. TLR-2 and -4 can be activated by both influenza and *S. pneumoniae* (Karlström et al., 2011).

TLR activation by RSV can also enhance the inflammatory response caused by allergens. For example, HDM contains β-glucans which can activate TLR4 and contribute to allergic asthma (Ryu et al., 2013). Murawski *et al.* (2009) also showed that TLR2 is a functional receptor for RSV. In several mouse models of asthma, TLR2 was shown to contribute to Th2 activation (Redecke et al., 2004, Dillon et al., 2004, Taylor et al., 2006), therefore, activation of TLR2 by RSV during on-going allergic airway inflammation may cause excessive Th2-associated inflammation. Although RSV infection initiates Th1 cytokine production as with other respiratory infections, RSV is also known to induce excessive Th2 responses, e.g. increased IL-5 production in allergic individuals (Zhang et al., 2009). RSV also synergises with Th2 cytokines to promote the production of chemokines such as thymus and activation

regulated chemokine (TARC) (Monick et al., 2007). In an early study in infants, RSV infection-induced RSV-specific IgE production, which correlated with histamine levels in nasal secretions and wheezing in RSV infection alone (Welliver et al., 1981). During on-going allergic inflammation, the addition of RSV-induced IgE may contribute to the exacerbation of symptoms. In a mouse model airway inflammation and AHR, transfer of RSV-specific IgE was shown to increase AHR (Dakhama et al., 2004), which suggests a role for RSV-specific IgE in airway inflammation and the possibility of the combination of RSV-specific and allergen-specific IgE increasing symptoms of asthma.

1.5.3.3 Altered immune response

Following any inflammatory event, changes to the capacity of the innate immune response to respond to further infection can occur, which can be due to the pathogen itself or due to host responses. For example, influenza-induced type I IFN responses cause suboptimal neutrophil responses to bacteria (Shahangian et al., 2009). Conversely, IFN responses in allergic asthmatics have been shown in some studies to be impaired, which could contribute to susceptibility of asthmatic individuals to viral infections (Wark et al., 2005). However, conflicting evidence was recently published, suggesting that IFN responses, i.e. IFN and interferon-stimulated gene (ISG) expression was comparable following *in vitro* RSV-infection of normal versus asthmatic bronchial epithelial cells (Patel et al., 2014). Further investigation into the existence and importance of anti-viral immunity is required.

In investigating the role of the immune status of the lung in secondary inflammatory events, it is important to consider the role of the most abundant leukocyte population of the lung, the alveolar macrophage. As discussed previously, alveolar macrophages play an essential role in maintaining the lung health. As a result of infection or allergic inflammation, however, changes to the macrophage population can occur, and these changes to this one population alone can have major effects on the susceptibility of the lung to further infections. These changes can either be quantitative, i.e. changes in cell frequency, or relate to the phenotype and function of the cells.

It has been observed that influenza virus infection depletes the alveolar macrophage population in the lung (Ghoneim et al., 2013). The authors found that over 90% of the alveolar macrophage population in the lung had diminished 1 week after initial influenza infection, and that this depletion resulted in impaired immunity against subsequent *S. pneumoniae* infection. A reduction in the proportion of alveolar macrophages found in the lung was also observed in a mouse model of HDM-induced allergic airway disease (Habibzay et al., 2012).

Alveolar macrophages isolated from the lungs of mice infected with influenza have been shown to have an altered phenotype. These alveolar macrophage exhibit characteristics such as impaired NF-κB activation in response to TLR ligation and increased expression of negative regulator receptor CD200R and, therefore, have a higher threshold of activation in order to mount an efficient immune response (Didierlaurent et al., 2008, Goulding et al., 2011, Snelgrove et al., 2008, Hussell and Cavanagh, 2009). This altered immune state persists for several months post-influenza infection, during which time the lung is more susceptible to bacterial infections (Didierlaurent et al., 2008). This phenomenon has also been demonstrated in a model of bacterial infection following HDM-induced allergic airway disease (Habibzay et al., 2012). Mice that were sensitised to HDM were more susceptible to bacterial infection and this was attributed to increased expression of negative regulators of TLR signalling, for example, A20 and Tollip. Axl, a member of the Tyro3, Axl, and Mer (TAM) receptor tyrosine kinases family, has recently been shown to be expressed exclusively on mouse airway macrophages and is important for the resolution of lung inflammation following influenza (Fujimori et al., 2015). In a model of RSV-induced exacerbation of fungal asthma, Axl blockade significantly ameliorated parameters of exacerbation, which suggests that the promotion of resolution by Axl promotes subsequent viral-mediated pathogenesis (Shibata et al., 2014). Production of anti-inflammatory cytokines IL-10 and TGF-β by the epithelium, promote wound healing and a return to homeostasis, but also suppress immune responses by macrophages, which allow for subsequent infection (van der Sluijs et al., 2004, Holt et al., 1993, Zdrenghea et al., 2015). The balance of resolution of initial lung inflammatory disease and the ability to mount an effective response to following subsequent infections is clearly a multifaceted topic, in which macrophages are an important cell type worthy of further study.

The balance of responsiveness to TLR ligands is not only important in primary infections and allergic inflammation, but also in determining susceptibility to secondary infections. Recently, the role of co-receptors in TLR signalling has been explored, with the aim of identifying therapeutic targets to reduce inflammation without compromising pathogen clearance. An important class of receptors, expressed on myeloid cells such as neutrophils and macrophages, and involved in fine-tuning the TLR response are the TREM proteins.

1.5.4 TREM proteins

TREM proteins were first identified by Bouchon *et al.* (2000). The authors found two receptors that associated with DNAX activation protein-12 (DAP12), which they named TREM-1 and TREM-2. A family of TREM proteins of the Ig superfamily has since been identified, which also includes TREM-3, TREM-like transcript (TLT)-1, TLT-2 and PDC-TREM (Chung et al., 2002, Washington et al., 2002, King et al., 2006, Watarai et al., 2008). The genes encoding TREM proteins are located on human chromosome 6p21.1 and mouse chromosome 17C (Allcock et al., 2003).

1.5.4.1 TREM-1 signals via DAP12

TREM-1 is a 30 kDa glycoprotein, consisting of an extracellular domain, a transmembrane domain and a short cytoplasmic region that lacks any signalling motifs (Bleharski et al., 2003). Crystallography of TREM-1 structure indicates that TREM-1 exists either as a dimer with two ligand binding sites or as a monomer with only one site for ligand binding (Radaev et al., 2003, Kelker et al., 2004). Haselmayer and colleagues (2007) found evidence of a binding partner for TREM-1 on human platelets. The authors were, however, unable to identify the ligand due to low affinity binding to recombinant soluble (s)TREM-1, rendering mass spectrometry and affinity chromatography unsuccessful. More recently, Wu *et al.* (2012) showed that TREM-1 binds to high-mobility group box 1 (HMGB1) and that it could potentially act as an agonist of TREM-1.

Because of a lack of signalling motifs, TREM-1 associates with DAP12 in order to induce signal transduction (Bouchon et al., 2000). A positively charged lysine residue within the transmembrane domain of TREM-1 forms a non-covalent interaction with an aspartic acid residue on DAP12 (Figure 1.3). This results in the formation of the immunoreceptor-tyrosine-based activation motif (ITAM) on the intracellular domain on DAP12 (Lanier and Bakker, 2000). Tyrosine residues on DAP12 are phosphorylated by proto-oncogene tyrosine-protein kinase (Src) kinases, which lead to the recruitment and phosphorylation of spleen tyrosine kinase (Syk). Subsequently, signalling cascades involving PI3 kinase (PI3K), phospholipase-C-γ (PLC-γ) and MAPK are activated. Gene expression is induced which leads to cytokine and chemokine production (e.g. CXCL8, TNF-α and IL-1β) and calcium mobilisation (Bouchon et al., 2000) .

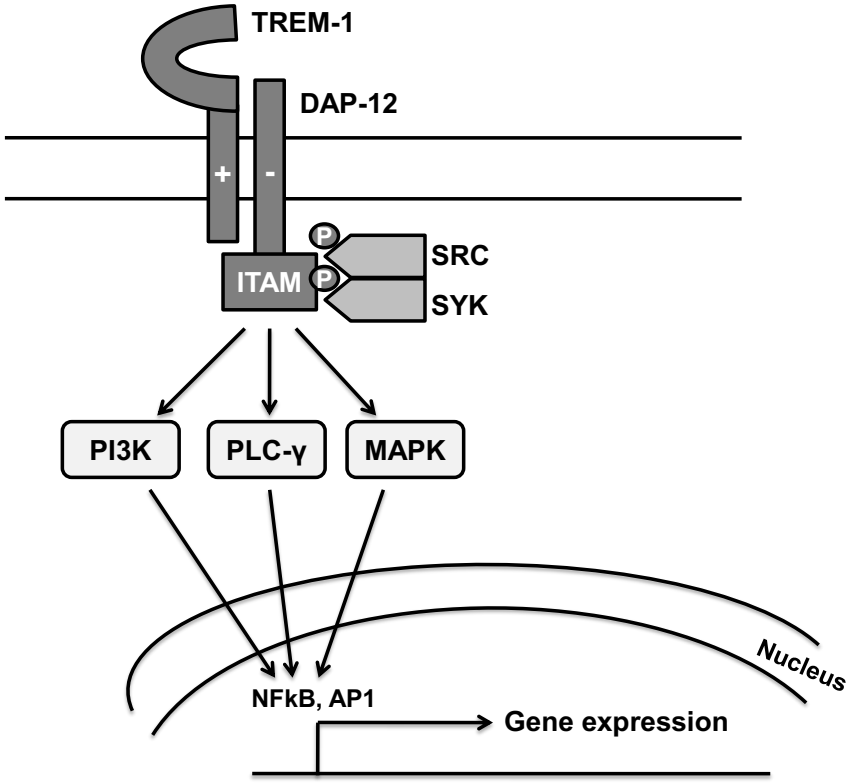

Figure 1.3 TREM-1 signalling pathway.

Engagement of TREM-1 with its as yet unknown ligand leads to association and activation of signalling adaptor DAP12. Phosphorylation of signalling adaptors result in the activation of intracellular signalling pathways leading to inflammatory mediator gene expression.

1.5.4.2 TREM-1 is an amplifier of inflammatory responses

TREM-1 is predominantly expressed on myeloid cells and amplifies inflammation by synergising with TLRs to induce proinflammatory cytokine expression (Bouchon et al., 2000). In addition to its direct role in promoting proinflammatory responses, TREM-1 plays diverse roles in the immune response. Cai and colleagues (2014) found that activation of TREM-1 by an agonistic antibody prevents the apoptosis of monocytes via extracellular signal-regulated kinase and PI3K/Akt signalling pathways. Another study showed that while both neutrophils and monocytes express TREM-1, there are differences in the role of intracellular pathways activated. Both cell types utilise the p38 MAPK and PI3K signalling cascades, but while monocyte activation requires the interaction of the two signalling cascades, independent MAPK and PI3K signalling result in neutrophil activation (Prüfer et al., 2014). TREM-1 also plays a part in tolerance, as signalling via TLR7 and TREM-1 is involved in the killing of alloreactive $CD8^+$ T cells by immature DCs (Zangi et al., 2012). TREM-1 expression has also been shown in non-myeloid cells. One research group reported expression of TREM-1 on both oral and airway epithelial cells (McMahon et al., 2011, Rigo et al., 2012). TREM-1 activation induces proinflammatory cytokine production in these epithelial cells, and, interestingly, TREM-1 expression is upregulated by vitamin D.

1.5.4.3 The role of TREM-1 in disease

Since its discovery in 2001, TREM-1 has been implicated in a plethora of inflammatory diseases, such as psoriasis and colitis (Hyder et al., 2013, Zhou et al., 2013). However, TREM-1 has been most studied in bacterial infections and sepsis. TREM-1 is expressed on myeloid cells from patients with bacterial infections and sepsis caused by a variety of bacterial species, including *Pseudomonas aeruginosa* and *Escherichia coli* (Wang et al., 2012, van Bremen et al., 2013). A study by Hommes and colleagues (2014) found that mice lacking TREM-1/3 were less able to phagocytose *S. pneumoniae* and produced lower levels of cytokines in response to infection. Conversely, in a mouse model of *P. aeruginosa*, TREM-1/3 knock-out mice had increased inflammatory cytokine levels and increased mortality due to impaired transmigration of neutrophils across the airway epithelium (Klesney-Tait et al., 2013). Clearly, the role of TREM-1 is not restricted to promoting

inflammatory mediator release, rather a diverse role in innate immunity is beginning to emerge.

1.5.4.4 sTREM-1 in diagnosis of disease and infection

In addition to TREM-1 cell surface expression, a soluble form sTREM-1 can be detected during inflammatory diseases, such as sepsis (Gibot et al., 2012, Horst et al., 2013). The expression of sTREM-1 is currently used as diagnostic tool in plasma and is even detectable in the urine as a marker of kidney injury (Derive and Gibot, 2011).

sTREM-1 is also a marker of severity in a various lung diseases, e.g. tuberculosis, community-acquired pneumonia and bacterial exacerbations of chronic obstructive pulmonary disease (COPD) (Huang et al., 2014, Tejera et al., 2007, Rohde et al., 2012). In one study, plasma sTREM-1 levels were found to be higher in asthmatic patients compared to healthy controls, and that the levels of sTREM-1 correlated with severity disease and numbers of neutrophils in the blood (Bucova et al., 2012). These studies suggest that the presence of sTREM-1 is not only an indicator of disease, but can also be indicative of disease severity.

1.5.4.5 TREM-2 plays a distinct role in immunity from TREM-1

Like TREM-1, the TREM-2 protein lacks a signalling motif and relies upon DAP12 to mediate intracellular signalling. Interaction of TREM-2 with DAP12 leads to phosphorylation of Syk kinase, and several signalling pathways can be activated, e.g. PI3K and ERK pathways (Peng et al., 2010). The authors propose that these pathways can in turn be inhibited upon TREM-2 activation by the recruitment of SHIP-1 to DAP12. However, unlike TREM-1, TREM-2 exhibits diverse functions dependent on the cell types on which it is expressed. In microglia, TREM-2 promotes phagocytosis (Takahashi et al., 2005), while TREM-2 has both activating and inhibiting effects in osteoclastogenesis (Klesney-Tait et al., 2006). Deficiency in TREM-2 or gene variants of TREM-2 are associated with a higher risk of developing neurological diseases such as Nasu Hakola disease, Alzheimer's and Parkinson's disease (Paloneva et al., 2002, Jonsson et al., 2013, Rayaprolu et al., 2013).

TREM-2 is also thought to play a role in bacterial infections. TREM-2 was identified as a phagocytic receptor for *E. coli* in Chinese hamster ovary cells (N'Diaye et al., 2009), and is important in protection against microbial sepsis (Chen et al., 2013). Triggering of PI3K signalling by TREM-2 activation results in the suppression of the inflammatory response, as well as the clearance of bacteria in models of *P. aeruginosa* infection (Sun et al., 2013, Zhu et al., 2014).

TREM-2 has been shown to exert anti-inflammatory effects in macrophages (Turnbull et al., 2006). In this study, the authors found TREM-2 expression on recently recruited macrophages and alternatively-activated (M2) macrophages, which have an anti-inflammatory function (Gordon and Martinez, 2010). TREM-2 activation also negatively regulates TLR signalling in DCs and macrophages (Ito and Hamerman, 2011, Peng et al., 2013). Furthermore, knock-down of TREM-2 in macrophages using siRNA increases LPS-induced TNFα production (Gao et al., 2013). TREM-2 is also involved in wound healing. A model of colonic mucosal injury in mice showed that mice lacking TREM-2 exhibited impaired wound healing, which was associated with reduced epithelial cell proliferation, increased IFN-γ and TNF-α, promotion of classical macrophage activation and reduced IL-4 and IL-13 expression (Seno et al., 2009). Thus, providing further evidence that TREM-2 is associated with M2 macrophages and wound repair, and of the distinct roles of TREM-1 and TREM-2.

The differences of TREM-1 and TREM-2 expression, i.e. amplification versus inhibition of inflammation, may be altered during lung inflammation, thereby impairing the immune response to secondary bacterial or viral infection. The expression of TREM-1 and TREM-2 on myeloid cells will be explored different models of inflammatory lung disease in this thesis.

1.6 Treatment of secondary complications of the lung

1.6.1 Secondary bacterial infections following influenza

In addition to the use of drugs such as antibiotics to treat secondary bacterial infections that reduce inflammation in general, anti-viral and anti-bacterial drugs have been studied to determine their effectiveness in reducing morbidity and mortality associated with secondary bacterial infections.

Anti-viral drugs are used in the treatment of influenza infection, for example, NA inhibitors, which prevent viral release. An example of which is oseltamivir. Tested in a randomised, double-blind trial, oseltamivir was able to reduce symptoms of influenza by 44% in children (Whitley et al., 2001). A study in adults showed that antibiotic use was reduced by 27% by oseltamivir treatment (Kaiser et al., 2003). Meta-analyses on the effects of oseltamivir on pneumonia have shown conflicting results (Gums et al., 2008, Piedra et al., 2009), however, there remains the possibility that boosting anti-viral activity will reduce susceptibility to bacterial infections caused by one of the aforementioned mechanisms of co-pathogenesis.

Therapies targeting the bacterial infection are also utilised in the treatment of secondary bacterial infections. Anti-bacterial therapy focuses on reducing excessive inflammation and the promotion of resolution (Metersky et al., 2012). Protein synthesis inhibitors (PSIs) are an example of drugs that prevent the production of bacterial toxins and inhibit bacterial lysis, thereby reducing the degree of inflammation induced. In a mouse model of secondary bacterial infection following influenza, PSI treatment reduced inflammation, specifically TNF-α-associated inflammation, without compromising bacterial clearance. Macrolides, a class of bacteriostatic antibiotics, were shown to reduce inflammation in influenza alone (Sato et al., 1998, Gielen et al., 2010). Studies have suggested that combined use of drugs, e.g. NA inhibitors and PSIs could hold promise for treatment of viral and bacterial co-infections; however, more studies are required before their use in the clinic (Metersky et al., 2012).

1.6.2 Asthma and asthma exacerbations

Glucocorticoids are the main treatment for asthma, however, conflicting evidence exists on whether they are beneficial for the treatment or prevention of viral exacerbations (Edwards et al., 2006). For example, glucocorticoid use increased the incidence of bacterial pneumonia in COPD patients (Calverley et al., 2007), while in another study, the authors found that long term glucocorticoid treatment reduced asthma exacerbation-related hospital admissions in preschool children (Guilbert et al., 2006).

Since impaired type I IFN responses have been observed in some asthmatic patients, promoting a robust anti-viral response in those patients has recently been tested as a new approach for treating viral exacerbations of asthma. A clinical trial of inhaled IFN-β in adults showed that treatment reduced asthma exacerbations if administered at the first sign of cold symptoms (Djukanović et al., 2014). Promotion of the anti-viral response to reduce viral replication and the reported effect of IFN-β inhibiting the Th2 response are two possible mechanisms by which exacerbations are prevented (Pritchard et al., 2012).

Another strategy in the treatment of viral exacerbations of asthma is vaccination against the pathogen itself. As described previously, palivizumab is a good candidate for reducing RSV infection when used prophylactically (Shadman and Wald, 2011). However, it is still not recommended for the prevention of RSV-induced asthma exacerbations except in preterm infants (AAP, 2014). Motavizumab is a monoclonal antibody that binds to RSV-F protein and reduces RSV burden more effectively than palivizumab, but has not been licenced for use in the USA. A recent trial reported its efficacy in preventing RSV infection in healthy infants, however, the vaccine had no effect on recurrent wheeze in healthy term born neonates, in contrast to palivizumab (O'Brien et al., 2015; Blanken et al., 2013).

1.6.2.1 Asthma therapies targeting inflammation

Given the prominent role of inflammation in the development of asthma and the multitude of cells and soluble mediators involved, it is of no surprise that inflammation has been at the centre of asthma treatment development. One such area of research is allergen-specific

immunotherapy (SIT). Known allergens can be utilised and administered to allergic individuals in order to induce immunological tolerance. SIT can alter the balance of allergen-specific IgA and IgG_4 versus allergen-specific IgE (Holgate and Polosa, 2008). $CD4^+CD25^+FoxP3^+$ regulatory T cells that produce high levels of IL-10 and TGF-β can inhibit various immune cells involved in the inflammatory response in asthma (Lloyd and Hawrylowicz, 2009), and SIT promotes such cells, thus, precipitating tolerance (Jutel and Akdis, 2011, Fujita et al., 2012). Immunotherapy based on a combination of T-cell antigens was shown to reduce OVA-specific IgE production and eosinophil production in a mouse model of allergic airway inflammation (Mackenzie et al., 2013). The benefits of SIT have also been tested in patients with established disease. A randomised controlled trial of house dust mite-SIT in children showed that SIT treatment enabled the reduction of corticosteroid doses in the control of allergic asthma (Zielen et al., 2010).

Cytokines involved in allergic inflammation have also been considered as targets for the treatment of allergic asthma. Monoclonal antibody (mAb) blockade of the IL-4 receptor α (IL-4Rα) reduces exacerbations and Th2 cytokine production upon withdrawal of glucocorticoids (Wenzel et al., 2013). Administration of the anti-IL-5 mAb reslizumab results in a trend towards reduced exacerbations in severe eosinophilic asthma (Haldar et al., 2009; Castro et al., 2011), while treatment of patients with asthma with a mAb targeting IL-13 improved lung function (Corren et al., 2011).

Other mediators involved in airway inflammation have also been targeted, such as IgE and TSLP (Gauvreau et al., 2014, Kopp et al., 2009). However, while these treatments show promise in their effectiveness in controlling allergic inflammation during asthma, it may take prolonged periods of time before effects can be seen, with treatment and the observation phase lasting many weeks. Moreover, the route of administration usually requires injection via the intravenous or subcutaneous route, which is neither as convenient nor as well received as an oral or inhalation route. The development of treatment that can be administered via the inhalation route that localises the action of the drug to the site of inflammation would be ideal, and this is an area that some drug development companies are exploring.

1.6.2.2 Targeting signalling pathways

One approach to modulating inflammation during allergic airway disease and exacerbations is to target signalling pathways involved in the immune cell activation. Signalling kinases that are important in initiating inflammatory responses, such as Src family kinases, PI3 kinases and p38 MAPK, are examples of molecules that have been experimentally inhibited in allergic inflammation.

Syk is a tyrosine kinase essential for the development of the lymphatic system and deficiency in Syk leads to embryonic lethality (Böhmer et al., 2010). Syk associates with the intracellular domains of a variety of cell-surface receptors of, for example, macrophages, neutrophils, mast cells and B cells (Taniguchi et al., 1991). Activation of Syk by phosphorylation leads to the initiation of signalling cascades leading to cytokine production and phagocytosis (Mócsai et al., 2010). With its wide-ranging function in a plethora of immune cells that are involved in inflammation, it is an attractive therapeutic target (Riccaboni et al., 2010), with an increasing number of patents for inhibitors filed in recent years (Norman, 2014). The Syk inhibitor R343 has been tested in an asthma challenge model (Allan et al., 2012). Two doses of R343 were given to patients before and after allergen challenge in steroid-naïve patients. At both a high (12 mg) and low dose (1.3 mg), R343 was able to prevent the early and late asthmatic response to the same degree as glucocorticoid fluticasone treatment and was well tolerated by patients.

Similarly, Src family kinases have been investigated for their effectiveness in controlling allergic inflammation. The Src family, including Lck and Fyn, are important kinases in T-cell development and activation (Palacios and Weiss, 2004). Rapid phosphorylation and activation of Src kinases can occur upon IgE cross-linking, in basophils and mast cells (Harvima et al., 2014), thus making Src inhibitors potentially good candidates for controlling asthma symptoms. Signalling through Src kinases is also involved in AHR (Katsumoto et al., 2013). Dasatinib (Bristol-Myers Squibb) is an inhibitor of Abl and Src kinases and is approved for use in chronic myeloid leukaemia, in which prevents leukaemic cell proliferation by inhibiting the oncogenic form of the tyrosine kinase c-Abl (Shah et al., 2004). In a mouse model of allergic dermatitis, dasatinib was able to inhibit inflammation by

suppressing the proinflammatory cytokines IL-1β, TNF-α and IFN-γ (Jung et al., 2013). *In vitro* studies also show the effectiveness of dasatinib in suppressing mast cell cytokine production and IgE-mediated basophil activation (Hantschel et al., 2007, Kneidinger et al., 2008).

Downstream signalling kinases p38 MAP kinase (and PI3K are also targets for allergic inflammation treatments. p38 MAPK signalling is involved in IgE-induced proliferation of ASM (Redhu et al., 2013), and regulates the production of Th2 cytokines in $CD4^+$ T cells (Dodeller et al., 2005). PI3Kγ and δ expression is generally restricted to leukocytes and they are important in macrophage, neutrophil and eosinophil migration, mast cell degranulation and Th1/Th2-cell differentiation (Marwick et al., 2010). PI3K inhibitors are also effective in reducing AHR and airway inflammation in models of allergic inflammation (Kwak et al., 2003, Lee et al., 2006). Clearly, pharmacological inhibition of various signalling kinases warrants further investigation in the search for new drugs for the treatment of asthma itself and for complications of asthma, e.g. exacerbations. Of particularly interest, would be the combination of PI3Kγ and δ inhibition, which could enhance the inhibition of inflammatory processes involved in asthma.

1.7 Urgent need for new treatments for respiratory diseases

There is undoubtedly a need for novel treatments for important respiratory conditions, such as secondary bacterial infections following influenza infection and exacerbations of asthma. Antibiotic resistance is becoming more prevalent and is a major global health issue, as demonstrated by the drafting of a global action plan on antibiotic resistance by the WHO in 2015 (WHO, 2015). While action on the issue is welcome, it is important that development of new therapies for diseases, such as secondary bacterial infection, do not focus solely on antibiotics, which are fast becoming redundant in many diseases (e.g. multidrug-resistant tuberculosis) (Miotto et al., 2015). Similarly, existing treatments for asthma and exacerbations of asthma are not effective in all patients (Masoli et al., 2004) and novel treatments that are not steroid-based are needed. One approach could be to develop drugs target signalling pathways that are central to the inflammatory response. TC1 and TC2 are two such small molecule inhibitor compounds that have been developed by drug discovery

for the treatment of airway diseases. TC1 specifically inhibits PI3Kγ and δ and has shown anti-inflammatory effects in a smoking and OVA-induced asthma mouse model and poly(I:C)-induced airway inflammation (Kimura et al., 2013). TC1 has been shown to suppress CXCL8 production from human peripheral blood neutrophils derived from patients with COPD (Ito et al., 2013b). TC2 is an inhibitor acts on Syk, Src and p38 MAPK and is also a promising candidate in controlling inflammatory conditions. TC2 was well tolerated in a phase I study and has demonstrated target engagement in COPD (Russell et al., 2015). Given their roles in various aspects of inflammatory lung disease, they represent potentially effective therapies for the treatment of respiratory diseases.

1.8 Hypothesis

Given the role of TREM-1 and TREM-2 in published models of bacterial and viral infection, it is hypothesised that these proteins will be expressed and differentially regulated in models of influenza, secondary bacterial infection, HDM-induced allergic airway disease and viral infection following HDM-induced allergic airway disease. Specifically, TREM-1 is hypothesised to be upregulated during peak inflammation, while TREM-2 will be upregulated during the resolution phase. Furthermore, sTREM-1 levels are hypothesised to correlate with peak inflammation.

Common signalling kinases, such as Src, Syk, MAPK and PI3K are involved in inflammatory processes in the lung; thus, it is hypothesised that inhibition of these kinases using small molecule inhibitors will reduce the signs of airway disease in the HDM and HDM/RSV models—i.e. inhibitor treatment will reduce AHR, allergy-induced cell infiltration, immunoglobulin production and cytokine production. Also, RSV infection of HDM-exposed mice is hypothesised to exacerbate parameters of allergic airway disease.

1.9 Thesis aims

1) To determine the expression profile of TREM-1, TREM-2 and sTREM-1 in mouse models of influenza and secondary bacterial infection and to determine the correlation of

TREM expression with disease pathogenesis (Chapter 3). The expression of TREM-2 will also be studied in Chapters 4 and 5.

2) To determine the effects of signalling kinase inhibition during a mouse model of HDM-induced allergic airway disease (Chapter 4).

3) To develop a mouse model of RSV infection during HDM-induced allergic airway disease and to determine the effects of signalling kinase inhibition in this model (Chapter 5).

Chapter 2: Materials and methods

Chapter 2: Materials and methods

2.1 Animals

6–8 week-old or 17–19 g female BALB/c mice were purchased from Harlan Olac (Bicester, UK). All mice were maintained in specific pathogen-free conditions at Bio Safety Level 2 and kept in accordance with institutional and UK Home Office guidelines.

2.2 Pathogen stocks

2.2.1 Influenza

Influenza A Puerto Rico/8/34 H1N1 (PR8) was a kind gift from Dr Andreas Wack (National Institute for Medical Research, Mill Hill) and was stored at -80°C in single-use aliquots.

2.2.2 *S. pneumoniae*

Wild type *S. pneumoniae* (serotype 2) strain D39 (NTCC 7466, National Collection of Type Cultures, London, UK) was a kind gift from Prof Jeremy Brown (Centre for Respiratory Research, UCL, London). Bacterial stocks were cultured at 37°C in 5% CO_2 in Todd-Hewitt broth (Sigma, Dorset, UK) supplemented with 0.5% yeast extract (OXOID, Basingstoke, UK) to an optical density OD_{600} of 0.4 (approximately 1 x 10^8 colony forming units (CFU)/mL) and stored at -80°C in 10% glycerol as single-use aliquots.

2.2.3 RSV

Plaque-purified RSV A2 strain was a kind gift from industrial collaborators. Viral stocks were generated by infecting HEp-2 cells monolayers in culture flasks at multiplicity of infection (MOI) 0.1. 48 hours post-RSV infection, virus was released from infected cells by sonication for 20 seconds in a sonicating water bath. Resultant cell debris and supernatant were snap frozen and stored at -196°C in liquid nitrogen in single-use aliquots. The RSV batch used in this thesis was produced by Dr Gaynor Campbell.

2.3 Mouse infection models

2.3.1 Influenza infection model

Female BALB/c mice were anaesthetised and intranasally inoculated with 15 plaque-forming units (PFU) PR8 in phosphate buffered saline (PBS) or 50 μL PBS alone as control. Disease severity and weight loss were monitored daily. Mice were culled 7 and 14 days post-infection, or when mice had reached humane endpoint (as defined by HO Project Licence) by intraperitoneal injection of 3 mg pentobarbitone (Animal Care, York, UK) and exsanguination via the femoral artery.

2.3.2 Secondary bacterial infection model

Female BALB/c mice were anaesthetised and inoculated with 7 PFU of PR8 intranasal or 50 μL PBS alone as a control. 7 days post-viral infection, mice were inoculated with *S. pneumoniae* i.n (10^6 CFU). Disease severity and weight loss were monitored daily. Mice were culled 24 or 48 hours post-bacterial infection, or when mice had reached humane endpoint, by intraperitoneal injection of 3 mg pentobarbitone and exsanguination via the femoral artery.

2.4 Mouse allergen challenge and infection models

2.4.1 Allergic airway disease model

Female BALB/c mice were anaesthetised and inoculated with 25 μg HDM extract intranasal (*Dermatophagoides pteronyssinus*; Greer Laboratories, Lenoir, NC, USA) dissolved in sterile PBS or PBS alone 3 times per week for 4 weeks. After 4 weeks of HDM challenge and 24 hours after the final HDM exposure, mice were either subjected to airway function measurements or culled by intraperitoneal injection of 3 mg pentobarbitone and exsanguination by cardiac puncture.

2.4.2 Viral challenge during allergic airway disease model

Female BALB/c mice were anaesthetised and inoculated with 25 μg HDM extract intranasally dissolved in sterile PBS or PBS alone 3 times per week for 4 weeks. After 3 weeks of HDM challenge and 24 hours after the previous HDM exposure, mice were intranasally inoculated with 1 x 10^6 or 1.28 x 10^6 PFU RSV or PBS as control. Disease

severity and weight loss were monitored daily. On day 3 or 6 post-RSV infection and 24 hours post final HDM exposure, mice were either subjected to airway function measurements or culled by intraperitoneal injection of 3 mg pentobarbitone and exsanguination by cardiac puncture.

2.5 Inhibitors

Female BALB/c mice were anaesthetised and intranasally inoculated with 1 mg/mL TC1, 1 mg/mL TC2, 2 mg/mL dasatinib (Bristol-Myers Squibb) or 2 mg/mL R343 (Rigel Pharmaceuticals) in 10% dimethyl sulphoxide (DMSO) (Sigma) vehicle or vehicle alone daily for 5 consecutive days prior to cull (Chapter 3 and Chapter 4) or for 5 consecutive days prior to RSV infection (Chapter 5). All inhibitors were provided by industrial collaborators.

2.6 Measurements of AHR

Measurements of dynamic resistance and compliance were performed using The Flexivent system (Scireq, Montreal, Canada). After induction of anaesthesia with 50 mg/Kg pentobarbitone (Sigma) intraperitoneal and 200 mg/Kg ketamine (Fortdodge Animal Health Ltd, Southampton, UK) by intramuscular injection, mice were tracheostomised and connected to the Flexivent ventilator via a blunt-ended 19-gauge needle. Mice were ventilated using the following settings; tidal volume of 7 mL/Kg weight, 150 breaths/min; positive end-expiratory pressure approximately 2 cm H_2O. Standardisation of the lung volume history was performed via two deep inflations. Measurements of resistance (R; ease with which air flows through the lumen), compliance (C; ability of the lung tissue and alveoli to expand on inspiration) and elastance (E; ability of the lung to recoil upon expiration, or stiffness of the lung) were determined from a user-defined protocol using the snapshot-150 perturbation, which is a single frequency sinusoidal waveform. Resultant data was fitted using multiple linear regression to the single compartment model to determine the above parameters. Measurements of tissue damping (G; a measure of energy dissipation in the alveoli) and tissue elastance (H; a measure of energy conservation in the alveoli) were determined using the quickprime-3 perturbation, which is a multiple frequency oscillatory waveform. The resultant data was fitted to the Constant-Phase model to determine the above parameters.

Changes in lung/airway function parameters were measured in response to increasing concentrations of nebulised metacholine from 3–100 mg/mL, delivered into the inspiratory line of the Flexivent ventilator. Airway resistance was expressed as $cmH_2O.s/mL$, compliance was expressed as mL/cmH_2O and elastance was expressed as cmH_2O/mL, tissue damping was expressed as cmH_2O/mL and tissue elastance was expressed as cmH_2O/mL.

2.7 Sample recovery and cell isolation

2.7.1 Serum recovery and cell isolation—Chapter 3

Mice were sacrificed using 3 mg of pentobarbitone anaesthetic, and exsanguinated via the femoral artery. Blood, bronchoalveolar lavage (BAL) fluid and lung tissue were harvested.

2.7.1.1 Whole blood and serum collection

Whole blood was allowed to clot overnight at 4°C before centrifugation at 5000 x g for 8 mins, and the supernatant stored at -80°C for future analysis. For CFU analysis, 90 μL of blood was prevented from clotting by addition of 10 μL of sodium heparin (Leo Pharma, Berkshire, UK).

2.7.1.2 BAL cell isolation

BAL was performed by inflation of the lung six flushes of 1.5 mL 5mM EDTA in Hanks' Balanced Salt Solution (HBSS) (PAA laboratories, Somerset, UK) via an intratracheal cannula. BAL fluid was centrifuged at 240 x g for 5 mins and the supernatant was stored at -80°C. Red blood cells were lysed with ammonium chloride potassium (ACK) buffer (0.15 M ammonium chloride, 1 M potassium hydrogen carbonate and 0.01 mM EDTA, pH 7.2) for 3 mins and washed with R10F (Roswell Park Memorial Institute RPMI-1640 medium (PAA) containing 10% v/v foetal calf serum (FCS) (Sigma) and 1% penicillin/streptomycin (Gibco, Paisley, UK)). Cell viability was assessed by Trypan blue exclusion and cell density adjusted to 1 x 10^6 cells/mL in R10F.

2.7.1.3 Lung cell isolation

One lung lobe was removed and finely chopped, incubated with 0.14 Wunsch units/mL Liberase (Roche, Welwyn Garden City, UK) and 50 μg/mL DNAse I (Roche) for 30 mins at

37°C, before passing through a 70 μM cell strainer. Cell suspensions were pelleted by centrifugation at 375 x g for 5 mins before red blood cell lysis with ACK buffer for 3 mins and washed with R10F media. Cell density was adjusted to 1 x 10^6 cells/mL in R10F following Trypan blue exclusion of dead cells.

2.7.1.4 Snap freeze

Lung lobes were snap-frozen in liquid nitrogen for future analysis.

2.7.2 Serum recovery and cell isolation—Chapters 4 & 5

Mice were sacrificed using 3 mg of pentobarbitone anaesthetic, and exsanguinated by cardiac puncture. In experiments with lung function studies, mice were anaesthetised with 50 mg/Kg pentobarbitone intraperitoneal and 200 mg/Kg ketamine intramuscular injection and exsanguinated by cardiac puncture following lung function measurements. Blood, BAL fluid and lung tissue were harvested.

2.7.2.1 Serum collection

Whole blood was collected in serum-separating BD Microtainer tubes (BD Diagnostics, Oxford, UK) tubes, left to clot overnight at 4°C before centrifugation at 5000 x g for 5 mins and stored at -80°C for future analysis.

2.7.2.2 BAL cell isolation

BAL was performed by inflation of the lung three times with 0.4 mL cold PBS via an intratracheal cannula. BAL fluid was centrifuged at 240 x g for 5 mins and the supernatant was stored at -80°C. Red blood cells were lysed with ACK buffer for 3 mins and washed with R10F media. Cells were counted using white blood cell counting fluid (crystal violet indicator in 1% v/v acetic acid in H_2O) and cell density adjusted to 1 x 10^6 cells/mL in R10F.

2.7.2.3 Lung cell isolation

One lung lobe was removed and finely chopped, incubated with 150 μg/mL Collagenase (Roche Diagnostics, Lewes, UK) and 25 μg/mL DNAse I (Roche) for 1 hour at 37°C, before passing through a 70 μM cell strainer. Cell suspensions were pelleted by centrifugation at 600 x g for 10 mins before red blood cell lysis with ACK buffer for 3 mins and then washed with

R10F media. Cells were counted using white blood cell counting fluid and cell density adjusted to 1 x 10^6 cells/mL in R10F.

2.7.2.4 Snap freeze

Two lung lobes were snap-frozen separately in liquid nitrogen for future analysis.

2.8 Lung histology

After dissection, one lung lobe was inflated with PBS and fixed in 10% neutral buffered formalin for 24 hours. Tissues were paraffin-embedded and 4 μm transverse sections were cut and fixed onto glass slides. Embedding, sectioning and staining with haematoxylin and eosin (H&E) and periodic acid Schiff (PAS) were performed by Lorraine Lawrence, Leukocyte Biology Histology Service, NHLI, Imperial College.

2.8.1 H&E staining

Slides were de-waxed through three changes of Histoclear (National Diagnostics, UK), then rehydrated through 100%, 90% and 70% ethanol and water before staining with H&E, dehydrated through increasing ethanol and mounted with cover slips.

2.8.2 H&E scoring

The extent of cellular infiltration was determined on H&E-stained lung sections using an arbitrary scoring system as previously described (Lloyd et al., 2001). Cellular infiltration into the lung tissue was scored (x40 magnification) according to Table 2 and the score for each whole section expressed as inflammatory score in arbitrary units.

2.8.3 PAS staining

Slides were de-waxed through three changes of Histoclear (National Diagnostics), then rehydrated through 100%, 90% and 70% ethanol and water before staining with periodic acid and Schiff's reagent. Slides were counterstained with haematoxylin, dehydrated through increasing ethanol and mounted with cover slips.

Table 2. Scoring system for H&E-stained lung sections.

Score	Observation
0	No infiltrate
1	One small pocket of infiltrate
2	Small infiltrate around more than 1 airway or vessel
3	Large infiltrate (more than 3 cells deep) around more than 1 airway or vessel
4	Large infiltrates around most airways and vessels
5	Large infiltrates around most airways and vessels, cells present in alveolar bed

2.8.4 PAS scoring

Mucus-containing cells were quantified on PAS-stained sections using an arbitrary scoring system based on a published protocol (Grünig et al., 1998), summarised in Table 3. The total scores for each section were divided by the number of airways examined (all airways per lung section; x40 magnification) and expressed as mucus score in arbitrary units.

Table 3. Scoring system for PAS-stained lung sections.

