ROLE OF EOSINOPHILS IN RESPIRATORY SYNCYTIAL VIRUS INFECTION

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"A thesis submitted for the degree of Doctor of Philosophy of The Australian National University."

DECLARATION OF AUTHORSHIP

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the Australian National University.

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Please note that Tom Townsend has drawn all images (except Figure 1.3) with instructions from the candidate, Dijana Townsend.

ABSTRACT

Eosinophils recruited to the lungs, in response to respiratory syncytial virus (RSV) infection, have been associated with, the pathophysiological sequel of infection and more recently, with accelerated virus clearance. Heparan sulfates (HS), on the cell surface, are utilised by RSV to initiate infection, making HS a potential therapeutics target. As there is no effective RSV vaccine currently available, controlling RSV has remained a formidable challenge.

The aims of this thesis were to investigate: (1) *in vitro* RSV effect on eosinophil morphology and eosinophil antiviral activity against RSV; (2) *in vivo* eosinophil antiviral activity expressed by four distinct mouse models following RSV infection and re-infection; and (3) the efficacy of HS mimetics treatment on RSV infection *in vitro* and *in vivo*.

Firstly, transmission electron microscopy analysis, major basic protein and eosinophil peroxidase assays, eosinophil chemotaxis and RSV kinetics assays were used to examine aim 1. *In vitro* findings indicated human RSV pathogen inability to infect murine eosinophils however; eosinophil exposure to RSV antigen or RSV infected HEp-2 supernatant resulted in eosinophil activation, degranulation and chemotaxis.

Secondly, four genetically modified BALB/s strains were infected and re-infected. Analysis of bronchoalveolar lavage (BALF), blood and lung histology was used to elucidate aim 2. These *in vivo* results revealed significant eosinophil mediated reduction in viral load following RSV primary infection of IL-5 Tg and IL-5 Tg Rag $2^{-/-}$ BALB/c. However, following the RSV re-infection, eosinophils in addition to NK cells and type 2 innate lymphoid cells (ILC) were unable to provide the same antiviral protection in both, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice. Results of $CD4^{+}$ cell adoptive transfer demonstrated a significant reduction of viral load recovered from IL-5 Tg Rag $2^{-/-}$ BALB/c mice re-infected with RSV.

Lastly, the antiviral potential of 53 HS mimetics was tested *in vitro* under four clinically relevant conditions. Following the *in vitro* findings, six HS mimetics were tested *in vivo* as a post RSV infection treatment and as a prophylactic treatment of RSV infection. Results revealed the first HS mimetic study examining and reporting on 53 HS mimetics RSV antiviral activity *in vitro*

followed by HS mimetics effect on viral load reduction *in vivo*. Moreover, HS 228 was found to be the best performing compound, both, as post RSV infection HS treatment and prophylactic treatment of RSV infection, while reducing RSV disease pathology *in vivo*.

Taken together, the findings of this thesis identify that RSV induced eosinophil activation results in targeted eosinophil response *in vitro* and *in vivo;* however, that eosinophil recruitment and activation following RSV re-infection appears to be dependent on stimulation by RSV-specific memory Th2 cells and/or sufficient cellular response involving NK cells and/or type 2 ILCs. In addition, exhaustive HS mimetics analysis has resulted in a comprehensive list of potential antiviral compounds that could be targeted for RSV therapeutic development. Furthermore, HS mimetics tested were found to activate an immune response even in the absence of viral infection, suggesting their potential in pre-emptive immune activation and modulation.

ABBREVIATIONS

6HB	six-helix bundle structure
AHR	airway hyperresponsiveness
APC	antigen presenting cell
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BODIPY	boron-dipyrromethene
BSA	bovine serum albumin
C/EBP	CCAAT-enhancer-binding protein
CCR	chemokine receptor
CD	cluster of differentiation
CR	chromotrope
CTAB	cetyl trimethylammonium bromide
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic lymphocyte-associated antigen
CX3CR1	fractalkine receptor
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulphoxide
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
EDTA	ethyenediaminetetraacetic acid
Eosinophilia	eosinophil infiltration
EPO	eosinophil peroxidase
ER	endoplasmic reticulum
F	fusion glycoprotein
FACS	fluorescence activated cell sort
FCS	fetal calf serum
FDA	Food and Drug Administration
FI	formalin-inactivated
G	attachment glycoprotein
GAG	Glycosaminoglycan
GATA	erythroid transcription factors
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBD	heparin-binding domain
HBSS	Hank's Balanced Salt Solution
HEp-2	human epidermoid cancer derived cells
HI	heat inactivated
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPS	histopathological score
HR	heptad repeat
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
HSV	herpes simplex virus

IC	inhibitory concentration
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	Innate lymphoid cells
IRF	interferon regulatory factor
kb	Kilobases
kDa	Kilodaltons
L	large polymerase protein
LMWH	low molecular weight heparins
LT	leukotriene
LTi	lymphoid tissue inducer
M	matrix protein
M2	internal virion protein
MBP	major basic protein
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein 1 alpha
MOI	multiplication of infection
mRNA	messenger RNAs
MTOC	microtubules organising centre
MyD88	myeloid differentiation primary response gene 88
N	nucleocapsid protein
NK	natural killer
NKT	natural killer T cells
NS	non-structural protein (e.g. NS1 or NS2)
OD	optical density
ORF	open reading frame
P	phosphoprotein
PAF	platelet-activating factor
PAS	periodic acid-Schiff
PBS	phosphate buffer solution
pfu	plaque forming units
PG	prostaglandin
PIV	parainfluenza virus
PMA	phorbol myristate acetate
PMD	piecemeal degranulation
PRRs	pattern-recognition receptors
PSN	penicillin-streptomycin-neomycin
PVDF	polyvinylidene difluoride
PVM	pneumonia virus of mice
RANTES	regulated upon activation normal T-cell expressed and secreted
RhoA	ras homolog gene family
RNA	ribonucleic acid
RNAi	ribonucleic acid interference

RNases	ribonucleases
RPMI	Roswell Park Memorial Institute medium
RSV	respiratory syncytial virus
RV	rhinoviruses
SH	small hydrophobic membrane protein
siNS1	small interfering non-structural protein 1
siRNA	small interfering ribonucleic acid
ssRNA	single stranded ribonucleic acid
STAT	signal transducers and activators of transcription
TBS	tris-buffed saline
TBST	tris-buffed saline supplemented with 0.1% Tween-20
TCR	T cell receptor
TEM	transmission electron microscopy
Tg	Transgenic
Th	T helper
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
TRIF	toll-interleukin receptor domain-containing adaptor-inducing interferon
US11	herpes simplex virus phosphoprotein
UV-RSV	ultraviolet-inactivated respiratory syncytial virus
VCAM	vascular cell adhesion molecule
VLA	very-late-antigen
WHO	world health organisation
WT	wild type

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Papers in internationally refereed journals:

Tauro, Sharyn; Su, Yung-Chan; Thomas, Sandra; Schwarze, Yurgen; Matthaei, Klaus I.; Townsend, Dijana; Simson, Ljubov; Tripp, Ralph A.; Mahalingam, Suresh (2008). Molecular and cellular mechanisms in the viral exacerbation of asthma. Microbes and Infection, Vol.10 (9), pp. 1014-1023.

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Chapter 1. General Introduction

1.1 RESPIRATORY SYNCYTIAL VIRUS (RSV)

Respiratory syncytial virus (RSV) is responsible for one of the most important pathogenic infections of childhood and is associated with significant morbidity and mortality (McNamara and Smyth, 2002). In 2005, RSV was found to be responsible for almost 34 million lower respiratory tract infections in children under 5 years of age worldwide, with 10% of infections requiring hospitalisation and as many as 199,000 deaths (Nair *et al.*, 2010). Approximately 99% of these deaths occur in developing countries, reflecting both a greater population of infants and the unjust consequences of inadequate resources. These morbidity and mortality numbers are most likely under-estimated, as RSV testing is generally incomplete and undercounted globally (Nair *et al.*, 2010).

Despite extensive RSV research covering epidemiology, clinical manifestations, diagnostic techniques, animal models and the immunobiology of infection, there is not yet a convincing and safe vaccine available. Numerous approaches to the development of RSV vaccines are being evaluated, as are the use of newer antiviral agents to mitigate disease. More research is urgently required to elucidate the mechanisms of RSV infection and pathogenesis to reduce the worldwide impact of this viral infection.

1.2 RSV DISEASE

1.2.1 History and RSV impact on society

Human RSV was first isolated in 1955 from a captive chimpanzee with upper respiratory tract illness (Morris *et al.*, 1956). It was quickly identified as a human virus and since 1956 RSV has been defined as a major paediatric respiratory pathogen causing serious lower respiratory tract infection in infants and young children worldwide (Chanock and Finberg, 1957; Chanock *et al.*, 1957). RSV also causes disease in adults, particularly in the elderly and in the immunocompromised (Walsh, 2011).

The World Health Organisation (WHO) estimates approximately 4 million deaths each year from infections that are transmitted via the respiratory tract, most of which occur in childhood (WHO, 2004). Viral infections of the respiratory tract are particularly serious during infancy and viral bronchiolitis is

the most common cause of infantile hospitalisation in the developed world (Henrickson *et al.*, 2004). It has been estimated to cause 91,000 hospital admissions per year in the USA alone, with associated hospitalisation costs adding up to $300,000,000 per year. RSV accounts for approximately 70% of all cases of viral bronchiolitis (Openshaw, 2002).

1.2.2 Epidemiology and evolution

RSV is a highly contagious virus and infects an individual multiple times throughout the life, resulting in worldwide epidemics of respiratory disease each year (Graham *et al.*, 2000). Humans are the only natural host for RSV, however the virus has been found to infect and cause severe disease in non-human primates (Kondgen *et al.*, 2008; Morris *et al.*, 1956). RSV occurs most frequently in areas experiencing greater seasonal temperature changes with RSV infections peaking in the winter months and extending into the spring (Arbiza *et al.*, 2005; Bolisetty *et al.*, 2005; Galiano *et al.*, 2005; Kuroiwa *et al.*, 2005; Mufson *et al.*, 1991; Wang *et al.*, 1995; Watson *et al.*, 2006; White *et al.*, 2005).

Circulating human RSV isolates have been classified into two antigenic groups, A and B (Mufson *et al.*, 1985) representing genetic divergence that has occurred approximately 350 years ago (Zlateva *et al.*, 2005). Multiple genotypes within subgroups A and B have been identified to co-circulate within the same season and community, with one or two dominant genotypes being replaced in successive years (Cane, 2001; Johnson *et al.*, 1987a). In addition, there can be shifts in the predominance of subgroup A versus B occurring in 1-2 year cycles (Waris, 1991). This reflects a modest advantage of the heterogeneous strain in evading previously induced immunity (White *et al.*, 2005). As a result, the RSV evolutionary pattern does not resemble that of influenza A virus, in which a strong immune selection dictates the fast replacement worldwide of dominant strains in a linear manner. Instead, RSV evolution resembles that of influenza B viruses, in which a less strong immune selection favours slower co-evolution of several branches of viruses (Rota *et al.*, 1992).

RSV A and B often co-circulate during an outbreak, however, group A is found to be associated with more severe disease (Domachowske and Rosenberg, 1999; McConnochie *et al.*, 1990). A study has shown that among all

infants, RSV strain A was 2.84 times more likely to cause severe disease than group B (Walsh *et al.*, 1997). Most children are infected by RSV during the first year of life and virtually all are infected by the age of two (McNamara and Smyth, 2002).

Re-infection is frequent during the first few years of life and is independent of antigenic differences in RSV isolates. Of the children who had been infected during the first year of life, 47% and 45% were re-infected during the second and third years of their life, respectively (Glezen *et al.*, 1986). Lower respiratory tract infection can occur during the first or second infections; however a considerable reduction in disease severity was observed with subsequent infections indicative of increasing protective immunity (Henderson *et al.*, 1979).

1.2.3 Groups at risk of RSV Infection

The incidence of RSV is greatest at the extremes of age, in infants and elderly (Openshaw and Tregoning, 2005). Children under 1 year of age are the most affected, with peak incidence between 2 to 4 months of age (Openshaw *et al.*, 2003). In children younger than 2 years of age, RSV infection is the most common cause of asthma attacks and is frequently associated with acute wheezing and respiratory distress (Zhao *et al.*, 2002). A number of factors can contribute to the risk of severe RSV disease early in life, including prematurity, low titres of maternal antibodies or lack of previous RSV infection (Cunningham *et al.*, 1991), underlying cardiopulmonary disease (Groothuis *et al.*, 1988; MacDonald *et al.*, 1982) and immunosuppression or immunodeficiency disorders (Fishaut *et al.*, 1980; McIntosh *et al.*, 1984). However, it is important to note that more than half of RSV hospitalisations occur in previously healthy, full term individuals (Falsey, 2007; Falsey *et al.*, 2005).

RSV re-infects healthy adults at a rate of approximately 5-10% per year, a rate that increases with increased exposure to the virus, such as with health care personnel (Falsey, 2007; Falsey *et al.*, 2005). Hospitalisation due to RSV in otherwise healthy non-elderly adults is rare, but RSV is considered second only to seasonal influenza as a cause of medically significant respiratory tract disease in adulthood. Morbidity and mortality due to RSV is substantially increased in the elderly, presumably due in part to immune senescence

(Thompson *et al.*, 2003). In countries that are more affluent, deaths due to RSV are more frequent in the elderly than in the paediatric population, whereas in less affluent countries the paediatric burden is greater.

Another group at risk are immunosuppressed individuals, including patients with an immune defect (e.g. complement deficiency, human immunodeficiency virus (HIV)) and patients who have undergone bone marrow transplantation (e.g. leukaemia patients) (Openshaw and Tregoning, 2005). Morbidity and mortality due to RSV is substantially increased in adults with underlying pulmonary or cardiac disease or immunosuppression (Falsey, 2007; Falsey *et al.*, 2005; Whimbey and Ghosh, 2000). In particular, the mortality rate associated with severe RSV infection in adults with profound immunosuppression due to leukaemia or hematopoietic stem cell transplant can be as high as 80-100% (Whimbey and Ghosh, 2000).

1.2.4 RSV disease and clinical symptoms

RSV is transmitted by respiratory secretions and by direct contact with contaminated surfaces or materials. Viral entry is usually through the nasopharynx and the eyes, thus the most common way of RSV transmission is by hand-to-hand, hand-to-mouth or hand-to-eye contact (Hall, 1983; Hall *et al.*, 1976; Tripp, 2004). Infection occurs in the lung epithelial tissue (Adams *et al.*, 2006; Openshaw, 2002) resulting in upper rather than lower respiratory tract infections (Openshaw, 2002). In addition, another path of transmission is through viral persistence where the host serves as a reservoir for transmission or re-infection. This path of infection contributes to the pathogenesis of asthma and chronic wheezing in children who have experienced bronchiolitis (Mahalingam *et al.*, 2006).

Clinical symptoms of RSV infected young children range from mild respiratory problems to severe cough, bronchiolitis and pneumonia (van den Hoogen *et al.*, 2001). These symptoms are usually followed by high fever, myalgia (muscle pain) and vomiting (van den Hoogen *et al.*, 2001). RSV infection is characterised by a slow, progressive increase in viral protein synthesis, where viral proteins first become detectable by 8 hours post infection and increase to a maximum by 18-24 hours, followed by the peak of virion morphogenesis (Collins *et al.*, 1996). Severe RSV infection has been strongly

associated with childhood asthma and repeated episodes of bronchospastic bronchitis which can continue into adulthood (Rosenberg *et al.*, 2007).

During early months of life, bronchiolitis may cause severe insult to the lungs and can cause long-term effects by delaying or preventing normal postnatal pulmonary changes. Consequently, this viral infection may lead to smaller underdeveloped lungs, which are more susceptible to disease later in life. Alternatively, neonatal infection may cause long-lasting changes in host immunity (Adams *et al.*, 2006).

It is thought that RSV disease arises from both direct viral damage and the host immune response, but the relative contributions of each remain controversial. However, it is generally accepted that there is a positive correlation between the level of virus replication and disease severity (DeVincenzo *et al.*, 2010; DeVincenzo *et al.*, 2005; Karron *et al.*, 1997b; Martin *et al.*, 2008), even though this was not observed in some studies (Bennett *et al.*, 2007; Hall *et al.*, 1976; Wright *et al.*, 2002).

In *in vivo* models, during an infection of several weeks, there was little visible damage to the tissue except that ciliary beating was usually impaired, in contrast to the rapid tissue destruction observed with influenza A virus (Wright *et al.*, 2005; Zhang *et al.*, 2002). This suggests that RSV is not inherently a highly cytopathic virus, although its effect on ciliary function facilitates the airway obstruction, which is characteristic of RSV disease.

RSV has also been shown to exacerbate asthma. Asthma is a chronic, episodic inflammatory disease of the airways where inflammation causes airway hyperreactivity (AHR) associated with narrowing of the small airways (known as bronchioles) and variable airflow response to triggering events such as allergens, viral infections and exposure to airway irritants (Beasley *et al.*, 2001). The asthmatic response is characterised by elevated production of immunoglobulin (Ig) E, cytokines, mucus hypersecretion, airway obstruction, eosinophil infiltration (eosinophilia) and enhanced AHR to spasmogens (Rothenberg, 1998; Zimmermann *et al.*, 2003). Episodes of virus induced exacerbations of asthma are accompanied by increased numbers of eosinophils in respiratory secretions and there is evidence of eosinophil degranulation (Handzel *et al.*, 1998). Eosinophil degranulation in airway tissues is thought to

significantly contribute to asthma pathogenesis, nasal polyposis, allergic rhinitis and eosinophilic pneumonia through the release of cytotoxic proteins (Walsh, 2001). Overexpression of interleukin (IL)-13 has also been found to induce multiple features of asthma, including eosinophilia, mucus overproduction and AHR (Pope *et al.*, 2005). The involvement of eosinophils in RSV infection will be discussed in more detail later on in this literature review.

The incubation period of RSV *in vivo* is typically 2 to 8 days, with virus replication spreading from the upper respiratory tract to the lower respiratory tract (Easton *et al.*, 2004; Wennergren and Kristjansson, 2001). In infants suffering from lower respiratory tract infection, signs of infection usually appear 1-3 days after the onset of rhinorrhoea, representing the viral spread into the bronchi and bronchioles. Clinical recovery from RSV bronchiolitis may occur during the continuous viral shedding from the upper respiratory tract. However, virus shedding stops with the emergence of specific secretory IgA at the time of clinical recovery (Domachowske and Rosenberg, 1999). Prolonged virus shedding from the respiratory tract is observed in immunocompromised individuals, which suggests that functional cell-mediated immunity is very important in the clearance of RSV infection (Domachowske and Rosenberg, 1999).

1.3 RSV VIRUS AND VIRAL GENOME

RSV is an enveloped, non-segmented, negative sense single stranded ribonucleic acid (RNA), cytoplasmic virus (Domachowske and Rosenberg, 1999; Olmsted and Collins, 1989; Spann *et al.*, 2004) with a genome of 15.2 kilobases (kb) in length (Olmsted and Collins, 1989; Teng *et al.*, 2001). The genome is comprised of ten genes which encode for eleven viral proteins (Collins *et al.*, 1996; Collins and Melero, 2011; Teng *et al.*, 2001) (Figure 1.1). These are; the major nucleocapsid (N) protein, phosphoprotein (P), large (L) polymerase protein, the non-glycosylated envelope associated matrix (M) protein, the fusion (F) glycoprotein, the attachment (G) glycoprotein, non-structural (NS1 and NS2) proteins, small hydrophobic (SH) membrane protein and internal virion protein (M2 (M2-1 and M2-2)) (Collins *et al.*, 1996; Olmsted and Collins, 1989). The viral replication cycle *in vitro* is relatively long (30-48 hours) (Collins and Graham, 2008) (Figure 1.2).

Figure 1.1. Genome structure of the Respiratory Syncytial Virus (RSV)

The RSV genome consists of 15,222 nucleotides. RSV ten major genes encode for eleven different viral proteins and these are: non-structural (NS1 and NS2) proteins, nucleocapsid (N) protein, phosphoprotein (P), matrix (M) protein, small hydrophobic (SH) membrane protein, attachment glycoprotein (G), fusion (F) glycoprotein, internal virion protein (M2 (M2-1 and M2-2)) and large (L) polymerase protein. Adapted from (McNamara and Smyth, 2002).

The respiratory syncytial virion is composed of the nucleocapsid enclosed within a lipid envelope (Easton *et al.*, 2004; Fields *et al.*, 1996; Smyth, 2002). The lipid envelope is derived from the plasma membrane of the host cell into which the three virus glycoproteins: F, G and SH, are embedded (Figure 1.2). The F and G proteins contribute to the 10- to 14-nm spikes present on the virion surface (Easton *et al.*, 2004).

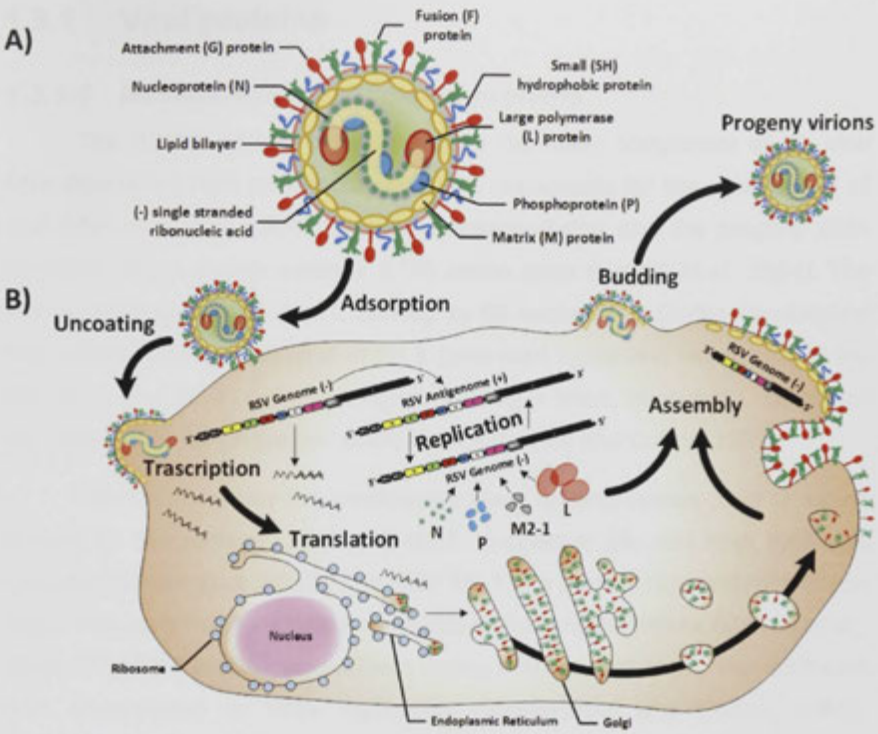


Figure 1.2. Schematic presentation of respiratory syncytial virus (RSV) virion structure and its replication cycle

A) RSV virion. The nucleocapsid of the RSV genome consists of the nucleocapsid protein (N), the phosphoprotein (P) and the large polymerase (L) protein. The nucleocapsid structure is surrounded by the matrix (M) protein, which forms a link between the nucleocapsid and the lipid membrane of the virus particle. Three virus glycoproteins are embedded in the lipid membrane, these are the attachment (G) glycoprotein, the fusion (F) protein and the small hydrophobic (SH) protein. **B) The RSV replication cycle.** The virus enters by direct fusion at the plasma membrane, and releases the encapsulated genome ribonucleic acid (RNA) and RNA dependent RNA polymerase into the cytoplasm. The polymerase uses the genome as a template to produce capped and polyadenylated messenger RNAs (mRNA), which are translated into viral proteins, and encapsulated antigenome and genome RNAs. The resulting encapsulated genomes are assembled with other viral proteins and bud from the plasma membrane to produce progeny virus particles. Adapted from (Bawage *et al.*, 2013; Jianrong and Yu, 2012).

1.3.1 Viral proteins

1.3.1.1 Nucleocapsid-associated proteins

The RSV L protein is thought to be the major component of the viral RNA-dependent RNA polymerase complex, responsible for the synthesis of all viral RNA, including mRNA, replicative intermediates and the progeny RNA genomes. The L protein contains 2,165 amino acids (Easton *et al.*, 2004). The last two RSV genes, M2 and L, overlap by 68 nucleotides. During transcription this overlap results in initiation at the L gene-start signal and termination at the M2 (M2-1 and M2-2) gene-end signal, yielding a short, truncated L mRNA as the major transcription product of the L gene (Fearns and Collins, 1999).

The M2 gene has two overlapping open reading frames (ORFs), which encode for two proteins, M2-1 and M2-2, involved in the viral RNA synthesis process (Blondot *et al.*, 2012). The RSV M2-1 is a transcription anti-termination factor important for the efficient synthesis of full-length mRNAs (Collins *et al.*, 1996). The M2-2 protein accumulates during infection and shifts RNA synthesis from transcription to RNA replication (Bermingham and Collins, 1999). Therefore, the M2-1 protein is essential for full viral transcription, while the M2-2 protein is responsible for viral replication (Cheng *et al.*, 2005).

The P protein is a key component of the viral RNA-dependent RNA polymerase complex (Lu *et al.*, 2002). Interaction of the RSV P protein with the N, L and M2-1 proteins promotes the formation of a transcriptase complex that is essential for viral RNA transcription and replication (Asenjo *et al.*, 2006; Garcia-Barreno *et al.*, 1996; Khattar *et al.*, 2001). As it has been determined with other paramyxovirus P proteins, the P protein of RSV likely acts as a cofactor that serves both to stabilise the L protein and to place the polymerase complex on the N protein RNA template (Horikami *et al.*, 1992). An *in vitro* study has also shown that P protein phosphorylation affects RSV budding and replication (Lu *et al.*, 2002). Another study found that interactions of P with M2-1 and RNA with M2-1 are required for efficient transcription activation by M2-1. M2-1 association with P is strictly required for recruitment to the viral RNA synthesis site (Blondot *et al.*, 2012).

The N protein forms an integral part of the nucleocapsid complex of the virion and is an essential component of the polymerase complex. It plays a

critical role in folding the genome into a semi-stable RNA helix. Recent studies have revealed novel ways that RSV prevents activation of cellular defences in response to infection. Studies have revealed that the RSV N protein uses multiple novel methods to inhibit induction and signalling of pro-inflammatory cytokines, the induction of apoptosis, and other aspects of cellular defence against infection (Groskreutz *et al.*, 2010; Valarcher *et al.*, 2003).

1.3.1.2 Non-structural proteins

The NS1 and NS2 proteins are relatively small, with the NS1 and NS2 proteins being 139 and 124 amino acids in length, respectively (Easton *et al.*, 2004). Deletion of the genes coding for NS1 and NS2, results in reduced viral replication in immune cells. These proteins play a very important role in attenuating host immune response. The NS1 and NS2 also activate pro-survival pathways in the infected cell, prolonging its survival and increasing viral yield (Bitko *et al.*, 2007).

1.3.1.3 Matrix protein

The viral nucleocapsid is surrounded by the M protein. Recent study, with other members of the *Paramyxoviridae* family, has shown that M protein at the later stages of infection is predominantly accumulated in the cytoplasm where it is able to bind to viral RNA. This finding suggests that the M protein may be capable of silencing viral RNA synthesis in preparation for packaging (Ghildyal *et al.*, 2003) as well as inhibition of the nuclear gene transcription (Ghildyal *et al.*, 2005). As previously mentioned the N, P and L proteins were also found to be necessary for RNA replication (Collins *et al.*, 1996).

1.3.1.4 Viral envelope proteins

The SH protein is a small integral membrane protein. The SH protein of RSV is the smallest of all *Pneumoviruses*, with a length of 64 amino acids (Easton *et al.*, 2004; Teng *et al.*, 2001). The function of the SH protein in RSV infection is unclear, however, it has been suggested that its role may be in evading the host's immune system or in providing an ancillary role in virus-mediated cell fusion (Rixon *et al.*, 2005). A study has shown that in the absence of G protein, the SH gene has enhanced the rate of virion entry, cell-to-cell fusion and plaque size (Techaarpornkul *et al.*, 2001). The SH protein shares structural features with a class of small hydrophobic proteins, the viroporins

(Gonzalez and Carrasco, 2003), that insert themselves into the membrane of infected cells and induce permeability to ions and small molecules. In line with this hypothesis, SH increases membrane permeability when expressed in bacteria (Perez *et al.*, 1997) and when incorporated into artificial membranes it forms pentameric and hexameric pore-like structures with cation channel-like activity (Carter *et al.*, 2010; Gan *et al.*, 2008).

The G protein is an attachment protein responsible for binding of RSV to the cell (Karron *et al.*, 1997a). The G protein is heavily glycosylated and helps the virus to evade host immune response by preventing the virus from being recognised as a foreign antigen (Domachowske and Rosenberg, 1999). An un-glycosylated region in the centre of the protein contains four cysteines held together by disulfide bonds in a cysteine noose (Gorman *et al.*, 1997; Johnson *et al.*, 1987b; Langedijk *et al.*, 1996), followed by a heparin-binding domain (HBD) (Feldman *et al.*, 2000; Feldman *et al.*, 1999). While in the endoplasmic reticulum (ER), RSV G protein is modified by the addition of multiple N-linked carbohydrate chains resulting in an increase in the molecular mass of G protein from 32 kilodaltons (kDa) to 60kDa. Maturation of the N-linked carbohydrates of the G protein occurs in the Golgi compartment, where a large number of O-linked carbohydrate chains are added, resulting in an 84-92kDa mature protein (Fernie *et al.*, 1985; Lambert, 1988; Levine *et al.*, 1987; Wertz *et al.*, 1985). The size variation of the G protein is presumed to be, in part, due to the difficulty in sizing heavily glycosylated molecules and variations in molecular mass markers. It is possible that a sheath of host-specified carbohydrate helps shield the G protein from immune recognition. Whether due to carbohydrates, its unfolded structure, or some other reason, G is a less efficient neutralisation and protective antigen compared to F (Olmsted *et al.*, 1986), and most individual monoclonal antibodies against G do not neutralise infectivity (Martinez *et al.*, 1997). The N- and O-linked glycosylation sites are clustered in two regions of the G protein, with amino acid content reminiscent of mucins. These two mucin-like domains in G are highly variable and contain multiple epitopes that are poorly conserved between strains. These two domains are separated by a central region to which conserved epitopes have been mapped (Martinez *et al.*, 1997). This conserved region includes a segment of 13 highly conserved amino acids that overlaps with a segment containing four closely spaced, invariant cysteine residues that are disulfide-bonded to form a cysteine noose (Gorman

et al., 1997; Johnson *et al.*, 1987b). The downstream pair of cysteine residues conform to a CX3C motif that is embedded to the CXC3 chemokine fractalkine sequence (Tripp *et al.*, 2001).

Studies in the mouse model comparing wild type with a mutant RSV lacking the CX3C motif revealed that fractalkine mimicry reduces the pulmonary influx of immune cells involved in innate and adaptive responses to RSV infection (Harcourt *et al.*, 2006; Tripp *et al.*, 2000; Tripp *et al.*, 2001). In addition, the cysteine-rich domain of G was shown to inhibit activation of toll-like receptor (TLR)-2, 4, and 9 in human monocytes, thus suppressing innate immune response (Polack *et al.*, 2005). In addition to these immune evasion strategies G protein in its secreted form, functions as an antigen decoy to help the virus escape neutralising antibodies and reducing antibody-mediated clearance by immune cells (Bukreyev *et al.*, 2008). Taken together these immune evasion strategies benefit not only virus replication but also viral persistence (Li *et al.*, 2006).

The F protein fuses the viral envelope with the host membrane, allowing the entrance of the viral nucleocapsid into the cell cytoplasm. It is also responsible for cell-to-cell fusion and the formation of syncytia (Walsh and Hruska, 1983). Syncytia are a mass of cytoplasm within a cell membrane, containing multiple nuclei and resulting from cell-to-cell fusion. Fusion of the virus to the cell membrane and formation of syncytia, are characteristic cytopathic effect of RSV, and are mediated by the viral F protein. It is still unclear what advantage RSV gains from forming syncytia through cell-to-cell fusion. However, it is believed that RSV may use syncytium formation to enable quick spread to neighbouring cells and/or to evade host defence mechanisms (Gower *et al.*, 2005). While penetrating through the cell membrane, RSV causes changes in the surface morphology of infected cells (Krzyzaniak *et al.*, 2013). As a result, the cell surface is covered with filamentous protrusions, which are coated with the viral envelope proteins F and G, suggesting a potential role for both proteins in forming cell-to-cell contact and syncytium formation (Gower *et al.*, 2005; Yao and Compans, 2000). The RSV F protein is necessary for infection, as mutant RSV lacking F protein cannot infect cells on its own but rather requires a helper virus to gain entry into cells (Batonick *et al.*, 2008). The F protein is post-synthetically cleaved and has other structural similarities to the

F proteins of other paramyxoviruses. In addition, some monoclonal antibodies directed to the F protein inhibit syncytium formation (Walsh and Hruska, 1983), confirming its role in fusion. The RSV-F protein has been shown to interact with intercellular adhesion molecule (ICAM)-1 expressed on the cell surface. Although it has not been definitively shown to be essential in viral fusion, ICAM-1 has been reported to bind the F protein and as such may still have a role in fusion (Behera *et al.*, 2001).

1.3.2 RSV receptors

RSV binding and entry is hypothesised to be a two-step process: the first involving the attachment of the virus to the cell membrane, which may be enhanced by electrostatic interactions with cellular glycoproteins/heparin and the viral G protein, and the second involving fusion to the cell membrane mediated by the viral F protein and a specific cellular fusion receptor (Tayyari *et al.*, 2011). Proposed RSV receptors include ICAM-1 (Behera *et al.*, 2001), heparin (Krusat and Streckert, 1997), annexin II (Krusat and Streckert, 1997), TLR-4 (Marr and Turvey, 2012) and fractalkine receptor, CX3CR1 (Harcourt *et al.*, 2006).

Binding of viral G protein in an electrostatic fashion to the cell surface may be the first step in efficient viral attachment prior to fusion via nucleolin. First described in 1973, nucleolin is a multifunctional protein found throughout the cell and primarily localised within the nucleolus (Bugler *et al.*, 1982; Orrick *et al.*, 1973). Nucleolin is involved in diverse biological processes including cell proliferation, growth, cytokinesis, replication, embryogenesis and nucleogenesis and is considered necessary for cell survival and proliferation (Srivastava and Pollard, 1999). Although nucleolin is typically thought of first and foremost as an intranuclear protein (Tajrishi *et al.*, 2011), there is abundant evidence that it can also be found within the cytoplasm and on the cell surface and may play the role of a "molecular shuttle" between these compartments (Hovanessian *et al.*, 2010; Srivastava and Pollard, 1999). The actin cytoskeleton modulates the entry of substances via nucleolin into the cytoplasm (Hovanessian *et al.*, 2010). Nucleolin also plays a role in viral replication and intracellular trafficking of viral components. For example nucleolin is required for herpes simplex virus (HSV) 1 DNA replication (Calle *et al.*, 2008) and also for trafficking of the herpes simplex

virus US11 protein (small HSV phosphoprotein) out of the nucleus (Greco *et al.*, 2012).

Viruses initially activate the innate immune system, which recognises viral components such as genomic DNA, single-stranded RNA and viral proteins through pattern-recognition receptors (PRRs) (Diebold *et al.*, 2004; Heil *et al.*, 2004). One group of PPRs involved in RSV infection are TLRs that are important for the production of type I interferons (IFNs) (Edelmann *et al.*, 2004; Ishii *et al.*, 2006; Stetson and Medzhitov, 2006). Detection of viral components by TLRs in immune cells activates intracellular signalling cascades result which in turn in secretion of type I IFNs, proinflammatory chemokines and increased expression of costimulatory molecules such as CD (cluster of differentiation) 40, CD80 and CD86 (Hochrein *et al.*, 2004; Hoebe *et al.*, 2003; Honda *et al.*, 2003). TLRs can be divided into subfamilies primarily recognising related pathogen-associated molecular patterns; TLR-1, TLR-2, TLR-4, and TLR-6 recognise lipids, whereas TLR-3, TLR-7, TLR-8, and TLR-9 recognise nucleic acids (Akira *et al.*, 2006; Latz *et al.*, 2007). Intriguingly, some TLRs are endowed with the capacity to recognise structurally and biochemically unrelated ligands, as exemplified by the ability of TLR-4 to recognise such divergent structures such as liposaccharide, the fusion protein of RSV (Akira *et al.*, 2006). The most important cell types expressing TLRs are antigen presenting cells (APCs), including macrophages, dendritic cells (DCs) and B lymphocytes (Janssens and Beyaert, 2003). Recently, transcripts encoding TLR-1, TLR-4, TLR-7, TLR-9, and TLR-10, all of which coordinate innate and acquired immune responses, were shown to be expressed constitutively by eosinophils (Nagase *et al.*, 2003) which as previously mentioned play very important role in RSV infection. Although TLR-3 and TLR-7 both recognise viral RNA, TLR-3 uses toll-interleukin receptor domain-containing adaptor-inducing IFN-β (TRIF) and does not require myeloid differentiation primary response gene 88 (MyD88), while TLR-7 signalling is MyD88 dependent (Diebold *et al.*, 2004). These differences in adaptor usage, along with the activation of different IFN regulatory factors (IRFs), are proposed to provide specificity in functional outcome (O'Neill *et al.*, 2003). In addition, the molecular characterisation of eosinophils supports the concept that these cells may contribute to the regulation of both, the innate and the adaptive immunity. As there is only limited direct evidence demonstrating eosinophil functional role in controlling of viral infection (Domachowske *et al.*,

1998a; Rosenberg and Domachowske, 2001), more research is required to elucidate the role of eosinophils in RSV infection.