Score	Percentage of cells staining magenta/purple
0	Less than 5%
1	5-25%
2	25-50%
3	50-75%
4	More than 75%

2.9 *In vitro* stimulation

NHBECs derived from two donors were seeded at 4 x 10^5 cells/well in a 24-well plate. Cells were stimulated with 100 µg/mL TLR2 agonist zymosan, 10 µg/mL TLR3 agonist poly(I:C), 400 ng/mL TLR4 agonist LPS, 0.1 µg/mL TLR7/8 agonist R848 and/or in combination with 5 µg/mL HDM extract, or PBS as control for 24 hours at 37°C, 5% CO_2. Cells were infected with RSV A2 MOI 0.1 or mock for 1 hour at 37°C. Cells were washed twice with PBS before

addition of fresh media and incubation for a further 24 hours at 37°C, 5% CO_2 before cells and culture supernatants were harvested.

2.10 Determining infectious load

2.10.1 RSV plaque assay

RSV titre was assessed from lung homogenate supernatants by titration on HEp-2 monolayers in 96-well plates. Briefly, serial tenfold dilutions of lung homogenate supernatants were added to HEp-2 monolayers in quadruplicate. Samples were incubated with cells at 37°C, 5% CO_2 for 4-7 days before assessing cytopathic effect (CPE). Viral titre was determined as TCID50/mL, i.e. the dilution at which CPE develops in 50% of replicate cultures multiplied the dilution factor of supernatants. RSV viral titre assays were performed by industrial collaborators.

2.10.2 CFU

Bacterial titres in the BAL, lung and blood were determined by counting CFU from serial tenfold dilutions of bacterial cell suspensions plated on Columbia blood agar plates supplemented with 5% defibrinated horse blood (TCS Bioscience, Buckingham, UK) incubated for 24 hours at 37°C. The total number of CFU was determined by multiplying number of colonies by dilution factor and volume of suspension.

2.11 Differential cell counts and Wright-Giemsa staining of leukocytes

BAL and lung leukocytes were applied to glass slides by cytocentrifugation of 5 x 10^4 cells for 4 mins at 16 x g (Cytospin 4, ThermoShandon, UK). Slides were allowed to air dry before fixing in absolute methanol for 5 mins. BAL and lung leukocytes were stained with Wright-Giemsa stain (Sigma) for 5 mins and rinsed in water. Percentages of macrophages, eosinophils, neutrophils and lymphocyte/monocytes were determined by counting under light microscopy at 40x magnification. Approximately 300 cells were counted in randomly selected fields and the number of each population divided by the total number of cells. Absolute numbers were obtained by multiplying the percentage of each cell type by the total number of cells recovered from BAL or lung tissue digest.

2.12 Flow cytometry

Cells isolated from BAL or lung tissue digest were stained with dead cell dye LIVE/DEAD® Fixable Dead Cell Stain (Invitrogen, Paisley, UK) for 20 mins before washing twice with PBA (PBS containing 0.1% sodium azide and 1% bovine serum albumin (BSA) (Sigma)) and centrifugation at 670 x g for 2 mins. Cells were then stained with antibodies (Table 4) for extracellular antigen markers in PBA for 30 mins at 4°C in the dark, washed twice and fixed with 2% formaldehyde/PBS. Data acquired on a BD FACS Canto II and 30,000 lymphocyte or myeloid events analysed with the FlowJo (Treestar, Ashland, OR) analysis programme. Variations in fluorescent levels were controlled for by including a fluorescence minus one (FMO) control for each fluorescent marker at each time-point. Expression of a particular marker was calculated by subtracting FMO fluorescence values from fluorescent antibody levels of the marker of interest.

Table 4. Antibodies for flow cytometry.

Name	Clone	Conjugation	Company	Concentration (µg/mL)
CD11b	M1/70	PerCP-Cy5.5	eBioscience	0.5
CD11c	N418	FITC, PE-Cy7		1
Ly6G	1A8	FITC		2
F4/80	BM8	FITC, PE-Cy7		2
CD3	145-2C11	PE-Cy7		2
CD4	RM4-5	PerCP-Cy5.5		2
CD8	53-6.7	APC		2
NKp46	29A1.4	PE		2
TREM-1	174031	APC	R&D Systems	2
TREM-2	237920	PE		2
CCR5	CTC5	APC		2
CCR7	4B12	APC		2

2.13 Soluble mediator detection

2.13.1 Enzyme-linked immunosorbent assay (ELISA)

Soluble mediators levels in BAL fluid, lung homogenate supernatant or serum were measured by sandwich ELISAs. Murine TREM-1, matrix metalloproteinase (MMP)-9 and IL-33 were measured by Duoset ELISA kits (R&D systems, Abingdon, UK) according to the manufacturer's instructions. Murine IL-13, IL-17A, TNF-α and IL-6 were measured by ELISA kits (eBioscience, Hatfield, UK) according to the manufacturer's instructions. Murine albumin was measured in the BAL using an ELISA kit (Bethyl Laboratories, UK) according to the manufacturer's instructions. Murine IgE, IgG_{2a}, IgG1, IL-4 and IL-5 were measured using paired antibodies sets (BD Biosciences, Oxford, UK). For HDM-specific IgE, plates were coated with 50 μg/mL HDM extract.

2.13.1.1 ELISA protocol

96-well EIA/RIA plates (Costar, High Wycombe, UK) were coated with capture antibody in coating buffer overnight at room temperature. Following 3 washes with wash buffer 0.05% Tween-20 (Sigma) in PBS, plates were blocked with blocking buffer for 1 or 2 hours at room temperature. Samples and standards of known concentration were then incubated for either 2 hours at room temperature or overnight at 4°C. After 3 washes, biotinylated detection antibody was incubated for 1 or 2 hours at room temperature in the dark. Following 3 washes, samples were incubated with streptavidin-HRP for 20 mins at room temperature, following by activation by tetramethylbenzidine (TMB) (Neogen, Ayr, UK). Reactions were stopped by addition of 0.19 M H_2SO_4, and optical densities were read at measurement wavelength 450 nm and reference wavelength 570 nm. Mean optical density of blank wells was subtracted from samples and standard values. A standard curve drawn by non-linear regression was used to calculate sample concentrations. For a list of buffers used see Table 5.

2.13.2 Luminex assay

Murine IL-4, IL-13, IL-33, IL-10, IFN-γ, IL-6, IL-1β, IL-17A were measured in BAL using a Th17 multiplex kit and eotaxin/CCL11, KC/CXCL1, CXCL10, CCL2 and MIP1β/CCL4 were measured using a chemokine multiplex kit (Millipore, Billerica, MA, USA) with detection using a multiplex platform (Luminex, Austin, TX). Luminex assays were carried out by industrial collaborators.

Table 5. Buffers used for ELISA.

ELISA	Coating buffer	Blocking buffer	Reagent diluent
TREM-1, IL-33	PBS	1%BSA/PBS	1%BSA/PBS
MMP-9	PBS	1%BSA/PBS	50 mM Tris, 10 mM $CaCl_2$, 0.15 M NaCl, 0.05% Brij35, pH 7.5
IgE, IgG_{2a}, IgG_1	0.1M $NaHCO_3$	3%BSA/PBS	1%BSA/PBS, 0.05% Tween-20
HDM-specific IgE	0.1M $NaHCO_3$	1%BSA/PBS	1%BSA/PBS, 0.05% Tween-20
IL-4, IL-5	0.1 M Na_2HPO_4	1%BSA/PBS	1%BSA/PBS, 0.05% Tween-20

2.13.3 Uric acid assays

Uric acid levels in BAL supernatant were measured using an Amplex Red Uric Acid Assay Kit (Molecular Probes, Invitrogen, Paisley, UK) according to the manufacturer's instructions.

2.13.4 LDH

Lactate dehydrogenase (LDH) levels in BAL or cell culture supernatant were measured using an LDH-based *in vitro* toxicology assay (Sigma) according to the manufacturer's instructions.

2.14 RNA analysis

2.14.1 Total RNA extraction from lung tissue

Total RNA was extracted from snap frozen lung tissue. 500 µL of Trizol (Invitrogen) was added to the lung lobe before homogenisation using a hand-held motorised pestle (VWR, Leicestershire, UK). 200 µL chloroform (Sigma) was added before mixing vigorously for 15 seconds. The mixture was left at room temperature for 2 mins before centrifugation for 15 mins at 7800 x g at 4°C. The upper aqueous layer was removed and transferred to a new tube, to which an equal volume of 70% ethanol (Sigma) was added and mixed gently. Total RNA was extracted using an RNeasy Mini kit (QIAGEN, Manchester, UK) according to manufacturer's instructions and stored at -80°C.

2.14.2 Total RNA extraction from epithelial cell cultures

NHBEC were harvested and lysed in Buffer RLT (QIAGEN). Total RNA was extracted using an RNeasy Mini kit (QIAGEN.) according to manufacturer's instructions and stored at -80°C.

2.14.3 cDNA synthesis

Total RNA was reverse-transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions and stored at -20°C.

2.14.4 Quantitative (q)PCR

cDNA was amplified in a 6 µL reaction containing 2.4 µL cDNA, 3 µL of 3 µL TaqMan Fast Advanced Master Mix, 0.3 µL 20x primers and 0.3 µL dH_20. qPCR reactions were performed on a Viia 7 (Applied Biosystems) with TaqMan primers (Applied Biosystems; Table 6) and gene expression was analysed using the change-in-threshold ΔΔCt-method.

2.15 Statistical analysis

Data graphs were plotted using Prism software (version 5, GraphPad). Graphs show mean data with error bars of the standard error of the mean (SEM), or box and whisker plots show median and range of data. Statistical significance was calculated using unpaired Mann-Whitney U test (Prism). p-values <0.05 were considered significant and are indicated on graphs as $*p<0.05$, $**p<0.01$, $***p<0.001$.

Table 6. TaqMan probes used in qPCR reactions.

Gene	Species	Probe	RefSeq interrogated sequence
Ifnb	Mouse	Mm00439552_s1	NM_010510.1
Irf7	Mouse	Mm00516793_g1	NM_016850.3
Oas2	Mouse	Mm00460961_m1	NM_145227.3
Ifi44	Mouse	Mm00505670_m1	NM_133871.2
Relma	Mouse	Mm00445109_m1	NM_020509.3
Arg1	Mouse	Mm00475988_m1	NM_007482.3
Il4	Mouse	Mm00445259_m1	NM_021283.2
Trem2	Mouse	Mm04209424_g1	NM_031254.3
Nos2	Mouse	Mm00440502_m1	NM_010927.3
Tnf	Mouse	Mm00443258_m1	NM_013693.3
Hprt	Mouse	Mm01545399_m1	NM_013556.2
ifnb1	Human	Hs01077958_s1	NM_002176.2
irf7	Human	Hs01014809_g1	NM_001572.3
oas2	Human	Hs00942643_m1	NM_002535.2
ifi44	Human	Hs00951349_m1	NM_006417.4
gapdh	Human	Hs02758991_g1	NM_002046.4

Chapter 3: TREM protein expression during lung inflammation

Chapter 3: TREM protein expression during lung inflammation

3.1 Introduction

TREM-1 and TREM-2 are innate immune signalling receptors involved in fine-tuning the inflammatory response. TREM-1 is involved in amplification of inflammation, while TREM-2 functions in the resolution of inflammation. While there are many publications investigating the role of TREM-1 in bacterial infections, fewer studies have investigated TREM-1 in viral infection immunity, particularly influenza. Several studies point to a role of TREM-1 in influenza infection; through transcriptome analysis, TREM-1 or genes associated with the TREM-1 signalling pathway were induced during influenza infection (Rosseau et al., 2007, Ivan et al., 2012, Askovich et al., 2013). Recently, TREM-1 deficiency was found to be beneficial in a model of influenza infection (Weber et al., 2014). TREM-$1^{-/-}$ mice had reduced morbidity and mortality upon influenza infection, while viral clearance was unaffected, suggesting that targeting TREM-1 could potentially minimise the inflammatory response that causes pathology, without compromising the ability of the host to control pathogen outgrowth. TREM-1 is, therefore, a potential target in reducing excessive inflammation, which leads to susceptibility to further infection, e.g. pneumococcal infection following influenza infection.

High levels of the soluble form sTREM-1 in serum have been correlated with poor prognosis of lung diseases such as allergic asthma and tuberculosis (Bucova et al., 2012, Huang et al., 2014). Given its usefulness as a diagnostic marker in various inflammatory diseases, understanding the source of sTREM-1 is of importance. sTREM-1 is thought to arise either from an alternative splice variant (Gingras et al., 2002a) or cleavage of surface expressed TREM-1. In support of the cleavage hypothesis, Gómez-Piña *et al.* (2012) found that a pan matrix metalloproteinase (MMP) inhibitor prevented the shedding of sTREM-1 from human monocytes. Furthermore, MMP-9 specifically leads to the shedding of sTREM-1 in a mouse macrophage cell line RAW 264.7 stimulated with TLR9 agonist CpG-oligonucleotide ODN (Molad et al., 2013). Analysis of the TREM-1 amino acid sequence reveals a potential MMP-9 cleavage site (Figure 3.1).

1	MRKAGLWGLL	CVFFVSEVKA	AIVLEEERYD	LVEGQTLTVK	CPFNIMKYAN
51	SQKAWQRLPD	GKEPLTLVVT	QRPFTRPSEV	HMGKFTLKHD	PSEAMLQVQM
101	TDLQVTDSGL	YRCVIYHPPN	DPVVLFHPVR	LVVTKGSSDV	FTPVIIPITR
151	LTER**PILIT**T	KYSPSDTTTT	RSLPKPTAVV	SSPGLGVTII	NGTDADSVST
201	SSVTISVICG	LLSKSLVFII	LFIVTKRTFG		

Figure 3.1 Amino acid sequence of murine TREM-1 with potential MMP-9 cleavage site.

The amino acid sequence of TREM-1 contains a possible MMP-9 cleavage site (underlined) Pro-X-X-Hy-(Ser/Thr); X=any amino acid, Hy=any hydrophobic amino acid.

MMPs are a family of extracellular enzymes that share characteristics such as having a zinc-containing active site and the ability to degrade extracellular matrix (ECM) protein (Parks and Shapiro, 2001). MMP-2 and MMP-9 are of particular interest in the lung, as they are known to be involved in inflammatory lung disease such as asthma and COPD (Chakrabarti and Patel, 2005). In the lung, MMPs act as modulators of inflammation and contribute to lung remodelling (Atkinson and Senior, 2003). MMP-9 mediates the migration of neutrophils into the airways during influenza infection (Bradley et al., 2012) and is able to modulate innate immune responses in macrophages *in vitro* by cleaving Sp-D, which regulates innate immune responses in the lung (Bratcher et al., 2012). Given that MMP-9 has already been shown to cleave regulators of the immune response and is secreted upon influenza infection, it is possible that MMP-9 exerts a regulatory effect by cleavage of TREM-1 that is upregulated during influenza and secondary bacterial infection.

It has been observed that alveolar macrophages have an altered phenotype following influenza. Molecules such as CD200R, TLR inhibitors and Axl are upregulated following influenza in order to promote resolution (Goulding et al., 2011, Didierlaurent et al., 2008, Fujimori et al., 2015). However, upregulation of such negative regulators of inflammation can impede the immune response to further infection, resulting in increased susceptibility to secondary bacterial infections (Goulding et al., 2011). TREM-2, unlike TREM-1, is associated with anti-inflammatory macrophages and is known to down-regulate TLR responses (Turnbull et al., 2006, Peng et al., 2013). Given its expression on M2 macrophages, it is possible that TREM-2 exerts anti-inflammatory functions following influenza infection in order to promote resolution in a similar fashion to receptors such as CD200R, thereby

contributing to increased susceptibility to subsequent infection, e.g. secondary bacterial infection.

3.1.1 Hypothesis

The inflammatory environment induced by respiratory viral infection has a lasting impact on the lung environment, increasing susceptibility to subsequent infections and also allergic inflammatory diseases, such as asthma. Therefore, the role of TREM proteins in different types of inflammatory lung disease warrants further investigation. Differential outcomes of inflammation may be attributed to this group of innate immune receptors.

Given that TREM-1 and TREM-2 play a role in modulation of an inflammatory state, it is hypothesised that TREM protein expression will be altered in murine models of inflammatory lung infections, such as influenza and *S. pneumoniae*. It is further hypothesised that TREM-1 and TREM-2 cell surface expression changes over time during an inflammatory response, with TREM-1 predominant in early stages of inflammation and TREM-2 associated with resolution. It is also hypothesised that neutrophils are a source of sTREM-1 and that MMP-9 plays a role in the cleavage of surface TREM-1 into sTREM-1.

3.1.2 Aims

1) To determine the expression profile of surface TREM-1 and TREM-2 receptors on myeloid cell populations during influenza single infection and secondary bacterial infection with *S. pneumoniae*, as well as sTREM-1 production in the airways.
2) To determine whether TREM protein expression is associated with severity of disease and if so, whether TREM-1 and TREM-2 play opposing roles in the initiation and resolution of inflammation in the lung.
3) To determine whether neutrophils are a source of sTREM-1, and the mechanism of its release.

3.2 Results

3.2.1 TREM protein expression during influenza infection

In order to determine whether TREM-1 and TREM-2 are expressed and differentially regulated during influenza, BALB/c mice were infected with 15 PFU of mouse-adapted influenza A/PR8 H1N1—hereafter referred to as PR8 influenza—and analysed 7 and 14 days post-infection (Figure 3.2A).

Infection with 15 PFU PR8 influenza resulted in loss of body weight from 5 days post-infection, which peaked at 15% on day 8 (Figure 3.2B). Weight loss recovery began 9 days post-infection until weight stabilised on day 12.

Cellular infiltration into the airway lumen (Figure 3.2C) and lung tissue (Figure 3.2D) followed the pattern of weight loss, with peak BAL and lung cell counts approximately 1 week post-infection, which were reduced to near baseline levels in BAL by day 14, however, the lung cell count remained higher than uninfected controls 14 days post-infection.

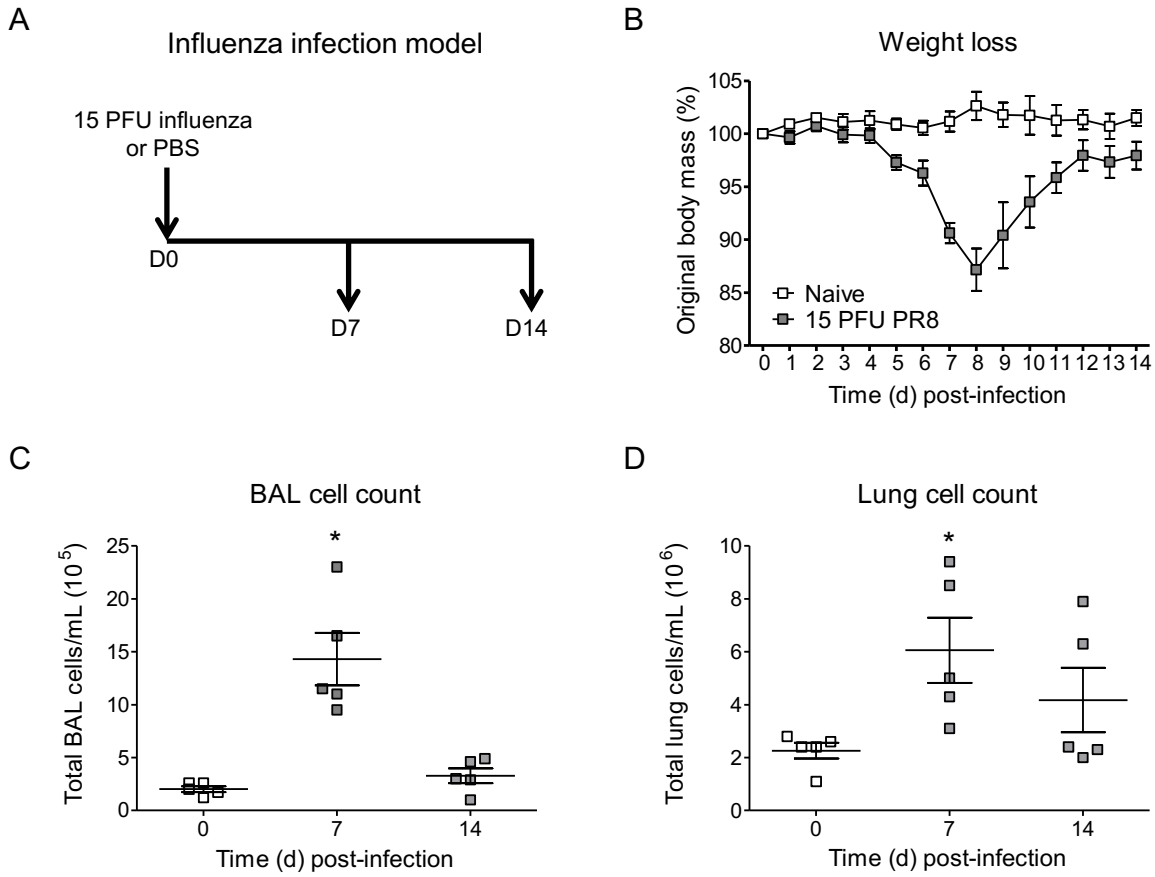

Figure 3.2 Weight loss and cellular infiltration during influenza infection.

BALB/c mice were infected with 15 PFU of PR8 influenza A virus and analysed at 7 and 14 days post-infection (A). Body weight was measured daily and is expressed as percentage of original body mass (B). At 7 and 14 days post-infection, mice were terminally anaesthetised, BAL performed and lung tissue harvested. Cells were isolated from BAL fluid and lung tissue and live cell enumerated by trypan blue exclusion (C, D). Naïve, uninfected mice were monitored as a control. Data are expressed as mean±SEM, n=5 mice per time-point, and are representative of two separate experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$.

3.2.1.1 Identification of myeloid cells in the airway during influenza infection

Myeloid cells are an essential cell population involved in the early innate immune response to pathogens, such as influenza virus (Iwasaki and Pillai, 2014). Thus, neutrophils and macrophages in the airway were analysed in this model of influenza infection. Myeloid cells were distinguished as neutrophils or macrophages by their expression of cell surface markers CD11c, CD11b, F4/80 and Ly6G (Figure 3.3). Leukocytes were selected by flow cytometry using size and granularity and doublets excluded (Figure 3.3A, B). Live cells were selected for by exclusion of cells staining positive for dead cell dye (Figure 3.3C). Cells were then characterised by their expression of CD11c, CD11b, F4/80 and Ly6G. $F4/80^{+}$ cells that expressed high levels of CD11c and intermediate levels of CD11b were termed alveolar macrophages and were subdivided into $CD11b^{+}$ or $CD11b^{int}$ (Figure 3.3D). Neutrophils were defined as $F4/80^{+}$, $CD11b^{+}$ and $Ly6G^{+}$ (Figure 3.3E).

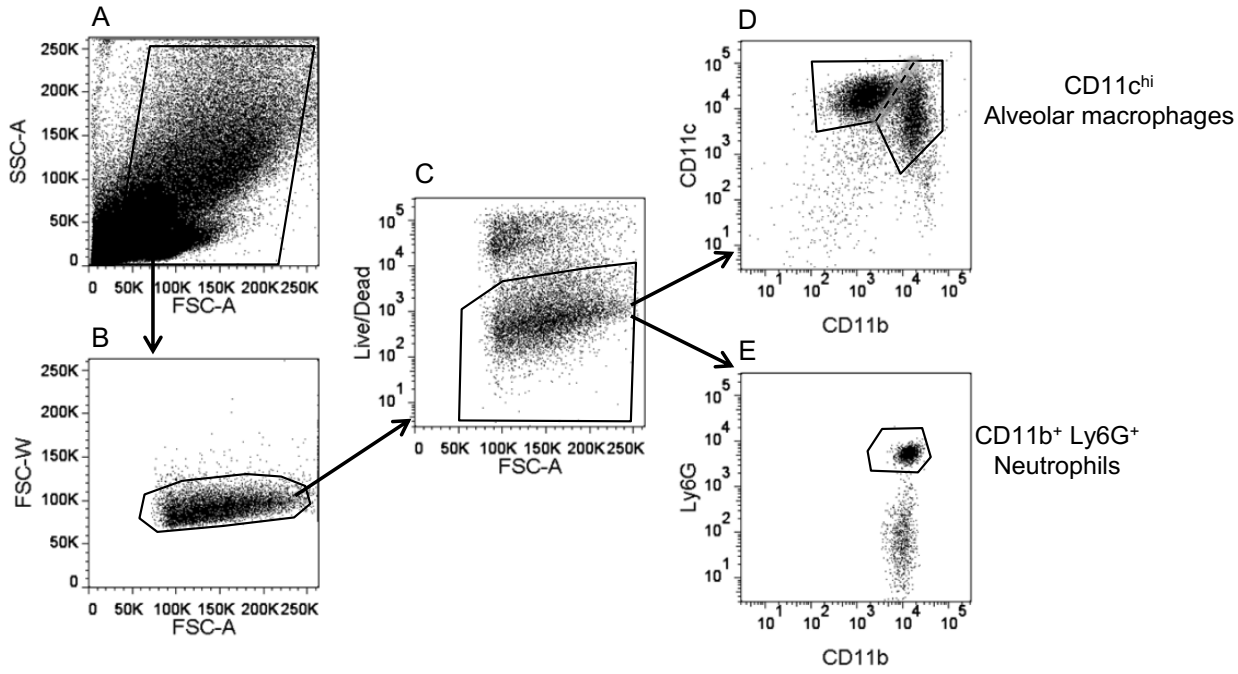

Figure 3.3 Identification of myeloid cell populations during influenza infection by flow cytometry.

Leukocytes were determined by forward (FSC) and side (SSC) scatter (A). Doublets were excluded by FSC-W versus FSC-A (B). Live cells were identified by live-dead staining (C). Live cells were further characterised by expression of CD11c, CD11b, F4/80 and Ly6G. Alveolar macrophages were defined as $CD11c^{+}F4/80^{+}$ and subdivided into $CD11b^{+}$ or $CD11b^{int}$ (D), while neutrophils were defined as $CD11b^{+}Ly6G^{+}$ (E).

3.2.1.2 Changes in alveolar macrophage populations during influenza infection

In naïve, uninfected mice, the major population of myeloid cells isolated from BAL fluid were $CD11c^{+}CD11b^{int}$ alveolar macrophages (Figure 3.4A). Upon influenza infection, cells expressing CD11b were recruited to the lung resulting in a 1:1 ratio of $CD11c^{+}CD11b^{int}$ versus $CD11c^{+}CD11b^{+}$ cells (Figure 3.4A-C). This was also seen in approximately comparable numbers of these two subsets of macrophages (Figure 3.4D, E). By day 14, the number of $CD11c^{+}CD11b^{+}$ macrophages was approximately threefold lower compared to day 7 (Figure 3.4E).

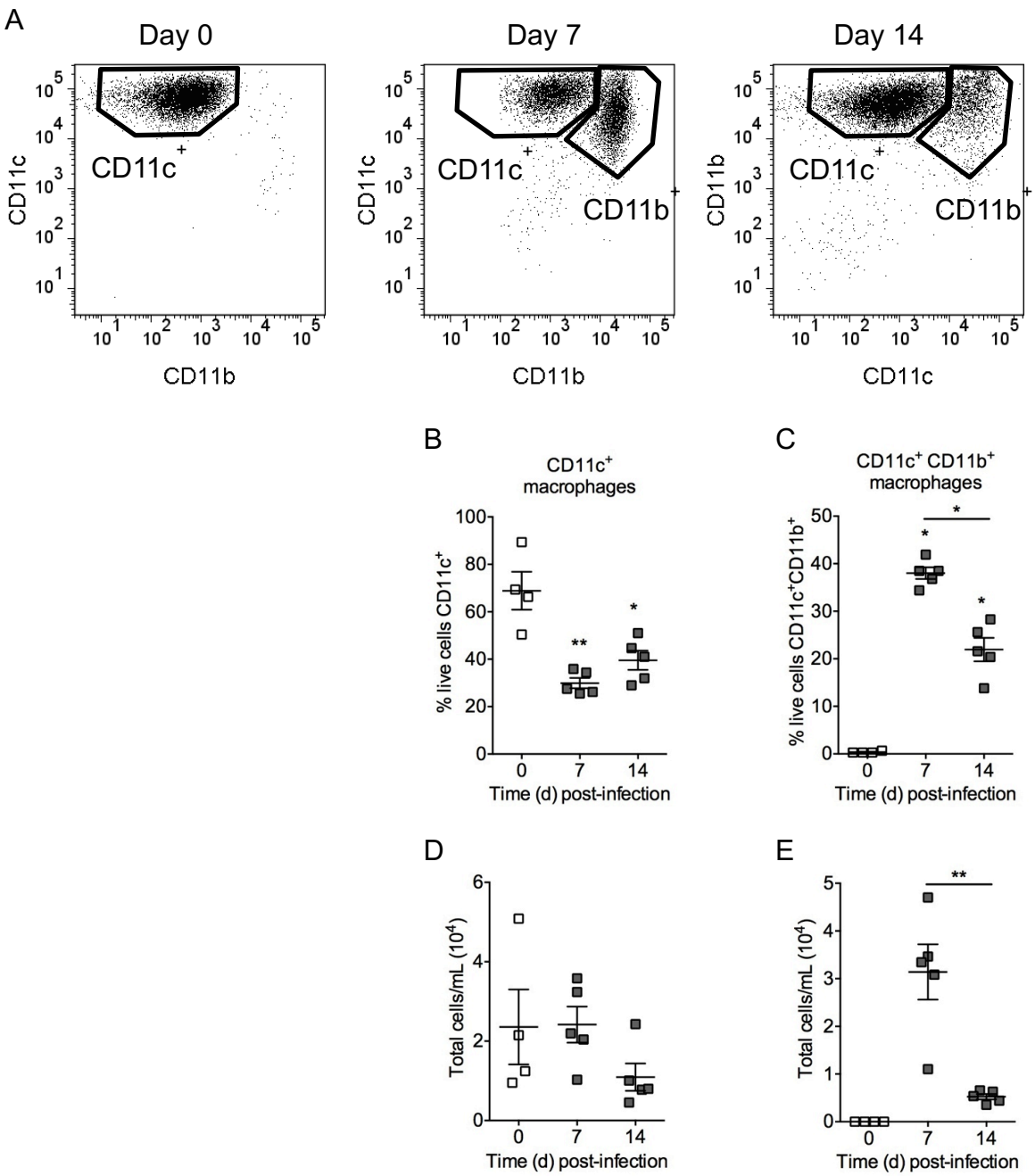

Figure 3.4 Alveolar macrophage populations in the airway following influenza infection.

BALB/c mice were infected with 15 PFU of PR8 influenza A virus and analysed at 7 and 14 days post-infection. Changes in $CD11c^{+}CD11b^{int}$ ($CD11c^{+}$) and $CD11c^{+}CD11b^{+}$ macrophage populations in the BAL fluid demonstrated by flow cytometry dot plots (A). The percentage and total cell count of $CD11c^{+}CD11b^{int}$ macrophages (B, D) and $CD11c^{+}CD11b^{+}$ macrophages (C, E) were determined by flow cytometry in BAL fluid at 7 and 14 days post-infection. Naïve, uninfected mice were analysed as a control. Data are expressed as mean±SEM, n=4/5 mice per time-point, and are representative of two separate experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$, **$p<0.01$.

3.2.1.3 Neutrophil recruitment to the lung during influenza infection

A negligible population of neutrophils was detected in the BAL of uninfected mice—less than 2% of live gated cells (Figure 3.5A). Although neutrophils were predominantly recruited early on during infection, i.e. around day 2/3 post-infection (based on a preliminary study, data not shown), they were still detected in the lung 7 days post-infection. The proportion of neutrophils increased following influenza infection and remained significantly higher than in naïve mice 14 days post-infection (Figure 3.5A), however, the total number of neutrophils significantly decreased from day 7 to day 14 (Figure 3.5B).

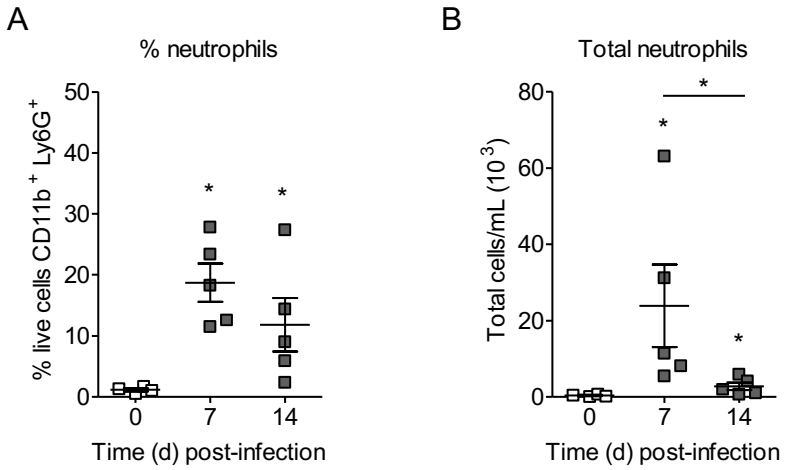

Figure 3.5 Neutrophils in the airway during influenza infection.

BALB/c mice were infected with 15 PFU of PR8 influenza A virus and analysed at 7 and 14 days post-infection. Percentage and total cell count of $CD11b^{+}Ly6G^{+}$ neutrophils (A, B) were determined by flow cytometry in BAL fluid at 7 and 14 days post-infection. Naïve, uninfected mice were analysed as a control. Data are expressed as mean±SEM, n=4/5 mice per time-point, and are representative of two separate experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$.

3.2.1.4 TREM protein expression on $CD11c^{+}CD11b^{int}$ alveolar macrophages during influenza infection

TREM expression was determined on $CD11c^{+}CD11b^{int}$ macrophages by flow cytometry. Less than 5% of $CD11c^{+}CD11b^{int}$ macrophages expressed TREM-1 on their surface, which translated to a low total number of macrophages in the airway expressing TREM-1, and low intensity of TREM-1 expression per cell (Figure 3.6A-D). In contrast, TREM-2 was

expressed on approximately 15% of $CD11c^{+}CD11b^{int}$ macrophages at homeostasis (Figure 3.6E, F). Interestingly, during peak inflammation following influenza infection, approximately 75% of all $CD11c^{+}CD11b^{int}$ macrophages expressed TREM-2 (Figure 3.6F). The total number and mean intensity of TREM-2 on $CD11c^{+}CD11b^{int}$ also peaked 7 days post-infection (Figure 3.6G, H). The percentage and total number of $CD11c^{+}CD11b^{int}$ expressing TREM-2 was significantly lower 14 days post-infection (Figure 3.6F, G).

3.2.1.5 TREM protein expression on $CD11c^{+}CD11b^{+}$ alveolar macrophages during influenza infection

Similar to $CD11c^{+}CD11b^{int}$ alveolar macrophages, a low proportion of $CD11c^{+}CD11b^{+}$ macrophages expressed TREM-1 upon influenza infection (Figure 3.7A-D). The percentage of $TREM\text{-}2^{+}CD11c^{+}CD11b^{+}$ macrophages was 10% higher than that of the $CD11c^{+}CD11b^{int}$ macrophages at peak inflammation—over 90% of $CD11c^{+}CD11b^{+}$ macrophages expressed TREM-2 (Figure 3.7E, F). This high proportion of TREM-2 expressing cells was sustained at 75% 14 days post-infection (Figure 3.7F), however, the total number and intensity of TREM-2 expression on $CD11c^{+}CD11b^{+}$ macrophages was reduced by approximately threefold and twofold respectively at day 14 compared to day 7 (Figure 3.7G, H).

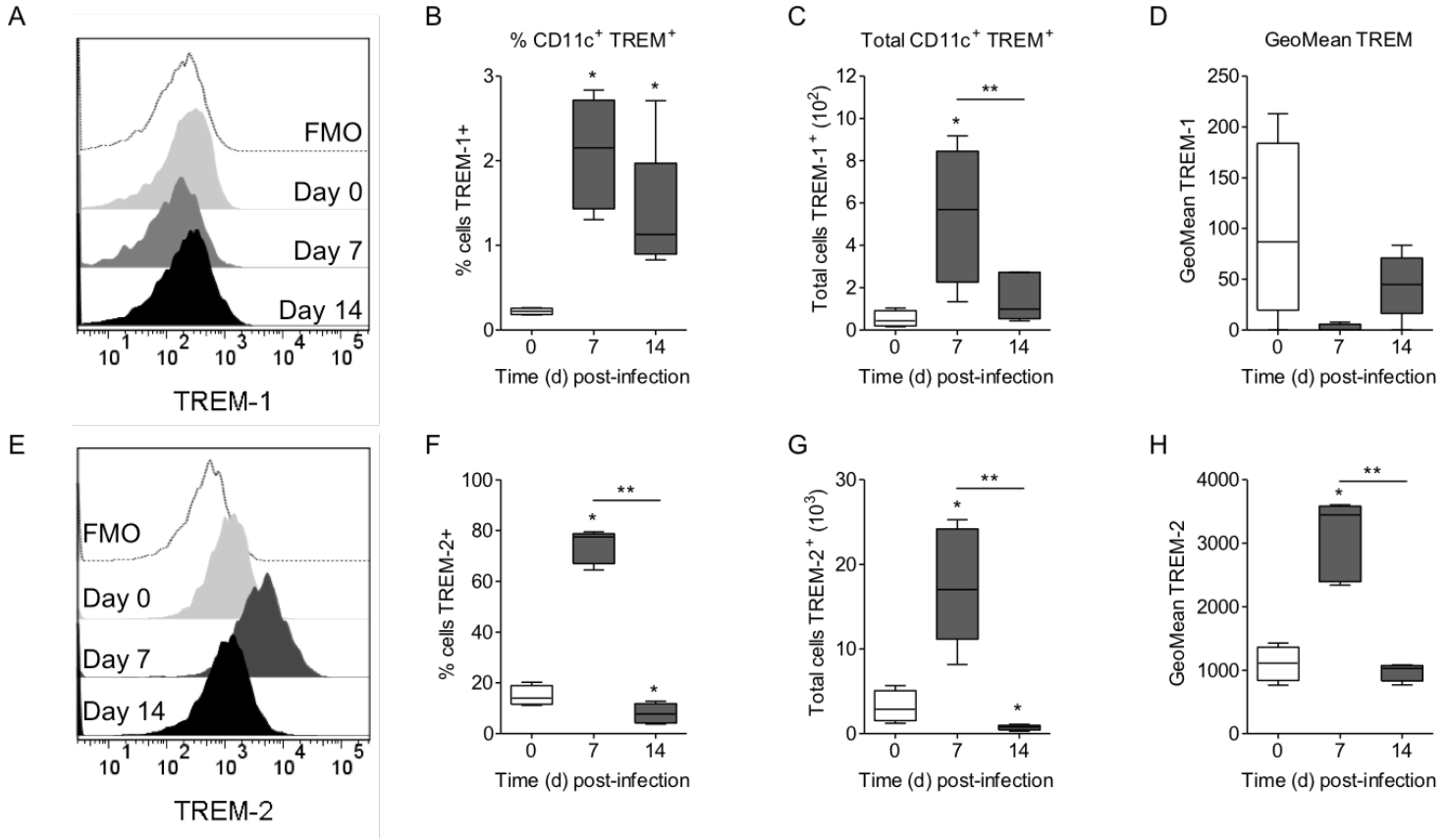

Figure 3.6 TREM expression on CD11c^{+}CD11b^{int} alveolar macrophages during influenza infection.

BALB/c mice were infected with 15 PFU of PR8 influenza A virus and analysed at 7 and 14 days post-infection. Percentage, total number and geometric mean of TREM-1^{+} (A-D) and TREM-2^{+} (E-H) CD11c^{+}CD11b^{int} (CD11c^{+}) macrophages were determined by flow cytometry in BAL fluid at 7 and 14 days post-infection. Naïve, uninfected mice were analysed as a control. Data are expressed as median, interquartile range (IQR) and minimum and maximum values, n=4/5 mice per time-point, and are representative of two separate experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups, *p<0.05, **p<0.01.

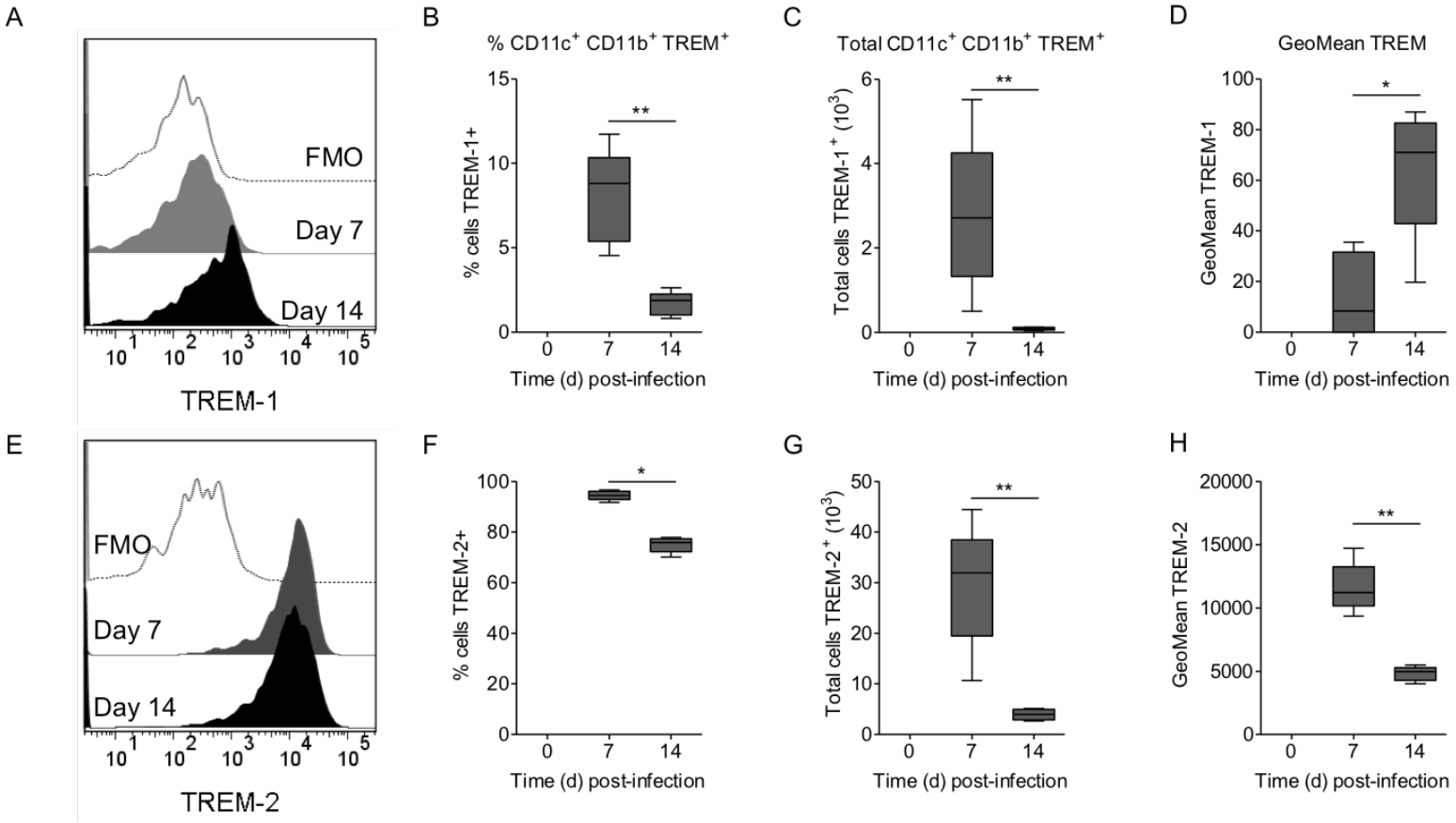

Figure 3.7 TREM expression on CD11c$^+$CD11b$^+$ alveolar macrophages during influenza infection.

BALB/c mice were infected with 15 PFU of PR8 influenza A virus and analysed at 7 and 14 days post-infection. Percentage, total number and geometric mean of TREM-1$^+$ (A-D) and TREM-2$^+$ (E-H) CD11c$^+$CD11b$^+$ macrophages were determined by flow cytometry in BAL fluid at 7 and 14 days post-infection. Naïve, uninfected mice were analysed as a control. Data are expressed as median, IQR and minimum and maximum values, n=4/5 mice per time-point, and are representative of two separate experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$, **$p<0.01$.

3.2.1.6 Double positive macrophages expressing TREM-1$^+$ TREM-2$^+$ during influenza infection

Analysis of alveolar macrophages double positive for TREM-1 and TREM-2 on day 7 post-influenza infection showed that while very few CD11c$^+$CD11b^{int} macrophages (less than 5%) expressed both TREMs, a higher proportion (12%) of CD11c$^+$CD11b$^+$ cells were double positive for TREM-1 and TREM-2 (Figure 3.8).

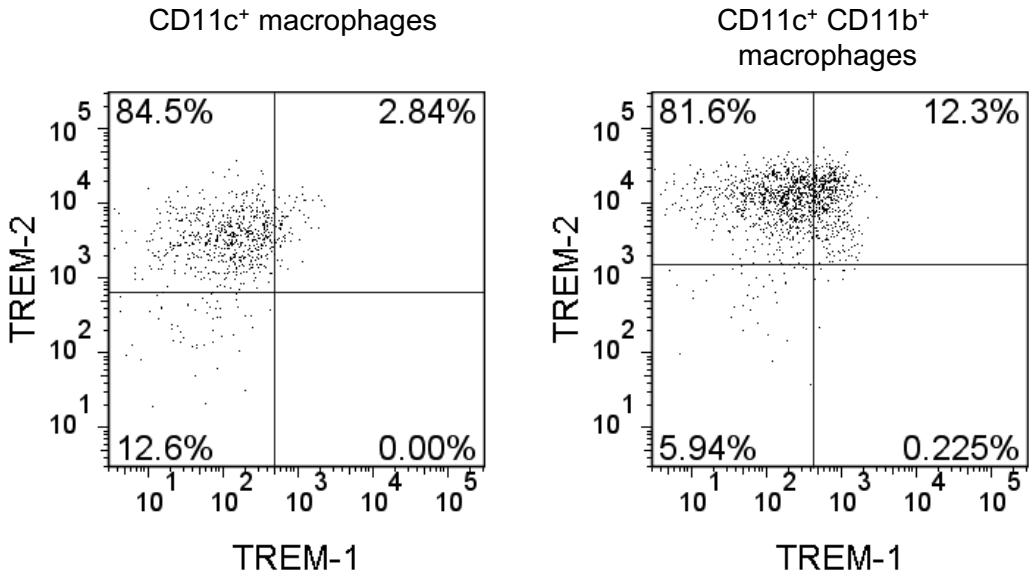

Figure 3.8 TREM-1$^+$ TREM-2$^+$ double positive macrophages during influenza infection.

BALB/c mice were infected with 15 PFU of PR8 influenza A virus and analysed 7 days post-infection. Dot plots showing proportions of TREM-1$^+$TREM-2$^+$ CD11c$^+$CD11b^{int} (CD11c$^+$) and CD11c$^+$CD11b$^+$ macrophages determined by flow cytometry in BAL fluid. Dot plots are representative of n=4/5 mice per time-point in two separate experiments.

3.2.1.7 CCR5 expression on TREM-2$^+$ alveolar macrophages during influenza infection

In order to determine whether TREM-2$^+$ macrophages have the potential to migrate to or from the lung, TREM-2$^+$ alveolar macrophages were stained for chemokine receptors CCR5 and CCR7 on day 7 and day 14 post-influenza infection. CCR5 mediates macrophage migration towards CCL4 (Samson et al., 1996), while CCR7 is expressed on macrophages derived from peripheral monocytes (Gordon and Taylor, 2005). Influenza infection resulted in the upregulation of CCR5 on approximately 20% of all TREM-2$^+$ CD11c$^+$CD11b^{int} macrophages on days 7 and 14, whereas almost all TREM-2$^+$ CD11c$^+$CD11b$^+$ macrophages expressed CCR5 (80% on day 7 and 85% on day 14) (Figure 3.9A, B), suggesting TREM-2$^+$

macrophages may have migrated to the lung following infection. CCR7 was only slightly upregulated (less than 5%) upon influenza infection in both subsets of macrophages (Figure 3.9C, D).

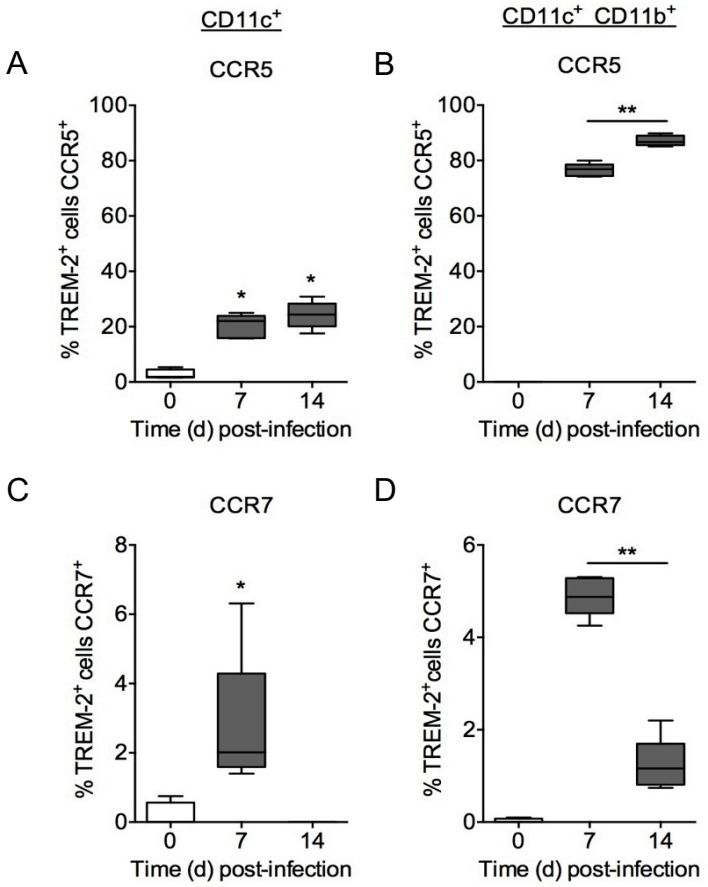

Figure 3.9 CCR5 and CCR7 expression on TREM-2$^+$ alveolar macrophages during influenza infection.

BALB/c mice were infected with 15 PFU of PR8 influenza A virus. Percentage of TREM-2$^+$ CD11c$^+$CD11b^{int} (CD11c$^+$) (A, C) and CD11c$^+$CD11b$^+$ (B, D) macrophages expressing CCR5 and CCR7 in BAL fluid was determined by flow cytometry at 7 and 14 days post-infection. Naïve, uninfected mice were analysed as a control. Data are expressed as median, IQR and minimum and maximum values, n=4/5 mice per time-point, and are representative of two separate experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups, *p<0.05, **p<0.01.

3.2.1.8 TREM-1 and TREM-2 expression on neutrophils during influenza infection

TREM expression was also determined on CD11b$^+$Ly6G$^+$ neutrophils. The number of neutrophils increased upon infection (Figure 3.5), and approximately a quarter of neutrophils

in the airway expressed TREM-1 7 days post-infection, while the proportion was 35% at day 14 (Figure 3.10A). The total number of TREM-1 expressing neutrophils occurred at day 7, however, neutrophils had higher TREM-1 expression per cell on day 14—approximately three times higher than day 7 (Figure 3.10B, C). Neutrophils also expressed TREM-2, with the highest percentage, number and intensity of TREM-2 expression on neutrophils 7 days post-infection—approximately 75% of neutrophils expressing TREM-2 amounting to 10^4 total cells (Figure 3.10D-F).

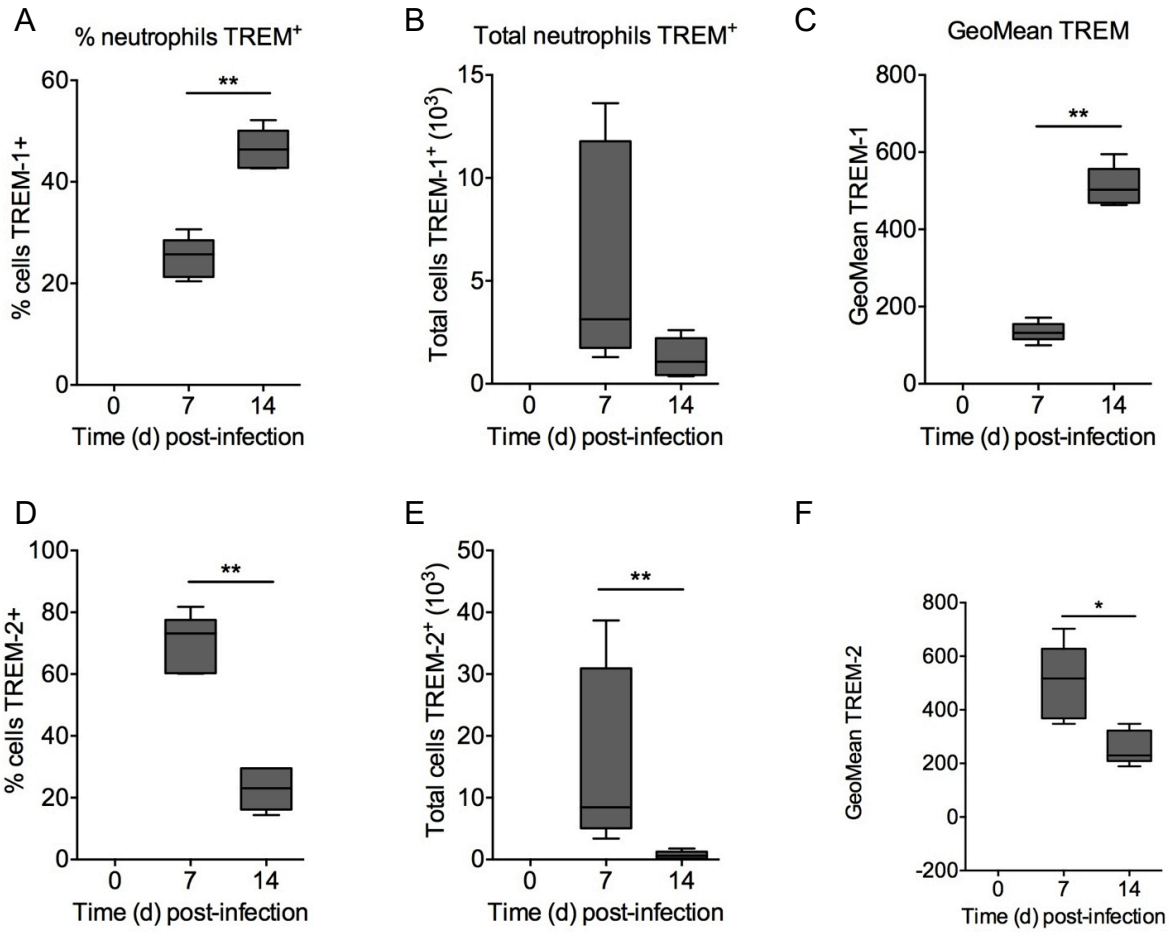

Figure 3.10 TREM expression on neutrophils during influenza infection.

BALB/c mice were infected with 15 PFU of PR8 influenza A virus and analysed at 7 and 14 days post-infection. Percentage, total number and geometric mean of TREM-1^+ (A-C) and TREM-2^+ (D-F) CD11b^+Ly6G^+ neutrophils were determined by flow cytometry in BAL fluid at 7 and 14 days post-infection. Naïve, uninfected mice were analysed as a control. Data are expressed as median, IQR and minimum and maximum values, n=4/5 mice per time-point, and are representative of two separate experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$, **$p<0.01$.

3.2.1.9 Levels of sTREM-1 during influenza infection

Since sTREM-1 is detected in various inflammatory diseases (Rohde et al., 2012, Bucova et al., 2012), sTREM-1 was measured in BAL fluid of mice infected with influenza on day 7 and 14 post-infection. On day 7, sTREM-1 levels in the BAL were approximately twofold higher compared to baseline levels in uninfected mice, however, this difference was not significant (Figure 3.11). sTREM-1 was not detected 14 days post-infection.

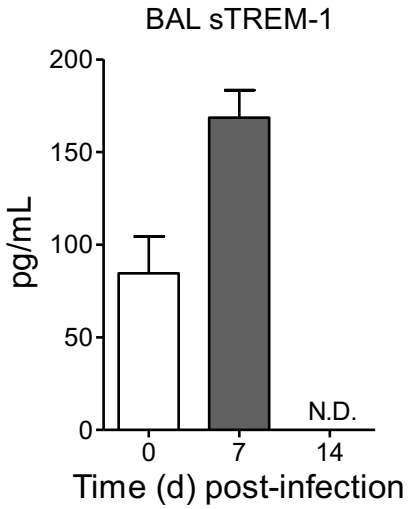

Figure 3.11 sTREM-1 levels in BAL fluid during influenza infection.

BALB/c mice were infected with 15 PFU of PR8 influenza A virus and analysed at 7 and 14 days post-infection. Levels of sTREM-1 in BAL were determined by ELISA. Naïve, uninfected mice were analysed as a control. Data are expressed as mean±SEM, n=4/5 mice per time-point, and are representative of two separate experiments. N.D.=not detected.

3.2.2 TREM protein expression during secondary bacterial infection following influenza

The Hussell group has previously shown that influenza infection renders the lung more susceptible to secondary bacterial infection (Goulding et al., 2011). A contributing factor to this predisposition to further infection is the interaction of inhibitory factor CD200R with its ligand CD200. Since TREM-2 is known to be anti-inflammatory (Turnbull et al., 2006) and upregulated on myeloid cells during influenza infection (Figure 3.6 and 3.7), TREM expression was analysed in a model of *S. pneumoniae* infection following influenza infection to determine whether TREMs could be contributing to susceptibility. BALB/c mice were infected with 7 PFU of PR8 influenza followed by infection with 10^6 CFU *S. pneumoniae* 7 days post-influenza infection. A lower dose of influenza was used compared with previous experiments in order to reduce the severity of infection prior to secondary infection with *S. pneumoniae.* Mice were analysed 24 and 48 hours post-bacterial infection (Figure 3.12A).

Influenza infection resulted in significant weight loss (10%) at day 8, while single infection with *S. pneumoniae* did not lead to weight loss (Figure 3.12B). *S. pneumoniae* infection following influenza infection caused a similar weight loss compared to single infections with either virus or bacteria at approximately 17%. Co-infection also resulted in significantly more cell infiltration into the airway lumen (Figure 3.12C) and lung tissue (Figure 3.12D), which was around twofold higher compared to influenza alone, 24 hours post-bacterial infection.

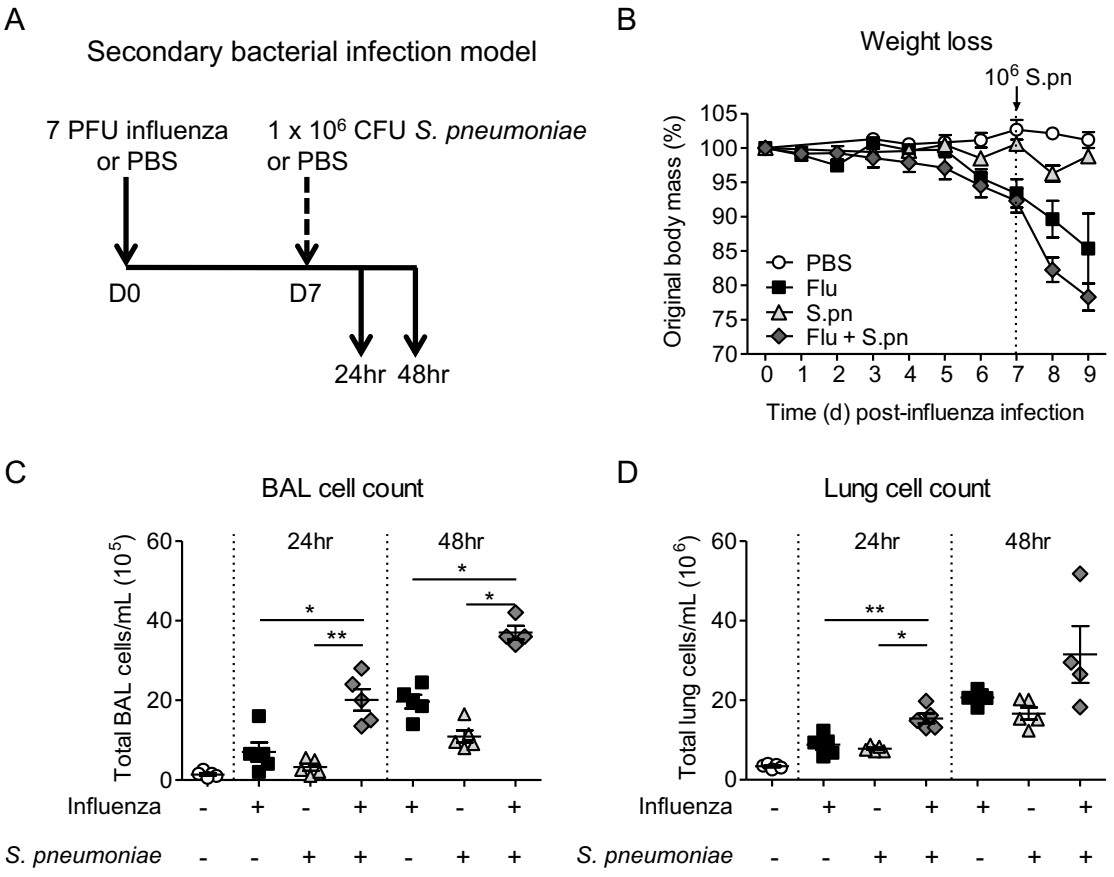

Figure 3.12 Weight loss and cellular infiltration during secondary bacterial infection.

BALB/c mice were infected with 7 PFU of PR8 influenza A virus, followed by 10^6 PFU *S. pneumoniae* on day 7 and analysed at 24 and 48 hours post-*S. pneumoniae* infection (A). Body weight was measured daily and is expressed as percentage of original body mass (B). At 24 and 48 hours post-*S. pneumoniae* infection, mice were terminally anaesthetised, BAL performed and lung tissue harvested. Cells were isolated from BAL fluid and lung tissue and counted (C, D). Naïve, uninfected mice were monitored as a control. Data are expressed as mean±SEM, n=4/5 mice per time-point and are representative of two separate experiments, except for the 48 hour time-point which was carried out once. Statistical significance was determined by Mann-Whitney U test between relevant groups, *p<0.05, **p<0.01.

3.2.2.1 Bacterial burden following secondary bacterial infection

Infection with *S. pneumoniae* alone led to very little bacterial dissemination from the airway lumen to the lung tissue and blood. However, prior infection with influenza resulted in recovery of bacteria from the airway, lung tissue and also dissemination into the blood (Figure 3.13A-C). Secondary bacterial infection following influenza abrogated bacterial clearance from the lung and enabled pathogen dissemination into the blood, which coincided with increased weight loss and inflammatory cell infiltration (Figure 3.12).

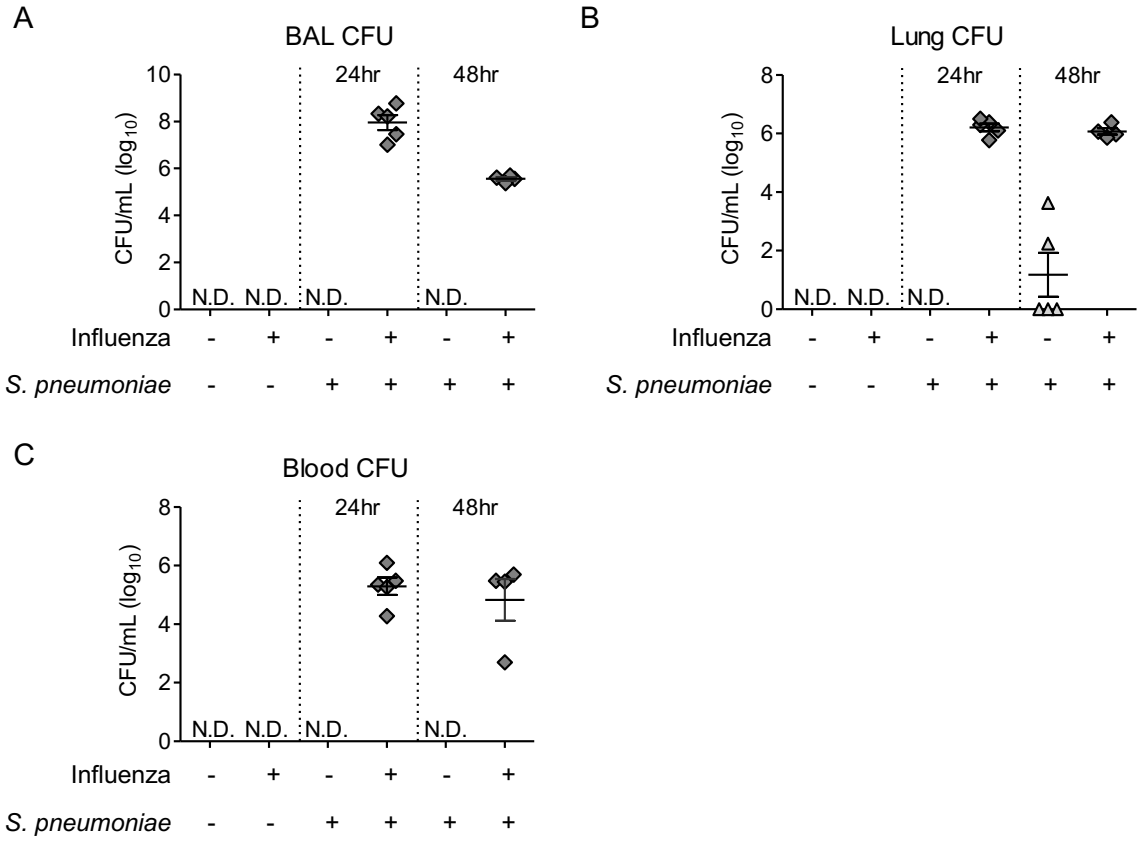

Figure 3.13 Bacterial burden during secondary bacterial infection.

BALB/c mice were infected with 7 PFU of PR8 influenza A virus, followed by 10^6 PFU *S. pneumoniae* on day 7 and analysed at 24 and 48 hours post-*S. pneumoniae* infection. Bacterial load in BAL fluid (A), lung tissue (B) and blood (C) were determined by CFU assay. Naïve, uninfected mice were monitored as a control. Data are expressed as mean±SEM, n=4/5 mice per time-point and are representative of two separate experiments, except for the 48 hour time-point which was carried out once. N.D.=not detected.

3.2.2.2 Proinflammatory cytokines in the airway during secondary bacterial infection

Proinflammatory cytokines IL-6 and TNF-α were measured in the BAL fluid during secondary bacterial infection following influenza. In line with increased weight loss, cellularity and bacterial burden, levels of IL-6 and TNF-α were significantly higher 24 hours and 48 hours post-*S. pneumoniae* challenge in co-infected mice compared to single infection groups (Figure 3.14A, B).

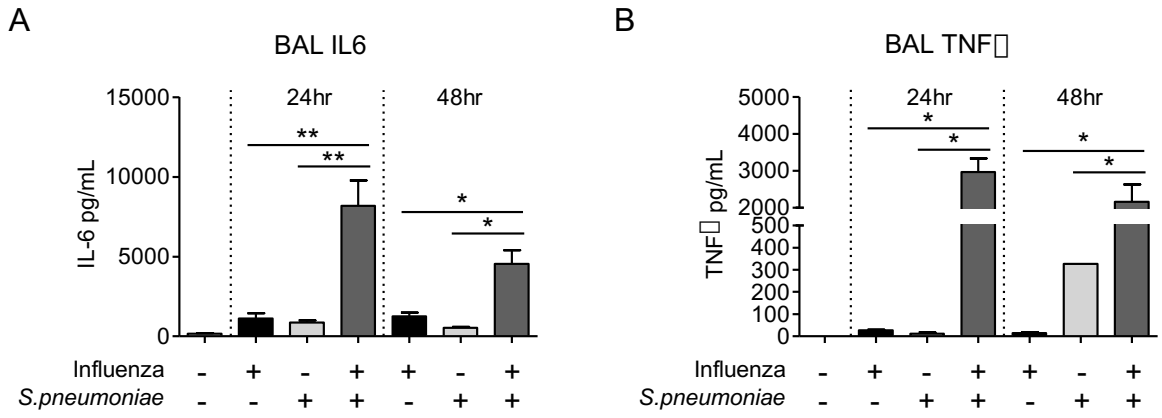

Figure 3.14 Proinflammatory cytokine production during secondary bacterial infection.

BALB/c mice were infected with 7 PFU of PR8 influenza A virus, followed by 10^6 PFU *S. pneumoniae* on day 7 and analysed at 24 and 48 hours post-*S. pneumoniae* infection. Levels of IL-6 (A) and TNF-α (B) were determined by ELISA. Naïve, uninfected mice were analysed as a control. Data are expressed as mean±SEM, n=4/5 mice per time-point, and are representative of two separate experiments, except for the 48 hour time-point which was carried out once. Statistical significance was determined by Mann-Whitney U test between relevant groups, *p<0.05, **p<0.01.

3.2.2.3 Myeloid cell populations during secondary bacterial infection

Total macrophage and neutrophil populations in the airway were determined by flow cytometry. While $CD11c^+CD11b^{int}$ macrophage counts were equal between groups 24 hours post-*S. pneumoniae* infection, an increase in macrophages was observed 48 hours post-bacterial infection in the single infection groups (Figure 3.15A). Total numbers of $CD11c^+CD11b^{int}$ macrophages were significantly lower (p=0.02) in the co-infection group, compared to bacterial infection alone 48 hours post-*S. pneumoniae* infection, a trend also observed in $CD11c^+CD11b^+$ macrophages (Figure 3.15A, B).

The total number of neutrophils increased between 24 and 48 hours post-*S. pneumoniae* infection, and the highest number of neutrophils was recovered from the BAL of co-infected mice (Figure 3.15C).

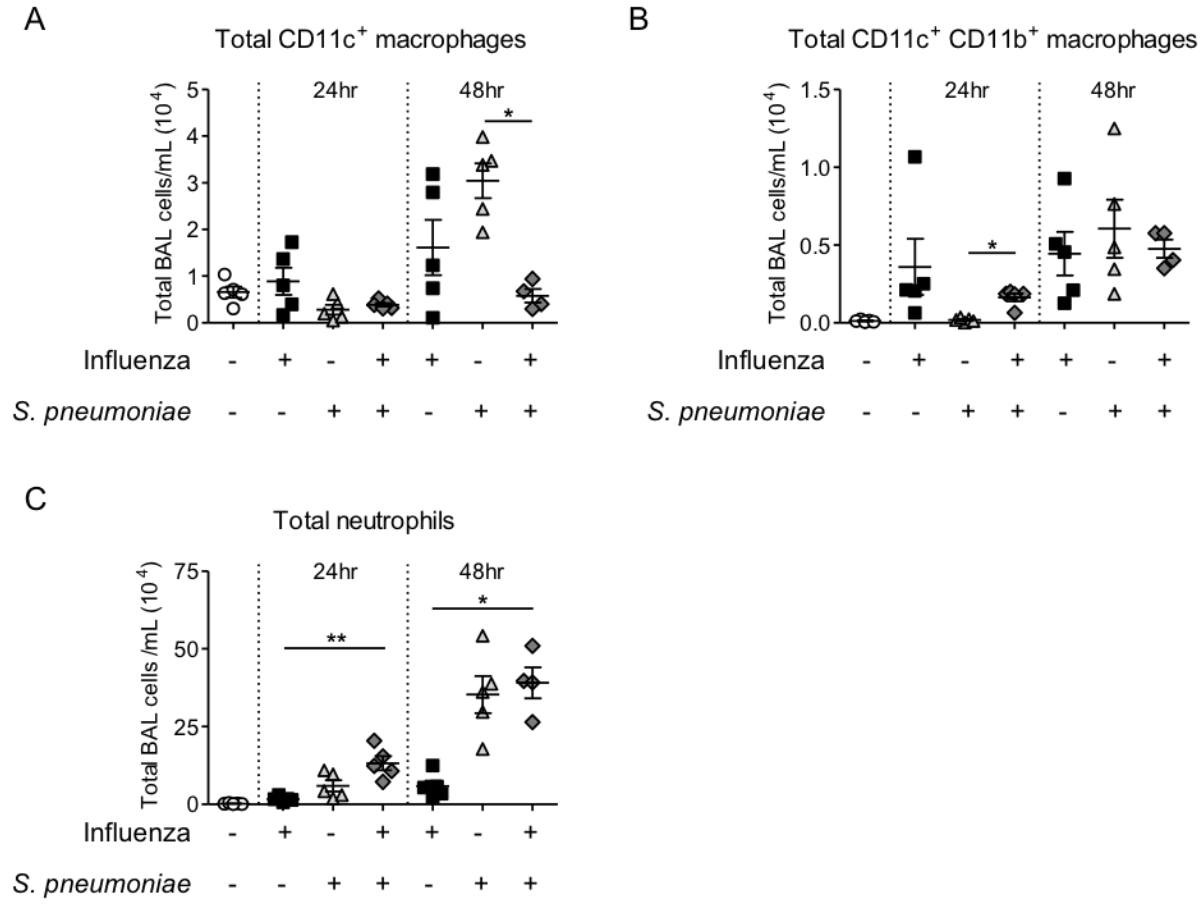

Figure 3.15 Myeloid cell populations during secondary bacterial infection.

BALB/c mice were infected with 7 PFU of PR8 influenza A virus, followed by 10^6 PFU *S. pneumoniae* on day 7 and analysed at 24 and 48 hours post-*S. pneumoniae* infection. Total cell count of $CD11c^+CD11b^{int}$ ($CD11c^+$) macrophages (A), $CD11c^+CD11b^+$ macrophages (B) and $CD11b^+Ly6G^+$ neutrophils (C) were determined by flow cytometry in BAL fluid at 24 and 48 hours post-infection. Naïve, uninfected mice were also analysed as a control. Data are expressed as mean±SEM, n=4/5 mice per time-point and are representative of two separate experiments, except for the 48 hour time-point which was carried out once. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$, **$p<0.01$.

3.2.2.4 TREM-2 expression on macrophages following secondary bacterial infection

The percentage and total number of $CD11c^{+}CD11b^{int}$ macrophages expressing TREM-2 and the intensity of expression was strongly increased in influenza-infected mice (Figure 3.6), while *S. pneumoniae* infection alone led to a significant decrease in TREM-2 expression compared to the untreated control group (Figure 3.16A-C). Secondary bacterial infection with *S. pneumoniae* resulted in a mild reduction in the expression of TREM-2 compared to influenza infection alone, though only significant in terms of the intensity of TREM-2 expression (Figure 3.16A-C), indicating that influenza and bacterial infections could be exerting opposing effects on the expression of TREM-2. $CD11c^{+}CD11b^{+}$ macrophages showed a similar pattern of TREM-2 expression in the different groups (Figure 3.16D-F).

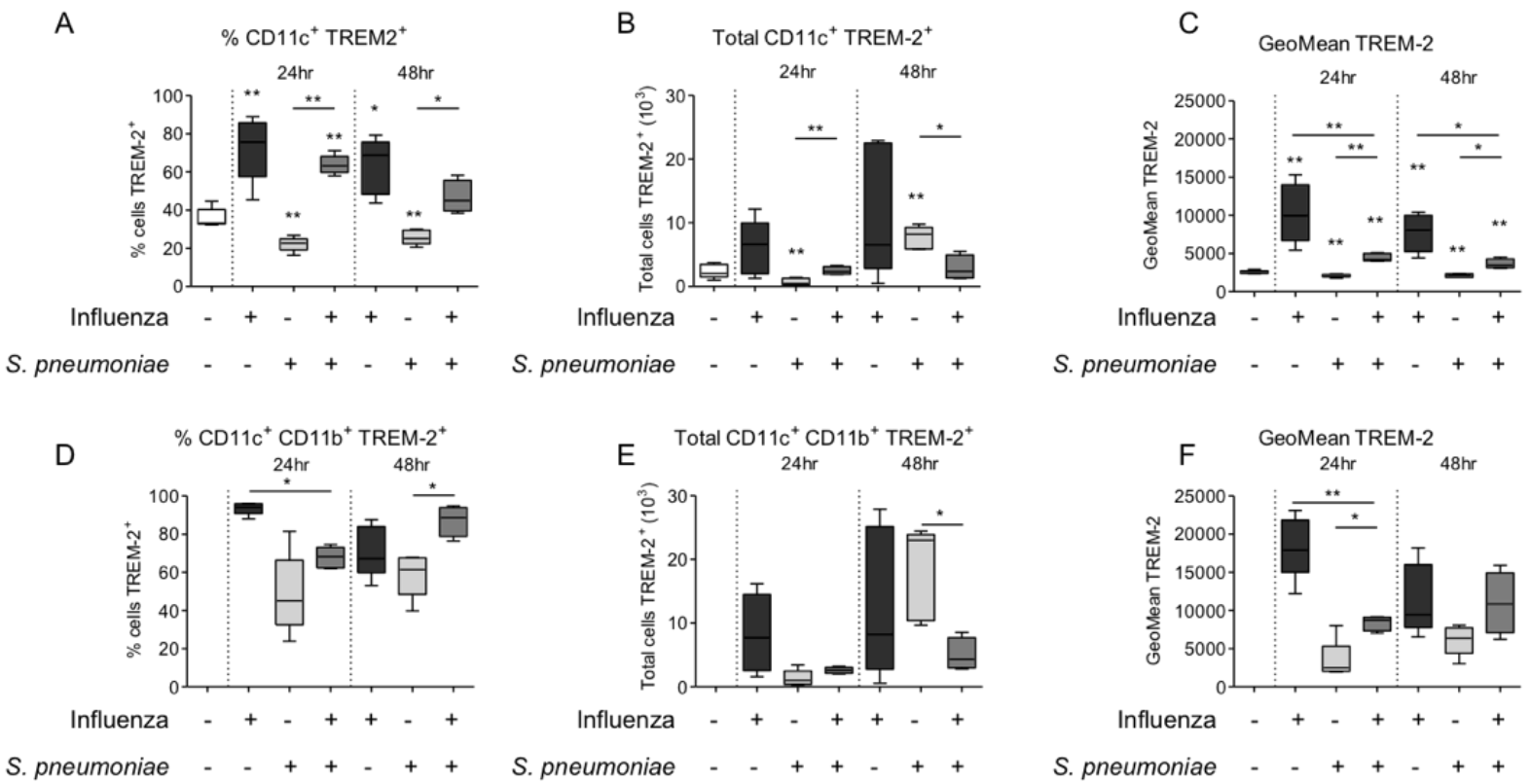

Figure 3.16 TREM-2 expression on macrophages during secondary bacterial infection.

BALB/c mice were infected with 7 PFU of PR8 influenza A virus, followed by 10^6 PFU *S. pneumoniae* on day 7 and analysed at 24 and 48 hours post-*S. pneumoniae* infection. Percentage, total number and geometric mean of TREM-2^+ $CD11c^+CD11b^{int}$ ($CD11c^+$) macrophages (A-C) and TREM-2^+ (D-F) $CD11c^+CD11b^+$ macrophages were determined by flow cytometry in BAL fluid at 24 and 48 hours post-infection. Naïve, uninfected mice were also analysed as a control. Data are expressed as median, IQR and minimum and maximum values, n=4/5 mice per time-point, and are representative of two separate experiments, except for the 48 hour time-point which was carried out once. Statistical significance was determined by Mann-Whitney U test between relevant groups, *p<0.05, **p<0.01.

3.2.2.5 sTREM-1 levels during secondary bacterial infection

As sTREM-1 has been suggested as a marker of bacterial infections, the next aim was to determine whether secondary challenge with *S. pneumoniae* had an impact on the release of sTREM-1 in influenza infected mice. While sTREM-1 levels were not higher in influenza single infection groups compared to untreated mice, there was a trend for increased sTREM-1 following *S. pneumoniae* infection in the BAL fluid and lung (Figure 3.17A, B), but not in the serum (Figure 3.17C). However, a striking increase in sTREM-1 levels in BAL, lung and serum was observed in co-infected groups (Figure 3.17A-C). This increase was most significant in the BAL, as 24 hours post-secondary bacterial challenge sTREM-1 levels were 5 times higher in co-infected mice compared to mice infected with *S. pneumoniae* alone.

sTREM-1 release has been attributed to cleavage from surface-bound TREM-1 by MMPs (Gómez-Piña et al., 2012). As we observed a potential MMP-9 cleavage site in the amino acid sequence of TREM-1 (Figure 3.1), and MMP-9 is released during inflammation of the lung, MMP-9 levels were measured in the BAL during secondary bacterial infection, where high levels of sTREM-1 are observed, to determine whether there was an association between sTREM-1 and MMP-9 secretion. MMP-9 secretion in the BAL mirrors sTREM-1 levels even in the single infections groups suggesting a link between the two. A particularly high concentration was MMP-9 was detected in the co-infected group, which, at 24 hours post-bacterial infection, was approximately sixfold higher than single bacterial infection ($p=0.0079$) (Figure 3.17D). This is a very similar to the trend seen in sTREM-1 at 24 hours (Figure 3.17A), which suggests that the release of MMP-9 in the airway might cause increased levels of sTREM-1 via a cleavage mechanism.