Feldman *et al.* (2000) have reported in their study that *cp*-52, the RSV mutant lacking its SH and G genes, binds to glycosaminoglycan (GAGs) on the cell surface of the target cell in a manner similar to wild type RSV (Feldman *et al.*, 2000). Furthermore, they found that the RSV F protein, like its G protein, binds to heparin-agarose, though the relative affinities were not explored. These results suggest that in the absence of the G protein, the F protein may be able to attach the virion to cellular GAGs initiating viral infection, highlighting the importance of heparan sulfate (HS) as an RSV receptor enabling RSV propagation. Li *et al.* (2006) have also shown that RSV is capable of infecting neuronal cells in the lungs of mice, however the RSV infection was reduced in the absence of the RSV G protein and the G protein CX3C motif (Li *et al.*, 2006).

1.3.2.1 Viral interaction with GAGs

1.3.2.1.1 Proteoglycans and GAGs

Proteoglycans (PGs) are important components in the extracellular matrix (ECM) and on the cell surface. They are composed of a core protein with one or more covalently O-linked GAG chain(s) (Bernfield *et al.*, 1999; Kjellen and Lindahl, 1991). GAGs are linear polysaccharide composed of repeating disaccharide units (Yamada and Kawasaki, 2005). The GAGs are divided into four classes: 1. HS/heparin; 2. Chondroitin sulfate (CS) and dermatan sulfate (DS); 3. Keratan sulfate (KS); 4. Hyaluronan (Figure 1.3) (Kjellen and Lindahl, 1991).

HS and heparin are composed of repeating disaccharide units of hexuronic acid (HexA) and glucosamine (GlcN) in -HexA/1,4-GlcN1,4- structure with sulfation at various positions (Lindahl and Li, 2009). Heparin, an intracellular GAG, is synthesised and stored in mast cells. Heparin shares high similarity with HS in structure, but is more sulfated. The biological functions of heparin in mast cells are not entirely clear, however it is known that heparin is crucial for expression and storage of histamine, proteases and other inflammatory mediators within mast cells granules (Kolset and Zernichow, 2008). HS is generally attached to core proteins, forming heparan sulfate

proteoglycan (HSPG). In this form, HS binds to a variety of protein ligands regulating a wide variety of biological processes including angiogenesis, blood coagulation, tumour metastasis and viral attachment (Jones *et al.*, 2005; Nangia-Makker *et al.*, 2000).

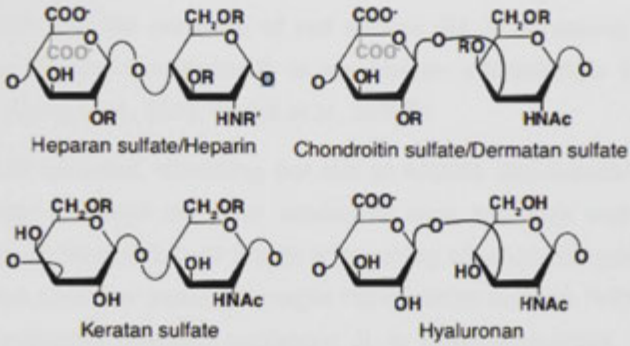


Figure 1.3. Disaccharide structure of glycoaminoglycans

R represents H or SO^{3-}, R' represents H, $COCH_3$ or SO^{3-}; GlcA of heparan sulfate/heparin and chondroitin sulfate can be epimerised to IdoA (in grey) (Kjellen and Lindahl, 1991).

GAGs found in the intracellular vesicles and on the outer face of the plasma membrane, are found to act as virus receptors. Several studies have shown that some bacteria and viruses use GAGs, particularly HS for attachment to, and entry into, cultured immortalised cells (Baldassarri *et al.*, 2005; Bousarghin *et al.*, 2005; Jones *et al.*, 2005; Rue and Ryan, 2002).

A number of viruses have demonstrated a great affinity for cell HSPGs, playing important roles in virus attachment and entry (Lee *et al.*, 2006). HS has been shown to bind the RSV G protein (Krusat and Streckert, 1997) on the cell surface of the human epidermoid cancer derived (HEp-2) cells, however it turns out that human airway epithelium does not express HS on the apical surface, the site of RSV attachment and cellular entry (Duan *et al.*, 1998). Therefore, the binding to heparin by the G protein may simply serve to demonstrate that the G protein has a general affinity for negatively charged carbohydrates on the cell surface (Guo *et al.*, 2008). Peptides from a conserved region of the G protein bind to heparin and to target cells, partially inhibiting viral infection (Feldman *et al.*, 2000). Furthermore, the G protein has been shown to bind immobilised

heparin (Krusat and Streckert, 1997). These findings further support the idea that the G protein is the RSV attachment protein and point to cell surface GAGs as the ligand. RSV infection is mediated partly by an initial interaction between attachment glycoprotein and highly sulfated heparin-like GAGs located on the cell surface (Crim *et al.*, 2007; Techaarpornkul *et al.*, 2002). Also, it has been suggested that the presence of cell surface GAGs containing iduronic acid, such as HS and chondroitin B, is required for efficient RSV infection in cell culture (Dong *et al.*, 2013; Hallak *et al.*, 2000b).

HS mimetics, mimicking the role of heparin and heparan sulfate, have the ability to inhibit the virus binding to cells and their antiviral efficiency depends on their molecular weight and number of sulphated groups present. A study has used low molecular weight HS mimetics such as PI-88, a mixture of highly sulphated mannose containing di- to hexa-saccharides, to inhibit HSV infection of cells and cell-to-cell spread (Nyberg *et al.*, 2004). Heparin, which is approximately six times larger than PI-88, appeared to be a better inhibitor of HSV infectivity, whilst PI-88 performed more efficiently in reduction of cell-to-cell spread of the virus (Nyberg *et al.*, 2004). This is indicative of the importance that the size of the HS mimetics compound has on the inhibition of intracellular transmission of HSV. PI-88 appears to inhibit HSV infection of cells by blocking the binding of the viral attachment glycoproteins to the cell surface (Nyberg *et al.*, 2004).

RSV interactions with cellular GAGs, *cp*-52 in particular, share similarities with other viruses, including alphaviruses (Byrnes and Griffin, 1998), flaviviruses (Chen *et al.*, 1997), and herpes viruses (Neyts *et al.*, 1992). Therefore, consideration of the model that was proposed for dengue virus binding to cells by Putnak *et al.* (1997) is appropriate (Putnak *et al.*, 1997). This model suggests that the initial interaction of the dengue virus E glycoprotein with cell surface GAGs provokes a conformational change allowing E glycoprotein to interact with a putative high-affinity receptor, triggering endocytosis. In a similar scenario, RSV-F interaction with cellular GAGs could result in a conformational change that exposes the fusion peptide or allows RSV-F to interact with a putative high-affinity receptor required for fusion. RhoA is a small GTPase of the Ras superfamily that has been shown to interact with RSV-F both *in vitro* and *in vivo* (Pastey *et al.*, 1999). Another study has shown

that RhoA facilitates virus induced syncytium formation; however, whether RhoA is expressed on the cell surface and serves as the RSV receptor, remains unclear. Also, their preliminary findings suggest that an RSV mutant lacking G-protein can still infect CHO*pgsA-745* cells, which are 99% GAG deficient, indicating that RSV F protein alone can infect cells by interacting with cellular membrane components other than GAGs (Feldman *et al.*, 2000).

In addition, dengue virus, a human pathogen that has re-emerged as an increasingly important public health threat, has been found to use HS for viral attachment (Chen *et al.*, 1997). Heparin and the polysulfonate pharmaceutical suramin have been found to effectively prevent dengue virus infection of target cells, indicating that the envelope protein-target cell receptor interaction is a critical determinant of infectivity (Chen *et al.*, 1997).

Developing pharmaceuticals that inhibit target cell binding to the virus may also be an effective strategy for the treatment of RSV infection. These could harness the similarities observed in the cell binding mechanisms between dengue virus and RSV, specifically the interactions with cellular GAGs (Chen *et al.*, 1997).

1.4 IMMUNE RESPONSE TO RSV INFECTION

Viral detection, clearance and the process of recovery from viral respiratory tract infection (as well as resistance to re-infection) is mediated by both the innate and adaptive host immune responses with cellular and humoral immune responses, acting directly to orchestrate viral clearance (Braciale *et al.*, 2012; Graham and Braciale, 1997). In animal models of RSV infection, both cytotoxic T cells and antibody responses play a pivotal role in RSV clearance from the lung (Braciale *et al.*, 2012; Yoo *et al.*, 2013).

1.4.1 Adaptive immunity

1.4.1.1 Cell-mediated immunity and T cell deficiency

Cell-mediated immune response plays an important role in RSV clearance (Domachowske and Rosenberg, 1999). It is classified according to the expression of lymphocyte surface antigens into, $CD8^+$ cytotoxic T lymphocytes (CTL) and $CD4^+$ T helper (Th) cells. Both types of cells possess antiviral and immunogenic capabilities.

$CD4^+$ Th cells are subdivided into Th1, Th2 and Th17 lymphocytes based on their cytokine production profile. The Th1 subset is characterised by the production of IFN-γ, IL-2, IL-12 and tumour necrosis factor alpha (TNF-α), while Th2-cells are characterised by the production of IL-4, IL-5, IL-10 and IL-13 (McNamara and Smyth, 2002). In the murine model, induction of different $CD4^+$ T cell responses may be dependent on the RSV antigen. It has been found that when mice are primed with vaccinia expressing F protein, a strong Th1 CTL response is induced (Graham *et al.*, 2000). However, when mice are immunised with RSV G protein, a Th2 response is generated which is associated with eosinophilic infiltration into the lung following subsequent RSV challenge resulting in increased clinical disease symptoms (Graham *et al.*, 2000). The third subset of $CD4^+$ Th cells, Th17, differs from the other two subsets in terms of their requirements for differentiation and expansion factors and in their target pathogens. Th17 lymphocyte are characterised by production of IL-17, which is known to induce mucus production in the respiratory tract and increase the expression of polymeric Ig receptors that facilitate the release of IgA and IgM antibodies into the respiratory tract (Jaffar *et al.*, 2009). Of the other cytokines produced by Th17 lymphocytes, IL-21 promotes Th17 proliferation and antibody production by B lymphocytes (Mitsdoerffer *et al.*, 2010). Th17 lymphocytes are present in the respiratory tract and there is evidence that they play a key role in responses to fungal infections. These cells, however, also contribute to inflammatory disorders that afflict the respiratory tract, such as asthma and chronic obstructive pulmonary disease (COPD). Increased production of the Th17-related cytokines, such as IL-17A, IL-22 and IL-23 in COPD patients reflects the involvement of Th17 lymphocytes in initiating and driving the disease process (Di Stefano *et al.*, 2009; Vargas-Rojas *et al.*, 2011). In addition, excess IL-17 production has been reported in animal models and human patients has been associated with neutrophil dominated asthma and with cortisone-resistant severe AHR (Wilson *et al.*, 2009; Zhao *et al.*, 2010). Th17 lymphocytes have also been implicated in effector mechanisms triggered in response to RSV and other types of respiratory viral infections (Faber *et al.*, 2012; Mukherjee *et al.*, 2011). Mukherjee *et al.* (2011) study found that blocking of IL-17 significantly decreased viral load and altered cytotoxic CD8 T-cell marker expression (Mukherjee *et al.*, 2011).

$CD8^+$ T cells play a critical role in mediating RSV clearance. However, as RSV is a leading cause of severe virus induced respiratory disease in individuals over the age of 65, it is important to note that immune response varies depending on age. While it is clear that T cell immunity declines with age, it is not clear to what extent the $CD8^+$ T cell response to RSV is altered. A study has found a decrease in the capacity of aged mice to induce a high magnitude acute $CD8^+$ T cell response, resulting in prolonged viral replication. This would help explain the increased disease severity of RSV infection observed for aged individuals (Fulton *et al.*, 2013). RSV-specific $CD8^+$ lymphocytes are important in recovery and pathogenesis of RSV disease, however, a too vigorous response can be harmful (McNamara and Smyth, 2002). Therefore, the key to effective immunity is in T cells regulation (Braciale *et al.*, 2012; Graham and Braciale, 1997).

In a study of RSV-infected infants, no correlation was found between clinical disease parameters and the level of the virus specific CTL response in bronchoalveolar lavage (BAL) fluid or blood (Heidema *et al.*, 2007). Moreover, only low numbers of T cells were detected in postmortem lung tissues of children with lethal RSV infections (Johnson *et al.*, 2007; Welliver *et al.*, 2007). In addition individuals with compromised T cell immunity can shed virus for months (Hall *et al.*, 1986) with prolonged virus shedding also observed in nude or irradiated BALB/c mice (Cannon *et al.*, 1987) and in mice depleted of both $CD4^+$ and $CD8^+$ cells (Graham *et al.*, 1991). In wild type mice, an RSV-specific $CD8^+$ T cell response provides protection against infection, but the effect is short-lived (Connors *et al.*, 1991; Kulkarni *et al.*, 1995). Experiments in the RSV mouse model have shown that T cells are necessary and sufficient for virus elimination and that T cell mediated immunopathology contributes significantly to the disease (Cannon *et al.*, 1988; Graham *et al.*, 1991; Ostler *et al.*, 2002). However, RSV is not a natural pathogen of mice, and only a limited respiratory infection can be established after intranasal inoculation of virus at high titres ($>10^5$ plaque forming units (pfu)) (Graham *et al.*, 1988). The significance of these findings for human RSV infection is therefore unclear.

Infection of mice with pneumonia virus of mice (PVM), a virus of the same genus as RSV, is increasingly used as a natural host experimental model for human RSV infection (Easton *et al.*, 2004). PVM replicates to high titres in

the mouse lung, causing a rapid onset of severe, and eventually fatal, granulocytic bronchiolitis at doses as low as 10^2 pfu (Domachowske *et al.*, 2000a; Domachowske *et al.*, 2002). Supporting a similarity in clinical presentation between RSV and PVM is evident in functional CTL inactivation observed in mice after PVM challenge (Bonville *et al.*, 2003; Claassen *et al.*, 2005; Domachowske *et al.*, 2001). A study using T cell deficient mice challenged with PVM showed that mice became chronic virus carriers in the absence of acute disease, illustrating the dual role of T cells for virus control and immunopathology (Frey *et al.*, 2008). Controlled variation of host and viral parameters showed a tight balance between beneficial and detrimental effects of T cells but also revealed pathways of disease that appeared to be T cell independent following high dose PVM infection (Frey *et al.*, 2008). Earlier studies found that the innate immune response to PVM in C57BL/6 mice (Easton *et al.*, 2004) resulted in a very poor pulmonary lymphocyte responses (Domachowske *et al.*, 2000b) while in contrast analysis of BALB/c mice at later time points following infection showed significant $CD8^+$ T-cell infiltrates (Claassen *et al.*, 2005). This data indicates a potential PVM mediated functional inactivation of T cells as well varying responses depending on a genetic makeup.

Analysis of the function of PVM specific T cells after re-stimulation with PVM infected APCs has resulted in a pulmonary T cell response to PVM that is comparable to other murine respiratory virus infections, including RSV (Ostler *et al.*, 2001). This supports observations that functional silencing of T cells in the alveolar space may not be to impair virus control but represents a physiological mechanism to limit pulmonary inflammation rather than a viral escape strategy (Ostler and Ehl, 2002; Vallbracht *et al.*, 2006). Frey *et al.* (2008) also showed that T cell deficient or T cell depleted mice could not eliminate PVM15 during an observation period of 49 days. They found that persisting virus titres in these chronically infected mice were almost 10-fold lower than peak titres, suggesting that T cell independent factors, e.g., components of the innate immune response, can provide some level of virus control but fail to eliminate the pathogen (Frey *et al.*, 2008). These results mirror previous findings in human and murine RSV infection in that infants with congenital T cell deficiencies fail to eliminate RSV (El Saleeby *et al.*, 2004; Fishaut *et al.*, 1980; Hall *et al.*, 1986), and depletion of T cells leads to persistent infection in BALB/c mice (Graham *et*

al., 1991). Adoptive transfer experiments have shown that T cells are necessary and sufficient to clear RSV from infected mice (Cannon *et al.*, 1988; Cannon *et al.*, 1987; Ostler *et al.*, 2002). Similar to the RSV model (Graham *et al.*, 1991), both $CD4^+$ and $CD8^+$ T cells contributed to the clearance of PVM from the lung and it would be important to assess if this applies to human model as well. Limitations of the cell mediated immune response to RSV seem to be crucial to RSV evasion tactics of the immune system, short lived immunological memory and partial viral clearance. Further research into RSV infection using T cell deficient model is required to assess the degree of RSV inhibition of the adaptive immunity as well as if enabling of the enhanced innate immune response is able to compensate for the absence of adaptive immune components.