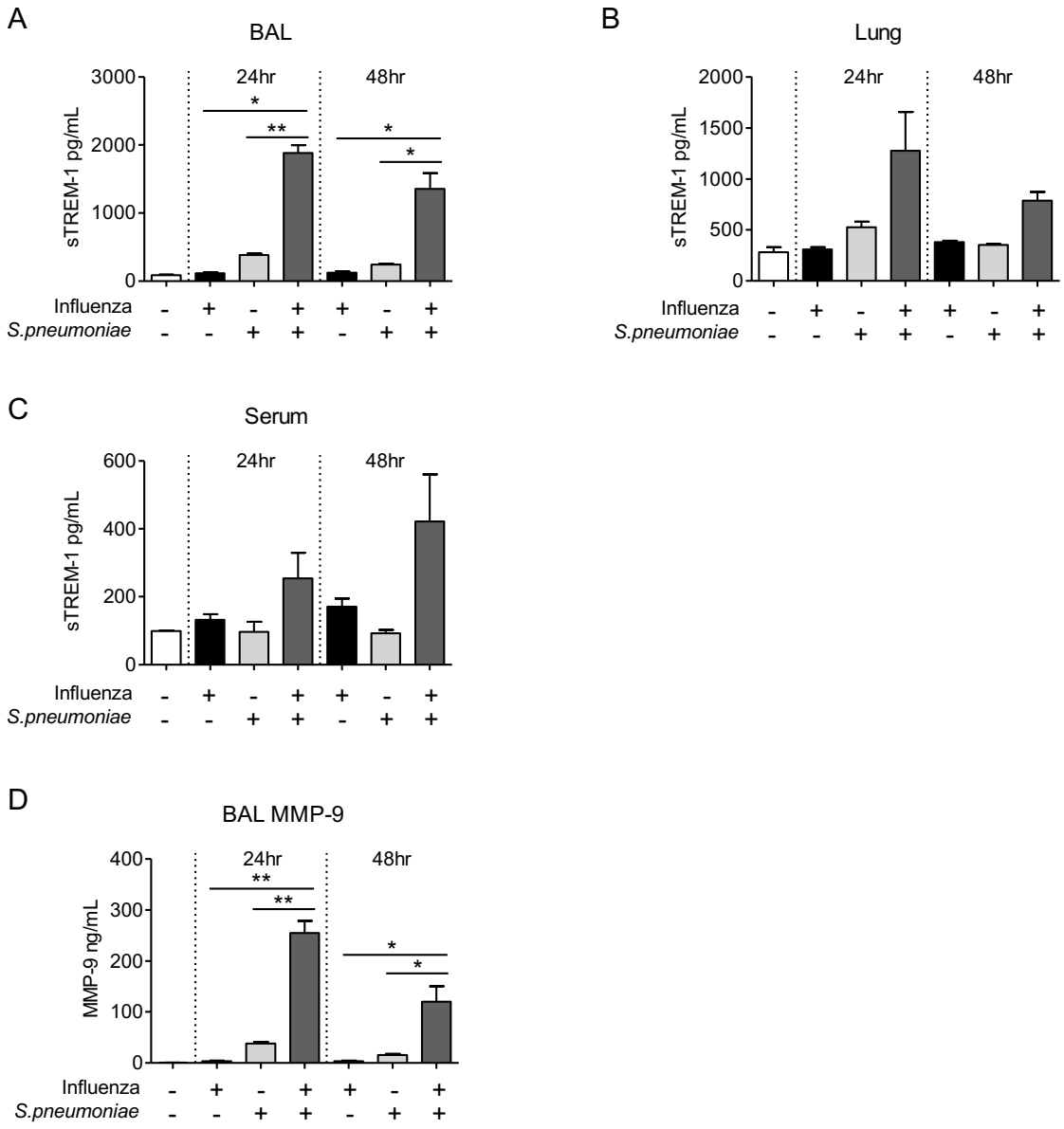

Figure 3.17 sTREM-1 and MMP-9 levels during secondary bacterial infection.

BALB/c mice were infected with 7 PFU of PR8 influenza A virus, followed by 10^6 PFU *S. pneumoniae* on day 7 and analysed at 24 and 48 hours post-*S. pneumoniae* infection. Levels of sTREM-1 in BAL fluid (A), lung (B) and serum (C) and BAL MMP-9 (D) were determined by ELISA. Naïve, uninfected mice were analysed as a control. Data are expressed as mean±SEM, n=4/5 mice per time-point, and are representative of two separate experiments, except for the 48 hour time-point which was carried out once. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$, **$p<0.01$.

3.2.2.6 sTREM-1 levels as an indicator of increased susceptibility

Comparison of sTREM-1 levels and weight loss 24 hours post-*S. pneumoniae* infection show that the extent of weight loss trends with increased sTREM-1 detected in the BAL (Figure 3.18A). sTREM-1 levels in the BAL also increase with decreasing percentage of neutrophils expressing TREM-1 (Figure 3.18B). The total number of TREM-1^{+} neutrophils 24 hours post-infection is again lowest in the co-infected animals (Figure 3.18C). This suggests that susceptibility to secondary bacterial infection coincides with a decrease in TREM-1 expressing neutrophils and a corresponding increase in sTREM-1 release into the airway. The marked increase in sTREM-1 levels could be a consequence of increased inflammation and disease severity (Fig 3.12).

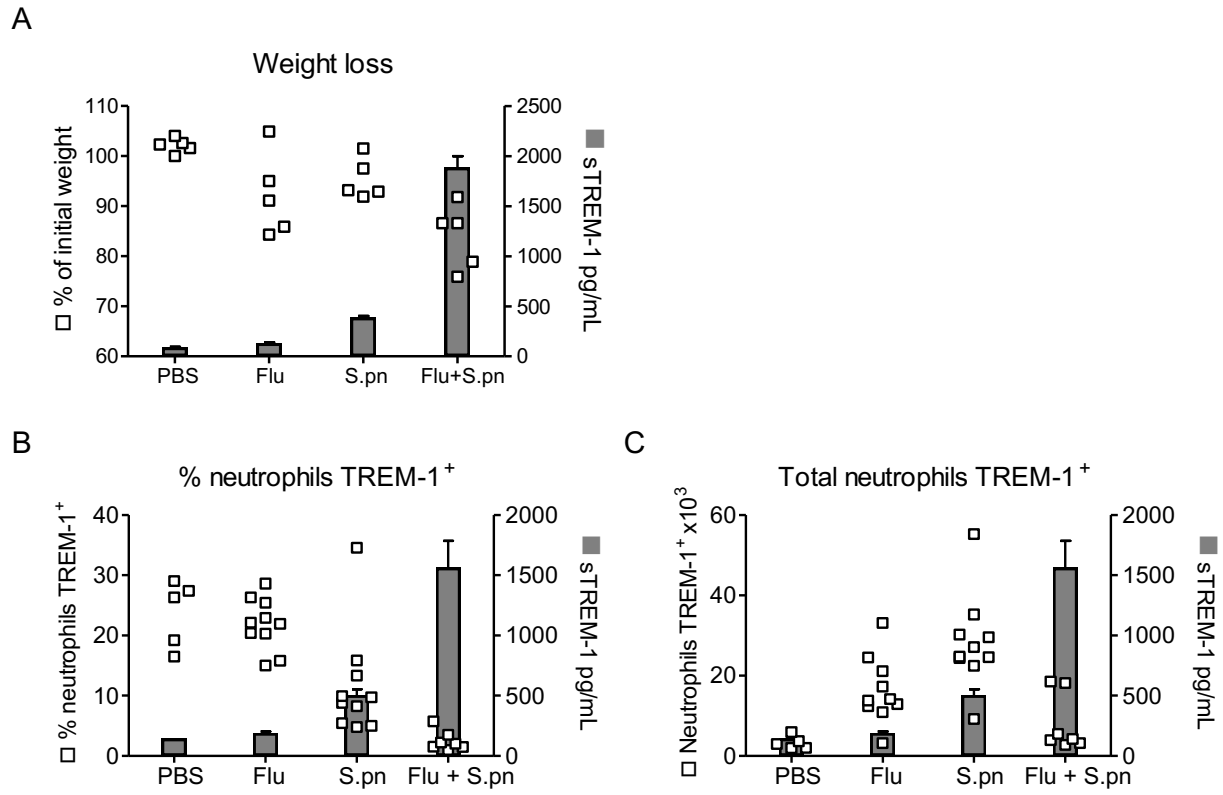

Figure 3.18 Comparison of BAL sTREM-1 levels with weight loss and TREM-1 expressing neutrophils.

BALB/c mice were infected with 7 PFU of PR8 influenza A virus, followed by 10^6 PFU *S. pneumoniae* on day 7 and analysed at 24 hours post-*S. pneumoniae* infection. Levels of sTREM-1 in BAL fluid were determined by ELISA. sTREM-1 (grey bars—■) compared to weight loss (open squares—□) 24 hours post-*S. pneumoniae* infection (A), sTREM-1 (grey bars—■) compared to percentage of neutrophils TREM-1^+ (open squares—□) (B) and sTREM-1 (grey bars—■) compared to total number of neutrophils TREM-1^+(open squares—□) (C). Naïve, uninfected mice were also analysed as a control. Data are expressed as mean±SEM, n=4/5 mice per time-point and are from one experiment.

3.3 Discussion

In this chapter, the expression patterns of TREM-1 and TREM-2 were studied in models of respiratory infections. TREM-1 is associated with various bacterial infections, but its expression pattern in viral infections has not been investigated, nor have TREM-1 or TREM-2 been studied in secondary bacterial infections. The expression of TREM proteins was also compared to other parameters of inflammation in order to determine whether TREMs are associated with disease severity.

3.3.1 TREM-1 and TREM-2 are differentially regulated during influenza infection

In a model of influenza infection, TREM-1 was predominantly expressed on neutrophils (Figure 3.5). However, only 5% of alveolar macrophages expressed TREM-1, which was surprising and may reflect the environmental regulation of airway macrophages described before (Hussell and Bell, 2014). The increase in TREM-1^+ neutrophils in the airway during influenza infection is interesting and suggests an important function during infection. Neutrophils are recruited to the lung in large numbers during influenza infection and can be activated by recognition of single-stranded viral RNA by TLR7 (Wang et al., 2008). Since TREM-1 is capable of amplifying inflammatory signals (Bouchon et al., 2001), increased TREM-1 expression during influenza could serve to amplify intracellular signalling that results in proinflammatory cytokine production. TREM-1 expression could also be contributing to the recruitment of neutrophils to the airway, as a recent study showed that TREM-1 was required for the transepithelial migration of neutrophils from the blood into the airway during pneumonia (Klesney-Tait et al., 2013). The reduction in absolute numbers of TREM-1^+ neutrophils at day 14 post-infection is likely due to the reduction in neutrophil number. Influenza virus could also be causing a reduction in TREM-1-expressing neutrophils, in the same way that filoviruses have been demonstrated to cause the shedding of TREM-1 from human neutrophils (Mohamadzadeh et al., 2006). TREM-1 may also be down-regulated by anti-inflammatory peptides present in the lung following influenza infection. Examples of peptides that are able to down-regulate TREM-1 are vasoactive intestinal peptide (VIP) and cathelicidin. VIP reduced LPS-induced TREM-1 mRNA expression in a dose-dependent manner in mouse lung cell lines (Sun et al., 2011), while cathelicidin reduced lipoteichoic acid (LTA)-induced TREM-1 expression on monocytes (Amatngalim et al., 2011). Expression of VIP has been demonstrated in respiratory disease (Sergejeva et al.,

2004) and cathelicidin is detected in the lungs of influenza-infected mice (Gaudreault and Gosselin, 2008). It would therefore be interesting to examine if these peptides are involved in the regulation of TREM-1 expression on neutrophils in this model.

sTREM-1 was detected in low levels in uninfected mice, and a trend for increased sTREM-1 during peak inflammation was observed (Figure 3.11). Although this enhancement of sTREM-1 production was not statistically significant, using more mice per group studied might have increased the significance of this finding. An increase in sTREM-1 detected 7 days post-infection mirrors an increase in TREM-1 expression on neutrophils, which supports the hypothesis that sTREM-1 is derived from shedding of TREM-1 from the surface of myeloid cells (Gómez-Piña et al., 2012, Molad et al., 2013). The mechanism by which this occurs requires further investigation. sTREM-1 was not detected in BAL of infected mice 14 days post-infection. This could be due to the reduction in TREM-1^{+} neutrophil numbers at this time-point, or may suggest that sTREM-1 is undetectable due to binding to its ligand. sTREM-1 may be acting as a decoy receptor to prevent signalling through TREM-1 which consequently inhibits inflammation as first suggested by Gibot *et al.* (2004).

TREM-2 was strongly expressed by alveolar macrophages during peak inflammation (Figure 3.6 and 3.7). While a high proportion of $CD11c^{+}CD11b^{int}$ alveolar macrophages—around 80%—were TREM-2^{+}, almost all $CD11c^{+}CD11b^{+}$ macrophages expressed TREM-2. The majority of $CD11c^{+}CD11b^{+}$ macrophages expressing TREM-2 also stained positive for chemokine receptor CCR5 (Figure 3.9). CCR5 mediates the migration of macrophages towards CCL4 and promotes survival of macrophages during viral infection (Samson et al., 1996, Tyner et al., 2005). Co-expression of TREM-2 and CCR5 on $CD11c^{+}CD11b^{+}$ macrophages suggests that this population may be recruited to the lung upon infection whereupon the macrophages express TREM-2 to ameliorate on-going inflammation. CCR5 may then promote the survival of TREM-2-expressing macrophages and therefore lead to the persistence of anti-inflammatory macrophages during peak inflammation. That there is a reduction in the TREM-2^{+} macrophage population supports the hypothesis that TREM-2 promotes resolution, and once inflammation is resolved, TREM-2 is then down-regulated on macrophages.

3.3.2 Decreased TREM-2 and increased sTREM-1 associated with susceptibility to bacterial infection following influenza infection

Resolution of inflammatory disease results in an altered homeostasis in the lung, which affects the responsiveness to subsequent pathogens (Goulding et al., 2011). In this chapter, the same susceptibility to bacterial infection in mice previously infected with influenza was observed in BALB/c mice. The mice exhibited increased weight loss, cellular infiltration to the lung, bacterial burden and proinflammatory cytokine production in secondary bacterial infection compared to single infections (Figure 3.12 and 3.14). In addition, the number of neutrophils was increased, and number of macrophages decreased during co-infection compared to influenza or *S. pneumoniae* single infections (Figure 3.15). Interestingly, the expression of TREM-2 on macrophages, which are likely playing an anti-inflammatory role, was reduced in the co-infected group (Figure 3.16). This could be a consequence of increased susceptibility to secondary bacterial infection and the proinflammatory environment it creates. Upon bacterial infection, TREM-2 expressed on long-lived macrophages during influenza infection is rapidly down-regulated in favour of boosting a proinflammatory response to bacterial infection.

Coinciding with potent proinflammatory cytokine production is a large influx of neutrophils. However, the number of TREM-1^{+} neutrophils was not correspondingly high. Instead, a very high concentration of sTREM-1 was detected in the airway and lung (Figure 3.17). It is suggested that sTREM-1 can be produced either by an alternative splice variant of TREM-1 mRNA (Gingras et al., 2002b) or by cleavage of TREM-1 from myeloid cell surfaces possibly by MMP enzymatic activity (Gómez-Piña et al., 2012). Furthermore, there is a potential MMP-9 cleavage site in the amino acid sequence of TREM-1 (Figure 3.1) and MMP-9 is known to be secreted by neutrophils during influenza infection (Bradley et al., 2012). As such, high sTREM-1 levels detected in the airway of co-infected mice were hypothesised to be related to MMP-9. Measurement of MMP-9 levels in the BAL—where sTREM-1 levels were the highest—showed that maximal MMP-9 release coincides with maximal sTREM-1 release (Figure 3.17). This suggests a role for MMP-9 cleavage of TREM-1 in this model of secondary bacterial infection following influenza infection.

3.3.3 sTREM-1 as a marker of susceptibility to secondary bacterial infection

sTREM-1 has been measured in a variety of diseases and is a good candidate as both a diagnostic and prognosis marker of diseases (Adly et al., 2014, Ye et al., 2014), but has not been measured in secondary bacterial infection after influenza infection. In our model of secondary bacterial infection, there was a trend for inverse correlation of sTREM-1 concentration in BAL with weight loss, and sTREM-1 was associated with neutrophil reduced surface TREM-1 expression, which could give impetus to the theory that sTREM-1 is released from neutrophils. Further work could reveal a novel mechanism by which neutrophils self-regulate TREM-1 to dampen inflammation during infectious disease by eliminating the amplifying effect of TREM-1 signalling on proinflammatory cytokine production. sTREM-1 in the airway might also sequester the ligand of TREM-1, further reducing inflammatory signalling and facilitating a return to homeostasis. This feedback loop could be important in returning the lung environment to homeostasis after exuberant inflammation.

Understanding of this interesting form of TREM-1 hold potential in the manipulation of TREM-1 either by blockade by synthetic peptides, e.g. LP17 or blocking antibodies. A recent study showed that mice lacking TREM-1 had reduced mortality and morbidity but intact viral clearance (Weber et al., 2014). This study demonstrates the value in targeting TREM-1 as an amplifier of inflammation to reduce disease pathology without compromising host defence against pathogens. Similarly, manipulation of the TREM-2 axis could be explored, in order to promote the anti-inflammatory effects of TREM-2.

3.3.4 Conclusion

TREM-1 and TREM-2 are differentially expressed on myeloid cells during influenza infection; predominantly expressed on neutrophils and macrophages respectively. Secondary bacterial infection induced high levels of sTREM-1 in the BAL and lung and correlated with MMP-9, thus suggesting a role of proteolytic cleavage of TREM-1 by MMPs. Interestingly, expression of TREM-2—an anti-inflammatory signalling receptor—was reduced during secondary bacterial infection, suggesting that TREM-2 down-regulation may occurs during the proinflammatory phase of bacterial infection. These findings confirm that TREM proteins are involved in influenza and bacterial co-infection, and pending further manipulation

studies, represent a class of proteins that could therapeutically targeted in order to control excessive inflammation in inflammatory lung disease. Studies investigating the mechanisms by which TREM signalling controls inflammation and the precise mechanisms by which sTREM-1 is produced during inflammation are on-going. The next chapter will investigate the targeting of signalling kinases in order to control inflammation and symptoms of allergic airway disease.

Chapter 4: Role of signalling kinase inhibitors during HDM-induced allergic inflammation

Chapter 4: Role of signalling kinase inhibitors during HDM-induced allergic inflammation

4.1 Introduction

Animal models of human disease are widely used in research with two main goals: 1) to identify the mechanisms of disease and 2) to assess the efficacy of new therapeutic candidate drugs prior to clinical testing. Many research groups have studied the allergic response in mice and since mice do not spontaneously develop asthma, such studies require the experimental induction of asthma with an allergen. Early studies utilised OVA as an allergen and, in this model, intraperitoneal sensitisation of mice with OVA followed by inhalation of OVA induced disease features such as Th2 cytokine production, allergen-specific IgE production and AHR, which are seen in human asthma (Lloyd et al., 2001). The OVA model is a good representation of type 2 asthma; however, OVA is not an allergen commonly associated with human asthma, nor is the short-term exposure and intraperitoneal sensitisation a true reflection of asthma development in man.

Mouse models using more clinically relevant allergens, such as HDM, have since been developed. Repeated intranasal exposure to HDM extract over a number of weeks results in the development of chronic airway inflammation, Th2 cytokine release, mucus secretion, AHR and airway remodelling (Johnson et al., 2004, Gregory et al., 2009). The HDM model has since been the subject of studies into the mechanisms underlying allergic airway disease. Various studies have, for example, shown that alveolar macrophages are critical regulators of the Th2 response, macrophage-derived HIF-α plays a role in driving airway remodelling and the role of vitamin D in the development of the allergy has also been explored in a neonatal model of HDM-induced allergic airway disease (Mathie et al., 2015, Byrne et al., 2013, Vasiliou et al., 2014). Since this established model of allergic airway disease exhibits many features seen in human asthma and relies upon an intranasal route of allergen challenge that more closely mimics human encountering allergen, it is a credible model in which the role of newly developed candidate drugs can be tested in the pre-clinical testing phase. Models such as the OVA and HDM model are only mechanistic models, so direct translation between *in*

vivo studies and that of human disease cannot be made. However, using such models allows for mechanistic studies into how candidate drugs function, providing insight before carrying out clinical studies in humans.

One class of drugs currently being explored in the treatment of chronic inflammatory lung disease are signalling kinase inhibitors. While glucocorticoid therapy is the main anti-inflammatory treatment used in asthma, there remains a subset of patients that do not respond to treatment. One cause of this steroid-insensitivity is thought to be increased signalling kinase activity (Adcock and Lane, 2003). Furthermore, increased understanding of the inflammatory component of asthma has increased the pool of potential drug targets in asthma treatment. Recently, the targeting of signalling components has been explored with a number of studies into the use of signalling kinase inhibitors in allergic disease and asthma. The kinases investigated include Src, Syk, p38 MAPK and PI3Kγ and δ. These kinases play roles in a plethora of inflammatory processes leading to asthma, including inflammatory cytokine production, T-cell activation and differentiation, basophil activation, leukocyte migration and ASM proliferation (Mócsai et al., 2010, Palacios and Weiss, 2004, Kneidinger et al., 2008, Redhu et al., 2013, Marwick et al., 2010). Moreover, pharmacological inhibition of these kinases has shown promise in the reduction of asthma disease parameters, for example, inhibition of PI3Kδ reduced AHR and airway inflammation in an OVA mouse model (Lee et al., 2006). Such studies have, however, mainly used OVA as an allergen, which, as previously discussed, has certain limitations in modelling human asthma. As such, we evaluated the effect of signalling kinase inhibitors in an alternative model of allergic airway disease, the HDM model, which is described in this chapter.

TC1 is a specific PI3Kγ and δ dual inhibitor that has shown anti-inflammatory effects in a smoking and OVA-induced asthma mouse model and poly(I:C)-induced airway inflammation (Kimura et al., 2013). TC1 also suppresses CXCL8 production from human peripheral blood neutrophils derived from patients with COPD (Ito et al., 2013b). TC2 is a narrow-spectrum kinase inhibitor (NSKI) that acts on Syk, Src and p38 MAPK and is also a promising candidate in controlling inflammatory conditions. TC2 was well tolerated in a phase I study and has demonstrated target engagement in COPD (Russell et al., 2015). This chapter will utilise the HDM model of allergic airway disease in order to investigate the efficacy of

signalling kinase inhibitors in *in vivo* allergic airway disease (Figure 4.1). Neither of the two compounds TC1 and TC2 has previously been tested in a model of chronic airway inflammation caused by HDM allergen, nor indeed has the effect of the inhibitors on airway function in mouse. Thus, it was proposed to investigate the effect of TC1 and TC2 in a model of HDM-induced airway inflammation.

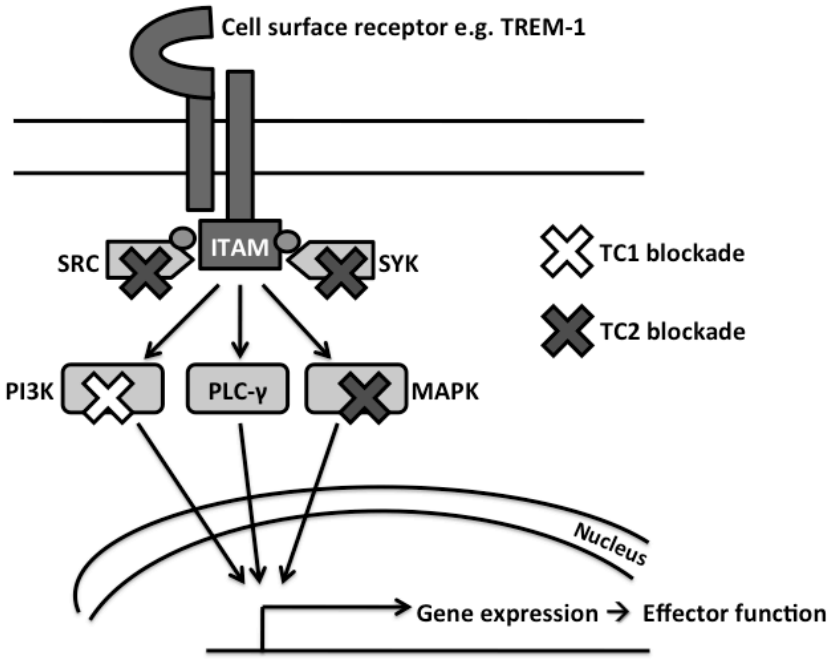

Figure 4.1 Signalling kinases blocked by TC1 and TC2.
TC1 inhibits PI3Kγ and δ; TC2 inhibits Src, Syk and MAPK.

4.1.1 Hypothesis

Given that PI3K, Src, Syk and p38 MAPK kinases play vital roles in the development of inflammation in general and specifically in the allergic lung, it was hypothesised that inhalation of kinases inhibitors TC1 and TC2 would abrogate inflammation in a model of HDM-induced allergic airway disease, thereby reducing the inflammatory parameters and impaired airway function that is characteristic of this particular mouse model of asthma. It is posited that inhibitor treatment will lead to a decrease in inflammatory mediators detected in the lung and BAL and that airway function will be improved.

4.1.2 Aims

The aim of this chapter is to determine whether inhaled kinase inhibitor treatment with TC1 and TC2 in a model of HDM-induced allergic airway disease alters:

1) airway function.
2) airway inflammation, i.e. analysis inflammatory mediator and lung inflammatory score.

4.2 Results

4.2.1 Signalling kinase inhibitors in HDM-induced allergic airway inflammation

The aim was to determine whether signalling kinase inhibitors are able to dampen HDM-induced allergic airway inflammation. Allergic airway inflammation was established with HDM exposure 3-times per week for 3 weeks (Gregory et al., 2009). Signalling kinase inhibitors TC1 (PI3Kγ/δ inhibitor), dasatinib (Src inhibitor), R343 (Syk inhibitor) in 10% DMSO or vehicle alone (10% DMSO in PBS) were administered intranasally for 5 consecutive days using a therapeutic regimen (Figure 4.2). AHR, leukocyte infiltration, serum Igs, and the production of cytokines and chemokines were analysed 24 hours after the final HDM dose (Gregory et al., 2009).

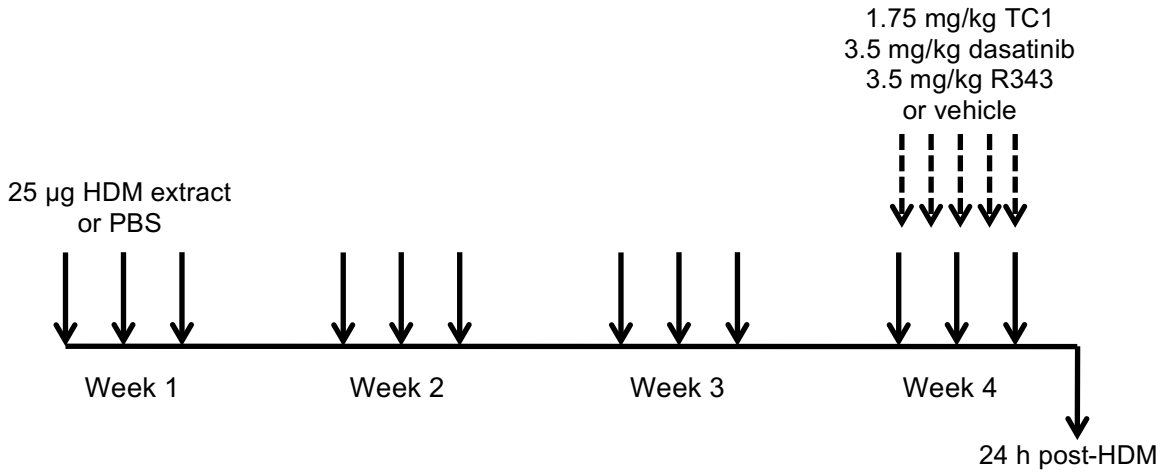

Figure 4.2 Signalling kinase inhibitor treatment during HDM-induced allergic airway inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC1, 3.5 mg/kg dasatinib, 3.5 mg/kg R343 in 10% DMSO vehicle or vehicle alone intranasally, 2 hours before HDM was given, for 5 consecutive days preceding analysis. Mice were analysed 24 hours post-final HDM exposure.

4.2.1.1 AHR and cellular recruitment following TC1, dasatinib and R343 treatment in HDM-induced allergic airway disease

HDM-treated mice that were given vehicle treatment alone developed AHR in terms of increased resistance and decreased compliance of the airway compared to PBS controls (Figure 4.3). Treatment with inhibitors TC1, dasatinib and R343 did not significantly alter airway resistance or compliance in HDM-exposed mice (Figure 4.3A-F). HDM exposure led to increased cell numbers in the airway and lung tissue in vehicle control mice compared to PBS controls (Figure 4.4A-F). While TC1 and dasatinib treatment did not alter cell counts in the lung or BAL fluid (Figure 4.4A-D), treatment with Syk inhibitor R343 significantly ($p<0.01$) reduced cell infiltration into the airway lumen (Figure 4.4E).

4.2.1.2 IgE production following TC1, dasatinib and R343 treatment in HDM-induced allergic inflammation

In line with previous studies from the group (Gregory et al., 2013), serum levels of total and HDM-specific IgE were elevated in animals treated with HDM and vehicle controls compared to PBS (Figure 4.5A-F). Dasatinib treatment significantly reduced total and HDM-specific serum IgE production, however, TC1 and R343 had no effect (Figure 4.5A-F).

4.2.1.3 Cytokine and chemokine production following TC1, dasatinib and R343 treatment in HDM-induced allergic airway disease

Levels of chemokines and cytokines were measured in the BAL fluid by Luminex assay. While HDM exposure led to an increase in various chemokine mediators, such as CCL11, CXCL10, CXCL1, CCL4, which are involved in the recruitment of leukocytes, and the Th2 cytokine IL-4, treatment with TC1, dasatinib and R343 did not significantly alter levels detected (Figure 4.6A-C). Other Th2 cytokines such as IL-13 and IL-5 were not detectable at this time-point in the BAL fluid (data not shown).

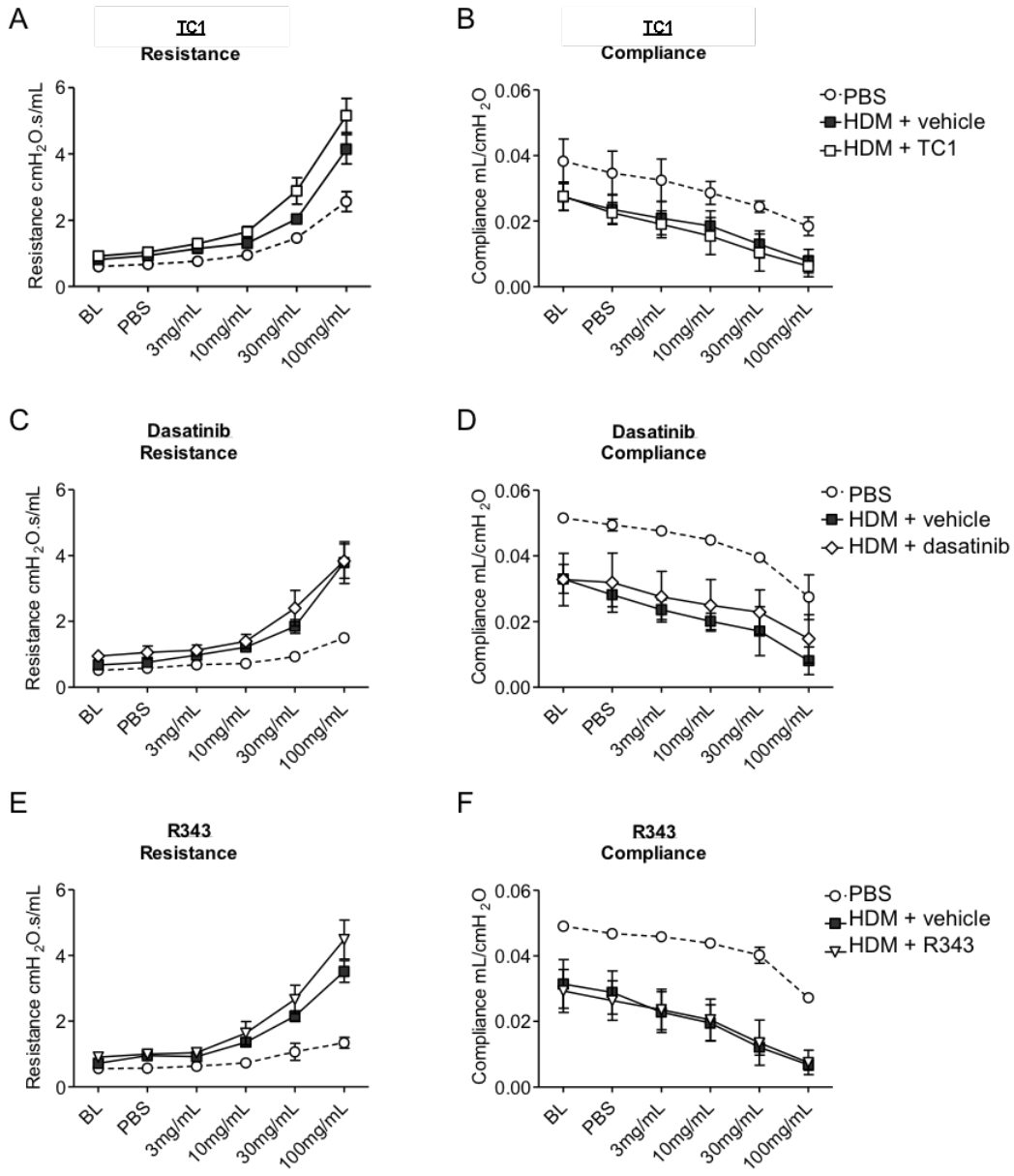

Figure 4.3 Effect of TC1, dasatinib and R343 on AHR during HDM-induced allergic inflammation

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC1, 3.5 mg/kg dasatinib, 3.5 mg/kg R343 in 10% DMSO vehicle or vehicle alone intranasally for 5 consecutive days preceding analysis. 24 hours post-final HDM exposure, mice were anaesthetised and subject to lung function studies. Airway resistance (A, C, E, G) and airway compliance (B, D, F, H) were measured in mice treated with TC1, dasatinib and R343. Naïve, untreated mice were analysed as control. Data are expressed as mean±SEM, n=3–8 mice per group. Data for TC1 are representative of two separate experiments. Dasatinib and R343 experiments were performed once. Statistical significance was determined by Mann-Whitney U test between relevant groups. No significant differences ($p<0.05$) were recorded.

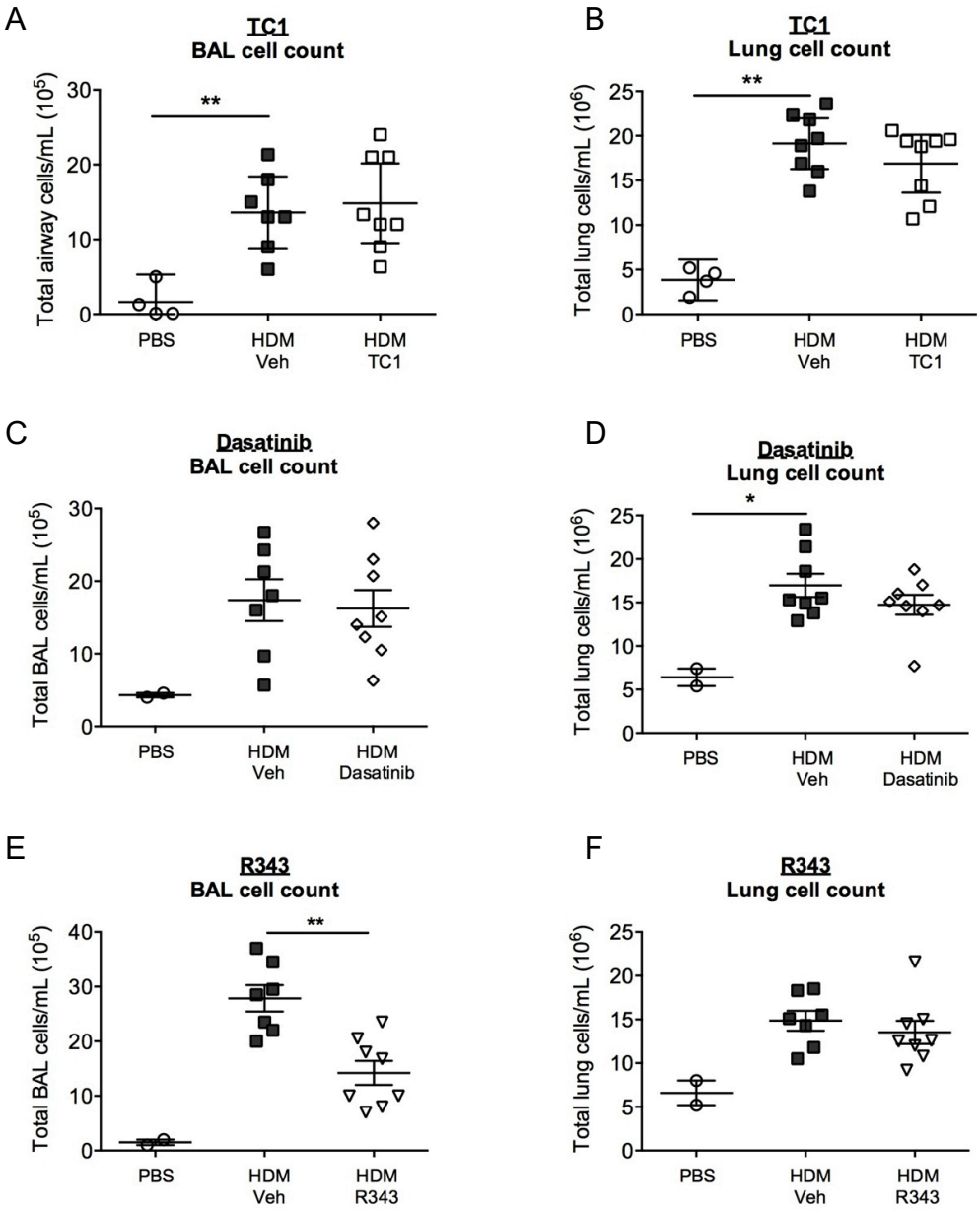

Figure 4.4 Effect of TC1, dasatinib and R343 on cellular infiltration during HDM-induced allergic inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC1, 3.5 mg/kg dasatinib, 3.5 mg/kg R343 in 10% DMSO vehicle or vehicle alone intranasally for 5 consecutive days preceding analysis. 24 hours post-final HDM exposure, following lung function analyses, BAL was performed and lung tissue harvested. Cells were isolated from BAL fluid (A, C, E) and lung tissue (B, D, F). Naïve, mice were analysed as control. Data are expressed as mean±SEM, n=2–8 mice per group. Data for TC1 are representative of two separate experiments. Dasatinib and R343 experiments were performed once. Statistical significance was determined by Mann-Whitney U test between relevant groups, **p<0.01.