1.4.1.2 Humoral immunity

Humoral immunity is mediated by B lymphocytes, which are responsible for antibody production. The presence of maternal IgA antibodies, within colostrum, provides limited protection against RSV in breastfed infants (McNamara and Smyth, 2002). The amount of antibodies in the newborn is similar to maternal levels and declines slowly during the first month of life. Serum and secretory antibodies are produced in response to RSV infection however, the total amount of antibodies produced in infants is very low (Yamazaki *et al.*, 1994). Antibody responses in young infants are often ineffective at neutralising virus as well as short lived compared to older individuals (Welliver *et al.*, 1980). Immunologic immaturity contributes to limited innate and adaptive responses observed early on in life (Adkins *et al.*, 2004; Levy, 2007). For example, the B cell response to RSV infection of young infants, which are less than 3 months of age, was found to have a biased antibody gene repertoire. Furthermore, it also expressed significantly reduced frequency of somatic mutations, limiting the host capacity to respond to new foreign antigens. These findings demonstrate how immature immunity contributes to the poor neutralising activity characteristic of responses in young infants (Williams *et al.*, 2009). Furthermore, the immunosuppression by RSV specific maternal serum antibodies, present in young infants, have been shown to suppress both serum and secretory antibody responses to RSV infection, but did not suppress the cell mediated response or priming for a secondary

antibody response (Crowe *et al.*, 2001; Murphy *et al.*, 1988). In contrast, a recent study examined a cohort of infants of less than 6 months of age with very low titres of RSV specific maternal antibodies and found that the titres of RSV neutralising serum antibodies, induced by primary RSV infection, were indistinguishable from those of individuals of 6-24 months of age (Shinoff *et al.*, 2008). This showed that, when the immunosuppressive effect of maternal antibodies is minimal, wild type RSV could induce a substantial antibody response even in young infants.

1.4.2 Innate immune response

The innate immunity is non-specific and provides the first line of defence against infection (Goldsby *et al.*, 2003). The innate immune response recruits effector molecules and phagocytic cells rapidly to the site of infection through the release of cytokines and chemokines, however, it lacks immunological memory (McNamara and Smyth, 2002). In the lung, pulmonary surfactant is the first line of defence producing a surface active complex of lipids and proteins that lines the alveolar surface. However, it has been shown that the concentration of surfactant proteins (A, B and D) is significantly reduced in severe RSV infection (Kerr and Paton, 1999). Surfactant protein A mediates processes such as opsonisation and complement activation. *In vitro,* surfactant protein A has been shown to neutralise RSV by binding to the F protein (McNamara and Smyth, 2002).

In RSV infection, the most important cells of the innate immune response are neutrophils, macrophages, DCs, eosinophils and natural killer (NK) cells. Literature suggests that DCs, as the primary APCs, are the first to encounter RSV and are likely to carry viral antigen from the respiratory tract to lymph nodes where DCs encounter and activate naïve virus specific T cells (Braciale, 2005). Macrophages and neutrophils are phagocytic cells whose main function is to engulf and destroy viruses and other foreign pathogens (Message and Johnston, 2001). Macrophages play an important role in controlling the immune response to viral infection. Both macrophages and epithelial cells encounter RSV in the airways (Kimpen, 2001b). Recent studies in mice showed that macrophages provide an immediate response of proinflammatory cytokines following RSV infection (Pribul *et al.*, 2008), and are a major producer of type I IFN (Kumagai *et al.*, 2007). Macrophages appear to be important both in

restricting the virus and in clearing debris that otherwise can promote further damage and inflammation (Reed *et al.*, 2008). During RSV infection, macrophages secrete cytokines (e.g. IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α) which further upregulate the immune response, increasing vascular permeability and recruiting and activating lymphocytes, neutrophils, natural killer cells and eosinophils to the site of infection (McNamara and Smyth, 2002). These activated cells have been found to secrete a number of chemokines whose primary function is the movement of cells within tissue and across endothelial barriers. Chemokines include monocyte chemoattractant protein (MCP)-3, MCP-4, regulated upon activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein 1α (MIP-1α) and eotaxin (Foster *et al.*, 1996).

Neutrophils are the predominant airway leukocytes in RSV bronchiolitis and they are activated in the presence of infection. A recent study of infants hospitalised for severe RSV disease showed that the appearance of neutrophil precursors in the peripheral blood, which precedes their influx into the lungs, closely followed the peak of virus shedding and was coincident with clinical symptoms, implying possible roles both in protection and disease (Lukens *et al.*, 2010).

Up until the last decade, NK cells were unique in being the only identified innate cell derived from a lymphoid progenitor. Recent developments have now classified NK cells as the earliest identified member of a family of hematopoietic effector cells termed innate lymphoid cells (ILCs) that are dependent on the transcription factor Id2. Currently, ILCs can be broadly classified into three groups: (a) NK cells, (b) the retinoic acid receptor-related orphan receptor γ t (Rorγt)-dependent ILCs (lymphoid tissue inducer (LTi) cells, ILC17, ILC22), and (c) ILC2 cells. These groups have recently been named ILC1, ILC3 and ILC2 respectively. These various ILCs have now been implicated in protection against infectious organisms, organogenesis of lymphoid tissue, tissue remodelling during wound healing and homeostasis in tissue stromal cells.(Cherrier *et al.*, 2012; Hwang and McKenzie, 2013; Moro *et al.*, 2010; Neill *et al.*, 2010; Price *et al.*, 2010; Spits and Di Santo, 2011). Because the key cytokines secreted by some ILCs mirror those of various T helper cell populations, it has been proposed that ILCs may represent the innate

counterparts of T helper lymphocytes, at least in terms of cytokine production (Cherrier *et al.*, 2012; Spits and Di Santo, 2011). NK cells (or ILC1) are recruited locally during the initial phases of virus infection (Hussell and Openshaw, 1998). NK cells accumulate in the lung in the first few days of RSV infection and produce cytokines that activate specific T cells. In addition, NK cells are responsible for most of the early production of IFN-γ, found to be important in preventing the development of lung eosinophilia (Hussel *et al.*, 1997a; Hussell and Openshaw, 1998). The main function of NK cells is to recognise and destroy virus infected cells on the basis of alterations that occur on the surface proteins on the normal cell (Message and Johnston, 2001). The ILC2 cells were independently discovered in 2010 by three separate groups, and were called nuocytes, natural helper cells (NHCs), and innate type 2 helper (Ih2) cells (Moro *et al.*, 2010; Neill *et al.*, 2010; Price *et al.*, 2010). Using a combination of flow cytometry and microarray analyses, ILC2 cells were shown to lack the expression of lineage defining surface markers for T cells, B cells, natural killer T (NKT) cells, DCs, macrophages, neutrophils, eosinophils, mast cells, basophils, and lymphoid tissue inducer (LTi) cells. ILC2 cells share a number of surface and functional similarities (Saenz *et al.*, 2010) and variability of surface expression markers may be attributed to the different tissues these ILC2 cells have been identified in: lungs, intestine, liver and bone marrow (Brickshawana *et al.*, 2011; Chang *et al.*, 2011; Mjosberg *et al.*, 2011; Monticelli *et al.*, 2011; Wong *et al.*, 2012; Yasuda *et al.*, 2012). All identified ILC2 cells are lineage negative, respond to treatment with either IL-25 and/ or IL-33, and can produce type 2 cytokines (IL-5 and/or IL-13). A report of particular interest by Mjösberg *et al.* (2001) characterised a possible human equivalent of mouse ILC2 cells (Mjosberg *et al.*, 2011). These human type 2 ILC cells share a similar phenotype, function with mouse ILC2 cells, are found in the fetal and adult lung and gut tissues. They also found these cells present in the peripheral blood, expressing the chemokine receptor type (CCR) 6 however, not producing type 2 cytokines. This suggests that human ILC2 cells may initially be released into the bloodstream in an inactivate form after which they home into the lung and gut tissue. There, they may mature, becoming activated *in situ* to start producing type 2 cytokines (Mjosberg *et al.*, 2011). Type 2 cytokines, such as, IL-25 and IL-33, are important in ILC2 cells activation and subsequent effector cytokines production. IL-25 (IL-17E) is a member of the IL-17 family that is associated

with Th2-like inflammation and disease (Fort *et al.*, 2001; Lee *et al.*, 2001). IL-25 mRNA transcripts are produced in Th2 cells and lung epithelial cells while the protein has been reported to be produced by alveolar macrophages, mast cells, eosinophils, and basophils (Angkasekwinai *et al.*, 2007; Ikeda *et al.*, 2003; Kang *et al.*, 2005). IL-25 upregulates the production of type 2 cytokines through the activation of eosinophils, mast cells, ILC2 and Th2 cells (Kondo *et al.*, 2008; Neill and McKenzie, 2011).

Eosinophils play a specialised role in innate host defence as the potential exists to mediate direct killing via the release of granule associated cytotoxic proteins and the eosinophil ribonucleases (RNases). Also, RSV infection of lung epithelial cells stimulates the expression of the eosinophil activating cytokines, IL-5 and IL-4 secreted by mucosal lymphocytes and chemokines such as eotaxin, RANTES and MIP-1 α secreted by RSV-infected epithelial cells (Matsuzaki *et al.*, 1996; Olszewska-Pazdrak *et al.*, 1998a). TLRs also play an important role in innate immunity and eosinophil activity, with eosinophils expressing TLR-1, TLR-4, TLR-7, TLR-9 and TLR-10 (Nagase *et al.*, 2003). Figure 1.4 summarises the innate and adaptive immune response to RSV primary infection and re-infection, as discussed above.

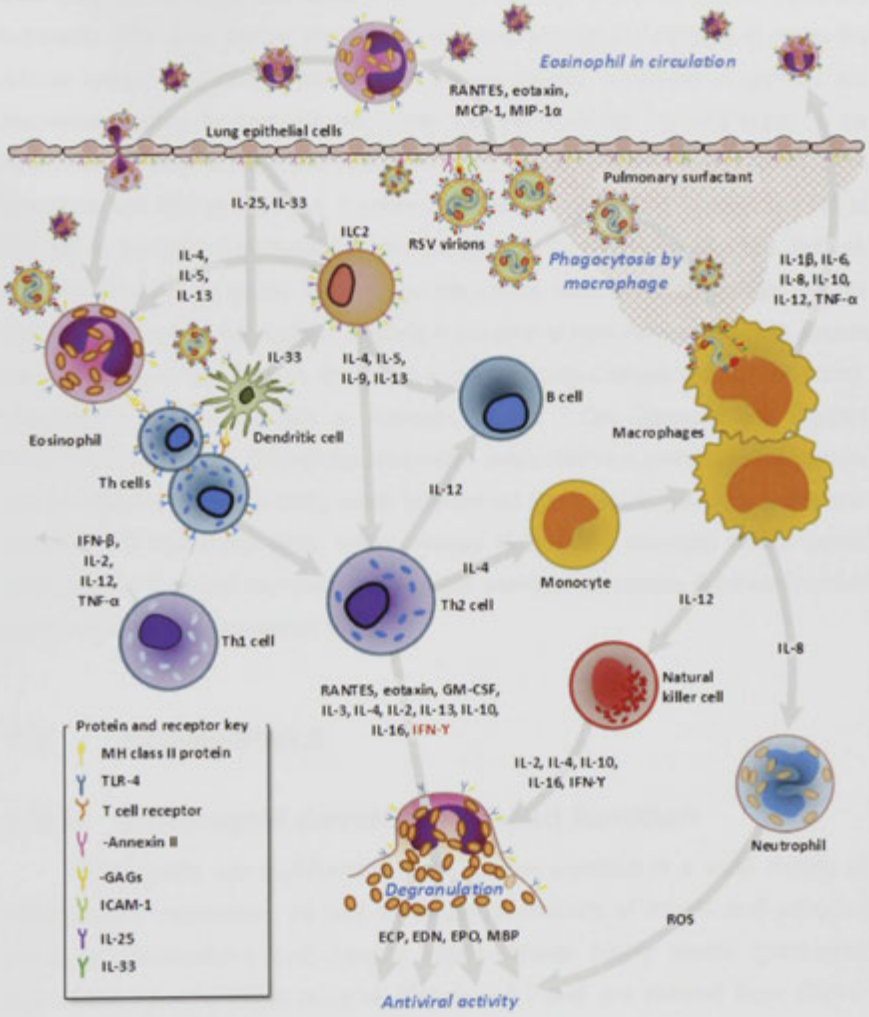

Eosinophil in circulation
RANTES, eotaxin,
MCP-1, MIP-1α
Lung epithelial cells
Pulmonary surfactant
IL-25, IL-33
ILC2
RSV virions
IL-4,
IL-5,
IL-13
Phagocytosis by
macrophage
IL-1β, IL-6,
IL-8, IL-10,
IL-12, TNF-α
IL-33
IL-4, IL-5,
IL-9, IL-13
B cell
Eosinophil
Dendritic cell
Macrophages
Th cells
IL-12
IFN-β,
IL-2,
IL-12,
TNF-α
IL-4
Monocyte
Th2 cell
IL-12
Th1 cell
IL-8
Natural
killer cell
RANTES, eotaxin, GM-CSF,
IL-3, IL-4, IL-2, IL-13, IL-10,
IL-16, IFN-Y
IL-2, IL-4, IL-10,
IL-16, IFN-Y
Neutrophil
Degranulation
ROS
ECP, EDN, EPO, MBP
Antiviral activity
Protein and receptor key
MH class II protein
TLR-4
T cell receptor
-Annexin II
-GAGs
ICAM-1
IL-25
IL-33

Figure 1.4. Detailed innate immune response following respiratory syncytial virus (RSV) infection

Upon entry into the lungs, RSV virion is met by the first line of immune defence, pulmonary surfactants. RSV virion can be eliminated at this point however if it manages to evade this defence system, RSV infects epithelial cells. Infection induces the release of cytokines and chemokines resulting in eosinophil recruitment from the blood into the lung tissue. In the meantime, RSV virion is recognised by dendritic cell or eosinophil via Toll-like receptor (TLR)-4 interactions with RSV glycoproteins. T helper (Th) cells once activated differentiate into Th1 or Th2 cell. In the case of RSV infection, this usually leads to Th differentiation into Th2 cell. Activated Th2 cells also release cytokines and chemokines, which can be upregulated (in black letters) or downregulated (red letters), resulting in activation of other immune cells. This process leads to eosinophil degranulation resulting in a granular content release and antiviral activity. Abbreviations defined: Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), interleukin (IL), macrophage inflammatory protein (MIP)-1 α, granulocyte-macrophage colony-stimulating factor (GM-CSF), innate lymphoid cell type 2 (ILC2), monocyte chemotactic protein 1 (MCP-1)interferon (IFN), tumour necrosis factor (TNF), eosinophil cationic protein (ECP), eosinophil derived neurotoxin protein (EDN), eosinophil peroxidase, major basic protein (MBP) and reactive oxygen species (ROS).

1.5 EOSINOPHILS

1.5.1 Eosinophil development and function

Eosinophils are multifunctional leukocytes involved in a wide variety of inflammatory responses, as well as being modulators of innate and adaptive immunity (Rothenberg and Hogan 2005). These highly motile granulated leukocytes have bi-lobed nucleus (Figure 1.5.) and are derived from $CD34^+$ haematopoietic progenitor cells in the bone marrow (Rothenberg, 2004). Eosinophils play an important role in biological processes such as; post-pubertal mammary gland development (Gouon-Evans and Pollard, 2001), oestrus cycling (Gouon-Evans and Pollard, 2001), organ rejection (Nagral *et al.*, 1998), and allergic inflammatory responses (Wardlaw *et al.*, 1986) protection against viral (Barends *et al.*, 2004) and parasitic infections (Klion and Nutman, 2004).

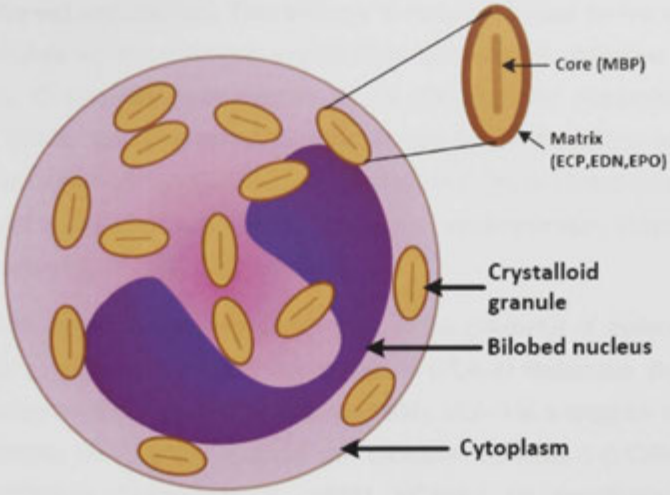


Figure 1.5. Eosinophil structure

The bi-lobed structure (in purple) represents the eosinophil nucleus containing the deoxyribonucleic acid, involved in transcription of information used for maintaining the role of eosinophils in innate immunity. The other structures are cytoplasm, cell surface membrane and secretion granules: major basic protein (MBP), eosinophil cationic protein (ECP) eosinophil derived neurotoxin (EDN) and eosinophil peroxidase (EPO) (Rothenberg and Hogan, 2005).

Eosinophils in the bone marrow mature for eight days under the control of transcription factors, erythroid transcription factors (GATA-1 and GATA-2) and CCAAT-enhancer-binding proteins (C/EBPs) (Rothenberg, 2004). A number of other inflammatory mediators have been implicated in regulating eosinophil accumulation at the site of infection and these include IL-1, IL-3, IL-4, IL-5, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) as well as the chemokines; RANTES, MCP-3, MCP-4, MIP-1α, eotaxin-1, eotaxin-2, eotaxin-3 and lipid mediators (platelet-activating factor (PAF) and leukotriene (LT) C4) (Foster *et al.*, 1996).

1.5.2 Adhesion and migration

Recruitment of circulating eosinophils from the blood stream into the mucosa involves several steps tightly regulated by chemoattractants, cell adhesion molecules (selectins and integrins) and Ig-like adhesion molecules. Firstly, eosinophils are recruited from the blood by vascular endothelial selectins

to the inflamed endothelium. This process is closely followed by the adherence of eosinophils to the vascular endothelium through the adhesion molecule activation. Eosinophils then migrate to the inflamed site (Desreumaux and Capron, 1996). Once recruited to sites of allergic inflammation, eosinophils become activated by signals produced through the Th2-mediated cascade and are thought to induce disease via the release of proinflammatory molecules and granular proteins (Foster *et al.*, 2002).

Adhesion of eosinophils also occurs in the presence of molecules from the integrin family such as very-late-antigen-4 (VLA-4) molecules, β_1 integrins and β_2 integrins (Bochner and Schleimer, 1994). VLA-4 is a receptor expressed by eosinophils that binds to vascular cell adhesion molecule 1 (VCAM-1) on in the endothelium (Symon *et al.*, 1994). VCAM-1 on endothelial cells is upregulated by IL-4 and IL-13. These two cytokines play an important role in allergic inflammation, and their increased expression was found to enhance eosinophil recruitment (Kita and Gleich, 1996). IL-13 is particularly important as it regulates multiple features of asthma, including IgE production, mucus overproduction, eosinophil recruitment and survival, AHR and expression of adhesion molecules and chemokines (Pope *et al.*, 2005).

1.5.3 Trafficking of eosinophils

Of all the cytokines implicated in modulating leukocyte recruitment, only IL-5 and eotaxin selectively regulate eosinophil trafficking (Rankin *et al.*, 2000). IL-5 regulates growth, differentiation, activation and survival of eosinophils. However, both IL-5 and eotaxin provide an essential signal for eosinophil expansion and mobilisation from the bone marrow into the lung following allergen exposure (Collins *et al.*, 1995). The mature cells shed L-selectin and migrate from the bone marrow to the blood (Gleich, 2000).

The finding that IL-4 and IL-13 are potent inducers of eotaxin by a signal transducers and activators of transcription (STAT)-6 dependent pathway provides an integrated mechanism to explain eosinophilia associated with Th2 responses (Kaplan *et al.*, 1996; Takeda *et al.*, 1996). Currently there is an increased focus on the importance of arachidonic acid metabolites, especially LTB4, LTC4, LTCD4 and LTE4, and prostaglandin (PG) D2, on eosinophil activation. Notably, the LT type 1-receptor antagonists (now approved for

asthma therapy), have been shown to reduce blood and lung eosinophilia (Tager *et al.*, 2000). A study has shown that mice with the targeted deletion of the LTB4 receptor also have markedly reduced allergen-induced eosinophilia (Tager *et al.*, 2000). Furthermore, eosinophils were also found to express high levels of the high-affinity PGD2 type II receptor. This receptor, also expressed by basophils and Th2 cells, appears to co-mediate Th2 cell and eosinophil/basophil recruitment. Eosinophils have also been shown to express high levels of histamine receptor 4 which mediates eosinophil chemoattraction and activation *in vitro* (O'Reilly *et al.*, 2002).

1.5.4 Proinflammatory and cytotoxic effects

Eosinophils are a potent source of proinflammatory mediators. These include cytotoxic proteins stored in eosinophil granules, lipid mediators newly formed following eosinophil activation, cytokines, chemokines, various eosinophil proteases and components of the respiratory burst (rapid release of reactive oxygen species), including superoxide and hydrogen peroxide (Walsh, 2001).

Eosinophil peroxidase (EPO), an abundant heme protein secreted from activated eosinophils, plays a central role in oxidant production by eosinophils (MacPherson *et al.*, 2001; Wu *et al.*, 1999). It is a member of the mammalian peroxidase subfamily and amplifies the oxidising potential of hydrogen peroxide produced during the respiratory burst by using it as a co-substrate to generate cytotoxic oxidants (MacPherson *et al.*, 2001; Wu *et al.*, 1999).

Eosinophil cationic protein (ECP) is a major component of the large secretory granules of human eosinophilic leukocytes (Domachowske *et al.*, 1998a). Gleich *et al.* (1986) were the first to note that the N-terminal of both ECP and eosinophil derived neurotoxin (EDN) showed distinct similarities to RNase A, a bovine ribonuclease that has since been identified as the prototype of the extensive gene superfamily (Beintema, 1997). Therefore, ECP and EDN are classified as ribonucleases having both neurotoxic and helminthotoxic activities, with EDN being more active than ECP (Gleich *et al.*, 1986; Slifman *et al.*, 1986).

Major basic protein (MBP) is localised to the core of the eosinophil granule while EPO, ECP and EDN are localised to the eosinophil granule matrix

(Gleich, 1996). MBP is a potent toxin which induces damage to various parasites, kills bacterial and mammalian cells, causes histamine release from basophils and mast cells, and activates neutrophils, platelets and eosinophils themselves (Kita *et al.*, 1995).

Eosinophil cytotoxic proteins are released through one of the following three mechanisms: *classical exocytosis*, whereby secondary granules fuse directly with the plasma membrane of the cell, to release their entire contents into the extracellular environment (Nusse *et al.*, 1990); *piecemeal degranulation (PMD)*, whereby small vesicles bud from the secondary granules and subsequently transport a subset of the granule proteins to the cell surface, resulting in the progressive loss of secondary granule constituents (Dvorak *et al.*, 1992; Dvorak *et al.*, 1991); and *cytolysis*, a highly organised process of cell death, where loss of the plasma membranes integrity leads to the release of cellular contents (Erjefalt *et al.*, 1998). The release of these cationic proteins and associated oxidants assists in the clearance of parasitic infection (Barnes *et al.*, 1998; Gleich and Loegering, 1984) and may assist in the clearance of viral infections. Eosinophils also induce the release of an array of inflammatory mediators and cytokines (e.g. IL-2, IL-4, IL-5, and IFN-γ) (Table 1.1) from epithelial cells, APCs or T cells, leading indirectly to cytotoxicity.

Table 1.1. Key factors involved in eosinophil-mediated immunity

Factors	Full name of factors	Key observations	References
PAF	Platelet activating factor	Enhanced leukotriene C4 release resulting in eosinophils influx; induces eosinophil chemotaxis	(Dimova-Yaneva *et al.*, 2004; Wardlaw *et al.*, 1986)
IL-2	Interleukin (IL)-2	Induces eosinophilia *in vivo*	(Barnes *et al.*, 1998)
IL-3	Interleukin (IL)-3	Stimulates colony formation in eosinophils; induces eosinophilia *in vivo*	(Foster *et al.*, 1996)
IL-4	Interleukin (IL)-4	Stimulates immunoglobulin class switching to immunoglobulin E; involved in elimination of parasitic infection; eosinophil growth	(Imai *et al.*, 2001)
IL-5	Interleukin (IL)-5	Induces eosinophil growth, differentiation and maturation; eosinophil release from the bone marrow; can induce eosinophilia	(Collins *et al.*, 1995)
IL-6, IL-8, IL-10, IL-12, TNF-α	Interleukin (IL)-6;Interleukin (IL)-8; Interleukin (IL)-10; Interleukin (IL)-12; Tumour necrosis factor (TNF)-alpha	Eosinophil recruitment; growth and differentiation	(McNamara and Smyth, 2002)
IL-12, IFN-γ	Interleukin (IL); Interferon-gamma	Reduces eosinophil influx after allergen exposure	(Barnes *et al.*, 1998)
IL-13	Interleukin (IL)-13	Eosinophil recruitment and survival	(Pope *et al.*, 2005)
RANTES, MIP-1 α, Eotaxin	Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES); Macrophage inflammatory protein (MIP)-alpha	Eosinophil activation, chemotaxis & degranulation	(Foster *et al.*, 2002; Rothenberg, 2004)
C/EBP,GM-CSF, LTC4	CCAAT-enhancer-binding protein (C/EBP); Granulocyte-macrophage colony-stimulating factor (GM-CSF); Leukotriene (LT) C4	Eosinophil chemotaxis	(Foster *et al.*, 2002; Rothenberg, 2004)

1.5.5 Eosinophilia

Eosinophilia occurs in a variety of disorders (Table 1.2.) and can range from mild, to severe. The most common cause of eosinophilia worldwide is helminthic infections, and the most common cause in industrialised nations is atopic disease such as allergic asthma (Rothenberg, 1998).

Table 1.2. Disease associated with eosinophilia (Adapted from Rothenberg, 1998)

Type of Disease	Eosinophilia		Examples of Causes
	Peripheral Blood	*Tissue*	
Respiratory	Present or absent	Present	Eosinophilic pneumonitis, asthma (Foster *et al.*, 1996)
Gastrointestinal	Present or absent	Present	Inflammatory bowel disease, eosinophilic gastroenteritis, allergic colitis (Beeken *et al.*, 1987; Jose *et al.*, 1994)
Allergic	Present or absent	Present	Allergic rhinoconjuctivitis, asthma, eczema (Ahlstrom-Emanuelsson *et al.*, 2004; Fauci *et al.*, 1982)
Systemic	Present	Present	Idiopathic hypereosinophilic syndrome, vasculitis (Fauci *et al.*, 1982)

Three processes lead to eosinophilia: eosinophil rolling, adhesion and migration however; degranulation, the forth process depicted in Figure 1.6, is a result of eosinophilia itself at the site of infection. As described in Table 1.2 eosinophilia has been associated with several health conditions. Accumulation of eosinophils, limited to specific organs, is a characteristic of particular diseases, including eosinophilic cellulitis or Well's syndrome. Additionally, eosinophilic pneumonias and Loffler's syndrome are also characterised by eosinophil infiltration and accumulation in the lung. Shulman's syndrome, which typically occurs in young adults, is another disease with characteristic eosinophil accumulation, with patients developing scleroderma-like skin indurations, predominantly on the extremities with joint contractures (Cottin and Cordier, 2005; Mosconi *et al.*, 2002; Van der Straaten *et al.*, 2006).

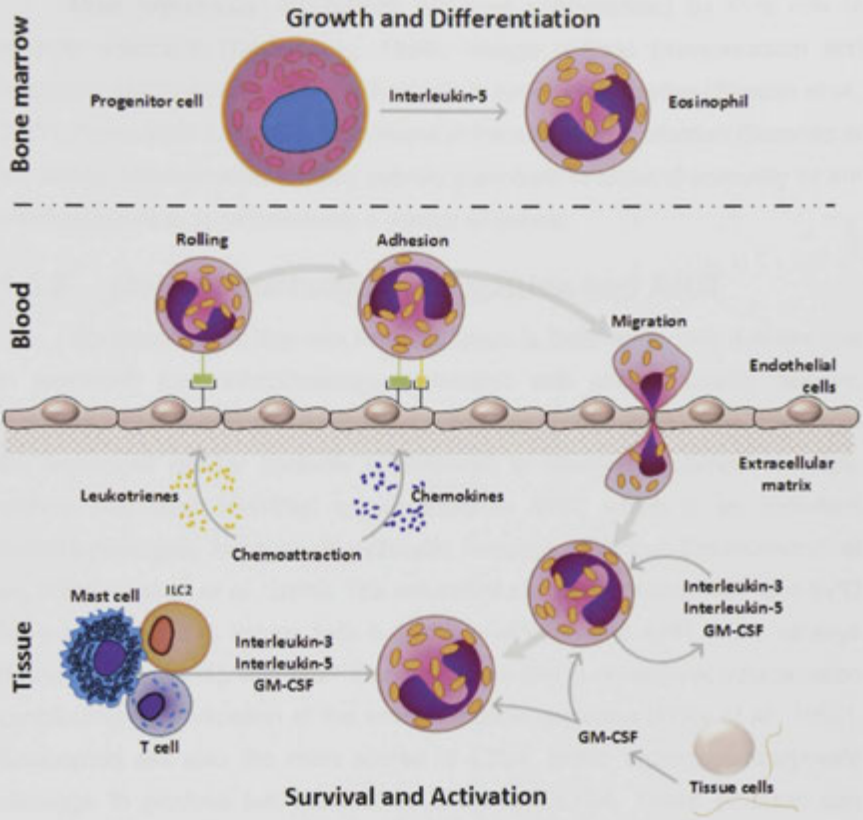


Figure 1.6. Processes involved in eosinophilia

Eosinophilia is initiated by T helper (Th) 2-mediated and/or innate lymphoid cell type 2 (ILC2) signals, namely interleukin-5 and eotaxin, driving the proliferation of eosinophils via the differentiation of progenitor cells in the bone marrow. Prolonged stimulation induces eosinophil mobilisation into the blood. Eosinophil recruitment from the blood involves rolling, adhesion and extravasation through the vascular endothelium, processes mediated by cell-surface integrins, selectins and adhesion molecules on the vascular endothelium and Th2-mediated chemoattractants. Eosinophils are then directed to the site of inflammation by chemokines and leukotrienes and induced to degranulation (Rothenberg, 1998).

Most importantly, eosinophils are best characterised by their role in parasitic infections (Rothenberg, 1998), allergic asthma (Kariyawasam and Robinson, 2007) and recently, by their role in tumour eradication (Simson *et al.*, 2007). These cells have also been found at the site of viral infection (Barends *et al.*, 2004), however whether they actively contribute to antiviral immunity or are mere bystanders, continues to be a source of debate.

1.5.6 Role of eosinophils in asthma and AHR

Eosinophil infiltration into the lung tissue is believed to play a major role in promoting the pathophysiology associated with allergic airway disease, including inflammation and AHR (Busse and Lemanske, 2001). The release of the eosinophil cellular contents in response to allergen challenge in atopic asthma has been identified to contribute to AHR, which is an important pathophysiological hallmark of asthmatic decease (Ahlstrom-Emanuelsson *et al.*, 2004; Kephart *et al.*, 2010). The eosinophil cationic proteins, MBP and EPO have been found to induce both bronchoconstriction and AHR in the airways (Gundel *et al.*, 1991), with MBP found to play a role in neurogenic inflammation contributing to modulation of the smooth muscle response (Fryer *et al.*, 1997). Eosinophils are also the main source of LTC4, which undergoes enzymatic cleavage to produce two metabolites LTD4 and LTE4. These products can induce mucus hypersecretion, AHR, oedema and bronchoconstriction (Kariyawasam and Robinson, 2007). These findings suggest that eosinophils play an important role in the pathogenesis of asthma.

1.5.7 Role of eosinophils in RSV infection

Eosinophils appear to have the potential to clear respiratory viruses, however they can also contribute to deleterious effects of viral infection as highlighted by their link to allergic airways disease and asthma (Busse and Lemanske, 2001). In addition the clinical similarities between viral bronchiolitis and asthma have led to speculation that these two diseases could have similar pathophysiological mechanisms, brought about by eosinophils (Domachowske and Rosenberg, 1999).

In the 1960s infants were immunised with formalin-inactivated (FI)-RSV vaccine (Kim *et al.*, 1969). Many of the vaccinated children became severely ill during a subsequent winter RSV epidemic resulting in several deaths (Olson

and Varga, 2007). The presence of eosinophils as a result of RSV infection became the main histological feature in the lungs and blood of the infants who died following FI-RSV vaccination and subsequent RSV infection (Lindemans *et al.*, 2006; Openshaw, 1995; Openshaw *et al.*, 2001). Laboratory-based research into this phenomenon using a mouse model confirmed that there was increased eosinophil influx in the lungs of mice following immunisation with FI-RSV and subsequent exposure to RSV (Openshaw *et al.*, 2001). It was unclear whether eosinophils were part of the host defence or were a sign of the immunopathology observed in response to RSV infection.

The recruitment of eosinophils to the lungs during RSV infection may be an initial immune response designed to reduce viral replication, since eosinophil cationic molecules, especially eosinophil RNases, have been shown to possess antiviral activity (Domachowske *et al.*, 1998a; Domachowske *et al.*, 1998b; Klebanoff and Coombs, 1996). Phipps *et al.* (2007) found that airway eosinophilia present in hypereosinophilic (IL-5 Tg) mice, or via transfer of eosinophils directly to the lungs, results in the enhanced clearance of RSV virions and a subsequent reduction in mucus hypersecretion and AHR (Phipps *et al.*, 2007). Eosinophils have the potential to play a beneficial role in RSV disease by clearing viral infections however when over-recruited, they exacerbate RSV inflammation, and cause subsequent tissue damage.

Further research studies have focused on elucidating the role of eosinophils in RSV infection and pathology. Studies by Garofalo *et al.* (1992) established that wheezing during RSV infection was associated with increased concentrations of LTC4 and ECP in respiratory secretions, both of which are mediators produced and secreted by activated eosinophils (Domachowske and Rosenberg, 1999; Garofalo *et al.*, 1992). Eosinophils were found to contribute to the inflammatory response through their chemokine expression profile (Olszewska-Pazdrak *et al.*, 1998a). Olszewska-Pazdrak *et al.* (1998) examined the secretion of chemokines by RSV-infected eosinophils *in vitro* and found that human eosinophils secreted significant amounts of RANTES and MIP-1α following their exposure to RSV (Olszewska-Pazdrak *et al.*, 1998a). Rosenberg and Domachowske (2001) also showed that eosinophils mediated a dose-dependent reduction in virus infectivity in the presence of the RSV-containing suspensions. They found that a 55kDa polypeptide ribonuclease inhibitor was

binding to the eosinophil granule proteins, EDN and ECP, with great affinity, causing a significant decrease in eosinophil activity and increase in viral infectivity (Rosenberg and Domachowske, 2001).

RSV infection induces expansion of virus-specific $CD4^+$ Th2 cells, resulting in the release of proinflammatory cytokines that contribute to eosinophilic airways inflammation (Openshaw *et al.*, 1992). A mouse study revealed that the RSV viral attachment G glycoprotein was linked to the release of Th2 cytokines and the development of eosinophilic pulmonary infiltrates (Harcourt *et al.*, 2004). This was also demonstrated by leukocyte chemotaxis experiments revealing G proteins ability to modulate the immune response and induce leukocyte migration via the CX3CR1-fractaline pathway. CX3CR1 is a chemokine receptor primarily expressed at the surface of the cytotoxic cells such as T cells, NK cells and monocytes/macrophages (Harcourt *et al.*, 2006). The CX3CR1 receptor binds to the chemokine fractalkine in murine and human models (Combadiere *et al.*, 1998). As fractalkine binds to the CX3CR1 chemokine receptor, it causes leukocyte adhesion and migration as well as a Th1 immune response (Harcourt *et al.*, 2006; Lee *et al.*, 2004). The RSV G protein contains a CX3C chemokine motif and it has been found to interact directly with the CX3CR1 receptor. Hence, the G protein is competing with fractalkine for binding to the CX3CR1 receptor. Upon binding, the RSV G protein is believed to modify the CX3CR1-fractalkine interaction resulting in a suppression of the Th1 response and upregulation of the Th2 immune response (Harcourt *et al.*, 2006) ultimately resulting in downstream eosinophil activation.

1.5.8 Role of eosinophils in PVM infection

PVM is the only virus of the family Paramyxoviridae and subfamily Pneumovirinae, which naturally infects mice and is the rodent pathogen most closely related to human RSV (Domachowske *et al.*, 2000a; Garvey *et al.*, 2005; Rosenberg and Domachowske, 2001). In 1939, Horsfall and Hahn (1939) were the first to identify PVM in mouse lungs (Horsfall and Hahn, 1939). In the absence of antiviral therapies, PVM infection results in increased viral replication and a profound inflammatory response which can lead to morbidity and mortality (Garvey *et al.*, 2005; Rosenberg and Domachowske, 2001).