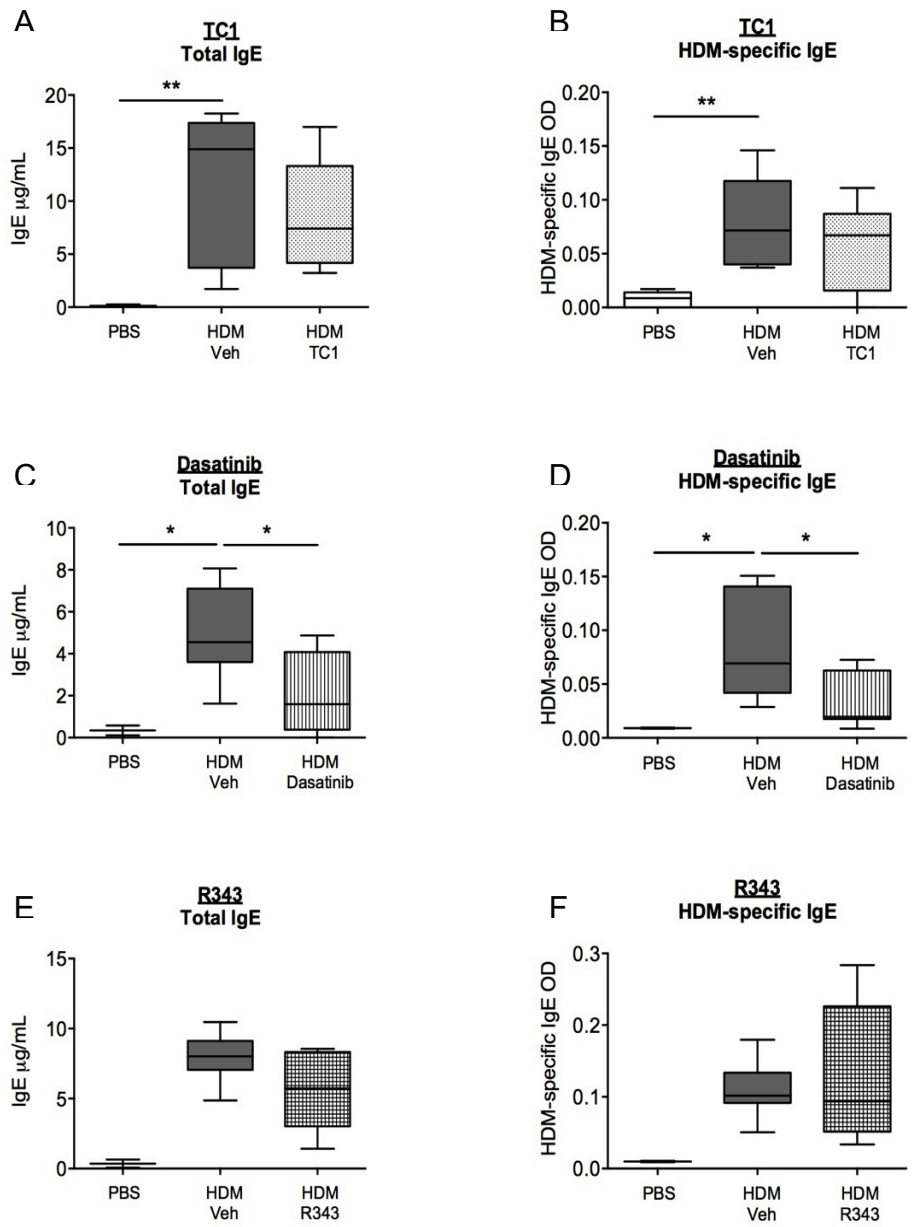

Figure 4.5 Effect of TC1, dasatinib and R343 on serum IgE production during HDM-induced allergic inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC1, 3.5 mg/kg dasatinib, 3.5 mg/kg R343 in 10% DMSO vehicle or vehicle alone intranasally for 5 consecutive days preceding analysis. Mice were analysed for total IgE (A, C, E) and HDM-specific IgE (B, D, F) in serum by ELISA. Naïve mice were analysed as control. Data are expressed as median, IQR and minimum and maximum values, n=3–8 mice per group. Data for TC1 are representative of two separate experiments. Dasatinib and R343 experiments were performed once. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$.

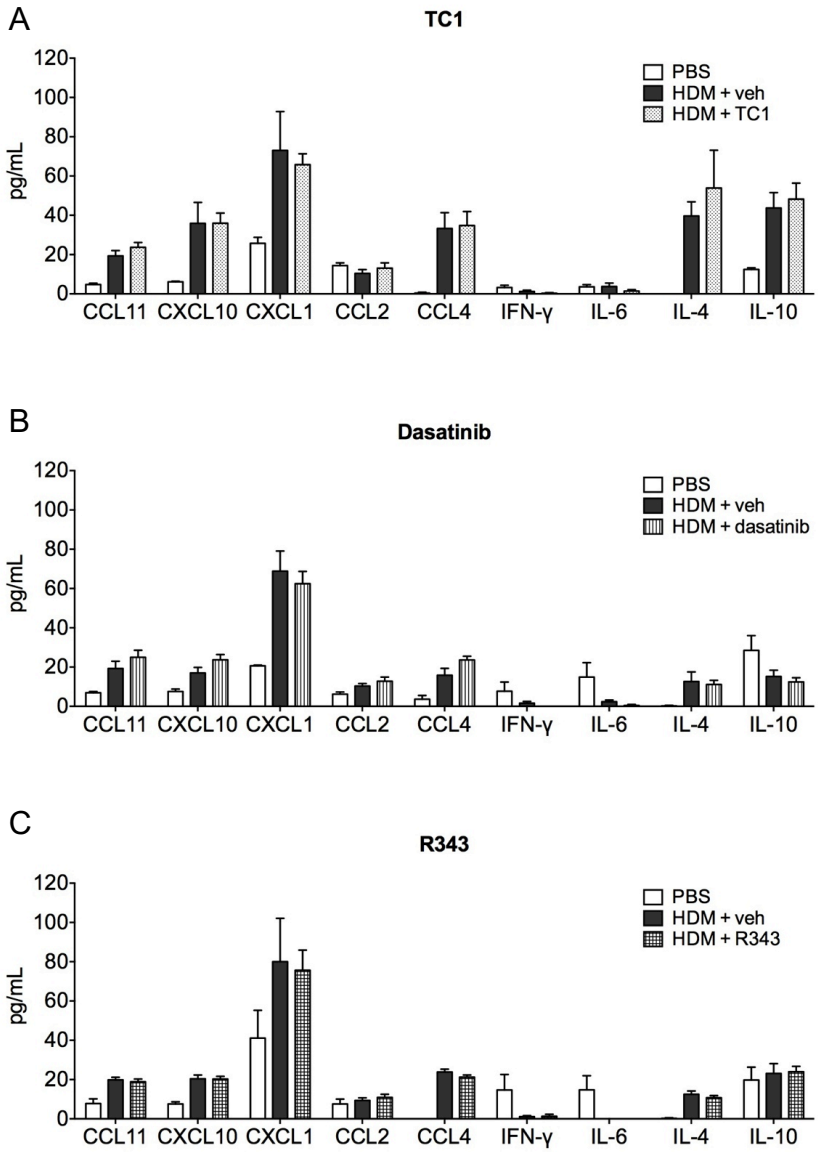

Figure 4.6 Effects of TC1, dasatinib and R343 on chemokine and cytokine production.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC1, 3.5 mg/kg dasatinib, 3.5 mg/kg R343 in 10% DMSO vehicle or vehicle alone intranasally for 5 consecutive days preceding analysis. 24 hours post-final HDM exposure, cytokines were analysed in BAL (A-C) by Luminex assay. Data are expressed as mean+SEM, n=3–8 mice per group. Data for TC1 are representative of two separate experiments. Dasatinib and R343 experiments were performed once. Luminex assays were carried out by industrial collaborators. Statistical significance was determined by Mann-Whitney U test between relevant groups.

4.2.2 Effect of TC2 on HDM-induced allergic airway inflammation

TC2, a NSKI blocking Src family kinases, Syk and p38 MAPK, was tested in the HDM model. BALB/c mice were exposed to HDM for 4 weeks 3-times weekly. In the final week TC2 was administered in 10% DMSO or vehicle alone intranasally for 5 consecutive days while HDM exposure was continued. Mice were analysed 24 hours after the final HDM dose (Figure 4.7).

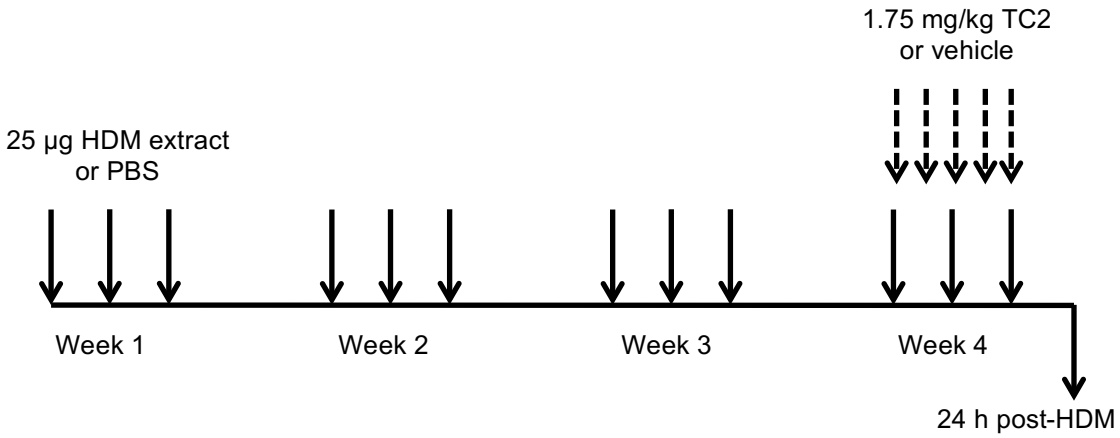

Figure 4.7 Inhaled TC2 treatment during HDM-induced allergic airway inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC2 in 10% DMSO vehicle or vehicle alone intranasally, 2 hours before HDM was given, for 5 consecutive days preceding analysis. Mice were analysed 24 hours post-final HDM exposure.

4.2.2.1 AHR following TC2 treatment in HDM-induced allergic airway disease

Exposure of mice to HDM led to increased airway resistance as compared to PBS treated mice. Treatment of mice with TC2 (for 5 days consecutive days prior to analysis) did not reduce airway resistance (Figure 4.8A). Similarly, TC2 did not have an effect on compliance as it was reduced in the same manner as exposure to HDM alone (Figure 4.8B). Treatment with TC2 also did not alter airway resistance nor airway compliance compared to HDM + vehicle control at 100 mg/mL metacholine challenge (Figure 4.8C, D), or at any other concentration of metacholine (Figure 4.8A, B).

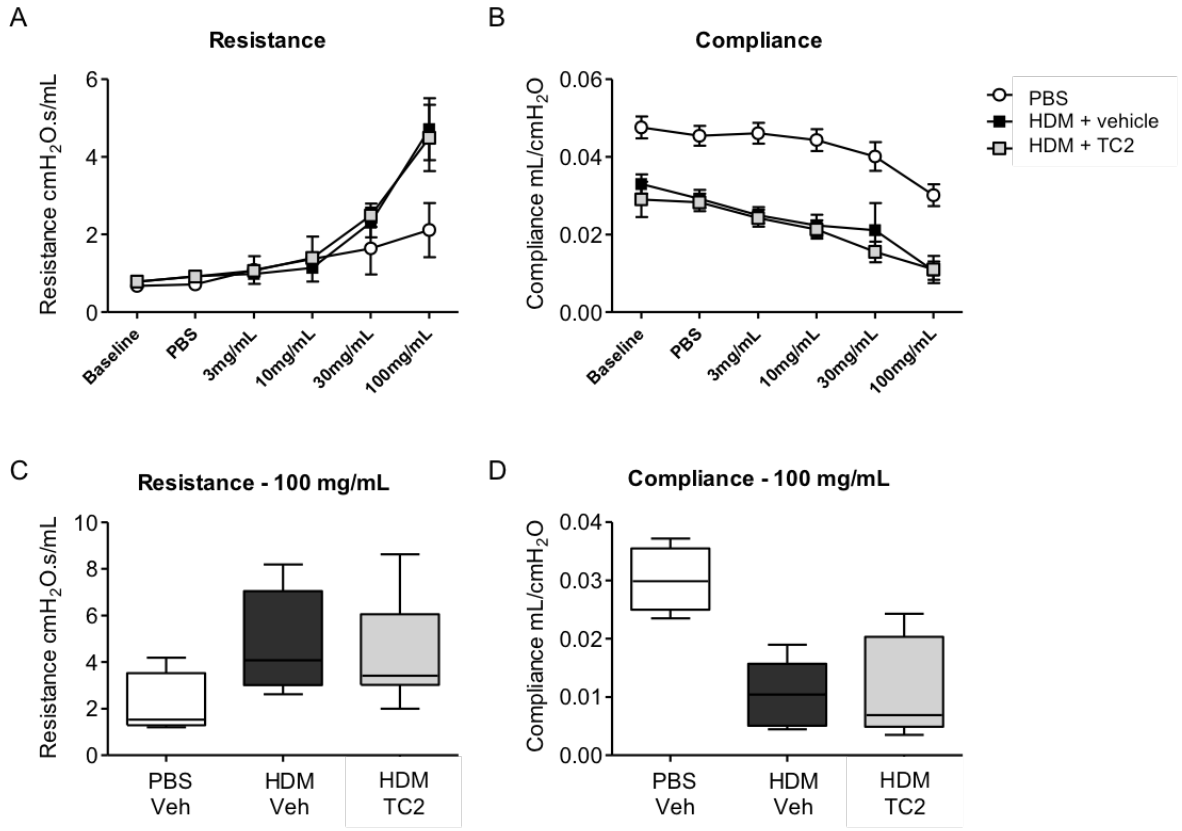

Figure 4.8 Effect of TC2 on HDM-induced AHR.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC2 in 10% DMSO vehicle or vehicle alone intranasally for 5 consecutive days preceding analysis. 24 hours post-final HDM exposure. Airway resistance (A) and airway compliance (B) were measured, data are expressed as mean±SEM. Airway resistance (C) and compliance (D) at 100 mg/mL of metacholine. Data are expressed as median, IQR and minimum and maximum values, n=4–8 mice per group and are pooled from 2 individual experiments.

4.2.2.2 Eosinophil recruitment following TC2 treatment in HDM-induced allergic airway disease

Total cell counts in the BAL and lung tissue were unaffected by TC2 treatment during HDM-induced allergic inflammation (Figure 4.9A, B). However, TC2 treatment significantly reduced the percentage of eosinophils ($p<0.05$) (Figure 4.9C) and the total number of eosinophils (Figure 4.9D). Neutrophils and lymphocyte/monocyte populations were unaffected by TC2 treatment.

4.2.2.3 Serum immunoglobulin production following TC2 treatment in HDM-induced allergic airway disease

TC2 treatment led to significant decreases in total IgE, HDM-specific IgE and total IgG_{2a} production in HDM-exposed mice compared to HDM-exposed mice treated with vehicle alone, $p=0.0041$, 0.029 and 0.02, respectively (Figure 4.10A-C). While not significant, a trend for decreased total IgG_1 was also observed in TC2-treated animals (Figure 4.10D).

4.2.2.4 Cytokine production following TC2 treatment in HDM-induced allergic airway disease

TC2 had no significant effect on the production of chemokines in the airway during HDM-induced airway inflammation (Figure 4.11A-E). Levels of proinflammatory cytokines, i.e. IFN-γ, IL-6, IL-1β and IL-17A, were unaffected by HDM exposure or TC2 treatment (Figure 4.12E-H). TC2 treatment also did not affect levels of Th2-associated cytokines such as IL-4 or IL-13, or other cytokines IL-10 or IL-33 (Figure 4.12A-D).

4.2.2.5 Effect of TC2 on TREM-2^+ macrophages

TREM-2 expressing macrophages are elevated in HDM-treated mice compared to PBS controls 3 days after final HDM exposure (Habibzay et al., 2012). TREM-2^+ macrophages were elevated compared to PBS controls 24 hours after the final HDM dose, however, TC2 treatment had no effect on the percentage of TREM-2^+ macrophages in the BAL (Figure 4.13).

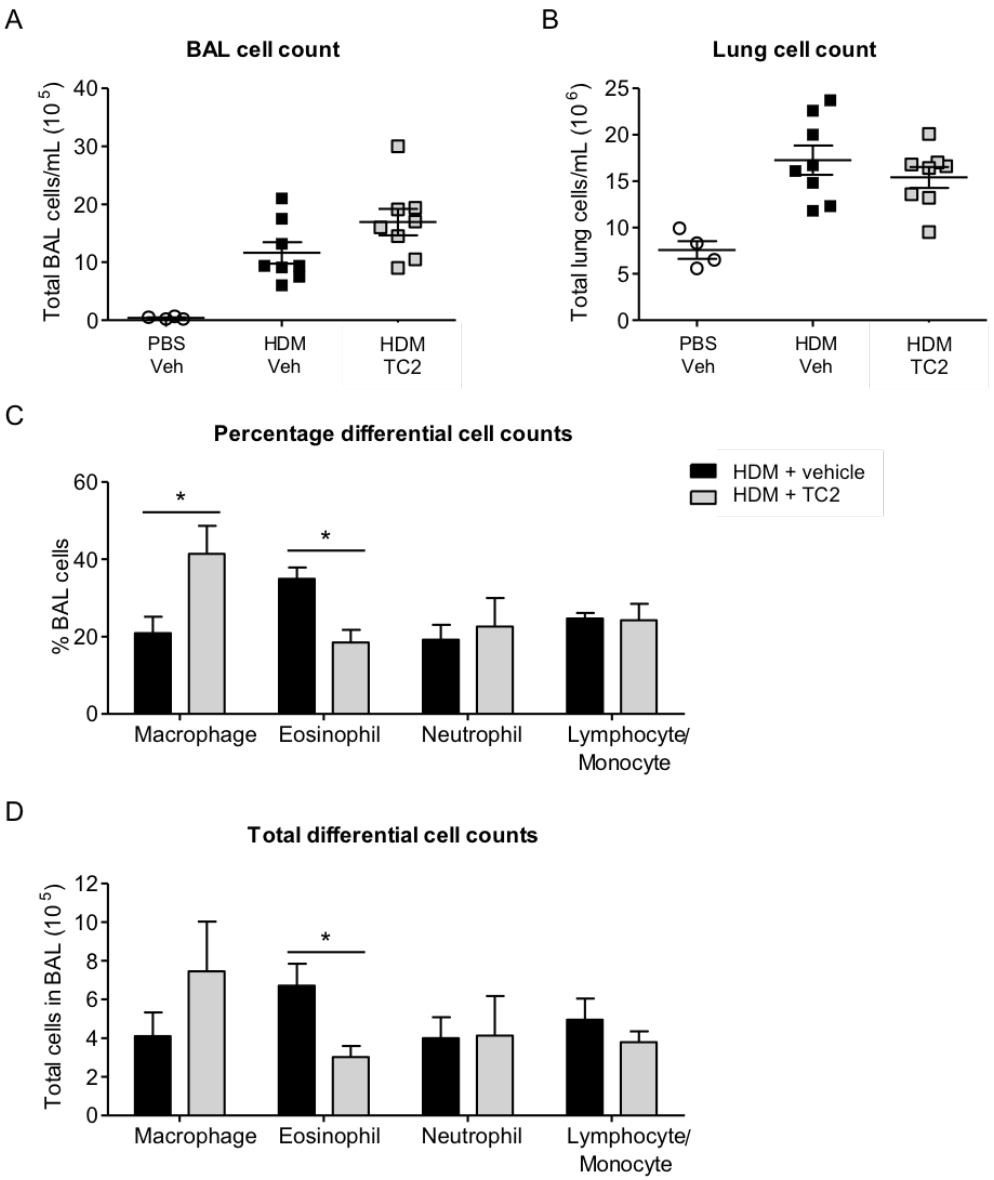

Figure 4.9 Reduced HDM-induced eosinophilia following TC2 treatment.

BALB/c mice were intranasally inoculated with 25 μg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC2 in 10% DMSO vehicle or vehicle alone intranasally for 5 consecutive days preceding analysis. 24 hours post-final HDM exposure, following lung function analyses, BAL was performed and lung tissue harvested. Cells were isolated from BAL fluid (A) and lung tissue (B) and counted. Percentage (C) and total number (D) of cell populations recovered from BAL were measured by differential cell counting of Wright-Giemsa stained cell samples. Data are expressed as mean±SEM, n=4–8 mice per group. Total cell counts are pooled data from 2 independent experiments. Differential cell count data are representative of two experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$.

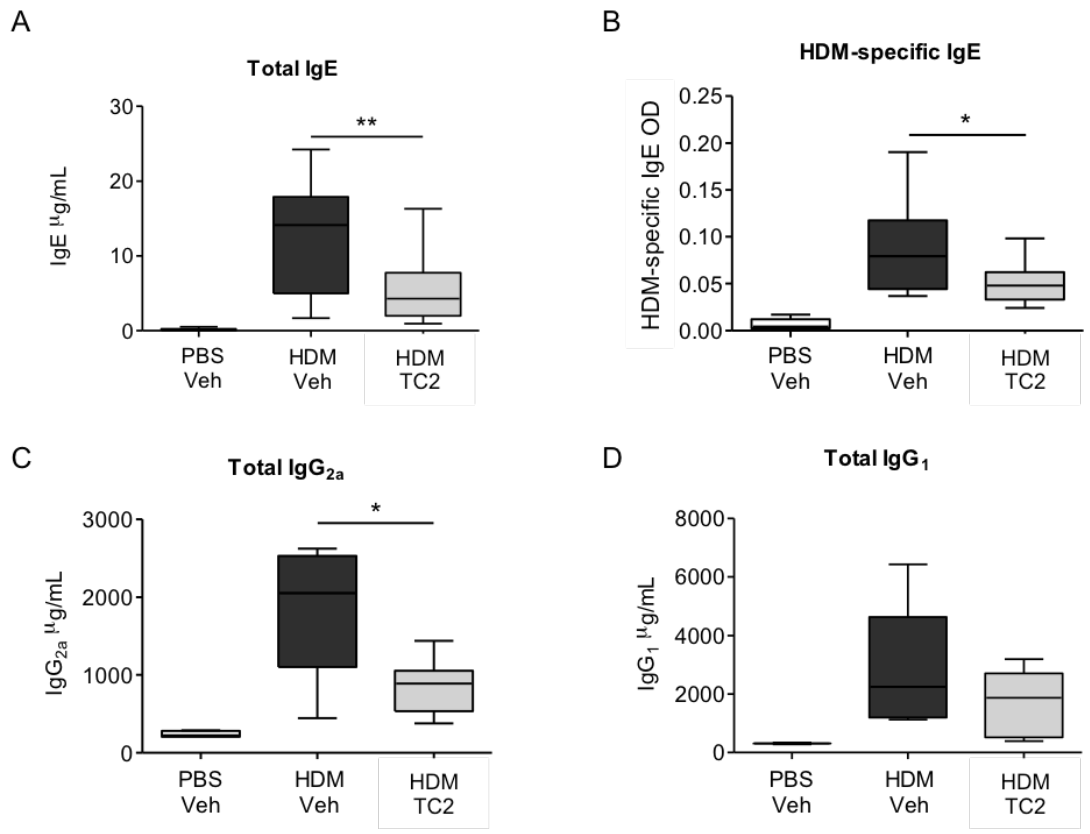

Figure 4.10 TC2 reduces IgE and IgG2a production in HDM-induced allergic airway disease.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC2 in 10% DMSO vehicle or vehicle alone intranasally for 5 consecutive days preceding analysis. 24 hours post-final HDM exposure, mice were analysed for total IgE (A), HDM-specific IgE (B), total IgG_{2a} (C) and total IgG_1 (D) in serum by ELISA. Data are expressed as median, IQR and minimum and maximum values, n=4–8 mice per group and are pooled data from 2 independent experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$, **$p<0.01$.

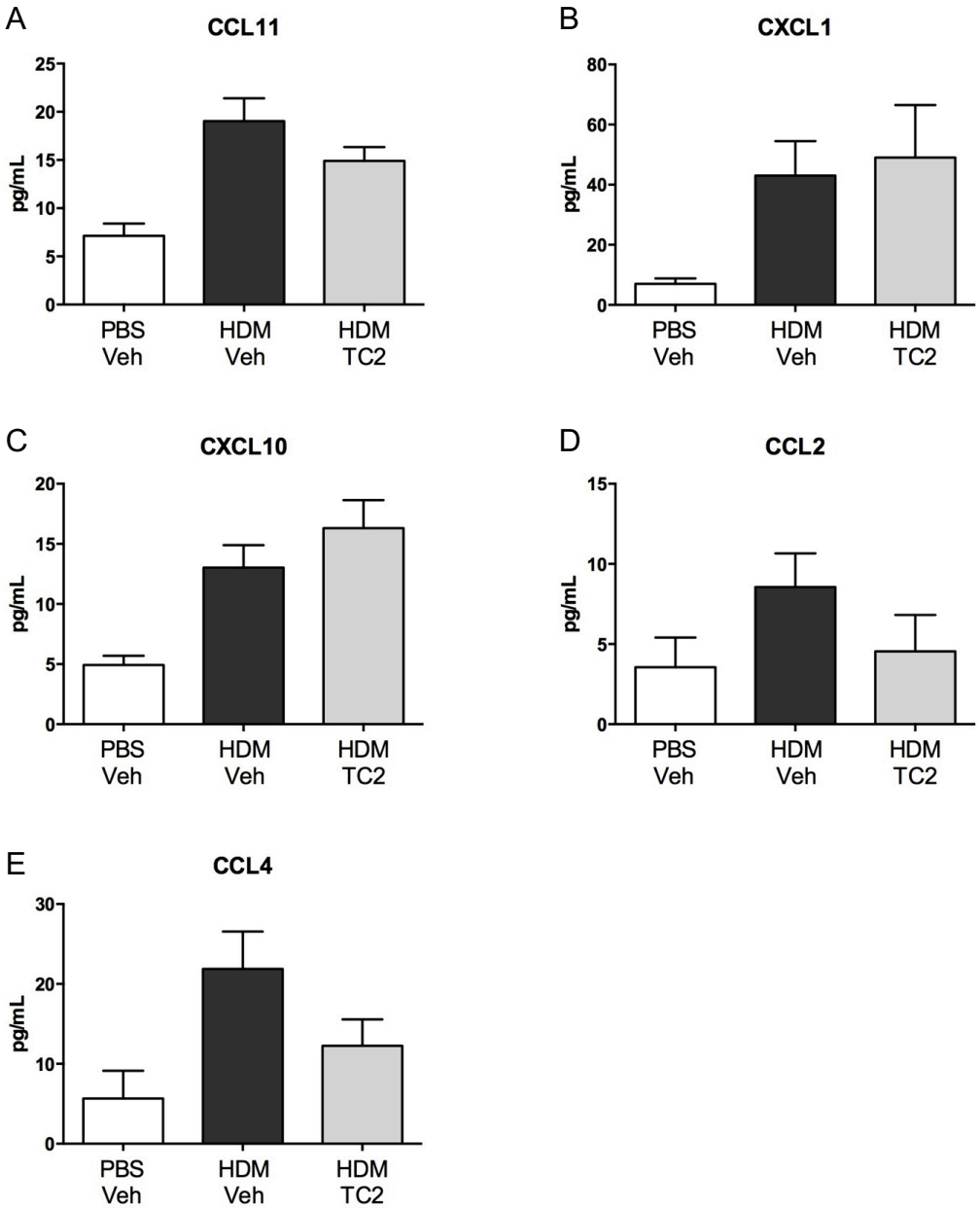

Figure 4.11 TC2 does not alter BAL chemokine production during HDM-induced airway inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC2 in 10% DMSO vehicle or vehicle alone intranasally for 5 consecutive days preceding analysis. 24 hours post-final HDM exposure, mice were analysed for BAL chemokines (A-E) by Luminex assay. Data are expressed as mean+SEM, n=4–8 mice per group and are pooled data from 2 independent experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups. Luminex assays were carried out by industrial collaborators.

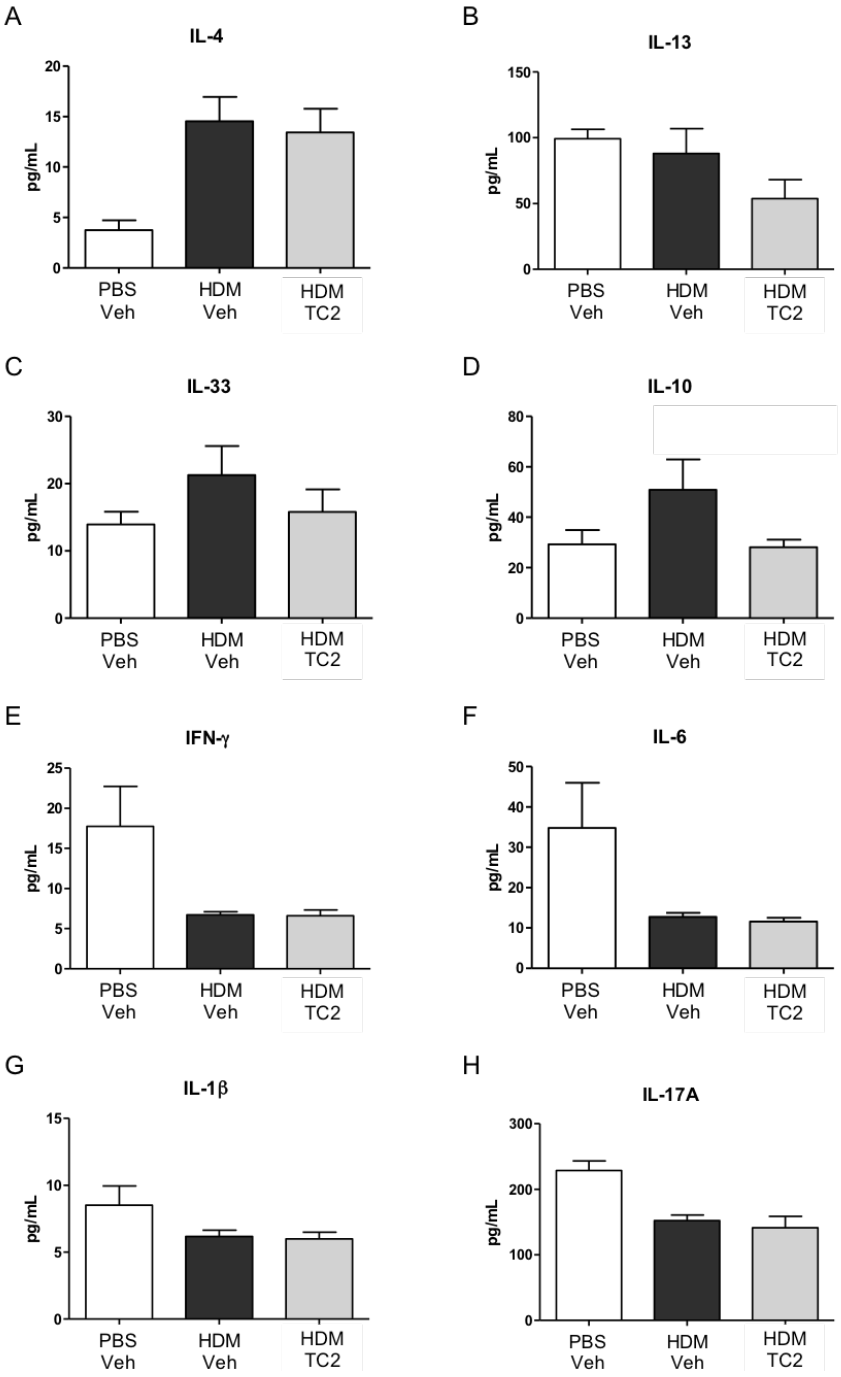

Figure 4.12 TC2 reduces IL-10 production in HDM-induced allergic airway disease.

BALB/c mice were intranasally inoculated with 25 μg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC2 in 10% DMSO vehicle or vehicle alone intranasally for 5 consecutive days preceding analysis. 24 hours post-final HDM exposure, mice were analysed for BAL cytokines (A-H) by Luminex assay. Data are expressed as mean+SEM, n=4–8 mice per group and are pooled data from 2 independent experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$. Luminex assays were carried out by industrial collaborators.

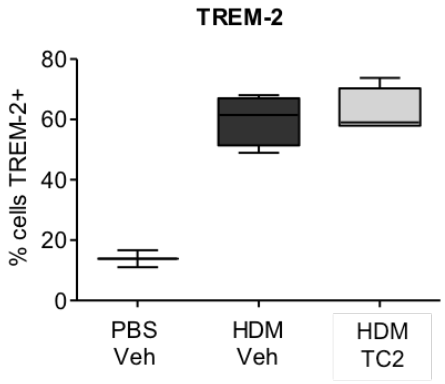

Figure 4.13 Effect of TC2 on TREM-2+ macrophage populations.

BALB/c mice were intranasally inoculated with 25 μg HDM extract 3-times weekly for 4 weeks. During week 4, mice were given 1.75 mg/kg TC2 in 10% DMSO vehicle or vehicle alone intranasally for 5 consecutive days preceding analysis. 24 hours post-final HDM exposure, percentage of TREM-2^{+} $CD11c^{+}CD11b^{int}$ macrophages were analysed in BAL by flow cytometry. Data are expressed as median, IQR and minimum and maximum values, n=2–4 mice per group and are from one experiment.

4.3 Discussion

In this chapter, the effect of the inhaled signalling kinases inhibitors TC1 and TC2 on HDM-induced allergic airway disease was explored. These inhibitors were administered to investigate whether AHR, cell infiltration, IgE production and cytokine and chemokine production could be modulated by the inhibition of specific signalling pathways in a murine allergic model of asthma.

4.3.1 PI3Kγ/δ inhibitor TC1 has no effect on HDM-induced allergic airway disease

Intranasal administration of PI3Kγ/δ inhibitor TC1 failed to modulate disease in this model, which suggests that specific inhibition of PI3Kγ/δ is not sufficient in affecting downstream effector functions that lead to HDM-induced allergic airway disease. Pan inhibition of PI3K using wortmannin and LY294002 has been shown to inhibit aspects of OVA-induced Th2 airway inflammation, e.g. AHR and eosinophilia (Ezeamuzie et al., 2001, Kwak et al., 2003, Lee et al., 2006). Furthermore, TC1 exhibited an anti-inflammatory effect in a steroid resistant model of allergic airway inflammation triggered by exposure to OVA and tobacco smoke. TC1 reduced AHR, the recruitment of inflammatory cells and levels of CXCL1 in BAL fluid in this model (Kizawa et al., 2013). It was, therefore, surprising that TC1 had no effect on the HDM model of allergic inflammation. A possible explanation for the lack of efficacy could be due to the extent of airway inflammation induced with this model. Continuous exposure to HDM in mice induces AHR after 3 weeks of exposure, which persists for up to 5 weeks (Gregory et al., 2009, Johnson et al., 2004). The animals in the TC1 study were analysed after 4 weeks of HDM exposure, therefore, despite the fact that PI3Kγ/δ is involved in many cell types during allergic inflammation, inhibition of this specific signalling pathway may not have been enough to overcome the severe inflammation caused by HDM exposure. That said, activation of the PI3K pathway was not evaluated in the current study. The therapeutic regime used might also have affected the effect of TC1 in this model of allergic inflammation, as prevention of disease is more easily achieved compared with treatment of established disease. Treating mice with TC1 prior to or at the beginning of disease induction might result in an effect. A study into the effects of TC1 on poly(I:C)-induced airway inflammation showed an effect when TC1 was given 2 hours before each poly(I:C) challenge (Kimura et al., 2013), whereas, TC1 was also shown to therapeutically modify airway inflammation in a model of tobacco smoke and allergen (OVA)-induced

asthma (Kizawa et al., 2013). Therefore, a different dosing regimen of TC1 in the HDM model of allergic airway disease—i.e. more doses or a higher concentration per dose—might still have an effect. Although not tested in this thesis, TC1 might have a role in preventing disease establishment if given before HDM challenge is started. Functional redundancy of other PI3K isoforms for functions specific to PI3Kγ/δ might also prevent TC1 from modulating inflammation in this model. Juss and colleagues (2012) reported functional redundancy of class I PI3K isoforms promoting neutrophil survival by GM-CSF stimulation, and targeting specific PI3K isoforms for inhibition in patients with COPD was unsuccessful in preventing exacerbations caused by prolonged neutrophil survival. Therefore, the effect of inhibiting PI3Kγ/δ may be compensated for by other class I PI3K isoforms or indeed other signalling kinases in our HDM model.

4.3.2 Src, Syk and p38 MAPK inhibitor TC2 reduces eosinophil numbers and IgE production in HDM-induced allergic airway disease

In contrast to TC1, administration of TC2 during HDM-induced allergic airway inflammation abrogated some parameters of allergic airway disease. Although AHR was unaffected by TC2 (Figure 4.8), TC2 treatment reduced eosinophil numbers in the airway (Figure 4.9) and total IgE and IgG_{2a} production in serum (Figure 4.10). Moreover, production of IgE specific for HDM was also inhibited following TC2 treatment.

As with TC1 treatment, intranasal administration of TC2 during allergic airway inflammation did not significantly affect any parameters of AHR. In line with previous studies, administration of 12 doses of HDM over a period of 4 weeks was sufficient to increase airway resistance and reduce airway compliance compared with PBS-treated mice (Figure 4.8). TC2 treatment in the 5 days preceding analysis had no effect on AHR. A number of studies point to a role for pathways involving signalling kinases p38 MAPK, Src and Syk in the ASM of patients with asthma (Krymskaya et al., 2004, Redhu et al., 2013). Use of inhibitors targeting Src and Syk kinases individually, i.e. dasatinib (Src inhibitor), R343 (Syk inhibitor) also did not alter lung function parameters (Figure 4.3). Therefore, although Src, Syk and p38 MAPK kinases are implicated in promoting ASM proliferation in the airways of patients with asthma, administration of an NSKI of these kinases via the intranasal route is not effective in preventing AHR in our mouse model of allergic airway disease. Although the

absence of an effect could be due to the ability of metacholine to induce mucus secretion (J. Schwarze, *pers. comms.*, 10 March 2016), which could be masking effects of any kinase inhibition. Moreover, immunohistological scoring of PAS-stained goblet cells in the airway (marker of mucus production) and inflammatory cell infiltration were not assessed in this experiment. Analysis of these factors may reveal changes to airway inflammation and mucus production caused by metacholine or, indeed, TC2 administration.

While the HDM model has certain advantages over other models, it is still a mechanistic model of asthma that may not recapitulate certain key molecular drivers of the disease across patient phenotypes. The relatively short period of dosing of TC2 may prevent the longer-lasting effects of signalling kinase inhibition. Future studies with longer dosing periods could expand upon the understanding of the effects of targeting specific kinases in allergic airway disease. The bioavailability of the drug may also be a limiting factor in this experimental model. Though intranasal instillation is widely used in mice and volumes of >25 μL under isoflurane anaesthetisation are thought to result in optimal delivery of substances to the lower respiratory tract (Southam et al., 2002, Miller et al., 2012), it is possible that the amount of inhibitor delivered to and accessible to the lung are sub-optimal. That TC2 treatment was able to modify some parameters of allergic inflammation suggests that drug delivery was successful, however, further optimisation of the dose of inhibitor may improve the efficacy of the drug and reveal further effects of TC2 treatment.

Intranasal inoculation of TC2 had no effect on the total inflammatory cell milieu in the BAL fluid and lung tissue. However, TC2 treatment reduced eosinophil numbers in the BAL. Given that a reduction in total BAL cells was observed using the single Syk kinase inhibitor R343, this suggests that at least Syk kinase could play a role in recruitment of cells into the airway during HDM-induced airway inflammation. Interestingly, in animals treated with TC2 BAL eosinophils were lower compared to control. A reciprocal increase in the percentage of alveolar macrophages was detected. These data suggest that inhibition of the Src, Syk and p38 MAPK signalling pathways disrupts the process of eosinophil recruitment into the lungs following HDM exposure. However, given that signalling kinases downstream of Src and Syk are involved in eosinophil migration (Marwick et al., 2010), targeting multiple upstream kinases Src and Syk using TC2 is sufficient to abrogate eosinophil accumulation in the BAL.

Another possibility is that TC2 treatment affects eosinophil viability—i.e. TC2 could cause the apoptosis of eosinophils in the lung, which would account for the reduced numbers of eosinophils isolated from the BAL. An *in vitro* TC2-induced eosinophil survival assay (e.g. by measurement of annexin V—a marker of cell apoptosis) could address this question. A downstream effector function that might be affected by TC2 is the production of eosinophil-recruiting mediators. IL-5 and CCL11 synergistically induce the movement of eosinophils out of the bone marrow into the blood and the site of inflammation (Palframan et al., 1998a; Palframan et al., 1998b). The levels of IL-5 and CCL11 were measured in BAL fluid supernatant but only CCL11 was detected (Figure 4.11A). A trend for a rise in CCL11 levels was detected in animals exposed to HDM. Src, Syk and p38 MAPK inhibition did not, however, alter the levels detected. Due to the low levels of these mediators detected, an alternative time-point, when levels are at their peak, may yet reveal changes induced by TC2. Indeed, in another mouse model, CCL11 was released soon after allergen challenge (6 hours) (Rosenwasser et al., 2003). Investigation into other eosinophil chemoattractants not included in this study, e.g. CCL5, MCP3/CCL7 and MCP4/CCL13 (Rosenberg et al., 2007), may also provide an explanation for this effect of TC2 on eosinophil recruitment. Another question related to this finding is whether or not eosinophil function is altered by TC2. Since the kinases targeted by this drug are widely expressed, it is possible that eosinophil effector functions might be abrogated upon inhibition. Eosinophil function could be studied, for example, by measuring myeloperoxidase activity to further elucidate the effects of TC2 on eosinophils.

TC2 treatment modulated another trait typical of the allergic response—production of antibodies from B cells. HDM exposure for 4 weeks led to an expected rise in IgE, HDM-specific IgE and IgG levels in the serum (Figure 4.10). Dasatinib treatment (Src inhibition) also had an inhibitory on IgE production, while Syk inhibitor R343 had no effect (Figure 4.5C-F). The data, therefore, suggest that Src may be a crucial kinase able to inhibit Ig production during allergic airway disease. A previous study showed that Src was important in initiating B-cell receptor signalling, which leads to activation of B cells (Gauld and Cambier, 2002). Blocking Src activity may, therefore, prevent B-cell maturation, proliferation and subsequently reduce the capacity of B cells to produce antibodies in response to allergen. p38 MAPK might also be preventing class-switching from IgM to IgE, since it has been shown to

be involved in this process (Zhang et al., 2002). Subsequent effects of IgE production may also be affected by TC2. As an example, IgE-induced histamine released by basophils was inhibited by dasatinib Src inhibition (Kneidinger et al., 2008).