Murine studies have found that eosinophils and neutrophils are among the earliest cellular responses to PVM infection (Domachowske *et al.*, 2000a; Rosenberg and Domachowske, 2001). Pulmonary eosinophilia in mice precedes the onset of symptoms but is not detectable once symptoms become obvious as it dissipates over time (Harrison *et al.*, 1999). Therefore, eosinophil products are more likely detectable earlier in infection (Rosenberg and Domachowske, 2001). Furthermore, although IL-5 normally recruits eosinophils to the site of infection, PVM infected mice appear to be completely devoid of IL-5, even in the presence of eosinophilia. Viral challenge of TLR7$^{-/-}$ mice induces all of the cardinal pathophysiologic features of asthma, including tissue eosinophilia, mast cell hyperplasia, IgE production, airway smooth muscle alterations, and airways hyperreactivity in a memory CD4^{+} T cell-dependent manner (Kaiko et al., 2013). Moreover, as PVM infection induces the release of MIP-1α, there appears to be correlation between the released MIP-1α, eosinophil infiltration and viral replication (Domachowske *et al.*, 2000a). PVM infection in MIP-1α and/or CCR3 deficient mice, which have no eosinophils and a reduced number of neutrophils detected in BAL fluid at the time of infection, resulted in minimal inflammatory response, increased viral load and accelerated mortality (Domachowske *et al.*, 2000a). These results demonstrate e that in the absence of eosinophils, MIP-1α/CCR3 deficient mice are overwhelmed by viral replication and potential death, thus suggesting the importance of eosinophils and neutrophils in antiviral activity.

Although PVM is the rodent pathogen most closely related to human RSV, a recent research study examining the role of eosinophilia in PVM infection has revealed some very interesting findings. A mouse study found that when mice are vaccinated with PVM they remained protected following virus challenge (Percopo *et al.*, 2009). These results indicate that the presence of eosinophils in the lung tissue and airways are associated with protection against virus infection. The same study repeated the vaccination and challenge protocol in eosinophil-deficient mice and found that eosinophil deficiency resulted in no change in viral titres and no change in the overall clinical symptoms (Percopo *et al.*, 2009). It is unclear as to why eosinophils did not promote viral clearance in PVM infection (Percopo *et al.*, 2009), despite evidence that they do promote antiviral activity in RSV infection (Phipps *et al.*, 2007). Most recent study by Percopo *et al.* (2014) has found that reduction in PVM viral load is dependent

on eosinophil activation. This study shows that eosinophils recruited to the airways in response to *Aspergillus fumigatus* (or ovalbumin) sensitisation and challenge do not degranulate on their own, but do so in response to subsequent activation with PVM (Percopo *et al.*, 2014). It is believed that this occurs in response to both, PVM replication in alveolar macrophages and bronchiolar epithelial cells as well as production of proinflammatory cytokines (Bem *et al.*, 2011; Dyer *et al.*, 2012; Rosenberg *et al.*, 2005).This is the first study to show that eosinophils have a profoundly antiviral role and promote survival in response to an otherwise lethal PVM infection (Percopo *et al.*, 2014). Taken together, these studies reveal a very promising and positive role of eosinophils in PVM and potentially RSV infection.

1.5.9 Eosinophils in Rhinovirus (RV) infection

Rhinoviruses (RV) cause the common cold which, in some cases, results in the hospitalisation of infected infants, the development of pneumonia in immunosuppressed patients and the exacerbation of asthma (Bartlett *et al.*, 2008). Currently, there is no effective treatment available for RV infection. Clinical studies have shown that RV infection results in the infiltration of eosinophils, neutrophils and lymphocytes in nasal and bronchial mucosa, causing airway inflammation (Bardin *et al.*, 1995; Levandowski *et al.*, 1988). Due to the involvement of RV in the exacerbation of asthma, Bartlett *et al.* (2008) investigated the interactions between RV infection and allergic airway inflammation using a mouse model. This study found that RV infection contributes to the exacerbation of eosinophilic airway inflammation, airway hyper-responsiveness, mucus secretion and the production of Th1 and Th2 cytokines. A Th2 immune response in a human system results in a release of cytokines such as IL-4, IL-5, IL-13 and GM-CSF. High levels of these cytokines can be correlated with increased eosinophil infiltration and greater disease severity (Kato *et al.*, 2011; Sasaki *et al.*, 2007). All of these responses appeared to be linked with a rhinovirus-induced exacerbation of asthma (Bartlett *et al.*, 2008).

There are approximately 100 different rhinovirus serotypes and about 90% of these, use the human ICAM-1 for cell attachment and entry. However, these viruses do not to bind to mouse ICAM-1 (Bartlett *et al.*, 2008). In a mouse study, Sasaki *et al.* (2007) found that virally infected respiratory epithelial cells

had increased expression of ICAM-1, resulting in eosinophil infiltration into the respiratory area. Moreover, the same study confirmed that the sites lacking ICAM-1 expression experienced less eosinophil infiltration (Sasaki *et al.*, 2007). Results from a study investigating ICAM-1 and RSV suggest that the regulation of ICAM-1 could potentially be used to inhibit rhinovirus infection (Traub *et al.*, 2013). Regulation of ICAM-1 could prevent cell adhesion and entry and at the same time reduce eosinophil infiltration, hence reducing the respiratory epithelium damage caused by cytotoxic proteins (e.g. MBP and EPO) released from the eosinophils.

1.5.10 Eosinophils as antigen presenting cells in viral infection

As early as 1960s, studies began documenting the capacity of eosinophils to internalise administered antigens and rapidly traffic them to regional lymph nodes. These studies revealed that within one hour of antigen injection, eosinophils containing the labelled antigens were localised within regional lymph nodes (Litt, 1964; Roberts, 1966; Shi, 2004). Although these studies did not establish that eosinophils were acting as APCs, they did show that eosinophils can be involved in the early uptake of antigen (Shi, 2004).

Naïve T lymphocytes require two signals from APCs to be activated. The first signal is provided through the interaction of the T cell receptor (TCR) with the MHC-II protein on APCs (Chambers and Allison, 1997; Linsley *et al.*, 1990). Although blood eosinophils do not usually express MHC class II protein, they have been shown to express MHC II in response to activation (Akuthota *et al.*, 2012; Linsley *et al.*, 1990). Allergen challenge has been shown to elicit an eosinophil influx into the airways resulting in recruited eosinophils expressing human leukocyte antigen (HLA)-DR (MHC II family member), which was not found on otherwise activated blood eosinophils (Mengelers *et al.*, 1994; Sedgwick *et al.*, 1992; Shi, 2004). Eosinophils in the sputum of asthmatics and airway have been found to express HLA-DR, but this expression was not found in blood eosinophils in chronic eosinophilic pneumonia patients (Shi, 2004).

To present antigen, a second co-stimulatory signal must be provided by APCs for the activation of CD28 and inhibition of cytotoxic lymphocyte-associated antigen (CTLA)-4 receptors on T cells (Shi, 2004). CD28 and CTLA-

4 are expressed by T cells and interact with the B7 molecules B7-1 (CD80) and B7-2 (CD86) (Freeman *et al.*, 1993; Linsley *et al.*, 1990), providing co-stimulatory signals necessary for T-cell activation. Blood eosinophils have no detectable levels of CD80 and CD86 while eosinophils extracted from IL-5 transgenic (Tg) mice from the peritoneal cavity have been found to express both CD80 and CD86 (Tamura *et al.*, 1996; Wang *et al.*, 2007). *In vitro* incubation of these cells with GM-CSF was found to increase CD80 and CD86 expression (Wang *et al.*, 2007). Hence, eosinophils appear to have the potential to act as APCs in allergic and viral infections. By activating rhinovirus-specific T cells, eosinophils may play an important role in the initiation of antiviral T cell responses, contributing to enhanced airway inflammation and increased asthma symptoms in susceptible individuals (Handzel *et al.*, 1998).

Although a number of studies support the role of eosinophils as potential APCs, there are studies that argue that even with MHC class II protein being expressed eosinophils are inefficient in activation of $CD4^+$ T cells by antigen (Mawhorter *et al.*, 1994). Therefore, the role of eosinophils as APCs remains a controversial topic debated intensely in the literature.

1.6 RSV PREVENTION AND TREATMENT

1.6.1 Prevention

Prevention is the most important aspect of healthcare; it can lead to reduction in morbidity and mortality, and lower the economic burden of RSV disease. Currently, there is no effective vaccine against RSV. Direct or indirect contact with the nasopharyngeal secretions or droplets (sneezing, coughing and kissing), fomites, and food from RSV infected patients can potentially transmit RSV as live virus can survive on surfaces for several hours (Hall, 1983; White *et al.*, 2005). Several approaches have been considered for developing an effective vaccine against RSV, however human immunisation against RSV has unfortunately failed (Graham *et al.*, 2000). In the 1960's a number of infants were immunised with a FI-RSV vaccine, who following the natural RSV infection experienced pulmonary eosinophilia, several deaths and high rate of hospitalisation (Graham *et al.*, 2000; Rakes *et al.*, 1999). An efficient RSV vaccine would be one with proper balance between immunogenicity and protection without any allergic response (Hacking and Hull, 2002). RSV F

protein has widely been accepted as the vaccine candidate due to its conserved nature among various strains as well as among the other paramyxoviruses (Hacking and Hull, 2002; Openshaw and Tregoning, 2005; Valarcher and Taylor, 2007; van Drunen Littel-van den Hurk *et al.*, 2007).

Both RSV, fusion and attachment, proteins are glycosylated and represent the targets of neutralising antibodies. The RSV F protein is potentially a good vaccine candidate due to its conserved and vital role in cell attachment. Passive immunisation with the monoclonal antibody specifically neutralising F protein has resulted in an effective protection against RSV. This licensed monoclonal antibody, named palivizumab (Synagis) is now used to provide passive protection for high risk infants from severe RSV (Ottolini *et al.*, 2002). Motavizumab, variant of palivizumab, has been found to neutralise RSV by binding the RSV fusion protein F following the RSV attachment to the host cell but before the viral transcription (Huang *et al.*, 2010). Palivizumab or motavizumab treatment is likely able to induce inhibition of cell-to-cell and virus-to-cell fusion, by preventing the conformational changes in the F protein required for viral fusion. The effective use of palivizumab is limited due to the cost, therefore its use reserved for infants that are at high risk of bronchiolitis (Harkensee *et al.*, 2006). Palivizumab, although effective, is costly and thus is not beneficial to the recipients especially during the periods outside RSV circulation. A cost effective means of producing RSV F neutralising antibodies was experimented in phages and plants. The efficacy of the plant derived palivizumab was found to be greater than the mammalian derived palivizumab (Zeitlin *et al.*, 2013).

The RSV genome codes structural and functional proteins that are immunogenic; and DNA based vaccines are developed based on these proteins. The process involves a DNA fragment coding part or whole protein of RSV being inserted into an appropriate expression plasmid vector under a constitutive promoter control. The initial work with this approach was successful in the expression in cells and *in vivo* murine models to eliminate the RSV infection, but the problem of RSV associated Th2 type immune response was persistent. To resolve this problem, Li *et al.* (200) attempted to manipulate the parameters of choice: the protein to be expressed, the expression vector, adjuvants, formulations and intracellular stability of the plasmid. Mice

challenged with the RSV-G construct had balanced systemic and pulmonary Th1/Th2 cytokines and RSV neutralizing antibody responses (Li *et al.*, 2000). However, the wild type RSV F protein expressed from DNA plasmid was poorly expressed (Ternette *et al.*, 2007) and has had low immunogenicity of F protein, the antagonistic activity of RSV for IFN and immunopathology (Martinez-Sobrido *et al.*, 2006). Consequently, Wu *et al.* (2009) developed a DNA vaccination strategy against RSV using a mucosal adjuvant (Wu *et al.*, 2009). The mice immunised with the DRF-412 vector contained less RSV RNA in lung tissue and induced a higher mixed Th1/Th2 cytokine immune response as well as better protection than those immunized with the DRF-412-P vector, which was confirmed by lung immunohistology studies (Wu *et al.*, 2009). Likewise, Mycobacterium bovis Bacillus Calmette-Gue'rin (BCG) vaccine was modified to carry RSV N or M2 and was found to establish the Th1 type immunity in RSV challenged mice (Bueno *et al.*, 2008). The recombinant vaccine also elicited the activation of RSV specific T cells producing IFN-γ and IL-2, along with reduction in weight loss and lung viral protein load, thus establishing a Th1-polarized immune response (Bueno *et al.*, 2008). This approach serves the purpose of naturally activating the immunostimulatory responses of the host while delivering the DNA vaccine.

The bitter episode of FI-RSV vaccine has impeded the vaccine development and in fact has raised serious concern over the use of native RSV or its components. Recently, several applications of nanotechnology have appeared in the development of vaccines popularly known as Nanovaccines. DNA vaccine is prone to rapid degradation when introduced into an animal system; so to increase the retention and increase the efficacy of the DNA vaccines, they can be encapsulated into a polymer that will protect and facilitate controlled release. Various synthetic or natural polymers are now experimented for targeted delivery and controlled release of the carrier (Glenn *et al.*, 2013; Smith *et al.*, 2012). Chitosan is a polymer of great interest in respiratory disease treatment, because of its mucoadhesive property and biodegradability, which balances of longer retention and controlled release of carrier molecules encapsulated. Thus, chitosan nanoparticles are being developed against RSV (Glenn *et al.*, 2013; Smith *et al.*, 2012).

In the case of RSV-naïve infants and children, it remains to be seen whether vaccine candidates will be sufficiently immunogenic in infancy to provide effective protection against RSV disease. It seems unlikely that complete protection against infection can be achieved however; a substantial reduction in RSV replication should be effective in controlling severe disease. The recent successful development of live-attenuated paediatric vaccines against rotavirus, which faced many of the same obstacles, gives hope for success.

1.6.2 Treatment

The effect of removing cell surface HS chains, on viral infectivity has recently been examined. The *in vitro* studies have shown that pre-treating of the cells with heparinase I results in reduction of the HS iduronic acids units, while heparinase II led to lower N-sulfation and heparinase III removed the N-sulfation units (Guo *et al.*, 2008). These findings suggest that low molecular weight heparin (LMWH) can inhibit positively charged RSV infection through cooperative electrostatic association (Guo *et al.*, 2008).

There are very limited treatment options available for RSV. However, there are many drugs for the symptoms associated with RSV infection. The target genes and proteins vital for RSV infection are important for developing preventative and treatment measures. The mode of action and potency of a drug determines the approach of prophylactic or curative application. Considering RSV life cycle, theoretically, there are numerous modes to interfere with RSV infection, but not all may be viable research. Replication, transcription and fusion are a few target processes for drug development against RSV. A focus is therefore on development of potent drug that holds conformity in the human trials.

Ribavirin or 1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1H-1,2,4-triazole-3-carboxamide is a widely used broad spectrum synthetic anti-viral drug for both DNA and RNA virus treatment. Oral and nasal administration of ribavirin can be used as a treatment of severe lower respiratory tract infections caused by RSV and influenza virus (Bocchini *et al.*, 2009). Ribavirin is phosphorylated in the cells and has been found to compete with adenosine-5'-triphosphate and guanosine-5'-triphosphate, for viral RNA-

dependent RNA polymerases in RNA viruses. The exact mechanism of anti-viral activity of ribavirin against RNA and DNA viruses is still not clear. The usefulness of ribavirin against viruses is believed to be not only due to its anti-viral activity but also due to its capability to modulate the immune system (Langhans *et al.*, 2012). The derivatives of ribavirin such as viramidine, merimepodib, and other inhibitory molecules like mycophenolate and mizoribine have shown antiviral activity against the hepatitis C virus, and hence, there is scope for investigating them as potential anti-RSV drugs (Chapman *et al.*, 2007; Gish, 2006).

A number of small molecule anti-RSV compounds have been described over the last 15-20 years. Most of them have been discovered by screening of chemical libraries of natural products using classic virology assays, modified for high throughput testing. Recent advances in the development of anti-viral drugs have seen the rise of fusion inhibitors. The fusion inhibitors are usually synthetic compounds or molecules interrupting the fusion of virus with the host cell usually by binding the fusion proteins. The fusion inhibitors have been widely studied as anti-viral agents in several viruses including henipavirus, hendra virus, nipah virus, paramyxovirus, metapneumoviruses, HIV and RSV (Bossart *et al.*, 2005; Deffrasnes *et al.*, 2008; Porotto *et al.*, 2007; Porotto *et al.*, 2006; Porotto *et al.*, 2010; Wang *et al.*, 2003; Wild *et al.*, 1993; Zhao *et al.*, 2000). The first reports of the use of peptide(s) as fusion inhibitors, include the development of DP-178, a synthetic peptide based on the leucine zipper region of the HIV fusion glycoprotein gp41 (Wild *et al.*, 1993) which achieved a 50% inhibition at 0.38 nM against HIV-1. Fusion inhibitors for the paramyxoviruses have also been developed based on the conserved region of the F protein. The F protein is widely known for its conserved nature among the Paramyxoviridae family. Lambert *et al.* (1996) developed the fusion inhibitors belonging to the conserved heptad repeat (HR) domains of F1 region of F protein which is analogous to the peptides DP-107 and DP-178 of HIV gp41 (Lambert *et al.*, 1996). These fusion inhibitors were tested against RSV, human parainfluenza virus (PIV) 3 and measles virus, and showed antiviral activity specific to the species of origin.

A series of benzimidazoles derivatives have been reported to interact with the F protein and, although dissimilar in structure, they all seem to bind to a

hydrophobic pocket of the trimeric coiled-coil made by HRA (amino acids 153-209) of the F1 chain (Zhao *et al.*, 2000). Binding to this pocket would interfere with the proper interaction of HRA with HRB (amino acids 482-520) and the formation of the six-helix bundle structure (6HB) that is required for completion of membrane fusion (Melikyan *et al.*, 2000). Diverse array of other benzimidazoles (e.g., JNJ-2408068) and chemically unrelated compounds (e.g., VP14637) with subnanomolar activities have been described. Interestingly they also seem to target the hydrophobic pocket of the 6HB (Douglas *et al.*, 2005; Lundin *et al.*, 2010). Other RSV inhibitors have been described besides those targeting the F protein. For instance, $NMSO_3$, a sulphated sialyl lipid, has been reported to inhibit virus binding, and mutants resistant to this compound had mutations in the G protein, identifying it as the presumptive target (Kimura *et al.*, 2004). Many negatively charged polymers, including heparin and dextran sulphate, also interfere with RSV binding, but their inhibitory concentrations are too high for application in the clinical setting.