The reduction in Ig release could also be due to upstream processes, such as Th2 cytokine production. Since activation of $CD4^+$ T cells and production of cytokines IL-4 and IL-13 are required to activate B cells and induce IgE production (Holgate, 2008), TC2 could inhibit these processes. There was, however, sparse evidence in the analysis of BAL cytokines suggesting that Th2 cytokine production was reduced. TC2 reduced IL-13 levels twofold, however, this result was not statistically significant (Figure 4.12B). Alternatively, analysis of cytokines present in the lung tissue by ELISA, or in cells following *in vitro* stimulation with phorbol 12-myristate 13-acetate and ionomycin, may reveal significant differences between TC2-treated and control animals.

In this study, we were able to confirm the increase in TREM-2 expression found on macrophages following HDM exposure observed previously in the group (Figure 4.13) (Habibzay et al., 2012), albeit using a slightly modified protocol. This suggests that TREM-2 is associated with type 2 inflammation and possibly alternatively-activated M2 macrophages, rather than type 1, which has been previously suggested (Turnbull et al., 2006). Signalling kinase inhibition did not effect TREM-2 expression.

4.3.3 Conclusion

While TC2 inhibition had no effect on AHR and cytokine or chemokine production in HDM-induced allergic inflammation in mice, TC2 treatment reduced eosinophil numbers in the BAL and serum levels of IgE and IgG. AHR was not affected by TC2 in established lung inflammation. However, there could still potentially be a role for kinase inhibition in an acute exacerbation of on-going inflammation, e.g. viral infection during allergic inflammation. This will be the focus of study in the next chapter.

Chapter 5: Role of TC2 and viral infection during HDM-induced allergic inflammation

Chapter 5: Role of TC2 and viral infection during HDM-induced allergic inflammation

5.1 Introduction

There is undoubtedly evidence for the involvement of RSV in both the priming of allergic airway disease, as well as a role for exacerbating disease. However, a lack of robust animal models of respiratory viral infections with relevant allergens has prevented clear conclusions about the role of lung infection in allergic asthma from being drawn and the development of new therapeutic strategies in the treatment of asthma exacerbations. Interesting and novel human viral challenge models are beginning to be utilised (DeVincenzo et al., 2010), but while they allow for greater characterisation of viral infections during allergic asthma, there is still a need for animal *in vivo* models in order to further understand the mechanisms underlying infection and to develop new treatments.

Models of RSV exacerbation of allergic asthma have been studied previously. Exacerbation of OVA-induced airway inflammation due to RSV infection has been observed (Peebles et al., 2001). Furthermore, Tourdout et al. (2008) observed RSV-induced airway remodelling in OVA-challenged mice that had not previously been systemically sensitised to OVA. In another model of OVA-induced allergic airway inflammation, RSV infection during the aerosol phase of OVA challenge resulted in an exacerbation of AHR (increased airway resistance), although populations of BAL leukocytes were not significantly altered (Newcomb et al., 2013). However, other studies have produced conflicting results, showing that RSV infection reduced AHR in OVA-induced allergic inflammation (Aeffner and Davis, 2012). Petersen et al. (2014) developed a model of RSV infection following cockroach allergen-induced airway inflammation. In this model, RSV infection did not cause significant changes in leukocyte numbers in the lung. mRNA expression of IL-4 and IL-13, however, were increased. Another study in which house dust mite (*D. farinae*) was used as an allergen, found that the viral dose impacted on the outcome of RSV infection (Kondo et al., 2004). Low dose (2×10^3 PFU) RSV infection attenuated airway inflammation and did not induce

AHR, whereas high dose (5 x 10^5 PFU) RSV infection exacerbated AHR. While this model utilises a clinically relevant antigen, the requirement of intraperitoneal sensitisation in the presence of an aluminium hydroxide adjuvant is not representative of the sensitisation of humans to allergens. Therefore, characterisation of a new model of RSV infection following HDM-induced allergic airway disease will be explored in this chapter. This model will also be used to test the effects of signalling kinase inhibitor treatment in a model of viral infection during allergic airway disease.

With viruses detected in approximately 80% of all asthma exacerbations (Papadopoulos et al., 2011), viral infection is a significant factor in the management of asthma. Although it was once recommended to double corticosteroid doses during exacerbations of asthma, little evidence was found to support this treatment, thus the practice is no longer endorsed (Harrison et al., 2004, BTS, 2008). Other approaches involve vaccination against respiratory viral infections, e.g. seasonal influenza vaccination to groups thought to be at high risk of contracting influenza (Department of Health, 2014). The use of type I IFNs to promote anti-viral responses during allergic asthma has been investigated. One such study using IFN-α combined with an anti-histamine and ibuprofen showed potential in reducing exacerbations, but resulted in some side-effects (Gwaltney et al., 2002). Inhaled IFN-β treatment in adults reduced asthma exacerbations if given at the first sign of cold symptoms (Djukanović et al., 2014). More specific targeting of inflammatory signalling pathways has been explored with NF-κB, a potential target for pharmacological inhibition (Edwards et al., 2006). Since the signalling kinases Src, Syk and MAPK are involved in RSV immunopathology (Thomas et al., 2002, Lindemans et al., 2006, Singh et al., 2007), inhibitors targeting these signalling kinases are potential candidates for the treatment of viral exacerbations of asthma.

The potential of other NSKIs for modifying viral exacerbations of respiratory disease has been explored. NSKIs, targeting a similar spectrum of kinases as TC2, have been shown to abrogate parainfluenza-induced exacerbations of AHR in OVA-sensitised guinea pigs (Broadley et al., 2011). Treatment with NSKIs has also been shown to reduce cytokine production and RSV replication in *in vitro* cell models of respiratory viral infection (Ito et al., 2013a, Ito et al., 2011). Therefore, given that TC2 was able to modify eosinophil recruitment and Ig production in HDM-induced allergic inflammation (Chapter 4), it may show promise

in modulating disease in a model of viral infection during HDM-induced allergic airway disease.

5.1.1 Hypothesis

It was proposed that a new model of RSV infection in the allergic lung, which utilised a physiologically relevant allergen such as HDM, was needed in order to further investigate the interactions between the two types of inflammatory lung diseases and to determine their reciprocal effects on one another.

The hypothesis to be tested is that RSV will increase parameters of HDM-induced allergic inflammation and that TC2 treatment will modulate the main disease parameters.

5.1.2 Aims

The aims of this chapter are:

1) To characterise a new model of RSV infection during HDM-induced allergic airway disease.
2) To determine the effect of TC2 in this model of RSV infection during HDM-induced allergic airway disease.

5.2 Results

5.2.1 Viral infection and TC2 treatment during HDM-induced allergic airway inflammation

Since TC2 inhibitor treatment reduced allergic parameters during a model of HDM allergic disease, the next aim was to test the effectiveness of this inhibitor in preventing viral exacerbations during on-going HDM-induced airway inflammation. The schedule of HDM exposure, RSV infection and TC2 treatment are shown in Figure 5.1.

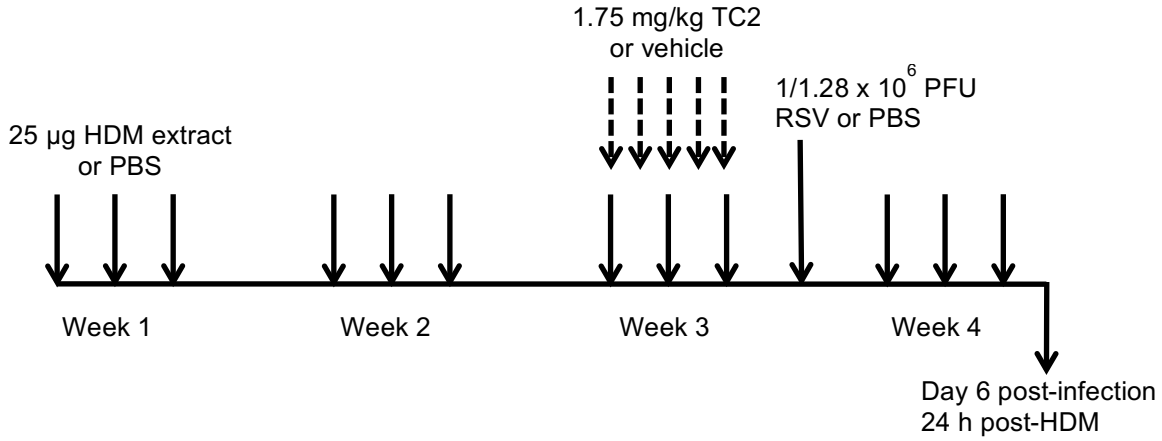

Figure 5.1 TC2 treatment during RSV infection and HDM-induced allergic airway disease.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS vehicle or vehicle alone, 2 hours before HDM was given, for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. It should be noted that PBS was used as a control in these experiments, rather than inactivated RSV or Hep2 cell lysate not containing RSV.

5.2.1.1 AHR following TC2 treatment in HDM-induced airway inflammation and RSV infection

RSV infection of mice with prior exposure to HDM led to an increase in airway resistance (resistance to flow in the airway) compared to uninfected, HDM-treated mice (Figure 5.2). However, other parameters of airway function, such as airway compliance (ability of the airway to expand) and elastance (stiffness of the airway), were not significantly altered by RSV infection (Figure 5.3, 5.4). Similarly, tissue elastance and tissue damping, which are indicative of structural changes to the airway, were not significantly altered by RSV infection (Figure 5.5, 5.6). Taken together, high dose RSV infection during HDM-induced airway

inflammation did not lead to a pronounced exacerbation of airway function, in terms of compliance and elastance, although resistance was increased.

Treatment with TC2 prior to RSV infection of HDM-exposed mice led to a significant increase in airway compliance (Figure 5.3) and reduced airway elastance, tissue elastance and tissue damping (Figure 5.4-5.6), compared to vehicle treatment ($p<0.05$). Airway resistance was reduced by TC2 treatment, but not significantly (Figure 5.2).

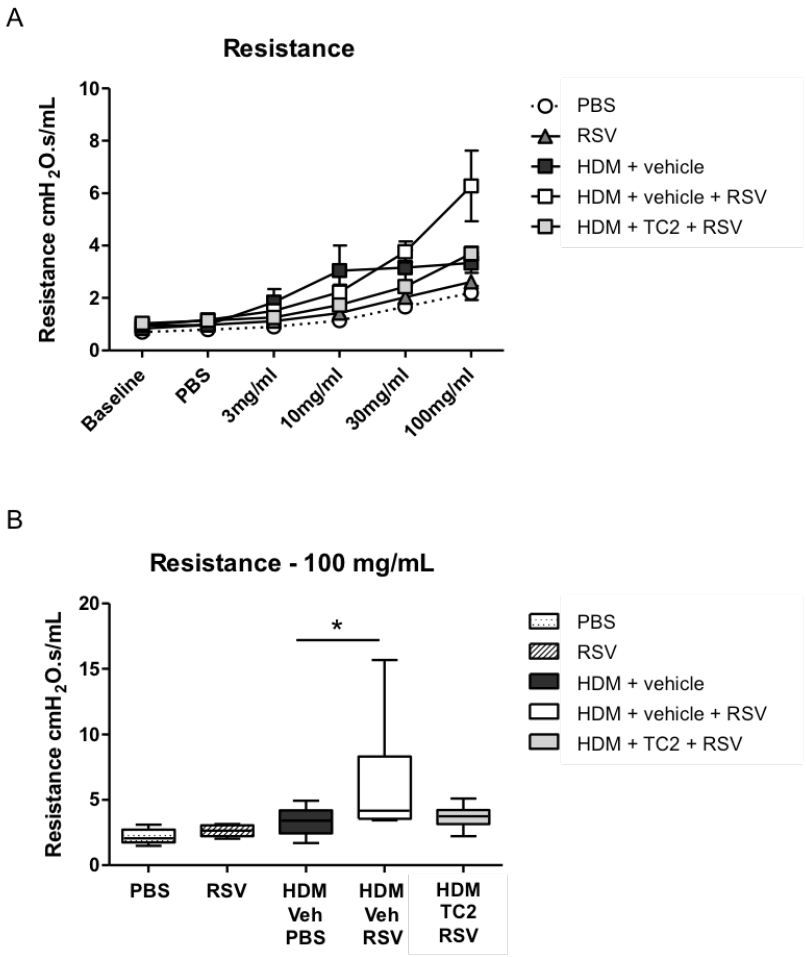

Figure 5.2 Effect of RSV infection and TC2 treatment on airway resistance during HDM-induced airway inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS vehicle or vehicle alone for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Airway resistance (A) was measured in lung function studies and is expressed as mean±SEM. Airway resistance at 100 mg/mL of metacholine (B) is expressed as median, IQR and minimum and maximum values. n=5–10 mice per group and are pooled data from two independent experiments carried out on separate days. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$.

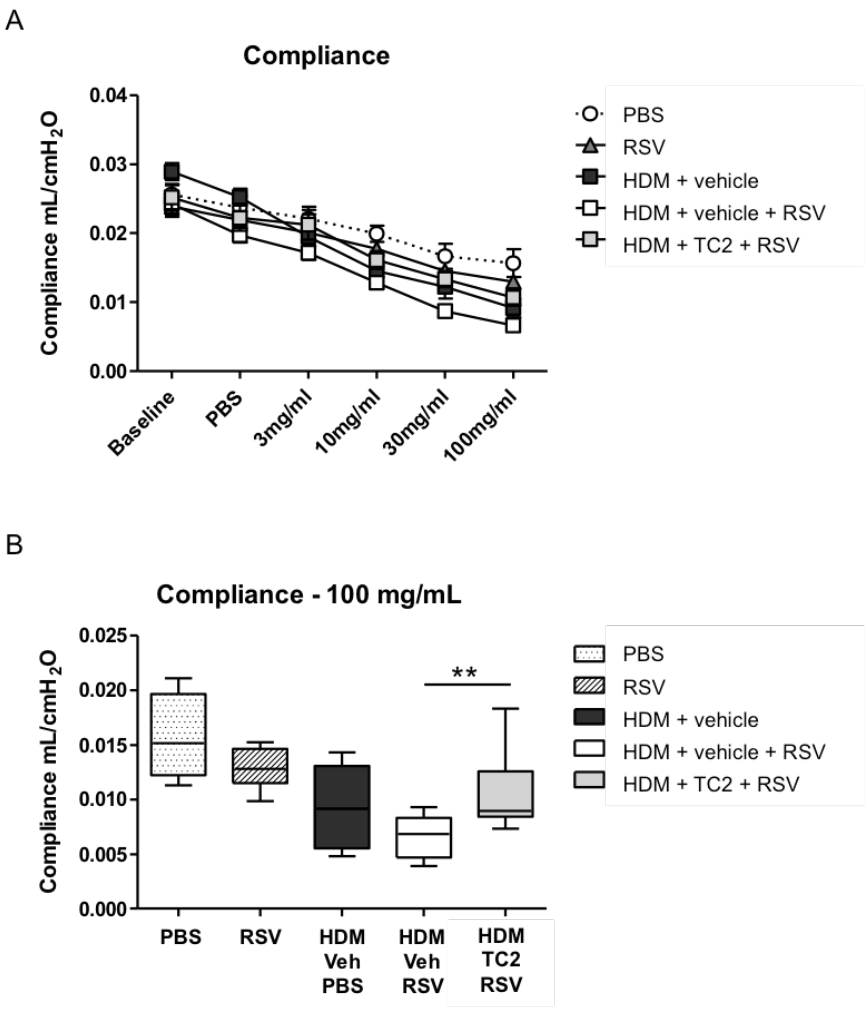

Figure 5.3 Effect of RSV infection and TC2 treatment on airway compliance during HDM-induced airway inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle alone for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Airway compliance (A) was measured in lung function studies and is expressed as mean±SEM. Airway compliance at 100 mg/mL of metacholine (B) is expressed as median, IQR and minimum and maximum values. n=5–10 mice per group and are pooled data from two independent experiments carried out on separate days. Statistical significance was determined by Mann-Whitney *U* test between relevant groups, **p<0.01.

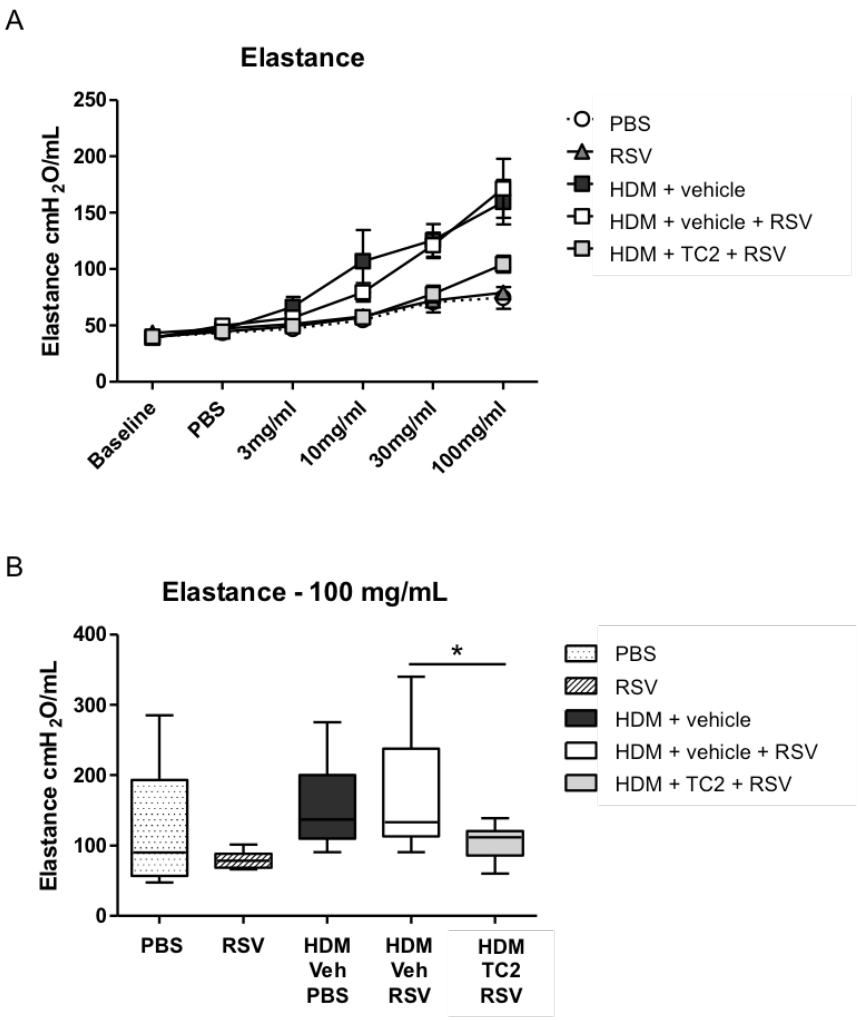

Figure 5.4 Effect of RSV infection and TC2 treatment on airway elastance during HDM-induced airway inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle alone for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Elastance (A) was measured in lung function studies and is expressed as mean±SEM. Elastance at 100 mg/mL of metacholine (B) is expressed as median, IQR and minimum and maximum values. n=5–10 mice per group and are pooled data from two independent experiments carried out on separate days. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$.

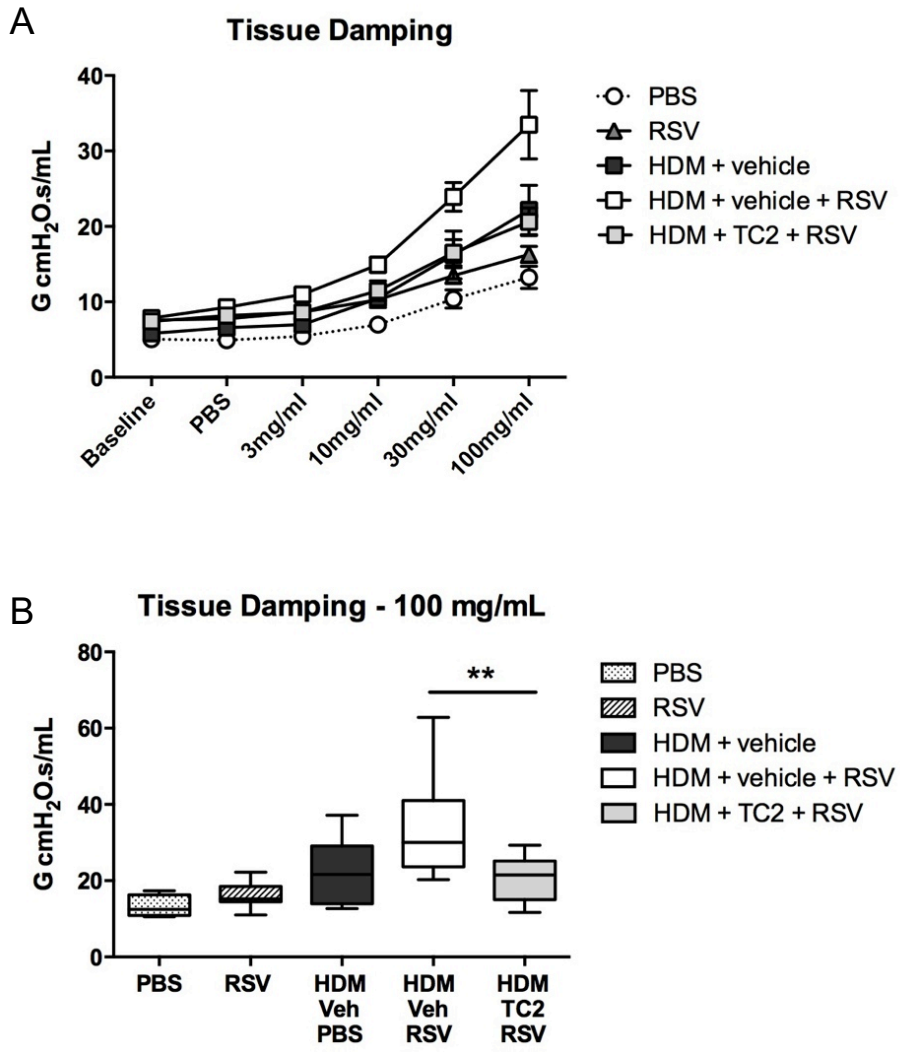

Figure 5.5 Effect of RSV infection and TC2 treatment on tissue damping during HDM-induced airway inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle alone for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Tissue damping (A) was measured in lung function studies and is expressed as mean±SEM. Tissue damping at 100 mg/mL of metacholine (B) is expressed as median, IQR and minimum and maximum values. n=5–10 mice per group and are pooled data from two independent experiments carried out on separate days. Statistical significance was determined by Mann-Whitney U test between relevant groups, **p<0.01.

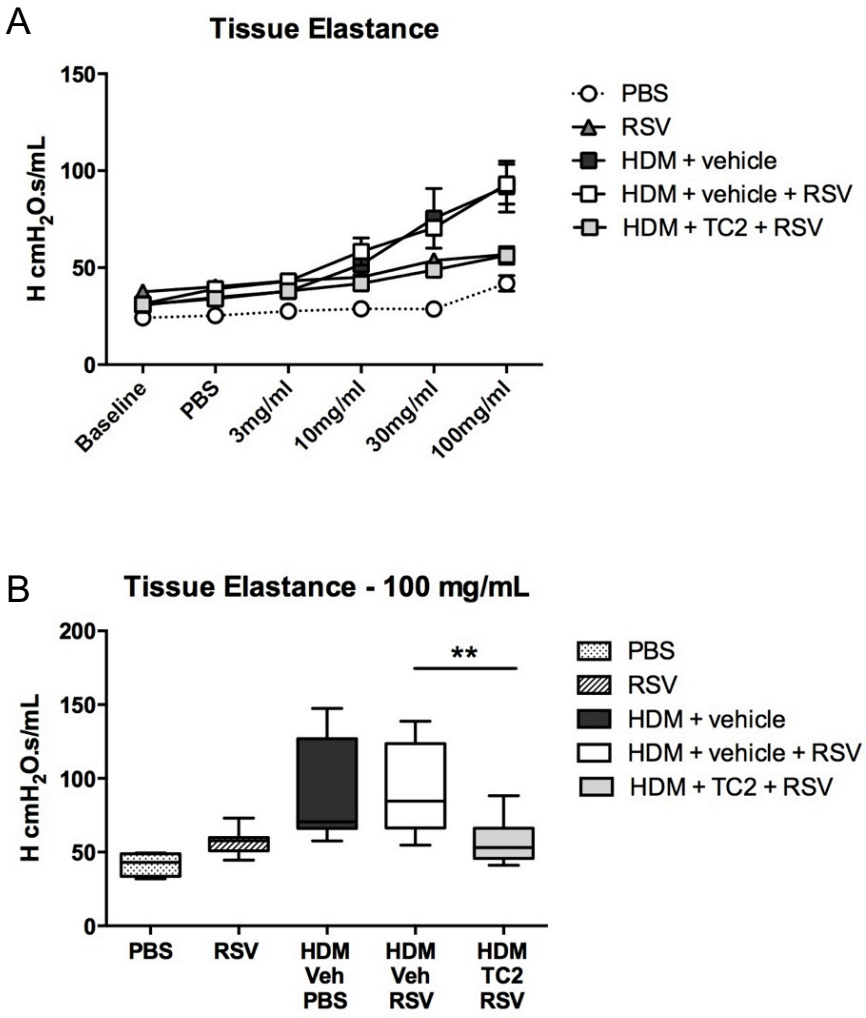

Figure 5.6 Effect of RSV infection and TC2 treatment on tissue elastance during HDM-induced airway inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle alone for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Tissue elastance (A) was measured in lung function studies and is expressed as mean±SEM. Tissue elastance at 100 mg/mL of metacholine (B) is expressed as median, IQR and minimum and maximum values. n=5–10 mice per group and are pooled data from two independent experiments carried out on separate days. Statistical significance was determined by Mann-Whitney U test between relevant groups, **p<0.01.

5.2.1.2 Inflammatory cell infiltration following TC2 treatment in HDM-induced airway inflammation and RSV infection

Total cell infiltrates in the airway lumen and lung tissue were unaffected by RSV infection during HDM-induced airway inflammation compared to uninfected HDM-treated mice (Figure 5.7A, B). The percentage of eosinophils was significantly reduced following RSV infection of HDM-exposed mice (accounting for approximately 20% of all differential cell types counted compared with 40% in HDM alone) (Figure 5.7C). However, this was not translated into changes in total numbers of eosinophils. The percentage of lymphocyte/monocytes was increased ($p=0.0135$) in RSV-infected HDM-exposed mice compared to uninfected HDM-treated mice (Figure 5.7C). TC2 treatment reduced the percentage and total number of lymphocyte/monocytes in the airway during RSV infection of HDM-exposed mice compared with mice treated with vehicle, $p=0.0062$ and $p=0.0078$, respectively (Figure 5.7C, D). TC2 had no effect on any other cell population analysed.

5.2.1.3 RSV-associated pathology following TC2 treatment in HDM-induced airway inflammation and RSV infection

HDM exposure prior to RSV infection led to a striking reduction in weight loss following RSV infection compared to infected mice that had not been exposed to HDM, with an approximate 10% difference between the mean weights of the two groups on day 6 post-infection (Figure 5.8A, B). Interestingly, this finding correlated with release of damage markers into the airway. Analysis of albumin, LDH and uric acid showed that HDM exposure prior to RSV infection reduced the levels of these markers detected in the BAL fluid by sevenfold, fivefold and 11-fold, respectively (Figure 5.8C-E). Treatment with TC2 prior to infection had an additive effect in preventing weight loss, compared to the effect of HDM alone (Figure 5.8A, B). Mice treated with TC2 during HDM exposure lost approximately 2% of their original body weight following RSV infection, compared with an 8% reduction in weight loss in the vehicle control group. However, TC2 treatment had no effect on damage marker levels in BAL when compared with vehicle-treated controls (Figure 5.8C-E).

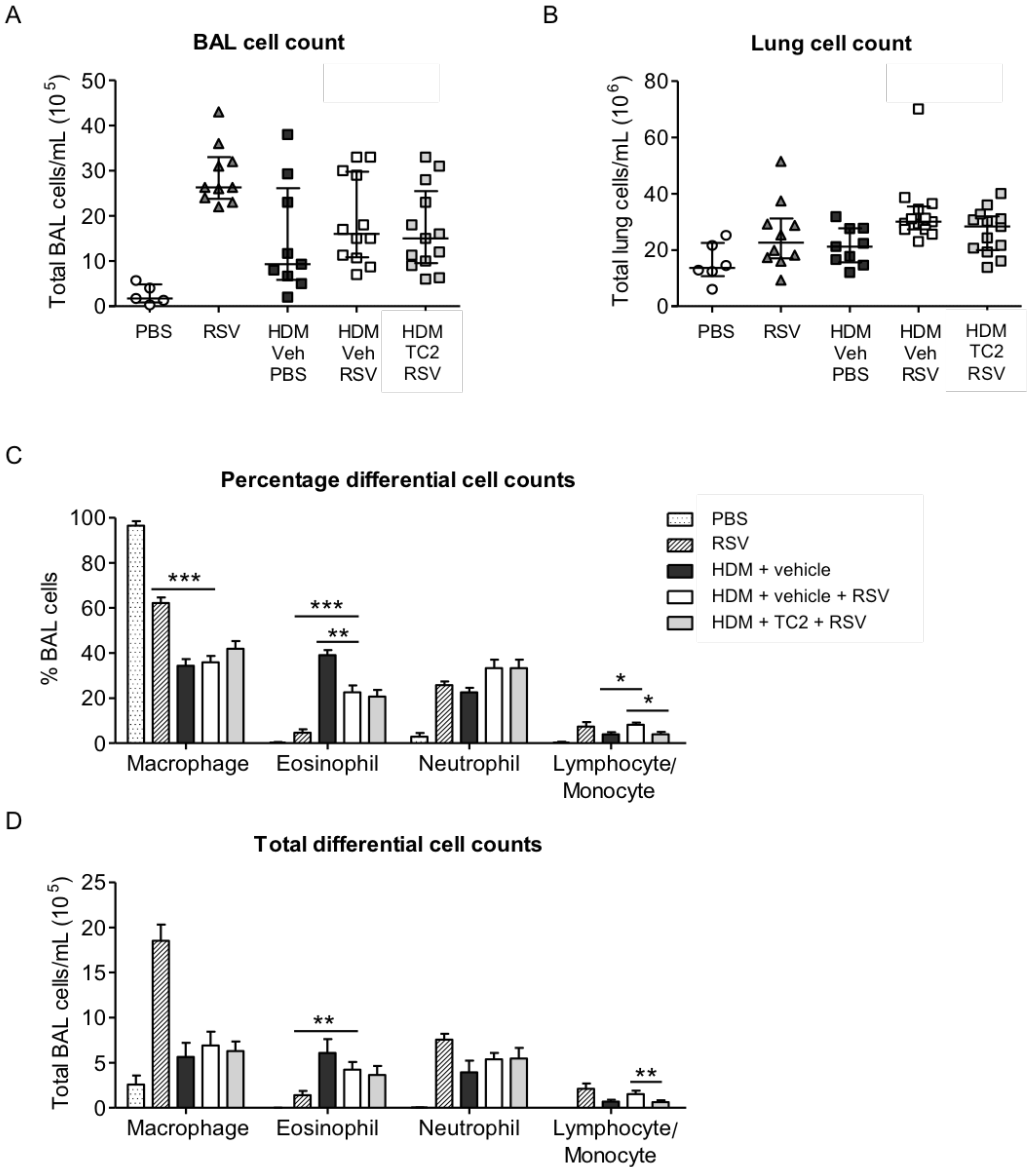

Figure 5.7 Effect of RSV infection and TC2 treatment on cellular infiltration during HDM-induced airway inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle alone for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Following lung function analyses, BAL was performed and lung tissue harvested. Cells were isolated from BAL fluid (A) and lung tissue (B) and counted. Percentage (C) and total number (D) of cell populations recovered from BAL were measured by differential cell counting of Wright-Giemsa stained cell samples. Data are expressed as mean±SEM, n=5–11 mice per group and are pooled data from two independent experiments carried on separate days. Statistical significance was determined by Mann-Whitney U test between relevant groups, *p<0.05, **p<0.01, ***p<0.001.

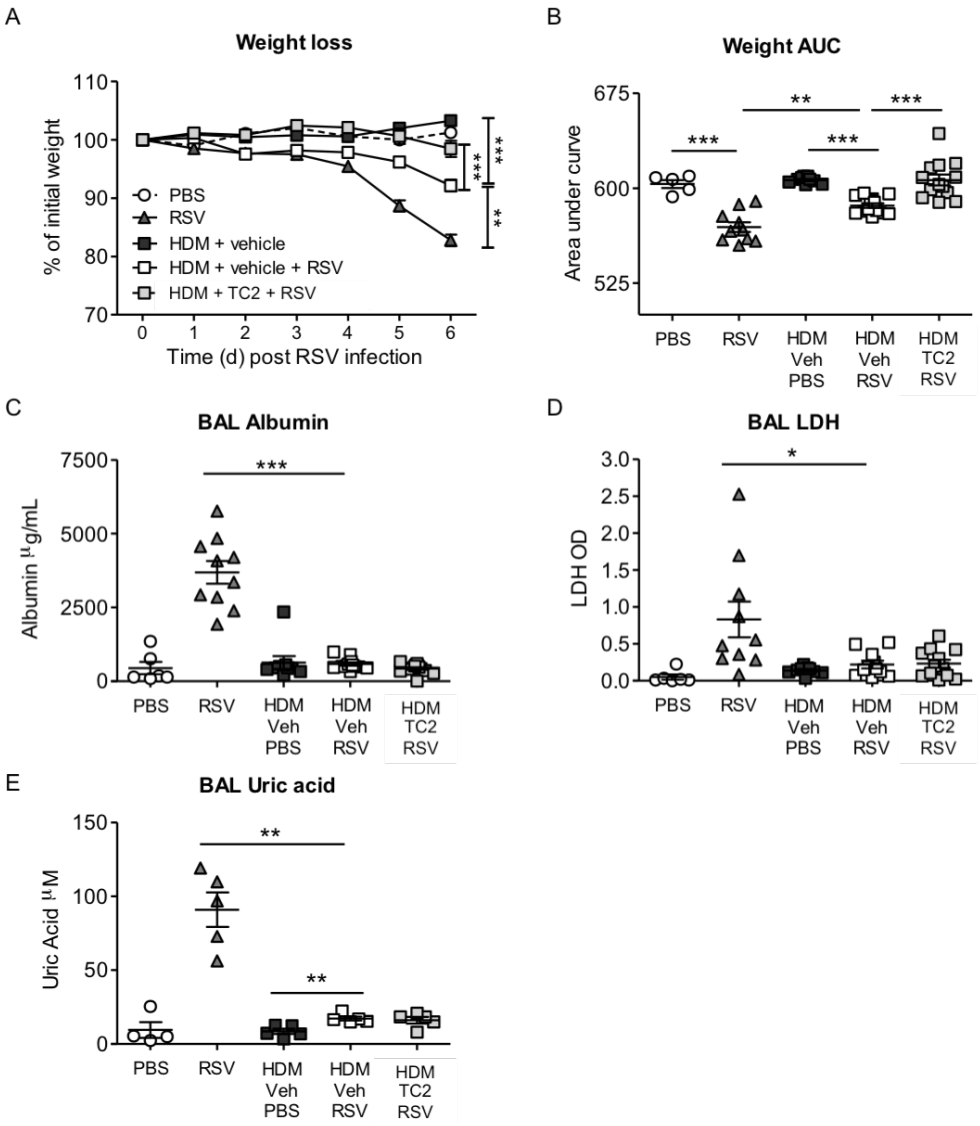

Figure 5.8 Effect of HDM and TC2 on RSV-associated weight loss and damage.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle only for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Body weight was measured daily during the infection phase. Weight is expressed as percentage of original body mass (A). Weight loss differences between groups were determined by comparisons of area under curve (AUC) (B). Levels of albumin (C) in BAL fluid were determined by ELISA. LDH (D) and uric acid (E) in BAL fluid was determined by colorimetric assays. Data are expressed as mean±SEM, n=4–13 mice per group and are pooled data from two independent experiments carried on separate days, except uric acid assay which is from one experiment. Statistical significance was determined by Mann-Whitney U test between relevant groups, *p<0.05, **p<0.01, ***p<0.001.

5.2.1.4 Inflammation and mucus score following TC2 treatment in HDM-induced airway inflammation and RSV infection

RSV infection alone led to a mild increase in inflammatory score (mean=2.4) compared to PBS alone, while HDM exposure alone induced a higher inflammatory score (mean=3.6). RSV infection following HDM exposure did not increase inflammation further (mean=3.7) (Figure 5.9A). The degree of goblet cell hyperplasia, as determined by scoring of PAS-stained lung sections, was not affected by RSV infection in mice treated with HDM (Figure 5.9B), nor was the expression of mucin gene *Muc5ac* (Figure 5.9C). TC2 had no effect on these parameters.

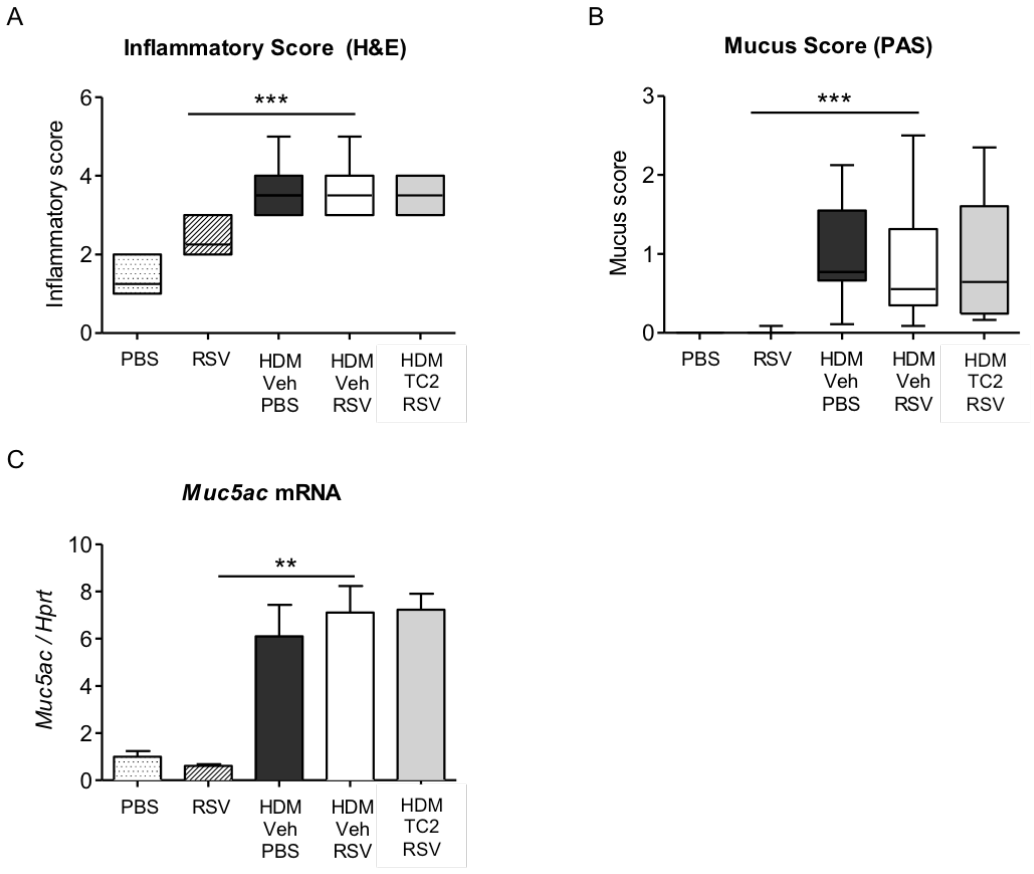

Figure 5.9 Effect of RSV infection and TC2 treatment on inflammation and mucus production during HDM-induced allergic airway inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle only for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Lung inflammation was quantified from H&E-stained lung sections using a semi-quantitative scoring system (A). Mucus production was quantified from PAS-stained lung sections using a semi-quantitative scoring system (B). Data are expressed as median, IQR and minimum and maximum values. Expression of *Muc5ac* mRNA in lung tissue was determined by qPCR, standardised to housekeeping gene *Hprt* and expressed relative to PBS control group. Data are expressed as mean±SEM. Pooled data from two independent experiments carried on separate days, n=4–13 mice per group, except *Muc5ac* qPCR which is from one experiment. Statistical significance was determined by Mann-Whitney U test between relevant groups, **p<0.01, ***p<0.001.

5.2.1.5 Immunoglobulin production following TC2 treatment in HDM-induced airway inflammation and RSV infection

RSV infection following HDM exposure led to a trend for increased total and HDM-specific IgE in serum, approximately a two-fold increase (Figure 5.10A, B). Total IgG_{2a} and IgG_1 were not significantly affected by RSV infection (Figure 5.10C, D). TC2 treatment significantly reduced total and HDM-specific IgE following RSV infection of HDM-treated mice (Figure 5.10A, B) in a similar fashion as HDM treatment alone (Figure 4.10A,B).

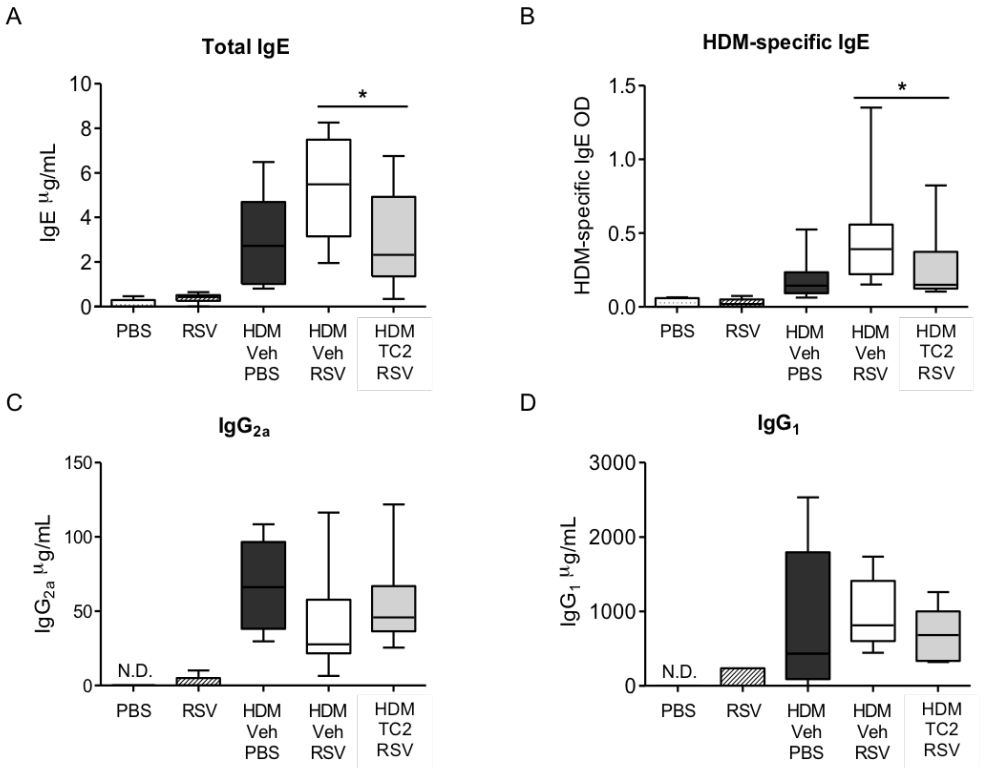

Figure 5.10 Effect of RSV infection and TC2 treatment on serum immunoglobulins during HDM-induced allergic airway inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle only for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Levels of total IgE (A), HDM-specific IgE (B), total IgG_{2a} (C) and total IgG_1 (D) in serum were determined by ELISA. Data are expressed as median, IQR and minimum and maximum values, n=5–13 mice per group and are pooled data from two independent experiments carried on separate days. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$.

5.2.1.6 Cytokine production following TC2 treatment in HDM-induced airway inflammation and RSV infection

RSV infection during allergic inflammation has been reported to cause asthma exacerbations (Johnston et al., 1995), however, in the present work, a preventative effect of HDM was observed as cytokines and chemokines induced by RSV were significantly reduced. Interestingly, HDM exposure prior to RSV infection reduced the levels of the chemokines CXCL10 and CCL2 in the BAL (fourfold and sevenfold reduction, respectively). Conversely, the neutrophil chemoattractant CXCL1 was increase in the HDM-exposed groups, while the eosinophil recruiting chemokine CCL11 and monocyte and NK cell chemoattractant CCL4 were not affected by HDM exposure (Figure 5.11A-E). Similarly, HDM exposure prior to RSV infection significantly blocked the expression of proinflammatory cytokines IFN-γ and IL-6 ($p=0.0044$ and $p=0.0015$, respectively) (Figure 5.12A, B).

RSV infection in HDM-exposed mice had an impact on the classical Th2-related IL-4 cytokine as levels in the BAL were significantly reduced (Figure 5.12C). Other Th2-associated cytokines, IL-5 and IL-13 were not detected in the BAL. However, this difference in IL-4 was not observed in the lung and levels of IL-5 and IL-13 were also unaffected by RSV infection (Figure 5.13A-C). IL-33 was measured in the lung at an earlier time-point of 3 days post-infection, but no effect of RSV was observed (Figure 5.13E). In the lung, IL-17A, which was not detectable in BAL (data not shown), was slightly increased in infected HDM-exposed mice compared to RSV alone (Figure 5.13D), however, levels were not significantly greater compared with uninfected HDM-treated mice.

In addition to the effect of prior HDM exposure on reducing RSV-induced IFN-γ, IL-6 and chemokine CXCL10, treatment with TC2 prior to infection further reduced levels of these mediators in RSV-infected, HDM-exposed mice ($p<0.01$) (Figure 5.11D, Figure 5.12A, B).

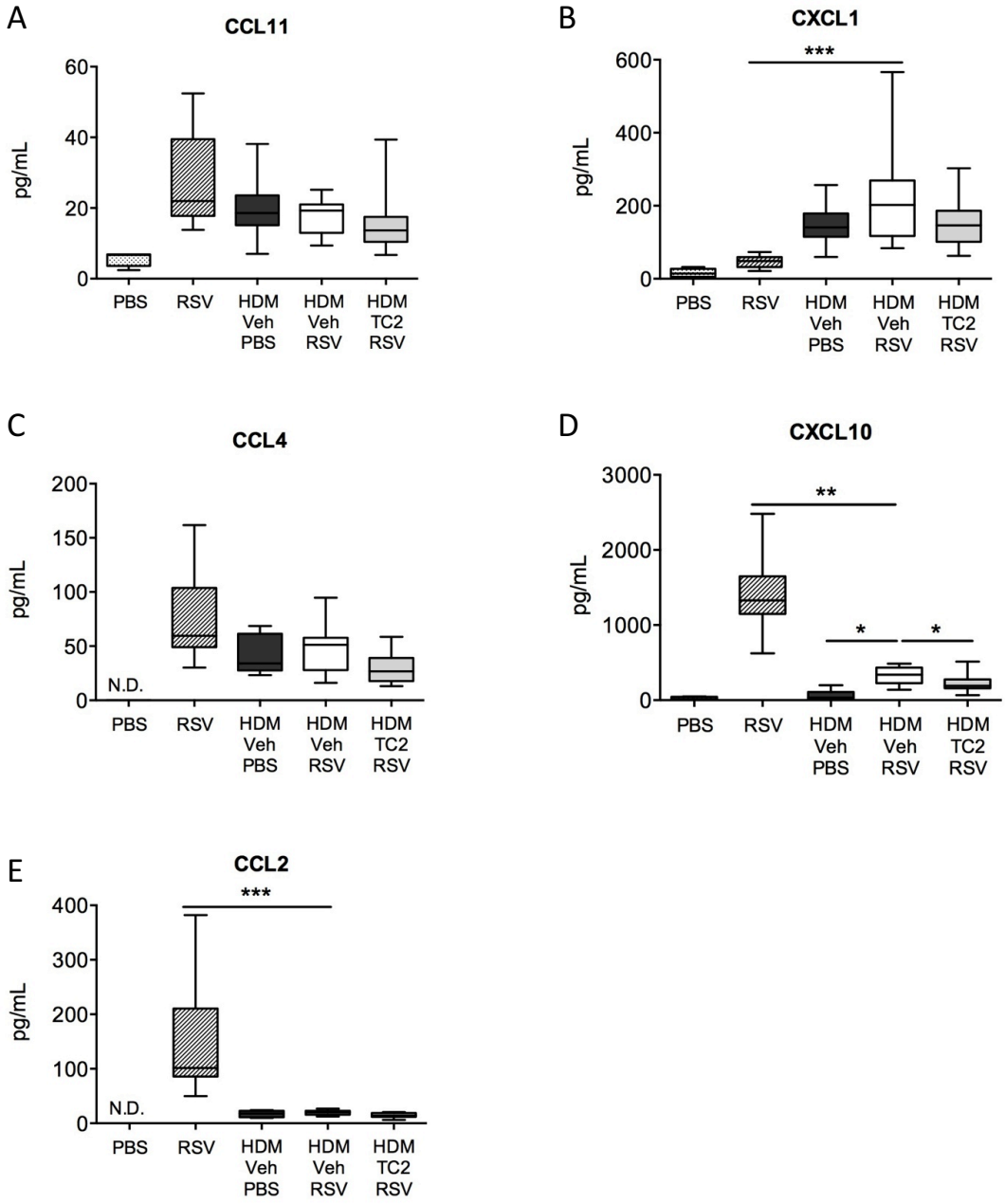

Figure 5.11 Effect of RSV infection and TC2 treatment on chemokine production during HDM-induced allergic airway inflammation

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle only for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Levels of chemokines (A-E) were measured in BAL fluid by Luminex assay. Data are expressed as median, IQR and minimum and maximum values, n=5–13 mice per group and are pooled data from two independent experiments carried on separate days. Statistical significance was determined by Mann-Whitney U test between relevant groups, *p<0.05, **p<0.01, ***p<0.001. Luminex assays were carried out by industrial collaborators.

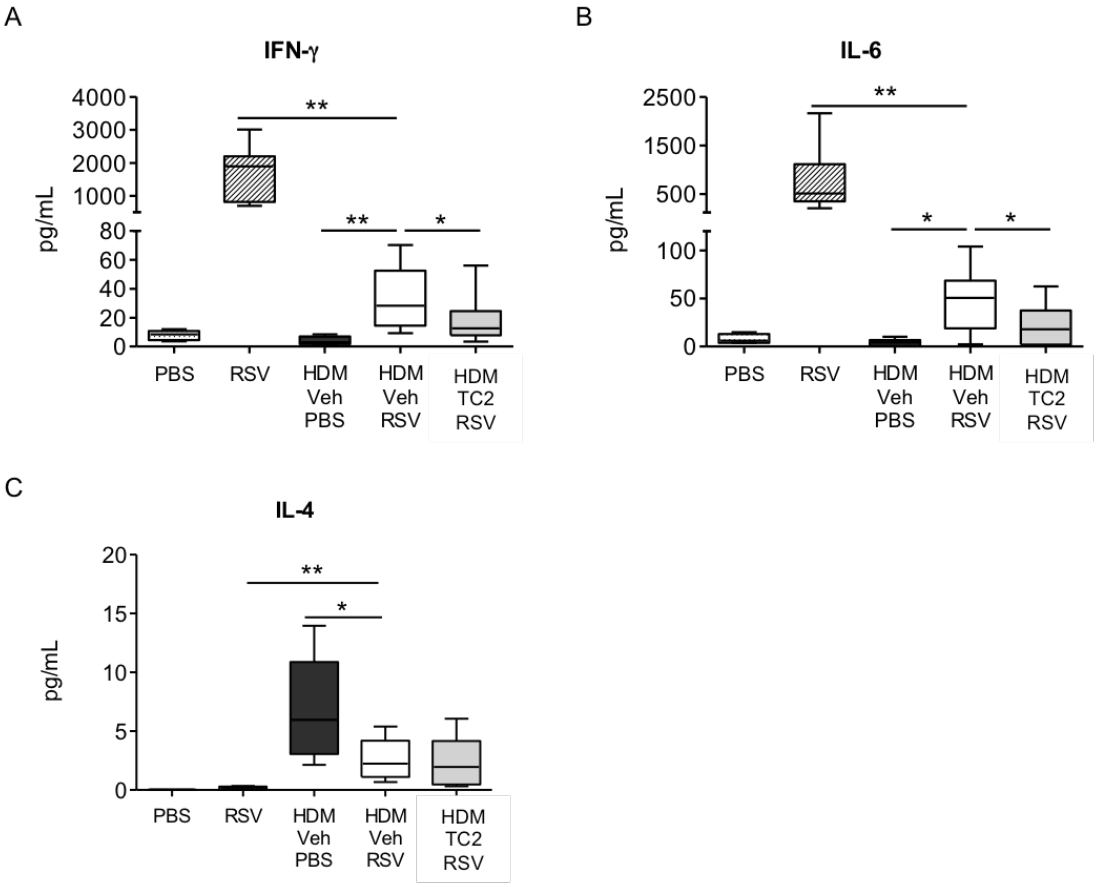

Figure 5.12 Effect of HDM and TC2 on cytokine production during RSV infection.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle only for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Mice were analysed for IFN-γ (A), IL-6 (B) and IL-4 (C) in BAL fluid by Luminex assay. Data are expressed as median, IQR and minimum and maximum values, n=5–13 mice per group and are pooled data from two independent experiments carried on separate days. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$, **$p<0.01$. Luminex assays were carried out by industrial collaborators.

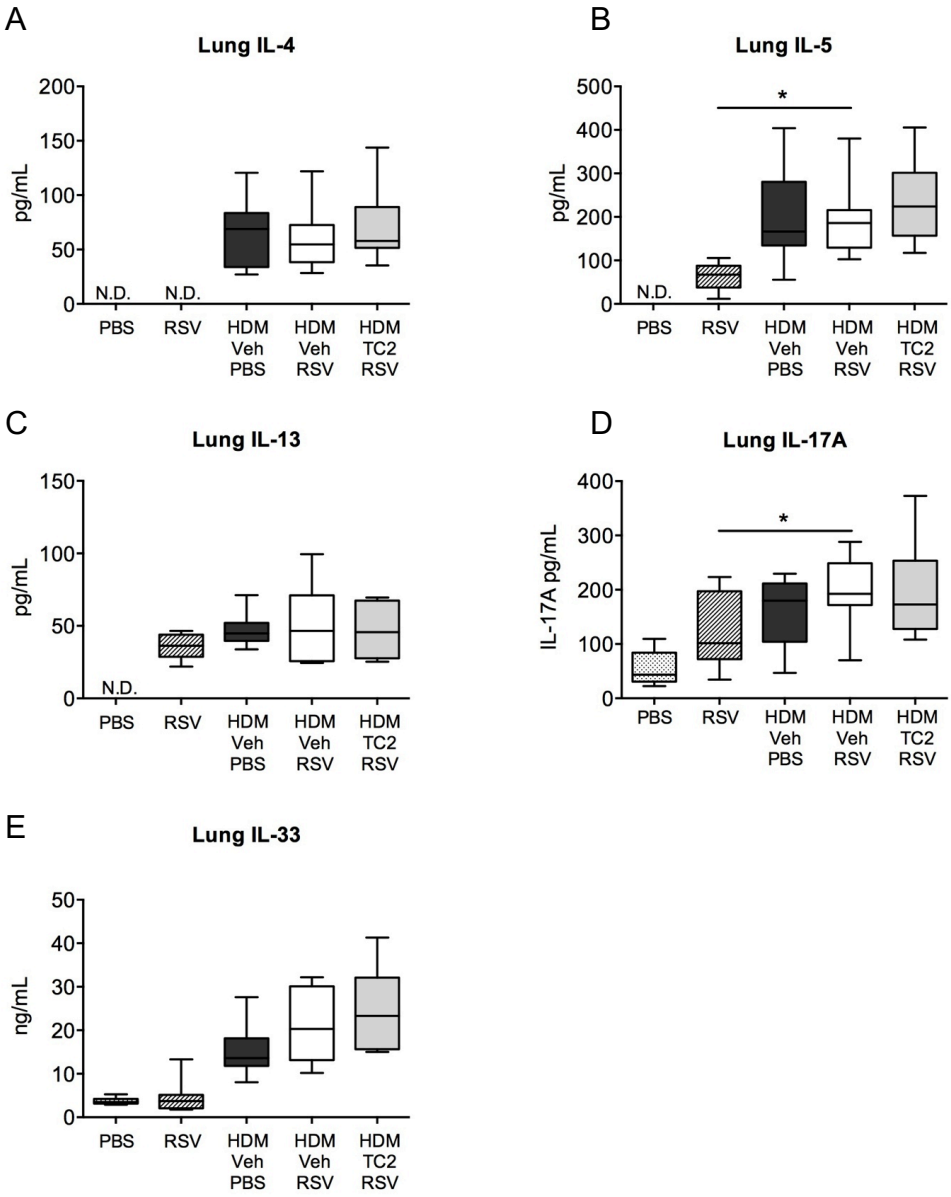

Figure 5.13 Effect of RSV and TC2 on Th2 cytokine production during HDM-induced allergic inflammation.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle only for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 3 (IL-33 only) or 6 days (IL-4, IL-5, IL-13, IL-17A) post-infection, 24 h post-final HDM exposure. Mice were analysed for cytokines (A-E) in lung homogenate supernatant by ELISA. Data are expressed as median, IQR and minimum and maximum values, n=5–13 mice per group and are pooled data from two independent experiments carried on separate days. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$.

5.2.1.7 Viral load and anti-viral gene expression following TC2 treatment in HDM-induced airway inflammation and RSV infection

Having observed the protective effect of prior HDM exposure in RSV-associated weight loss and damage and a general reduction in proinflammatory mediators, the next aim was to investigate whether HDM challenge prior to RSV infection modulates viral replication and the anti-viral response.

HDM-challenged mice were analysed 3 days post RSV infection. A significant reduction in lung viral burden, as measured by cytopathic effect in Hep2 cells, was observed in mice that had been exposed to HDM prior to RSV infection (Figure 5.14A). Corresponding to reduced viral load, expression levels of anti-viral genes *Ifn-β, Irf7, Ifi44* and *Oas2* were analysed in RNA extracted from whole lung tissue were all significantly lower in infected mice exposed to HDM than in mice infected with RSV alone ($p<0.01$) (Figure 5.14B-E). TC2 had no effect on viral load or anti-viral gene expression in the lung.

5.2.1.8 M2 macrophage marker genes following TC2 treatment in HDM-induced airway inflammation and RSV infection

In order to determine whether RSV infection of HDM exposed mice changes the phenotype of macrophages, M1 "classical" macrophages marker and M2 "alternatively" macrophage markers were analysed 3 days post RSV infection in RNA extracted from whole lung tissue. HDM exposure led to a significantly increased expression of M2 markers *Relmα*, *Arg1,* and *IL-4*, and M1 marker nitric oxide synthase 2 *Nos2* (Figure 5.15A-E). However, *Relmα* is not exclusively a macrophage marker, because it is also expressed at low levels on pulmonary epithelial cells (Holcomb et al., 2000). M1 associated cytokine *Tnfα* was not upregulated by HDM exposure. RSV infection following HDM exposure reduced M2 macrophage marker gene expression compared to HDM exposure in the absence of infection, but not to levels as low as RSV infection alone—levels of resistin-like molecule-α (*Relmα),* arginase-1 *(Arg1)* and *Il-4* were significantly higher than the RSV infection alone group. *Tnfα* expression was increased following RSV infection of HDM-exposed mice. Treatment with TC2 had no effect on the levels of any genes analysed.

5.2.1.9 Effect of prior HDM exposure and TC2 treatment on TREM expression in HDM-induced airway inflammation

Trem2 mRNA was measured in lung tissue 3 and 6 days post-infection by qPCR. HDM significantly induced *Trem2* expression at both time-points, but significantly down-regulated 1.8-fold following RSV infection on day 6 (Figure 5.16A, C). On day 3, mRNA levels correlated with the percentage of TREM-2^{+} macrophages in the BAL determined by flow cytometry (Figure 5.16A, B), but on day 6, RSV infection alone resulted in the highest proportion of macrophages expressing TREM-2 (approximately 70%) while mRNA levels were not correspondingly high (Figure 5.16C, D). Interestingly, TC2 treatment served to increase the percentage of TREM-2^{+} macrophages during early infection on day 3, while TC2 reduced the proportion of TREM-2 expressing macrophages during peak inflammation (Figure 5.16B, D).

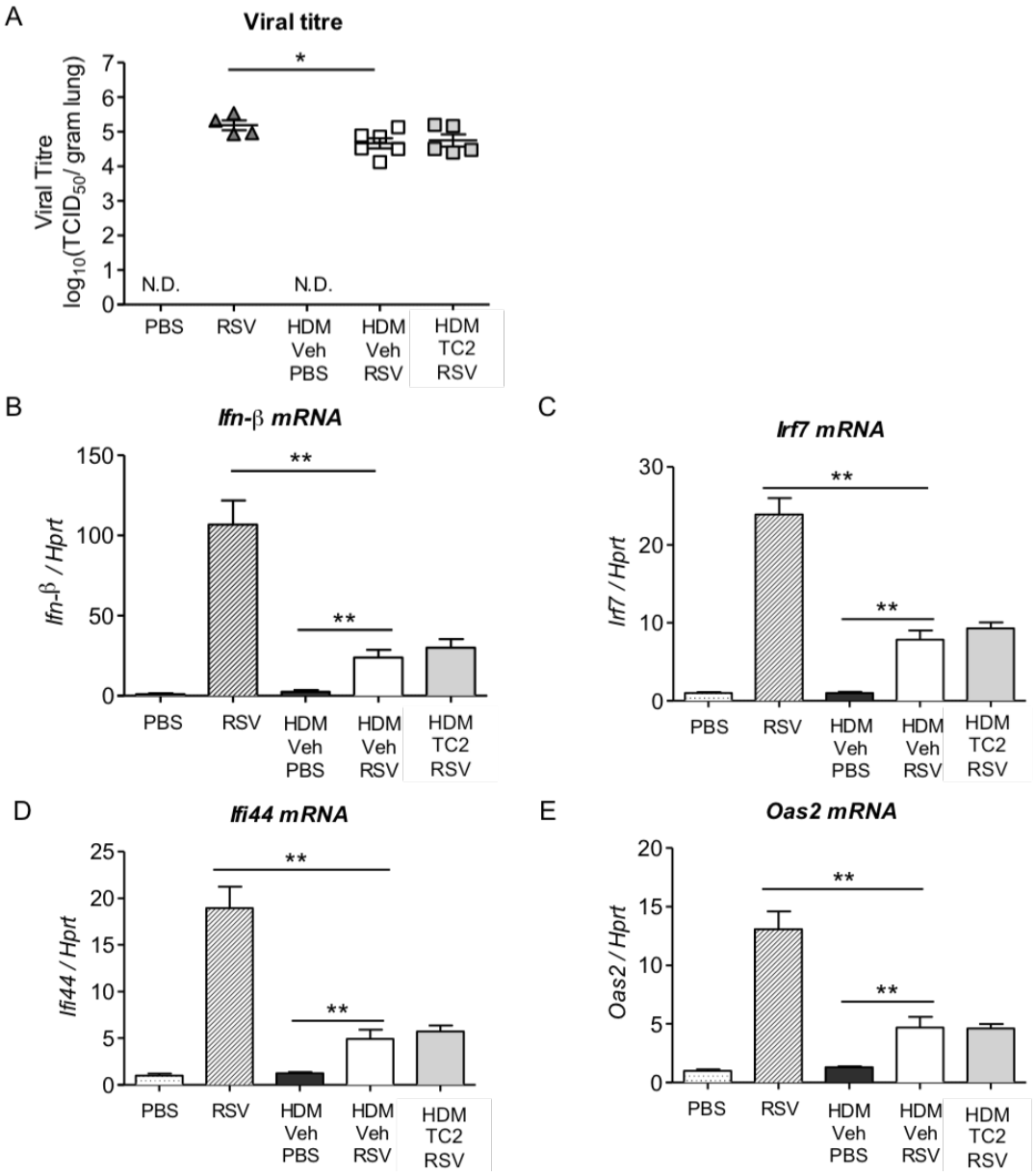

Figure 5.14 Effect of HDM and TC2 treatment on viral load and anti-viral gene expression.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle only for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 3 days post-infection, 24 h post-final HDM exposure. Viral load in lung tissue was determined by plaque assay (A). Expression of *Ifn-β* (B), *Irf7* (C), *Ifi44* (D) and *Oas2* (E) mRNA in lung tissue was determined by qPCR, standardised to housekeeping gene *Hprt* and expressed relative to PBS control group. Data are expressed as mean±SEM, n=4/5 mice per group and are representative of two separate experiments. Statistical significance was determined by Mann-Whitney U test between relevant groups, *p<0.05, **p<0.01. Plaque assays were carried out by industrial collaborators.

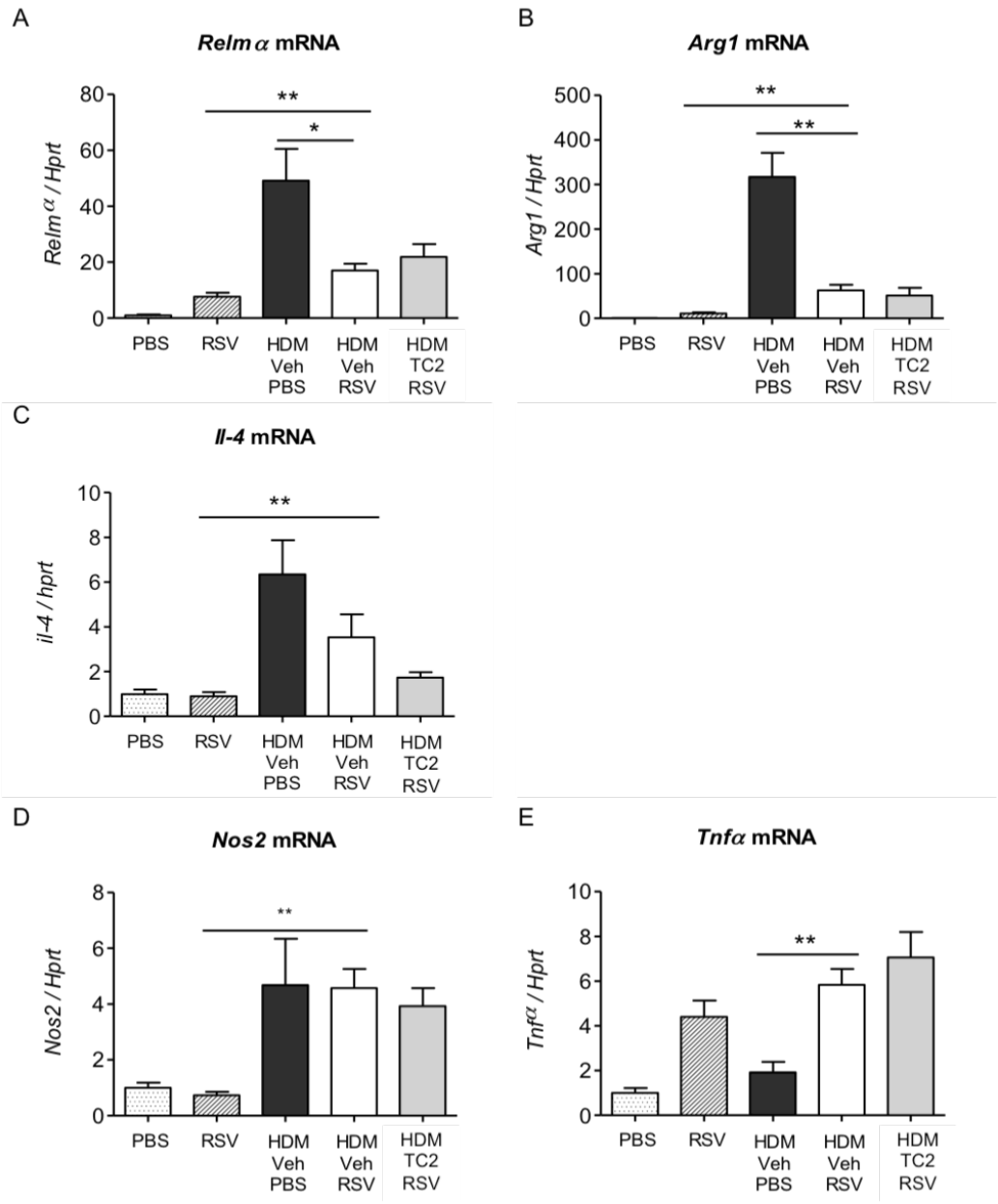

Figure 5.15 Effect of HDM and TC2 on M1 and M2 macrophage markers during RSV infection.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle only for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 6 days post-infection, 24 h post-final HDM exposure. Expression of *Relmα* (A), *Arg1* (B), *Il-4* (C), *Nos2* (D) and *Tnfα* (E) mRNA in lung tissue was determined by qPCR, standardised to housekeeping gene *Hprt* and expressed relative to PBS control group. Data are expressed as mean±SEM, n=4/5 mice per group and are from one experiment. Statistical significance was determined by Mann-Whitney U test between relevant groups, *p<0.05, **p<0.01.

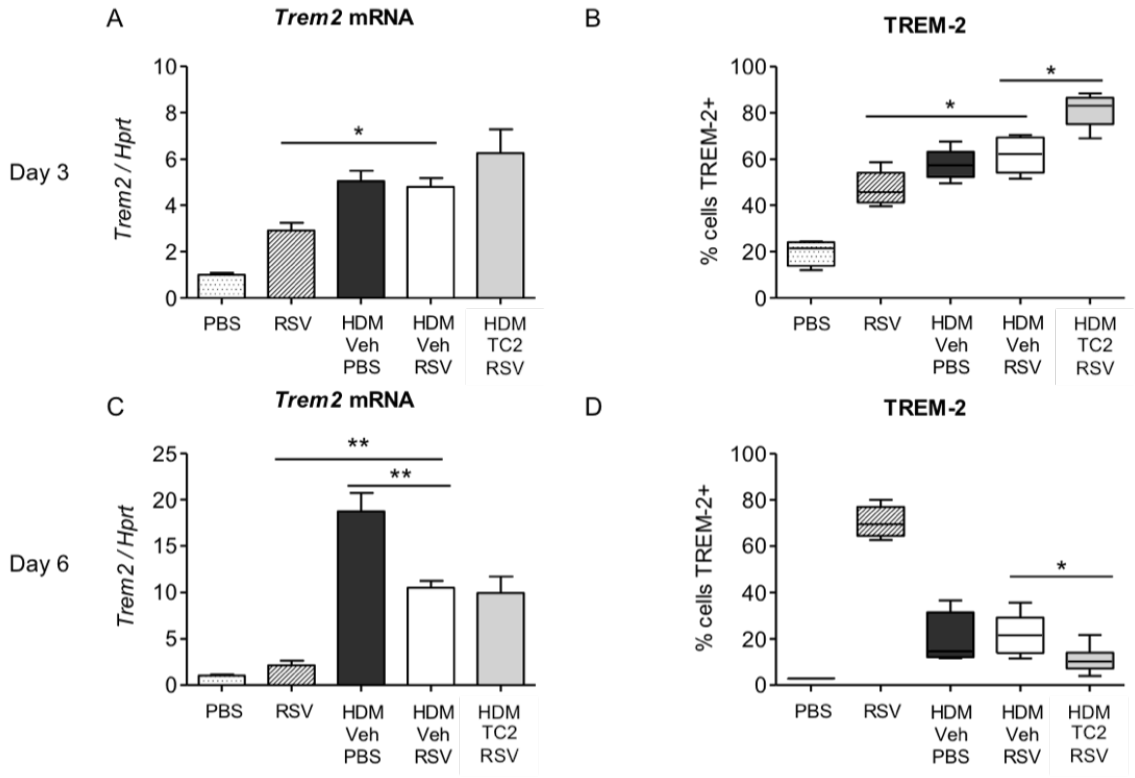

Figure 5.16 Effect of HDM and TC2 treatment on TREM expression during RSV infection.

BALB/c mice were intranasally inoculated with 25 µg HDM extract 3-times weekly for 4 weeks. During week 3, mice were given 1.75 mg/kg TC2 in 10% DMSO/PBS or vehicle only for 5 consecutive days before intranasal infection with 1/1.28 x 10^6 PFU RSV A2. Mice were analysed 3 or 6 days post-infection, 24 h post-final HDM exposure. Expression of *Trem2* mRNA in lung tissue was determined by qPCR 3 days (A) and 6 days (C) post-infection, standardised to housekeeping gene *Hprt* and expressed relative to PBS control group. Data are expressed as mean±SEM, n=4/5 mice per group and are from one experiment. Percentage of TREM-2^+ CD11c^+CD11b^{int} macrophages were analysed in BAL 3 days (B) and 6 days (D) post-infection. Data are expressed as median, IQR and minimum and maximum values, n=4/5 mice per group and are from one experiment. Statistical significance was determined by Mann-Whitney U test between relevant groups, *$p<0.05$, **$p<0.01$.

5.2.2 Effect of HDM and TLR agonist stimulation on RSV infection in human bronchial epithelial cells

In order to determine whether HDM has a similar protective effect in RSV infection in human cells, NHBECs from healthy control donors were cultured and pre-stimulated with HDM for 24 hours, prior to RSV infection. NHBEC cells were also pre-stimulated with various TLR agonists, zymosan, poly (I:C), LPS and R848, prior to RSV infection either alone or in combination with HDM to determine whether the protective effect of HDM in RSV infection was 1) due to HDM or any TLR stimulation, and 2) whether the protective effect could be augmented by a combination of HDM and TLR stimulation.

5.2.2.1 Effect of HDM and TLR stimulation and RSV infection on LDH release

In NHBEC from 2 donors, RSV infection resulted in a trend for increased LDH release, a sign of reduced cell viability (Figure 5.17A, B). LDH release from cells treated with LPS was reduced compared to PBS pre-stimulation, while HDM, R848 (TLR7/8 agonist) and poly(I:C) (TLR3 agonist) pre-stimulation led to a trend for reduced LDH release (Figure 5.17). Zymosan (TLR2 agonist) pre-stimulation alone did not affect LDH release (Figure 5.17), however, pre-stimulation of zymosan in combination with HDM reduced LDH release compared to HDM alone (Figure 5.18). There is however, disparity between the results derived from cells from the different donors.

5.2.2.2 Effect of pre-stimulation with HD and TLR agonists on RSV-induced anti-viral gene expression

NHBEC stimulated with HDM or TLR agonists prior to infection with RSV did not significantly (>twofold compared to PBS) upregulate *ifnβ*, *oas2* or *ifi44* (Figure 5.19A, B, E-H). While RSV alone did not lead to an upregulation of *irf7* (Figure 5.19C, D), pre-stimulation with HDM with zymosan, LPS or R848 before RSV infection led to an approximately twofold increase in *irf7* expression (Figure 5.20C, D).

The effect of RSV infection on anti-viral gene induction was donor-specific, since RSV resulted in induction of *ifnβ* in donor 1, but not in donor 2 (Figure 5.19A, B). HDM in combination with LPS increased *irf7, oas2* and *ifi44* above RSV infection alone in donor 1, but not in cells derived from donor 2 (Figure 5.20C-H).

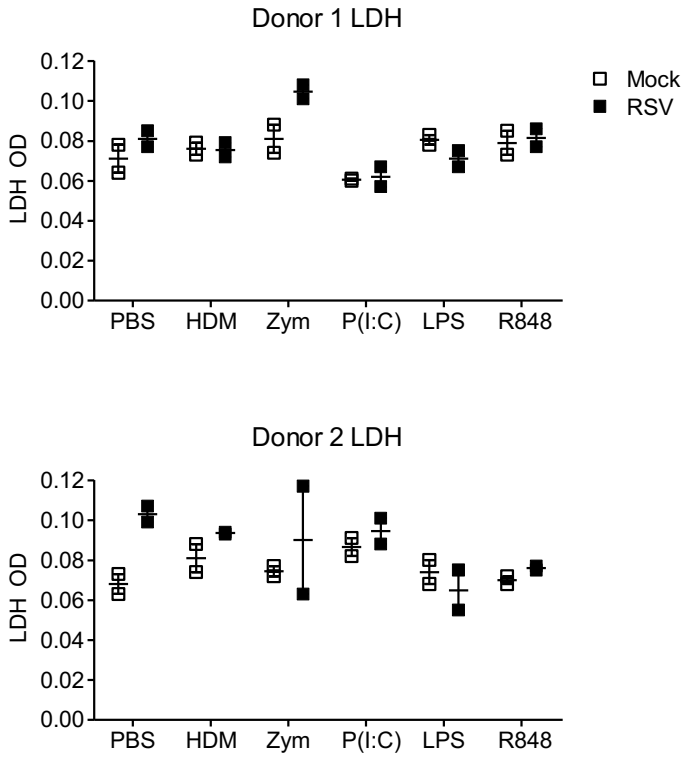

Figure 5.17 Effect of HDM or TLR agonists on RSV-induced LDH release in NHBEC.

Human bronchial epithelial cells derived from two healthy donors were seeded at 4 x 10^5 cells/well. Cells were stimulated with 5 µg/mL HDM extract, 100 µg/mL TLR2 agonist zymosan (Zym), 10 µg/mL TLR3 agonist poly(I:C) (P(I:C)), 400 ng/mL TLR4 agonist LPS, 0.1 µg/mL TLR7/8 agonist R848 or PBS as control for 24 h, before infection with RSV A2 MOI 0.1 or mock infection. Stimulations were performed in duplicate wells. Cell supernatants were tested for LDH by colorimetric assay in treated cells from two donors (A, B). Data are expressed as mean±SEM of duplicate wells.

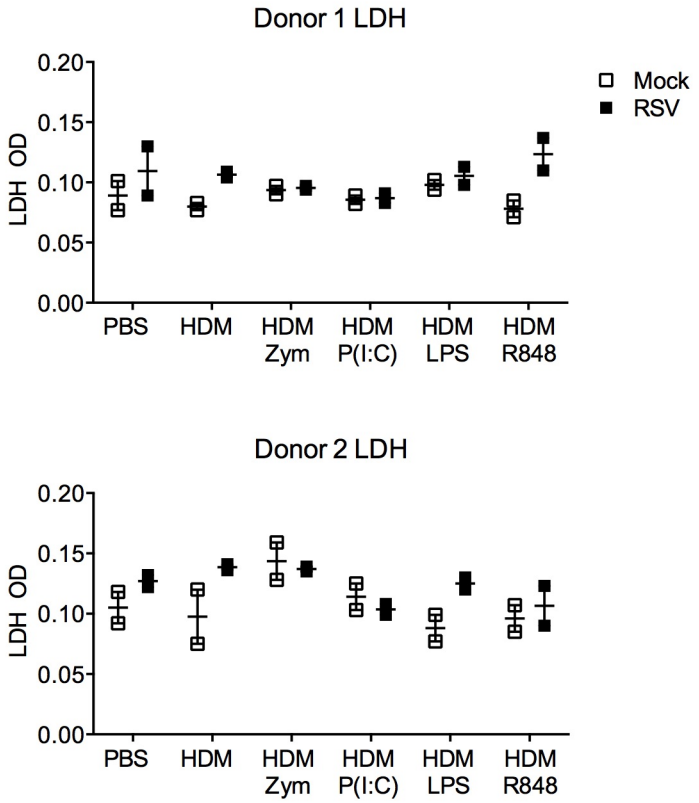

Figure 5.18 Effect of HDM in combination with TLR agonists on RSV-induced LDH release in NHBEC.

Human bronchial epithelial cells derived from two healthy donors were stimulated with 5 µg/mL HDM extract in combination with 100 µg/mL TLR2 agonist zymosan (Zym), 10 µg/mL TLR3 agonist poly(I:C) (P(I:C)), 400 ng/mL TLR4 agonist LPS, 0.1 µg/mL TLR7/8 agonist R848 or PBS as control for 24 h, before infection with RSV A2 MOI 0.1 or mock infection. Stimulations were performed in duplicate wells. Cell supernatants were tested for LDH by colorimetric assay in treated cells from two donors (A, B). Data are expressed as mean±SEM of duplicate wells.