Sudo *et al.* (2005) discovered a benzazepine derivative (YM-53404) that worked relatively late in the replication cycle and that selected mutations in the L gene (Saito *et al.*, 1997). Another benzodiazepine compound (RSV604) that worked late in the replication cycle was shown to select mutations in the N gene, pointing to this molecule as the drug target (Chapman *et al.*, 2007). Interestingly, these mutations were located in the long β hairpin of the RSV N structure noted above that may mediate interaction between N protein in the ribonucleoprotein (RNP) and the polymerase complex (Tawar *et al.*, 2009).

There are several novel nonbenzimidazole based compounds, showing anti-RSV activity *in vitro*, but a more polar compound thiazole-imidazole 13 was selected due to compound potency, moderate permeability, and low metabolic rate in rats, and more detailed *in vivo* studies are further anticipated (Pryde *et al.*, 2013).

Besides chemical compounds, there are other ways to inhibit RSV infectivity. RNA interference (RNAi) which is a normal cellular event has become a powerful means of controlling gene regulation. The interference mediated by small interfering RNA (siRNA) was used against human immunodeficiency virus, poliovirus, hepatitis C and PIV in cell culture (Barik, 2004; Lee *et al.*, 2002; Zhang *et al.*, 2010). The concept of inhibiting RSV

infection using targeted antisense mechanism was applied by Jairath *et al.* (1997) by silencing the RSV-NS2 gene (Jairath *et al.*, 1997). Following the RNAi approach, Bitko *et al.* (2005) designed siRNA against the P gene of RSV and PIV which protected mice against individual and mixed infections upon intranasal administration (Bitko *et al.*, 2005). The effectiveness of siRNA action was observed with and without the use of transfection reagents. This approach was also effective when targeting the RSV-F gene (Bitko and Barik, 2001). Similar work on HEp-2 cell lines was replicated using four siRNA, designed to silence RSV F gene, which showed inhibitory action against RSV at various concentrations (Vig *et al.*, 2009). Silencing different RSV genes too had an inhibitory action on the RSV, a plasmid encoding siRNA which was complexed with chitosan targeting RSV-NS1 gene decreased RSV infection in BALB/c mice and Fischer 344 rats and also reduced the associated inflammation (Kumar *et al.*, 2002; Zhang *et al.*, 2005). Zhang *et al.* (2006) showed that siRNA nanoparticle targeting RSV NS1 gene resulted in increased IFN-β and IFN-inducible genes in A549 cells and in human DCs, and increased differentiation of $CD4^+$ T cells to Th1 cells (Zhang *et al.*, 2005). In addition, mice treated with small interfering NS1 (siNS1) nanoparticles exhibited significant decrease in lung viral titres and inflammation.

Alvarez *et al.* (2009) came up with a new RSV-NS1 gene specific siRNA (ALN-RSV01) having a broad spectrum of antiviral activity that targeted the nucleocapsid gene of RSV (Alvarez *et al.*, 2009). *In vivo* BALB/c murine studies demonstrated that intranasal dosing of ALN-RSV01 resulted in a 2.5-3.0 log-unit reduction in RSV lung concentration. To scale up this molecule for RSV treatment in humans, the safety, tolerability, and pharmacokinetics were tested on healthy adults, demonstrating its safety and tolerance in human subjects (DeVincenzo *et al.*, 2008). In the clinical trials of ALN-RSV01, healthy subjects were administered either, a placebo or ALN-RSV01 nasal spray for RSV. There was a 44% reduction in the RSV infection following ALN-RSV01 administration. Thus, this study in real terms has established a unique "proof-of-concept" for an RNAi therapeutic agent in RSV treatment (DeVincenzo *et al.*, 2010). ALN-RSV01 proved to be safe and was effective against RSV even in a complex clinical situation like lung transplants, which was a remarkable achievement (Zamora *et al.*, 2011). ALN-RSV01 has recently completed phase II b clinical trials.

Another approach of RNAi treatment to combat RSV is to decelerate the adverse effects of RSV mediated Th2 type immune response as the aggravated host immune response can be more harmful than RSV infection itself. Particularly in neonates, RSV bronchiolitis increases IL-4 receptor α levels, which results in increased Th2 response, so an antisense oligomer was synthesised for the local silencing of the IL-4 receptor α gene. Intranasal application of the antisense oligomers into a neonatal murine model reduced the Th2 type mediated pulmonary pathological signs of inflammation and lung dysfunction (Ripple *et al.*, 2010). A combinatorial approach of the anti-sense oligomer against RSV and IL-4 receptor α would control RSV infection and the adverse effects of RSV mediated inflammation.

Currently, there is no vaccine and effective treatment against RSV. Prevention of RSV infection at present is limited to only high risk individuals with a limited efficacy. New preventive research like DNA vaccines, subunit vaccines and nano-vaccines has reached animal trials. In addition, RSV treatment approaches using antisense oligomers, fusion inhibitors, and benzimidazole drug have proceeded into clinical trials. The challenges associated with RSV management are categorically numerous. However, at the current pace of scientific research and development and with the implementation of scientific, commercial, and program recommendations to develop epidemiological strategies, it seems optimistic to have an effective diagnosis, prevention and treatment solution for RSV in near future.

1.7 AIMS OF THIS THESIS

RSV is one of the most important pathogens of childhood respiratory infections (McNamara and Smyth, 2002). Since the failed FI-RSV vaccine trail, RSV disease had been characterised by an influx of eosinophils to the site of viral infection where eosinophils were believed to contribute to RSV pathogenesis. None the less, the role of eosinophils in RSV infection has been debated vigorously and in 2007, Phipps *et al.* (2007) demonstrated that eosinophils play a crucial role in clearance of RSV infection (Phipps *et al.*, 2007). In addition, RSV can re-infect the same individual several times throughout their life. Re-infection is characterised by an initial influx of NK cells followed by recruitment of helper $CD4^+$ and $CD8^+$ lymphocytes to the site of infection (Olson *et al.*, 2008; Olson and Varga, 2007; Stevens *et al.*, 2009). To complicate matters $CD4^+$ and $CD8^+$ T lymphocyte subsets contribute to clearing of the RSV infection, but that both also contribute to RSV disease pathogenesis (Graham *et al.*, 1991). A number of viruses have demonstrated to have a great affinity for cell HSPGs, playing important roles in virus attachment and entry (Lee *et al.*, 2006). HS has been shown to bind the RSV G protein on the cell surface of the HEp-2 cells (Krusat and Streckert, 1997). In addition, HS mimetic, PI-88, has been shown to inhibit HSV infection of cells by blocking the binding of the viral attachment glycoproteins to the cell surface (Nyberg *et al.*, 2004).

It is important to note that there are several limitations to the previous literature findings as well as RSV-induced limitations due to its heterogenic nature which provides this virus with an ability to evade previously induced immunity (White *et al.*, 2005) as adaptive immunity in response to RSV have been found to be limited, incomplete and short lived. As RSV infects mostly young children and elderly, it is important to note that a capacity to induce appropriate immune response is somewhat reduced at early and late stages of life (Adkins *et al.*, 2004; Fulton *et al.*, 2013; Levy, 2007). In addition, the role of eosinophils in RSV and viral infections in general is yet to be explored in depth. The depth of research into a positive role of eosinophil is still limited with only a handful of research groups focusing on the potential of these cells in viral clearance. Although there have been extensive studies of epidemiology, clinical manifestations, diagnostic techniques, animal models and the immunobiology of infection, there is not yet a convincing and safe vaccine available. For RSV the

effective use of palivizumab treatment is limited due to the cost and thus only currently used in infants with high risk of bronchiolitis (Harkensee *et al.*, 2006). Therefore, inexpensive and more widely effective treatments are required urgently and as HS involvement in RSV infection is underexplored, it could potentially lead to the development of new, more effective and more affordable RSV therapeutics.

Therefore, aims of this thesis are:

Aim 1: To examine the *in vitro* effect of RSV on eosinophil morphology, release of eosinophil activation markers and eosinophil antiviral activity

Aim 2: To evaluate eosinophil antiviral activity in four distinct mouse models following RSV infection and re-infection.

Aim 3: To assess the efficacy of HS mimetics treatment on RSV infection *in vitro* and *in vivo*.

Chapter 2. General Methods

2.1 HEAT INACTIVATION OF FETAL CALF SERUM

In this procedure, fetal calf serum (FCS) (Sigma, Australia) is heated to 56°C in a water bath to destroy heat-labile complement proteins prior to use in cell growth medium.

Briefly, heat inactivation procedure involves placing a 500 mL bottle of FCS from the -80°C freezer in the refrigerator to thaw overnight. The following day, completely thawed was placed in a 37°C water bath allowing serum to equilibrate at 37°C. The temperature was then raised to 56°C and the bottle of serum was incubated for 35 minutes with inversion every 10 minutes. Once bath reached 56°C, the serum was incubated for 30 minutes during which the bottle was again inverted every 10 minutes. The serum was allowed to cool at room temperature for 30 minutes. Heat inactivated (HI)-FCS was then aliquoted into 50 mL tubes and stored at -20°C.

2.2 CELL CULTURE

Immortal cell lines were used for *in vitro* experiments. HEp-2 (originally derived from an epidermoid carcinoma of the larynx) (ATCC CCL-23) and Vero E6 (originally isolated from kidney epithelial cells extracted from an African green monkey) (ATCC CRL-1586) cell lines were obtained from the American Type Culture Collection (ATCC, USA). Briefly, once cells were 80% confluent, culture medium was removed and discarded. The cell monolayer was washed twice with phosphate buffer solution (PBS) (ANU, Australia) to remove any serum residues. Cells were detached by covering cell monolayer with 0.25% (w/v) Trypsin ((Merck, Australia) with 0.53 mM ethyenediaminetetraacetic acid (EDTA) in PBS (ANU, Australia)) and incubating for 5-15 minutes at 37°C. Cell suspension was diluted in complete growth medium (Dulbecco's Modified Eagle's Medium (Gibco, Australia) supplemented with 5% of HI-FCS (Sigma) (Dulbecco's modified eagle's medium (DMEM) with 5% HI-FCS) and usually one fifth of the cell suspension was transferred to new culture flask at required seeding density.

2.3 THAWING OF CELLS FROM LIQUID NITROGEN STORAGE

Cells were taken out from the liquid nitrogen storage and rapidly thawed in a water bath at 37°C. The cell suspension was transferred to a 10 mL sterile tube (Sarstedt, Australia) and media added to dilute the dimethyl sulphoxide (DMSO) (Univar, Asia Pacific Specialty Chemicals). Cells were then centrifuged at 670 x *g* (HD Scientific) for 5 minutes, supernatant was removed and fresh media added to resuspend the pellet appropriately. The cell suspension was then transferred to a T-25 tissue culture flask (Iwaki) and left to incubate at 37°C in a humidified atmosphere supplemented with 5% CO_2.

2.4 CRYOPRESERVATION OF CELLS

Cells were frozen down using freezing medium made up of 95% of DMEM with 5% HI-FCS and 5% of DMSO (Univar). Cells were aliquoted into sterile cryovials (Iwaki) and stored at -80°C overnight. The following day cells were transferred to liquid nitrogen for long-term storage.

2.5 CELL VIABILITY ASSAY

Cell viability was tested using trypan blue dye (Invitrogen, Australia). The cytoplasm of dead cells is susceptible to trypan blue dye staining, due to cell membrane permeability, rendering all unstained cells as viable. The cell sample was mixed with 0.4% trypan blue (Invitrogen) solution at 1:1 ratio, the mix was then used to fill the haemocytometer chamber. Cells were counted under the microscope across four 1 x 1 mm squares of one chamber and the average number of cells was determined per square. The following formula was used to calculate number of cells per mL of cell suspension:

$$\text{Number of viable}\frac{\text{cells}}{\text{mL}} = \frac{\text{Total number of viable cells counted}}{\text{4 squares}} \text{ x 2 (dilution factor) x } 10^4$$

Equation 2.1. Calculation of a number of cells per mL of cell suspension

2.6 CYTOSPIN PREPARATION

A cell suspension was prepared at 2 x 10^4 cells/mL final concentration. Slides were mounted behind the filter cards (Thermo electron corporation, USA) and cuvette in the metal holder of the centrifuge. Approximately 200 µL of cell suspension was loaded in the cuvette and spun at 800 x *g* (Shandon cytospin centrifuge, USA) for 5 minutes. The cuvette and paper were detached from the slide without damaging the fresh cytospin, which was then air-dried. Slides were stained using the May-Grunwald Giemsa staining procedure for differential leukocyte analysis (section 2.7).

2.7 MAY-GRUNWALD AND GIEMSA STAINING

Prepared cytospin and blood smear slides were fixed in 100% methanol (Scharlau, Spain) for 15 minutes. Slides were incubated in May-Grunwald staining solution for 5 minutes. May Grunwald staining solution was prepared by mixing equal parts of the May Grunwald stock solution (Australian Biostain, Australia) with buffered distilled water. Buffered distilled water was prepared as per instructions detailed in *Appendix 1*. Slides were then transferred into Giemsa staining solution for 10 minute incubation. Giemsa stain was prepared by mixing 1 part of Giemsa stock solution (Australian Biostain, Australia) with 9 parts of buffered distilled water. Subsequently, slides were then washed by transferring through three changes of buffered distilled water followed by a final, 10 minute incubation in the last change to ensure cell differentiation. Slides were air-dried and analysed under a light microscope (Nikon) for different leukocyte populations present.

2.8 RSV PROPAGATION

Approximately 1 x 10^7 Vero E6 cells were seeded in a T-150 flask (Iwaki) and left to incubate overnight at 37°C with 5% CO_2. The following morning cell monolayer was checked for approximately 80% confluence. Once ready, the flask was rinsed with 10 mL of Hank's Balanced Salt Solution (HBSS) (ANU, Australia) followed by A2 RSV strain (provided by Dr. Ralph Tripp) inoculation at MOI of 0.5 prepared in serum free DMEM (Gibco). The cell monolayer was allowed to adsorb virus for 2 hours at 37°C with 5% CO_2. After 2 hours, 8 mL of DMEM with 5% HI-FCS was added to the flask, and cells were incubated for 2-3 days at 37°C with 5% CO_2. The flask was checked daily for signs of syncytia

formation. The cells were deemed ready for harvest once there was 25-50% syncytium present with very little monolayer death. An optimal infection would result in syncytia evenly distributed across the entire flask. At the time of the harvest, approximately 80% of media was removed from the flask. The cell monolayer was scraped with a cell scraper (Thermo Fisher, Australia) and cell suspension collected in a pre-chilled 50 mL centrifuge tube. The cell suspension was sonicated 5 times for 5 seconds, at 25W in a biosafety cabinet. In between each sonication, the tube containing the cell suspension was chilled on ice for 2 minutes. Cell debris was removed by centrifugation at 3000 rpm for 7 minutes at 4°C. Supernatant was removed and aliquoted into sterile cryovials (Iwaki). Virus stock was stored -80°C until further processing.

Ultraviolet-inactivated RSV (UV-RSV) was generated by exposing RSV to UV radiation (Laftech Bio-Cabinets, Top Safe Class II, Australia) for 40 minutes. Immunostaining RSV viral titres assay (section 2.10) confirmed the absence of viable virus.

2.9 PREPARATION OF 2% METHYLCELLULOSE

In a shott bottle, 75 mL of HBSS (ANU) was added and brought to the boil in the microwave. This was then placed on a heated stirring block and 10 g of methylcellulose (Sigma, Australia) was slowly added, allowing powder to dissolve before adding more. The mixture was autoclaved at 15 psi for 20 minutes. While autoclaving, HBSS (ANU), HI-FCS (Sigma), DMEM (Gibco) and penicillin-streptomycin-neomycin (PSN) (Invitrogen, Australia) were pre-warmed in a 37°C water bath. When the autoclave run was finished, the mix was allowed cool down to 37°C whilst stirring. Once at 37°C, 40 mL HBSS (ANU), 10 mL HI-FCS (Sigma) and 10 mL 50X PSN solution was added to the methylcellulose mixture. The preparation was finalised with addition of 400 mL of serum free DMEM (Gibco). The solution was allowed to mix for at least 30 minutes at room temperature before being stored at 4°C until required.

2.10 IMMUNOSTAINING RSV PLAQUE ASSAY

Approximately 2 x 10^5 Vero E6 cells (ATCC) per well were seeded in 24-well plate (Iwaki) to obtain 80-90% confluence. Vero E6 cells (ATCC) were cultured in DMEM (Gibco) with 5% HI-FCS (Sigma) overnight at 37°C with 5% CO_2. The following day, serial dilutions of RSV samples (10^{-1} to 10^{-6}) were

prepared in serum free DMEM (Gibco) and 200 µL of the corresponding sample was loaded in quadruplicates onto the 24-well plate. Inoculated cells were allowed to adsorb virus for 1 hour at 37°C with 5% CO_2. After 1 hour, 1 mL of previously prepared, 2% methylcellulose was added to each well as an overlay and cells were left to incubate at 37°C with 5% CO_2 for 6 days. On day 6, methylcellulose was removed and cell monolayer fixed with 60:40 ice-cold acetone (LabScan, Thailand): methanol (Scharlau) for 10 minutes. Plates were air dried and blocked with 5% skim milk powder (Diploma, Australia) diluted in PBS (ANU) for 30 minutes at room temperature on a rocker. The block was removed and 200 µL of RSV monoclonal anti-F antibody 131-2A (Merck, Australia) was added per well, prepared at 1:1000 dilution in 5% skim milk blocking solution. Cells were incubated with primary antibody for 2 hours at 37°C with 5% CO_2. Cell monolayer was rinsed 3 times, 5 minutes each, with 0.5% Tween20 (Sigma, Australia) in PBS (ANU). Cells were then incubated with 200 µL/well of secondary antibody, goat anti-mouse IgG-alkaline phosphatase (Sigma, Australia) prepared at 1:1000 dilution in 5% skim milk blocking solution. Cells were incubated for 1 hour at 37°C with 5% CO_2. Cell monolayer was rinsed 3 times, 5 minutes each with 0.5% Tween20 (Sigma) in PBS (ANU). Cell monolayer was stained for RSV plaques with Vector black alkaline phosphatase substrate (Vector Laboratories, Europe). Reaction was stopped with deionised water. Stained RSV plaques were counted under a light microscope. To calculate total number of plaque forming units (pfu) (or viral titres) per 1 mL of RSV sample the following formula was used:

$$\frac{\text{number of plaques in quadriplicates}}{4} \text{ x dilution factor x } 5 = \text{plaques per mL}$$

Equation 2.2. RSV plaques (or viral titres) per 1mL of RSV sample

2.11 MICE STRAINS

Male BALB/c mice aged 6 to 8 weeks were obtained from the pathogen free facilities at the University of Canberra (UC) animal facility and Australian National University (ANU) animal services. Animals were housed in approved containment facilities and treated in accordance with approved UC animal experimentation guidelines. BALB/c mouse strains used were wild type (WT) BALB/c, IL-5 Tg BALB/c, Rag 2 knockout ($^{-/-}$) BALB/c and IL-5 Tg Rag $2^{-/-}$ BALB/c.