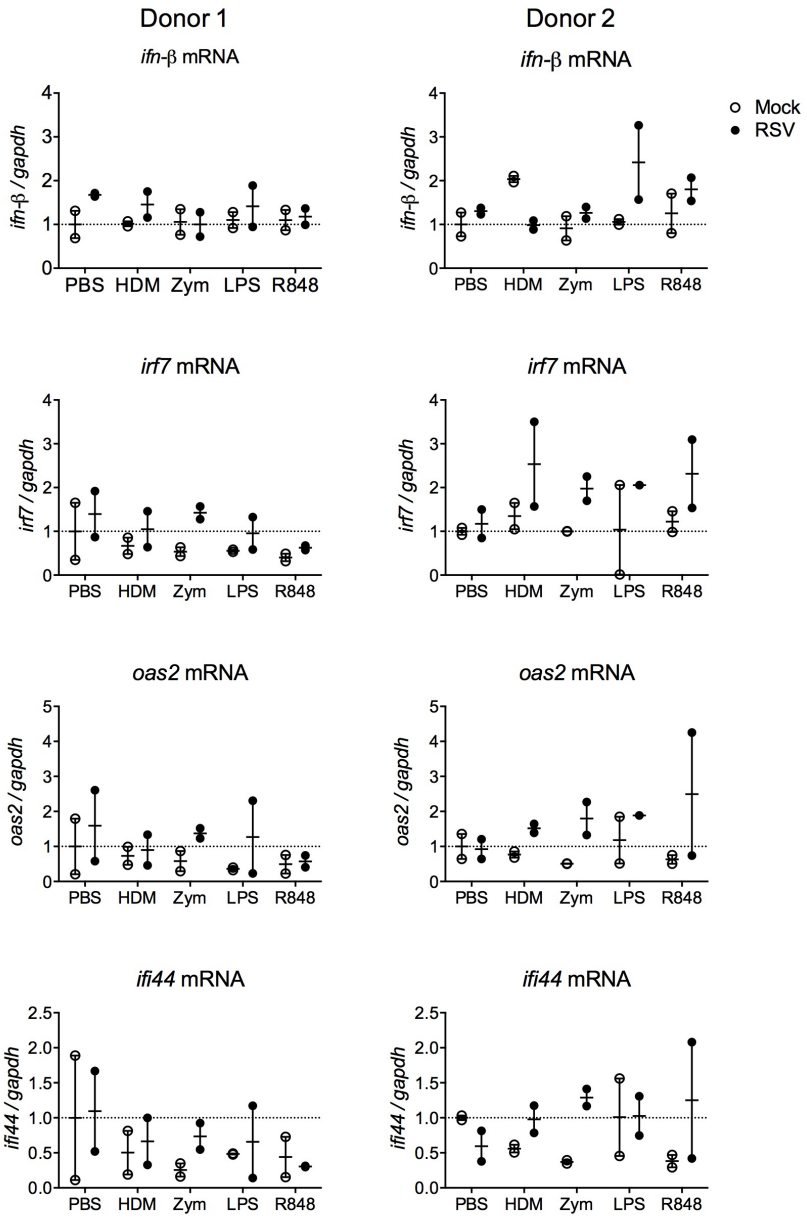

Figure 5.19 Effect of HDM or TLR agonists on RSV-induced anti-viral gene expression in NHBEC.

Human bronchial epithelial cells derived from two healthy donors were stimulated with 5 µg/mL HDM extract, 100 µg/mL TLR2 agonist zymosan (Zym), 10 µg/mL TLR3 agonist poly(I:C) (P(I:C)), 400 ng/mL TLR4 agonist LPS, 0.1 µg/mL TLR7/8 agonist R848 or PBS as control for 24 before infection with RSV A2 MOI 0.1 or mock infection. Stimulations were performed in duplicate wells. Expression of i*fn-β* (A, B), *irf7* (C, D), o*as2* (E, F) and *ifi44* (G, H) mRNA in cell lysates was determined by qPCR, standardised to housekeeping gene *gapdh* and expressed relative to PBS control. Data are expressed as mean±SEM of duplicate wells.

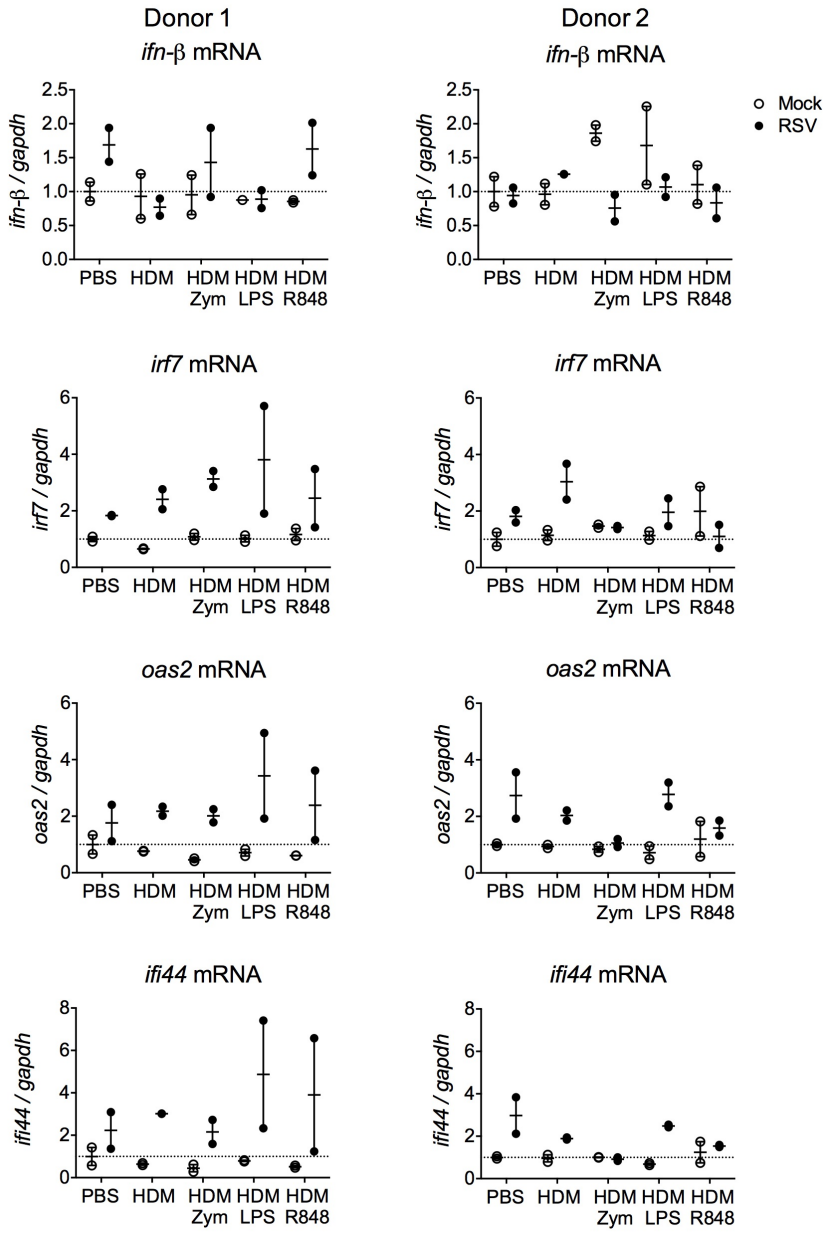

Figure 5.20 Effect of HDM or TLR agonists on RSV-induced anti-viral gene expression in NHBEC.

NHBECs derived from two donors were stimulated with 5 µg/mL HDM extract in combination with 100 µg/mL TLR2 agonist zymosan (Zym), 10 µg/mL TLR3 agonist poly(I:C) (P(I:C)), 400 ng/mL TLR4 agonist LPS, 0.1 µg/mL TLR7/8 agonist R848 or PBS as control for 24 h before infection with RSV A2 MOI 0.1 or mock infection. Stimulations were performed in duplicate wells. Expression of i*fn-β* (A, B), *irf7* (C, D), o*as2* (E, F) and *ifi44* (G, H) mRNA in cell lysates was determined by qPCR, standardised to housekeeping gene *gapdh* and expressed relative to PBS control. Data are expressed as mean±SEM of duplicate wells.

5.3 Discussion

In this chapter, the effect of TC2 administration in a new model of viral infection during on-going allergic airway disease was investigated. Mice that had been exposed to HDM and developed allergic airway disease were infected with RSV and treated with TC2. Allergic airway disease related parameters, such as AHR and airway inflammation, were tested, as were features of the anti-viral response. The aim was to characterise the model of viral infection during active airway inflammation and to determine whether TC2 treatment could reduce pathogenesis in either or both aspects of lung disease in this model.

5.3.1 Effect of HDM exposure prior to RSV infection

RSV infection in the HDM model of allergic inflammation did not significantly exacerbate AHR. While a small increase in airway resistance was observed, no other parameters of AHR were altered by RSV infection. This was an unexpected result given the evidence for RSV exacerbation in humans and indeed other mouse models (Nicholson et al., 1993, Johnston et al., 1995, Kondo et al., 2004). A possible reason for this observation is that the AHR induced by HDM challenge alone is difficult to increase further with other stimuli as it is already a robust response. Lowering the dose of HDM used to challenge the mice might induce milder AHR, which could then be manipulated with RSV infection. The dose of virus used might also play a role, as Kondo et al. (2004) found that low grade RSV infection was also not sufficient to increase AHR whereas high dose viral infection was. Development of a model of AHR exacerbation could be achieved by increasing the viral dose used in infection. The failure of RSV infection in exacerbating AHR could also be due to the time-point of infection and/or the time-point chosen for analysis. While RSV infection took place after 3 weeks of HDM exposure and analysis at day 3 and 6 post-infection, other models were analysed at different time-points (Petersen et al., 2014, Kondo et al., 2004). A more detailed representation of the effects of RSV infection in a mouse model of allergic airway disease could be achieved by a time-course analysis. Analysis of the acute infection phase until resolution of inflammation may reveal exacerbation of AHR which were not observed in the time-points of this study.

It may also be the case that, in this particular model, RSV infection neither contributed to nor exacerbated the pathology associated with pre-existing allergic airway disease and in fact

RSV might serve to attenuate airway inflammation. While no difference in total inflammatory cell populations or histological inflammatory score were observed, many other parameters of RSV-induced disease pathology were milder in the HDM-exposed groups compared with non-allergic animals. The most striking effect seen was the protection from RSV-induced weight loss in HDM-exposed animals in comparison with animals that had no prior HDM exposure. This finding corroborates with other studies in which induction of allergic inflammation prevents subsequent viral-induced weight loss (Peebles et al., 2001, Samarasinghe et al., 2014). This finding suggests that prior exposure to HDM protects the lung from viral pathogenesis. Recently, a number of studies have also observed that prior allergic inflammation reduces parameters of viral infection. In a study by Samarasinghe and colleagues (2014), mice were infected with influenza during peak *A. fumigatus*-induced allergic inflammation. Similar to the present study, weight loss was prevented and lung viral burden reduced, which indicates that in these particular models, RSV does not exacerbate existing allergic pathogenesis. Another study showed that HDM exposure prior to RV infection failed to exacerbate AHR (Rochlitzer et al., 2014). The authors concluded that the HDM-exposed mice had impaired anti-viral responses as they showed lower type I IFN levels than PBS-treated RV-infected mice. However, as the authors were unable to determine viral load in the lung due to high background, it may be that type I IFN levels were reduced due to lower viral burden in the lung. Taken together with lack of AHR exacerbation and reduced anti-viral gene expression and viral burden observed in this study, the Rochlitzer study would certainly concur with this present study. Rather than having an impaired anti-viral response, our findings of reduced weight loss and tissue damage marker production, combined with the reduction in anti-viral parameters and AHR suggest that prior allergen challenge protects mice from viral-induced exacerbation, pathology and tissue damage. The Th1 proinflammatory response to RSV was also abrogated in HDM-exposed animals compared with PBS controls. This correlated with other studies in which IFN-γ and IL-12 induction was reduced following HDM allergen challenge (Kondo et al., 2004, Hirose et al., 2008). This suggests that the effects of RSV, or viral infection in general, vary depending on the model used. In this study, HDM allergen challenge appears to prime the lung to a more appropriate immune response to RSV. As a result an excessive inflammatory response is avoided and no exacerbation is observed. The potent Th2 environment might be preventing pathological Th1 inflammatory response from developing, thus demonstrating the importance of a balance

between efficacious immune responses and excessive inflammation and pathology. RSV is known to skew the immune balance towards Th2 inflammation in order to evade the host response and enhances allergic inflammation in some models (Becker, 2006, Barends et al., 2003). A study in human airway epithelial cells demonstrated that Th1/Th2 predisposition prior to RSV infection greatly influenced inflammatory outcomes (Yamada et al., 2011). Th2-primed cells upregulated 62 genes in response to RSV infection, while Th1-primed cells did not, and the most highly upregulated gene was CCL2, suggesting its role in increasing pathogenesis of RSV infection in a Th2-primed lung environment. Interestingly, our findings appear to show the opposite effect. Prior allergen challenge and a Th2 environment prevented severe RSV-induced pathogenesis and resulted in a reduction in Th1 proinflammatory cytokine production. While the importance of a Th1 versus Th2 airway environment warrants further investigation in our model, the relationship between CCL2 and RSV-induced pathogenesis is replicated in this model. We found that CCL2 levels were significantly reduced in mice that had exhibited reduced weight loss and viral burden. CCL2 is a chemoattractant for various cells including neutrophils, monocytes and T-cell, but not eosinophils (Rose et al., 2003). Therefore, CCL2 could be a potential target in the prevention of immunopathology in RSV infection. Other chemokines not studied in this project, such as CCL3 which is also a determinant of pathology during RSV infection (Tregoning et al., 2010), could also account for the reduced pathology observed in this model.

5.3.2 Mechanisms conferring protection against RSV-associated pathology

The reduction in RSV-induced inflammation, anti-viral activity and pathology in HDM-exposed mice could either suggest that there is a barrier to infection, i.e. lung cells are infected by RSV to a lesser extent than in the non-allergic lung, or that the immune response mounted to RSV infection is more effective in clearing the virus compared with the naïve lung. The first possibility would suggest that there could be a mechanical, biochemical, or immunological barrier to infection. Since the HDM model is known to induce goblet cell hyperplasia (Johnson et al., 2004), it could be suggested that mucus may be reducing the access of virus to target cells, thus with a reduced degree of infection, associated weight loss, pathology and inflammatory parameters are accordingly reduced. The differences in cell types present in the allergic versus naïve lung may also impact on the ability of the virus to infect target cells. The influx of inflammatory cells induced by HDM challenge could simply

be acting as a physical barrier, preventing access to epithelial cells and macrophages which are the main cell targets for RSV (Rivera-Toledo and Gómez, 2012, Mastronarde et al., 1996). The cell types present in the lung also affect the immune response against the virus. Allergic inflammation has previously been shown to reduce IFN-γ production in response to RSV (Peebles et al., 2001), albeit in an OVA model of allergic inflammation. This finding was also observed in the present study. With a reduced propensity for the lung to produce IFN-γ, the general inflammatory response mounted as a result of RSV infection is likely to be muted compared with RSV infection of naïve mice. Conversely, TREM-2, which has previously been shown to be upregulated during the acute phase of inflammation, was significantly upregulated following HDM exposure and during the early stages of RSV infection. Since TREM-2 is expressed on anti-inflammatory M2 macrophages, the larger population of anti-inflammatory macrophages could be regulating the proinflammatory response and reducing subsequent pathology compared with RSV infection alone. In order to deduce whether the reduced viral burden was simply due to reduced access to target cells, GFP labelled-RSV could be used in the study, in order to track the localisation of the RSV and to determine whether the proportion of infected cells differs from infection of naïve versus allergic mice. The effect of specific cells on the immune response to RSV following HDM exposure could be addressed with adoptive transfer of various cells types. For example, adoptive transfer of naïve macrophages (i.e. not previously exposed to HDM) would reveal whether the phenotype of macrophages induced by HDM exposure are a contributing factor in the restrained inflammatory response and improved outcome.

Another potential cause of the reduction in the proinflammatory and anti-viral response to RSV is the desensitisation of TLRs as a result of HDM exposure. The HDM extract used is a crude extract known to contain varying amounts of endotoxin, therefore great care is taken to ensure the levels of endotoxin between different batches are comparable. Previous studies have demonstrated that prolonged exposure to HDM extract results in increased expression of TLR inhibitors such as A20 in the lung, leading the desensitisation of TLR2 and TLR4 (Habibzay et al., 2012). This study also showed that susceptibility to bacterial infection ensues as a result. The recognition of RSV antigens via various TLRs induces the innate immune response against the virus, including TLR2 and TLR4 (Kurt-Jones et al., 2000, Murawski et al., 2009), which are both also activated by HDM exposure (Liu et al., 2013). It

is possible that desensitisation of TLRs occurs following HDM challenge, resulting in impaired innate immune response induction upon subsequent RSV infection. Desensitisation of TLRs may explain the failure of RSV to exacerbate AHR in this model. In an OVA model of allergic airway inflammation, Starkhammar and colleagues (2014) found that TLR3 or TLR4 ligation alone could induce AHR in the absence of airway inflammation. Therefore, desensitisation of TLRs by HDM exposure could explain the lack of AHR exacerbation in our RSV infection model. However, the authors also observed a dampened inflammatory response to stimulation with LPS or poly(I:C), in terms of neutrophilia and proinflammatory cytokine production, which is similar to the diminished inflammatory response in our model. This might suggest that TLR stimulation is a direct cause of the reduction in inflammatory parameters analysed in our model, which contradicts a concept of TLR desensitisation. In order to establish the importance of TLR stimulation or desensitisation in our mouse model, TLR and TLR inhibitor gene expression could be investigated.

In order to determine whether TLR desensitisation plays a role in inhibiting the anti-viral response in human cells, HDM and TLRs were incubated with human bronchial cells prior to RSV infection. The aim was to determine whether HDM exposure resulted in a protective effect against RSV infection in terms of 1) cell damage and 2) anti-viral gene expression. However, due to suboptimal cell numbers and a high degree of variability between the responses of the cells from different donors, conclusions could not be drawn from these experiments. In order to test the hypothesis that HDM exposure confers protection against subsequent RSV infection, increasing replicates and sample numbers would yield more reliable results, as would initial experiments in epithelial cell lines, which are less variable than primary cells. This *in vitro* human model does not replicate the *in vivo* scenario in which alveolar macrophages are the prime target for desensitisation by prior stimuli. Thus an effect is more likely to be observed in the more complex *in vivo* environment.

Studies of other viral infections have demonstrated similar protective or impaired responses to viral infection following allergic inflammation. Ishikawa et al. (2012) suggest that allergic airway disease induces NK-cell proliferation which prevent influenza infection. While an *ex vivo* study of bronchial cells isolated from steroid-resistant asthmatic children found these cells had impaired type I IFN responses to RV (Edwards et al., 2013). Although no difference

in type I IFN responses was observed in another report studying adult asthmatics versus controls (Patel et al., 2014). In terms of this study, it is likely that the reduction in the inflammatory, anti-viral and histopathological responses is due to timing. Other studies have demonstrated similar protective effects of allergic airway disease in respiratory viral infection and a clear difference between the effect of acute and chronic allergic inflammation has been observed (Samarasinghe et al., 2014, Rochlitzer et al., 2014). While the model developed in this study cannot be transferred or used directly as a model of human disease, further investigation into the mechanism of this protection conferred by HDM exposure could provide potential targets for manipulating inflammatory lung disease associated with viral infections. Furthermore, the model still allowed for the testing of candidate drugs and demonstrated the benefits of inhibiting signalling kinases in a model of viral infection following HDM exposure.

5.3.3 Signalling kinase inhibition modifies parameters of airway inflammation

Though the model did not exhibit features of exacerbation, interestingly, TC2 treatment did modify some parameters of disease. Particularly noteworthy is that TC2 treatment reduced AHR in HDM-exposed RSV-infected mice compared with vehicle-treated controls. Airway compliance was increased, while tissue elastance and tissue damping, which are associated with structural changes in the lung, were reduced with TC2 treatment. Therefore, while inhibition of signalling kinases with TC2 failed to modify HDM-induced AHR alone, it is efficacious in a complex inflammatory setting of viral infection following HDM challenge. Other parameters of disease reduced by TC2 treatment were RSV-induced weight loss, IL-6 and IFN-γ production, CXCL10 production and total and HDM-specific IgE. Thus confirming the benefit of targeting Src, Syk and p38 MAPK on both the allergic response and RSV-induced proinflammatory response and weight loss. Another NSKI has previously been shown to reduce cytokine production by influenza-infected nasal epithelial cells (Ito et al., 2013a). RSV replication was also reduced with NSKI treatment in bronchial epithelial cells isolated from cystic fibrosis patients. *In vivo* treatment with a NSKI treatment reduced parainfluenza-induced exacerbations of OVA-induced allergic inflammation in guinea pigs. Therefore, it is likely that TC2 is exerting inhibitory effects on kinase signalling in both arms of the airway inflammatory environment (allergic and anti-viral) present in this model. Thus confirming the benefits of using signalling kinase inhibitors to reduce airway inflammation

and AHR during complex inflammatory airway disease. However, further extensive testing is required in order to characterise all of the effects of TC2 in this model. The parameters analysed in this study were selected in order to determine specific effects of TC2 in the model. Any potential harmful effects in this model were not assessed. Increased susceptibility to further infection is a possible side effect of dampening the immune response as is seen with other signalling kinases inhibitors, e.g. dasatinib (Src inhibitor) can cause neutropenia which could impair innate immune responses to infection (Wong, 2009). Further work could also be carried out in order to determine the possibility and effects of systemic dissemination of the compound in this model. However, extensive pre-clinical and clinical safety assessments showed no safety concerns of TC2 treatment (C. Stevenson 2015, *pers. comm.*, 29 October 2015). Overall, TC2 treatment shows promise in the regulation of airway disease associated with viral infection and allergic airway inflammation.

5.3.4 Conclusion

A new model of viral infection following allergic airway disease induction was developed. Rather than exacerbating allergic inflammation and AHR, the inflammatory, anti-viral and histopathological response to RSV was attenuated, suggesting a protective effect of prior HDM exposure. The model was used to test the effect of signalling kinase inhibitor TC2, and TC2 was found to modulate parameters of both the allergic and anti-viral/proinflammatory response.

Chapter 6: Discussion

Chapter 6: Discussion

6.1 Summary and impact of findings

Understanding the events leading to inflammatory lung disease is vital for the development of new therapeutic strategies. In many cases, inappropriate, excessive inflammation can create a lung environment that increases susceptibility to subsequent infection, as is seen in secondary bacterial infections following influenza infection or viral exacerbations of asthma.

While major advances in the field of immunological research have been achieved recently, a large knowledge gap still exists and further research into the immune interactions underlying airway inflammation is essential to enable the translation of new basic science findings into clinical practice. This thesis explored the role of innate immune receptors—TREM-1 and TREM-2—in animal models of lung inflammation, and the potential of pharmaceutical inhibition of kinases in ameliorating airway disease in animals. In this chapter, a summary of the findings presented in this thesis and their implications will be discussed.

6.1.1 Mechanisms for controlling inflammation in airway disease

Having been discovered just 15 years ago by Bouchon *et al.* (2000), proteins of the TREM family are relatively new players in the field of inflammation. Their importance, however, cannot be disputed. Many studies have associated the upregulation of TREM-1 as a prominent marker of an on-going proinflammatory response, particularly in human and animal studies of sepsis and other bacterial infections, e.g. Su *et al.* (2012) and Gibot et al. (2012). Several studies have linked TREM-1 production with respiratory viruses such as influenza (Rosseau et al., 2007, Ivan et al., 2012, Askovich et al., 2013), and Weber et al. (Weber et al., 2014), found that TREM-1 contributes to pathogenesis in a mouse model of influenza infection. We found that TREM-1 was not expressed on airway macrophages, but rather on recruited neutrophils at the peak of inflammation. In this model of acute, self-resolving influenza, TREM-1 is likely to be induced in order to support TLR signalling, thus leading to amplification of the inflammatory response, supported by the upregulation of TREM-1 on neutrophils at the peak of inflammation, cell infiltration and weight loss. Unexpectedly, TREM-2 was also upregulated during peak infection on both resident and

infiltrating macrophages. A similar upregulation of TREM-2 on lung macrophages was observed during infection (Wu et al., 2015). TREM-2 may have a role in promoting survival of macrophages, as described in the study by Wu et al. (2015) in which TREM-2 was shown to promote IL-13-induced M2 macrophage differentiation. TREM-2 could be functioning to abrogate the proinflammatory environment by expanding the M2 macrophage population and facilitating repair. Thus, TREM-2 could be a significant contributor to 'innate immune rheostat' theory, as described by the Hussell group (2009), where the balance of positive and negative regulatory receptors dictate the immune reactivity in the lung.

Manipulation of TREM-1 has been explored as a method of controlling infection. In our model, bacterial infection strongly induced sTREM-1 production after influenza infection, to a much greater extent than viral infection alone. This correlates with *in vivo* and patient studies in which sTREM-1 is recognised as a marker of bacterial infection in the lung (Gibot et al., 2004, Ye et al., 2014). These data suggest that sTREM-1 could be used as a diagnostic marker of secondary bacterial infection in the lung, either via its measurement in BAL or more easily available serum samples from patients suspected, but unconfirmed, of secondary bacterial infection. The ability to accurately distinguish between a viral disease and those complicated by bacterial infection is becoming important in reducing inappropriate use of antibiotics in clinical practice (Roca et al., 2015). The marked upregulation of sTREM-1 leads to the question of how and why this upregulation of sTREM-1 occurs. Gómez-Piña and colleagues (2007, 2012) found that sTREM-1 is likely to be cleaved by an MMP, through MMP inhibitor studies. The MMPs that are relevant in our model, i.e. produced during lung inflammation, are MMP-9 and MMP-2, and a cleavage site for MMP-9 exists in the TREM-1 amino acid sequence. Indeed, when MMP-9 was measured in the BAL, the concurrent production of MMP-9 with sTREM-1 suggested a relationship between the two proteins, and could be utilised to manipulate TREM-1 function (Figure 6.1). However, the data in this study only suggests an association exists rather than proving this to be the case.

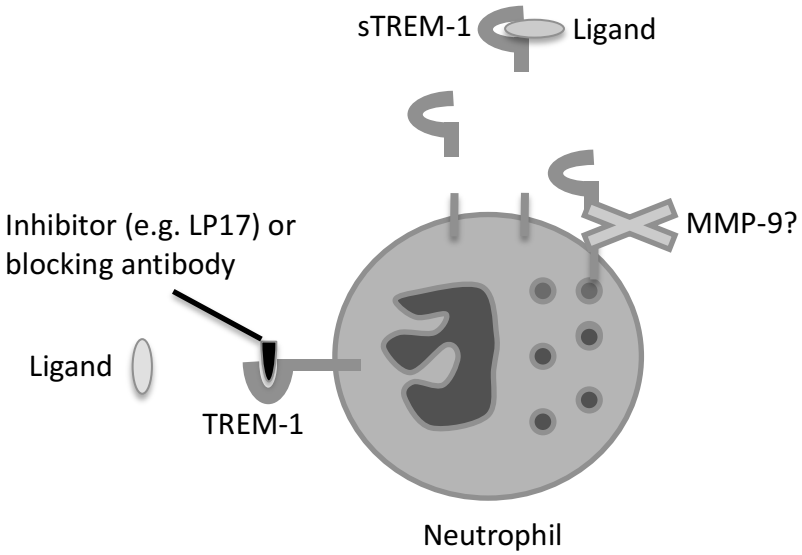

Figure 6.1 Potential manipulation of TREM-1 function.

The inflammatory response induced by ligation of TREM-1 with its ligand could potentially be manipulated to reduce the amplification of inflammation. An inhibitor of TREM-1 (e.g. LP17 or a blocking antibody) could block the binding of TREM-1 ligand with TREM-1, thus abrogating inflammatory signalling. The mechanism of cleavage of surface TREM-1 into sTREM-1 by MMP-9 could be utilised as a method of reducing TREM-1 signalling, and sTREM-1 released from the cell surface could act as a decoy by sequestering TREM-1 ligand and preventing TREM-1 activation.

6.1.2 Therapeutic manipulation of inflammatory lung disease

Various lung diseases could benefit from targeted therapies that serve to reduce the extent of or ameliorate inflammation. A common feature in the lung conditions studied in this thesis is excessive inflammation. Secondary exacerbations of lung disease are dependent in part on the vigour of this inflammatory response (Snelgrove et al., 2008, Hallstrand et al., 2014). The search for novel targets and therapies to treat such inflammatory lung conditions are on-going and one such area explored in this thesis was the signalling pathways leading to inflammation. Signalling kinases play a vital part in the activation of the inflammatory response in the lung (Adcock et al., 2008), and the inhalation route is likely to be the most appropriate method of drug delivery to the airways (Barnes, 2010).

Administration of the broad-spectrum kinase inhibitor TC2 was found to modify parameters of allergic disease in a HDM model and reduce selected hallmarks of the allergic and anti-

viral response in a model of viral infection during on-going allergic disease. As a result of this investigation, new, *in vivo* effects of TC2 have been found, including a reduction in eosinophil recruitment, IgE and IgG production, RSV-induced weight loss, proinflammatory cytokine production and CXCL10 production. The data in this thesis highlight the possible mechanisms by which TC2 functions in a mouse model that could potentially be replicated in the clinical setting—e.g. in the on-going phase 2 clinical trial of TC2. The direct translation of data derived from *in vivo* studies is, however, somewhat limited by the physiological differences between man and mouse (Mullane and Williams, 2014). However, animal studies are crucial in basic science and directly impact on clinical research.

6.1.3 Translational research: animal models and human disease

Fundamental basic research has long-since used various models to investigate biological systems. Major advances in technologies, such as computational modelling of lung diseases, have added to the information traditionally derived from *in vitro* and *in vivo* models (Burrowes et al., 2014). However, there is still a need for the *in vitro* and *in vivo* models to bridge the gap between computational models and human disease.

A plethora of mouse models have been developed to investigate respiratory disease. Two established models of inflammatory lung disease—secondary bacterial infection following influenza and HDM-induced allergic airway disease—were used in this thesis. These well characterised models have allowed for the investigation into the expression pattern of TREMs in influenza and secondary bacterial infection, which had not previously been studied. The potential of signalling kinase inhibition in modulating HDM-induced allergic airway disease was also explored in this thesis.

The potential of such experimental animal models in yielding translatable scientific findings is convincing. However, researchers must be cautious when developing animal models, as they might not reflect human disease. A new mouse model was developed to mimic respiratory virus infection during on-going allergic airway disease. Though an exacerbation of AHR was hypothesised based on the human response to such viral challenge, this was not observed in the model presented in this thesis. Instead, HDM exposure altered the nature of inflammation in the lung, reducing viral-induced disease parameters. Although fundamental

experimental differences in the models may account for these contrasting study results of animal models, the results could provide more insight into the role of the lung environment on subsequent immune responses—i.e. that previous inflammation in the lung can either increase or reduce subsequent inflammatory events, and the effect is likely to be dependent both on the type and extent of initial inflammation.

6.1.4 Importance of lung environment on subsequent lung response

Several studies since 2014 have suggested the importance of the existing lung environment on subsequent respiratory viral disease. Both the severity of existing immune response in the lung at the time of infection and the time-point at which infection occurs are likely to be important factors on the subsequent lung response. Rochlitzer and colleagues (2014) showed that RV did not result in an exacerbation of chronic airway inflammation in terms of Th2 inflammation or AHR, similar to the findings in RSV infection following HDM exposure reported here. A difference in the time-point of infection was postulated as a reason for the lack of disease, while another study suggested the importance of TLR sensitisation in the development of AHR (Starkhammar et al., 2014). The authors discussed that AHR did not necessarily correlate with cellular inflammation, therefore, the definition of 'exacerbation' should be considered for both aspects of disease independently. This was also noted in a model of HDM and poly(I:C) challenge (Clarke et al., 2014), and indeed the results presented in this thesis. Researchers studying the so-called viral exacerbation of allergic disease must be clear, both in their hypotheses and analysis of results, about which aspects of the overall allergic disease can be exacerbated. Indeed, the most thorough investigation should consider AHR, Th1 inflammation, Th2 inflammation, airway remodelling and the anti-viral response as separate aspects of allergic airway disease that might be exacerbated by viral infection.

Another novel finding from the work in this thesis is that HDM exposure prior to RSV infection significantly reduced viral-induced pathology. These results suggest that the lung environment induced by HDM exposure could protect the lung from viral infection, resulting in the reduction of factors associated with acute RSV infection, which was observed in the study by Samarasinghe *et al.* (2014). The mechanisms behind this phenomenon could be wide-ranging, for example, Fujimura *et al.* (2014) found that pet-associated house dust exposure induced increases of certain species of gut bacteria, which subsequently attenuated

allergen-induced inflammation. The protective effect of HDM exposure on RSV-induced pathology could also be due to accessibility of the virus to the cells. Our model of HDM-induced allergic airway disease shows a characteristic increase in mucus production in the airways, and this could be acting as a physical barrier to infection. Alternatively, the severity of disease likely depends on direct viral pathogenesis and the induction of an excessive Th1 cytokine polarised immune response. Since the allergic lung is dominated by Th2 cytokines, this may protect against immunopathology.

While the mechanisms behind this protective effect of HDM challenge prior to RSV infection have yet to be determined, the development of this model represents a starting point, and further characterisation and subsequent functional studies could potentially provide further insight into the role of prior lung inflammatory events on subsequent allergen or pathogen challenge.

6.2 Limitations and future work

6.2.1 Function and source of TREM proteins in inflammatory lung disease

While efforts were made to address the main hypotheses posed in this thesis, some limitations exist and questions remain. For example, the majority of the TREM studies analysed protein expression only, whereas gene expression and protein expression on other cell types could provide information on where, when and how TREM protein is produced and, as is the case for TREM-2, stored. Additional time-points post-infection could provide a more detailed understanding of the kinetics of TREM protein expression, which would allow for more accurate estimates of when best to target TREM-1 or TREM-2 for therapeutic modulation.

Manipulation of MMP-9 activity will clarify whether or not there is a direct, causal relationship between MMP-9 and sTREM-1. A strategy for the study of this would be to administer an inhibitor of MMP-9 during secondary bacterial infection and measure sTREM-1 release. Upon establishment of a direct relationship between MMP-9 and sTREM-1, it would be interesting to see whether preventing sTREM-1 production, by way of preventing its cleavage by MMP-9, affects the disease outcome, e.g. whether MMP-9 inhibition indirectly affects weight loss, inflammation and tissue damage associated with excessive

inflammation during secondary bacterial infection. This could potentially provide a means by which inflammation can be controlled without affecting pathogen clearance. The secondary effects of inhibiting MMP-9 function would, of course, need to be taken into account in such studies.

Further to determining the source of sTREM-1 in our model, an extension of the results presented in this study would be to determine whether manipulation of TREM-1 could help to control excessive inflammation in during secondary bacterial infection. Firstly, it would need to be determined whether sTREM-1 is present merely as a consequence of inflammation, or whether it plays an active part in regulating inflammation in our model. Whether sTREM-1 production is directly caused by TLR signalling upon pathogen recognition could, for example, be determined *in vitro* by incubating cells with TLR agonists and production of sTREM-1 measured by ELISA. The specific signalling cascade required to induce sTREM-1 release could be determined in such an experiment protocol. Alternatively, if TLR signalling alone fails to induce sTREM-1 release, it might be the case that other stimuli or an *in vivo* setting is required.

Simple blockade of TREM-1 via an antagonistic mAb could reveal which is the case. Furthermore, a small molecule peptide that mimics the extracellular domain of TREM-1 (LP17), thus preventing its dimerisation and downstream signalling has been used to block TREM-1 function by acting as an antagonist (Gibot et al., 2006). Use of this peptide in our model could uncover the therapeutic potential of TREM-1 manipulation. For example, if preventing TREM-1 signalling ameliorates the damaging inflammation in our model of secondary bacterial infection, it could be considered as a potential therapy in severe cases of secondary bacterial infection. However, pharmacokinetic studies would need to be undertaken to determine the optimal dose that enables reduction of inflammation without compromising bacterial clearance. Also, the administration route would be an important consideration. While LP17 has mostly been given as an intraperitoneal injection, the most efficacious route is likely to be intranasal or intratracheal. Administration of small molecules to a localised area is optimal for localising drug effects and preventing adverse effects in the periphery.

Availability of TREM-2 knock-out mice or a TREM-2 blocking antibody could allow for further clarification of the role of TREM-2 in our model of secondary bacterial infection following influenza infection. In light of the results obtained so far, and their correlation with other published data, it is likely that TREM-2, in both its surface expressed and soluble forms, contribute to an anti-inflammatory environment during both viral and bacterial infections. However, any attempts to manipulate TREM-2 for therapeutic treatment of excessive inflammation must also take into account the role of TREM-2 in the phagocytosis of bacteria (N'Diaye et al., 2009, Hommes et al., 2015). Cell-selective TREM-2 knockout mice could enable the investigation of TREM-2 effects on specific cell populations.

6.2.2 Mechanisms of TC2 modulation of models of allergic airway disease

Further optimisation of signalling kinase inhibition would be useful in maximising the effects of TC2. The pharmacokinetics and pharmacodynamics of TC2 were not studied in the models used in this study, nor was dose-optimisation for this particular strain of mouse and model carried out, and such pharmacological data would be beneficial in optimising the use of TC2 in mouse models, and indeed for using the lowest possible doses required to produce an effect to reduce the drug toxicity.

More detailed analysis of the signalling pathways and adaptors, mediators and cells types affected could be carried out through gene and protein expression (qPCR, flow cytometry, western blot analysis), which could elucidate the precise mechanism of TC2 action in our model. Understanding the precise mechanism of action, e.g. if only one particular pathway is involved, could allow for more specific inhibitors to be developed, which would be preferred compared with a broad-acting inhibitor, reducing the likelihood of adverse drug effects. Limitations associated with the use of specific housekeeping genes (i.e. variable expression in difference tissue or cells under different treatment conditions; Kozera and Rapacz, 2013) must, however, be considered. In this study, only one housekeeping gene was used per qPCR reaction. Use of a panel of housekeeping genes could help to eliminate the risk of bias caused by particular housekeeping genes.

An important consideration in the use of TC2 in the models presented in this thesis is the relatively high percentage of DMSO (10%) that was required to dissolve the powder

formulation of TC2. While 10% DMSO was used as a control in all relevant comparisons, there is evidence that DMSO can cause toxicity even at low doses of 2–4% *in vivo* (Galvao et al., 2013), therefore, an alternative solvent might eliminate the potential toxic effects in our model. This problem could also be avoided using devices that aerosolise dry powder formulations, an example of which is currently being developed for laboratory animals (Tonnis et al., 2014). The availability and established use of dry powder inhalers in humans eliminates this problem in the clinical administration of TC2.

6.2.3 Protective effect of prior HDM exposure on RSV infection

Since the HDM/RSV infection model was developed during the course of this study, many aspects of the model have yet to be characterised. One important consideration is that the model used PBS as a control for RSV infection rather than mock RSV (i.e. Hep2 cell lysate incubated without RSV) or inactivated RSV. However, the findings in the study are not likely to be dramatically different to the results presented here using PBS as a control, since studies in the lab where Hep2 lysate was used as a control also showed a protective effect of HDM in subsequent RSV infection.

Production of secreted mediators from different subsets of cells isolated from lung tissue could help to determine whether the protective effect of HDM exposure on RSV-induced pathology is due to a particular cell type. Adoptive transfer or selective depletion could determine the specific function of certain cell subsets, and establish their effects on disease outcome. In this study, analysis took place at two time-points of 3 and 6 days post- RSV infection. A full time-course ranging from hours post-infection to resolution of infection-associated inflammation may reveal key events in the infection process that are affected by prior exposure to HDM.

The possibility that mucus is acting as a physical barrier to viral infection could be addressed by tracking fluorescently labelled RSV *in vivo* to confirm whether RSV is able to infect target cells. Such a technique, combined with *in vivo* imaging systems, could also allow real-time tracking of RSV infection, giving an indication of the dynamics of RSV infection in our model compared with animals that do not have an on-going allergic inflammatory response.

As several studies have shown similar protective effects of prior allergic inflammation on respiratory viral infection (Rochlitzer et al., 2014, Samarasinghe et al., 2014), it would also be interesting to study the effect of HDM-induced allergic airway disease on other respiratory viral infections, such as influenza, to determine whether HDM also protects against other viral infections or whether the effect is unique to RSV alone.

6.3 Conclusion

New scientific findings are dependent on well established and well characterised models. In this thesis, it was observed that TREM-1 and TREM-2 were differentially expressed in established models of influenza and secondary bacterial infection. Modulation of TREM-1 and TREM-2 signalling pathways represents a potential method of controlling excessive inflammation during viral infection alone and secondary bacterial infection following viral infection. The association of sTREM-1 with bacterial response could be used in the diagnosis of bacterial infection, facilitating the selection of therapeutics appropriate to the specific infection present in the airway. In an example of targeting therapies against inflammatory lung disease, inhibition of selected signalling kinases using intranasally administered pharmacotherapeutics modified allergic parameters of HDM-induced allergic airway disease. A new model of RSV infection during on-going allergic inflammation failed to exacerbate AHR, and viral-induced pathology was reduced in animals with previous exposure to HDM. Experimental animal models of human disease remain an indispensible component of scientific research, aiding both the pursuit of knowledge of the mechanisms of disease and bridging the gap between basic science and clinical use. Development and thorough characterisation of new models that encompass both aspects of knowledge gathering and translatability are essential for the future of inflammatory lung disease research.

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