2.12 GENERAL ANIMAL HANDLING PROCEDURES

2.12.1 Euthanasia

At the conclusion of the experiment or if mice received a clinical score greater than 5, they were euthanised by CO_2 asphyxiation procedure. Briefly, mice were placed into a tightly closed plastic container connected via a tube to CO_2 which was slowly turned ON. Once animals would slow down/stopped moving, CO_2 was turned OFF and container remained closed for another few minutes to ensure that the animal has been euthanised properly. Once no pedal reflexes were detected, the animal was dissected and samples collected.

2.12.2 *In vivo* mouse monitoring procedures

Mice were monitored daily for any change in body weight and symptoms of RSV disease. Clinical scores (Table 2.1) were assigned to each mouse daily and if at any point of the experiment mice scored 5 or higher, they were euthanised immediately as per protocol outlined previously (section 2.12.1). If mice received a clinical score of 3-5, they were provided wetted food on the cage floor.

Table 2.1. Respiratory syncytial virus clinical scores for *in vivo* studies

Numerical scores	Physical symptoms displayed for infected mice
1	Healthy (no obvious signs of illness)
2	Barely ruffled fur and active
3	Ruffled fur, less active than normal
4	Ruffled fur, less active than normal and less than 20% weight loss
5	Hunched posture
6	20% weight loss of the starting weight-point of euthanasia
7	Death

2.12.3 Daily animal weighing procedure

Mice were weighed daily and monitored for any signs of weight loss. A mouse was gently picked up by the tail and placed inside a plastic container sitting on an analytical balance. Weight was recorded and the mouse returned to its cage.

2.12.4 Anaesthesia

For intranasal inoculation mice were lightly anaesthetised with isofluorane (PharmaChem, Australia) using the open drop jar method of administration. Approximately 2-3 drops of isofluorane (PharmaChem) were applied to a cotton wool placed inside a transparent jar with a lid. To minimise mice coming into a direct contact with the anaesthetics and systemic absorption, cotton wool was covered by a mesh. Mice were placed over the mesh in the jar and removed as soon as consciousness was lost. Once no pedal reflexes were detected, we proceeded with intranasal inoculation.

2.12.5 Intranasal inoculation

Anaesthetised mice were restrained manually with the head tilted down at 35-40° angle. A volume of less than 50 µL of fluid (or RSV stock) was placed a drop at a time on the nostril of the mouse causing the mouse to aspirate the virus into the nose, throat and lungs while coughing reflex was negated by anaesthesia.

2.12.6 Intraperitoneal inoculation

The animal was restrained manually and tilted head down at 35-40° angle allowing the intestines to fall away from the injection site. A 27-gauge needle was inserted into the lower right quadrant of the abdomen, slightly off the midline, anterior to the bladder. Mice received 100 µL intraperitoneal injections of 10 mg/mL/kg HS mimetics previously prepared in sterile saline.

2.13 *IN VIVO* SAMPLE COLLECTION

2.13.1 Bronchoalveolar lavage fluid (BALF) collection

Lungs were dissected free and the bronchus leading to the left lobe (apical, azygous, cardiac and diaphragmatic lobe) was temporarily tied up, while the right lobe was cannulated in order to perform BAL. The BALF was collected by gentle washing with 3 x 0.5 mL of sterile PBS and the sample placed on ice until further processing for differential leukocyte counts through cytospin preparation.

2.13.2 Histological analysis

The left anatomical lobe of the non-lavaged lung was fixed in 10% formalin (Sigma), embedded in paraffin and 3 mm sections stained with periodic acid-Schiff (PAS) or chromotrope (CR) stains (processed by A. Prins, John Curtin School of Medical Research, Australian National University). Slides were scored by an observer, blinded to the treatment groups for: parenchymal pneumonia (quantified on a 0 - 5 scale, 0 = none, 1.5 = minimal, 3 = medium and 5 = heavy); airways mucus occlusion (quantified on a 0 - 3 scale 0 = no occlusion, 1 = small areas of luminal accumulation of mucus, 2 = partial occlusion of at least one small airway and 3 = complete occlusion of at least one small airway); perivascular infiltrates (percentage of sites) (quantified on a 0 - 3 scale, 0 = none, 1 = few (< 10%), 2 = many (10-50%) and 3 = majority (> 50%)); peribronchiolar/bronchial infiltrates (percentage of sites) (quantified on a 0 - 3 scale, 0 = none, 1 = few (< 25%), 2 = many (25-75%) and 3 = all (> 75%)); and quality of peribronchiolar/bronchial infiltrates (quantified on a 0 - 3 scale, 0 = none, 1 = mild, 2 = moderate and 3 = severe). A combined histopathological score (HPS) (out of 17) was determined based on the sum of scores for these five observations. Histology slides were also evaluated for goblet cell count

(average number of goblet cells counted over 10 high power fields per sample) and parenchymal eosinophil count (average number of eosinophil counted over 10 high power fields per sample).

2.13.3 Homogenising lung tissue

Lungs were aseptically removed from mice and placed in a 2.0 mL microcentrifuge tube containing 1 mL of PBS (ANU) prepared with PSN (Invitrogen) (at 1X PSN final concentration) and one 5 mm steel ball (Procureit Australia). Tubes were snap frozen and stored at -80°C until further processing or were processed the same day. If frozen, the lung samples were partially thawed on ice. Samples were loaded into the two TissueLyser adapter blocks (pre-chilled at 4°C for at least 2 hours). Once blocks were balanced, samples were homogenised for 1 minute at a frequency of 30 Hz followed by centrifugation at 1700 x g (Eppendorf, Australia) for 1 minute at 4°C. In a biosafety cabinet, the supernatant of each sample was transferred into sterile 1.5 mL tubes and stored at -80°C until processed using the immunostaining RSV plaque assay (Section 2.10).

2.13.4 Blood smear

A drop of blood was placed onto a slide and smeared, creating a thin layer of blood. Dried slides were fixed in 100% methanol (Scharlau) for 10 minutes and processed with May-Grunwald and Giemsa staining (section 2.7). Leukocyte cell differentiation was performed under a light microscope (Nikon).

2.13.5 Total leukocyte count

Following heart puncture, 5 µL of blood was mixed with 95 µL of methylene blue (StemCell, Australia). Red blood cells were lysed using red cell lysis buffer (BD Pharmingen, Australia) for 5 minutes and total leukocytes were counted using haemocytometer. Following differential leukocyte analysis of blood smears, total leukocyte count was used to present a number of cells per mL of blood sample.

Chapter 3. Effect of respiratory syncytial virus infection on eosinophil biology

3.1 INTRODUCTION

As mentioned in Chapter 1, viral bronchiolitis has been associated with eosinophilic inflammation as demonstrated by epidemiological studies linking severe RSV bronchiolitis with the development of asthma later on in life. In past, accumulation of eosinophils at the site of viral infection has been perceived in a negative light however, the specific role played by virus elicited eosinophils-negative, positive or neutral-remains unclear. As a result, the role of eosinophils in RSV infection has been debated vigorously over the past few decades, with many researchers taking opposing stands on this issue. The aim of this study was to examine the *in vitro* effect of RSV on eosinophil morphology, release of eosinophil activation markers and eosinophil antiviral activity

RSV is a major cause of viral bronchiolitis and hospitalisation in infants under six months of age (Openshaw *et al.*, 2003). In addition, early RSV infection is known to be associated with development of respiratory problems such as asthma and wheezing later in life (Openshaw, 2002). RSV disease has been characterised by pulmonary eosinophilia elicited in response to primary RSV infection; observed in both, human infants and mouse neonates (Rosenberg *et al.*, 2009). As eosinophils are considered to be the main line of defence against parasites (Costa *et al.*, 1997; Hitoshi *et al.*, 1991) some researchers have focused their investigation on eosinophil antiviral ability.

The first RSV vaccine trial with FI-RSV yielded disastrous results in young vaccine recipients, following natural RSV infection during the subsequent winter. The vaccine trial resulted in recipients developing enhanced pulmonary disease leading to hospitalisation and in some cases, death of a few vaccine recipients (Chin *et al.*, 1969; Fulginiti *et al.*, 1969; van Hagen *et al.*, 1999). Studies with WT BALB/c mice have provided some insight into the mechanisms that may have contributed to FI-RSV enhanced pulmonary disease. These studies have found that WT BALB/c mice vaccinated with vectors expressing RSV G glycoprotein, purified G glycoprotein, or FI-RSV develop extensive enhanced pulmonary disease characterised by pulmonary eosinophilia, weight loss, exaggerated Th2-type cytokine responses and selective priming of $CD4^+$ T cells (Graham *et al.*, 2000; Hancock *et al.*, 1996; Openshaw *et al.*, 2001; Tripp *et al.*, 2000; van Hagen *et al.*, 1999; Varga *et al.*, 2001). G glycoprotein was also shown to compete with the chemokine, fractalkine for binding to CX3CR1

receptor, and to inhibit fractalkine mediated leukocyte chemotaxis (Tripp *et al.*, 2001). These studies highlight that RSV G glycoprotein has immune modulatory activities associated with the CX3C motif. Consequently, based on the findings from these FI-RSV studies, eosinophils are believed to contribute to the pathology of RSV disease.

However, very few research groups have focused on elucidating and understanding the potential that eosinophils may have in viral clearance. The first indication that eosinophils might have the means to function in promoting antiviral host defence came from a series of studies performed in the late 1990s. In this work, it was determined that eosinophils had a potential to reduce RSV infectivity of target epithelial cells *in vitro* (Domachowske *et al.*, 1998a; Soukup and Becker, 2003) as well as inhibit RSV infection in tissue culture (Rosenberg *et al.*, 2009). Interestingly, another study also revealed that RSV infected ΔdblGATA mice (eosinophil deficient) exhibited increased clinical illness similar to that observed in RSV infected WT BALB/c mice (Castilow, Legge, & Varga, 2008). Their findings also suggest that eosinophils are not required for increased weight loss, clinical illness and impaired lung capacity, the disease parameters most often associated with RSV vaccine enhanced disease (Castilow *et al.*, 2008). Finally, Rosenberg and Domachowske (1999 and 2001) were among the first groups to suggest that eosinophil recruitment and activation may lead to a positive host-defence (Domachowske and Rosenberg, 1999; Rosenberg and Domachowske, 2001). The possibility that eosinophils may be recruited in part to promote primary antiviral host defence is a very exciting concept (Rosenberg *et al.*, 2009).

Previous studies have also shown eosinophil activation in the presence of RSV infection (Domachowske *et al.*, 1998a; Phipps *et al.*, 2007). Eosinophil activation could result in piecemeal degranulation (PMD), a process known to lead to successful clearance of parasitic infections (Shamri *et al.*, 2011) through a selective release of eosinophil granule content. Eosinophil granules contain four cytotoxic proteins: (1) MBP, which is located in the eosinophil granule core; (2) EPO, (3) ECP and (4) EDN found in the matrix of the eosinophil granule (Rosenberg *et al.*, 2009). PMD leads to small vesicles budding from the secondary granules and subsequent transport of the granule protein subset to the cell surface, with a progressive and selective loss of secondary granule

constituents (Dvorak *et al.*, 1992; Dvorak *et al.*, 1991). This process of eosinophil degranulation has been well characterised with the help of transmission electron microscopy (TEM) studies. TEM analysis revealed that MBP forms crystals characterised by a very regular pattern of electron-dense stripes, visualised as a dark line while the other three eosinophil proteins (ECP, EDN and EPO) are located in the surrounding matrix, and appear light grey in colour (Dvorak *et al.*, 1993; Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999).

RSV clearance may be assisted through a process of eosinophil recruitment and migration, involving both, cytoskeletal activation and reorganisation (Morales-Ruiz *et al.*, 2000). Eosinophil migration is known to involve the formation of unique and highly dynamic adhesion structures called podosomes (sites of extravasation of the plasma membrane) (Siddiqui *et al.*, 2012). Podosomes are comprised of a core of short actin filaments and actin-associated proteins, enabling eosinophil migration from the blood into a tissue (Linder *et al.*, 2000). Migration is also enabled by activation of the microtubular network which can be visualised though fluorescence microscopy analysis (Remijsen *et al.*, 2011).

The activation of an immune response resulting in a positive resolution of RSV infection remains the most desired mechanism for the prevention and inhibition of RSV disease. This chapter aimed to examine the *in vitro* effect of RSV on eosinophil morphology, release of eosinophil activation markers and eosinophil antiviral activity. As previous studies have looked into RSV-induced eosinophil degranulation, in terms of ECP protein release only (Garofalo *et al.*, 1992; Olszewska-Pazdrak *et al.*, 1998b; Saito *et al.*, 1997), this chapter has focused on investigating early signs of eosinophil degranulation using MBP as the marker of eosinophil degranulation. In addition, extending on from the studies by Phipps *et al.* (2007) investigating eosinophil activation using EPO as the activation marker (Phipps *et al.*, 2007), in this study, the effect of RSV on eosinophil chemotaxis, through the activation of the eosinophil cytoskeletal components, α-tubulin and F-actin was examined. Furthermore, the effect of RSV on eosinophil morphology with an in depth TEM analysis as well as the RSV kinetics in mouse eosinophils was also examined.

3.2 METHODS

3.2.1 Eosinophil fluorescence activated cell sort

The leukocyte population was collected from IL-5 Tg BALB/c mice by peritoneal lavage. Mice were sacrificed by CO_2 asphyxiation (section 2.12.1) and the peritoneal cavity exposed by an incision through the abdominal skin. The cavity was washed 3 times with 5 mL of sterile HBSS (ANU) using an 18-gauge needle (Terumo, Australia). Lavage was collected and centrifuged at 240 x *g* (Sigma 3-16 PK) for 5 minutes. The supernatant was discarded and the cell pellet resuspended in red blood cell lysing buffer (BD Pharmingen, Australia) for 5 minutes. Cells were re-centrifuged at 240 x *g* (Sigma 3-16 PK) for 5 minutes followed by removal of supernatant and resuspended in sterile HBSS (ANU), with a maximum density of 1 x 10^7 cells/mL. All cell clumps and debris were removed prior to sorting, by filtration through a cell strainer (40 µm) (BD, Australia), then transferred into sterile 4 mL FACS tubes (BD Labware, Europe) and placed on ice prior to cell sorting.

The fluorescence activated cell sorter (FACS) (BD Biosciences FACSVantage SE, USA) was used to sort mature eosinophils from the peritoneal cell mix, based on forward (FSC) and side scatter (SSC) parameters and polarised light as well as green (FL1) and red (FL2) fluorescence measurements. Polarised light is especially important in sorting eosinophils as measuring depolarised light scattering (dSSC) in combination with normal SCC, enables eosinophils to be sorted with high precision. Electronic gates were set in order to include eosinophils only, achieving approximately 97% cell purity. Eosinophils were sorted into DMEM (Gibco) with 30% HI-FCS (Sigma) and kept on ice prior to use.

3.2.2 Eosinophil transmission electron microscopy (TEM) analysis

Approximately 6 x 10^6 freshly sorted eosinophils (per sample) were treated with Vero E6 cell lysate (negative control) or RSV at multiplication of infection (MOI) of 1. RSV was allowed to adsorb for 2 hours, followed by change of RSV containing supernatant for fresh media. Samples were collected at 3, 6, 12, 18, 24 or 48 hours post incubation and fixed using 2% glutaraldehyde/0.1M Sodium cacodylate (Sigma, Australia) overnight at 4°C followed by further processing at the Microscopy and Cytometry Resource Facility (Ms C. Gillespie, The Microscopy and Cytometry Resource Facility, JCSMR, ANU) in accordance with detailed protocol described in Appendix 2. Briefly, following primary fixation with 2% glutaraldehyde in 0.1M Sodium cacodylate (Sigma) for at least 2 hours, samples were secondary fixed with 1% Osmium textroxide (PriSciTech, Australia) for 1.5 hours followed by three 15 minute washes with 0.1M Sodium cacodylate and one 15 minute wash with distilled water. Samples were then dehydrated in acetone (Univar) (concentrations included: 15 minutes in each 30, 50, 70, 90, 95% acetone and three 15 minute lots at 100% acetone), followed by embedment in Spurr's resin (PriSciTech, Australia). Sections of each sample were cut and stained with 2% uranyl acetate (PriSciTech, Australia) and Reynold's lead citrate (PriSciTech, Australia). Samples were analysed and photographed using a Hitachi H7000 transmission electron microscope at 75kV.

Secondary granules of each eosinophil in TEM sample were counted and classified into four categories (type I, intact; type II, loss of crystal core; type III, loss of matrix; and type IV, loss of crystal core and matrix). The extent of degranulation was quantified by calculating the percentage of degranulating cells using the following Equation 3.1:

$$\%\ \text{of degranulating cells} = \frac{\text{no. of activated granules (type II} - \text{IV)}}{\text{total no. of granules}} \times 100$$

Equation 3.1. Quantification of degranulating eosinophils

3.2.3 Eosinophil degranulation assay

The eosinophil degranulation assay was performed in 24-well tissue culture plates (Iwaki) as previously described by Clark (2004). Plates were coated with 2.5% w/v bovine serum albumin (BSA) (Fraction V) (Sigma, USA) diluted in PBS (ANU) (100 µL/well) and incubated overnight at 37°C with 5% CO_2. The next day, 250 µL of purified eosinophils (section 3.2.1) prepared in DMEM (Gibco) at 5 x 10^5 cells/mL concentration, was added to each well, followed by the same volume of stimulus (Vero E6 cell lysate (negative control), UV-RSV (additional negative control), 0.1 mM (final concentration) phorbol myristate acetate (PMA) (Sigma, Australia) or RSV at an MOI 1 (Sigma). The plates were incubated at 37°C with 5% CO_2 for 4 hours, after which, they were spun down at 240 x *g* (Sigma 3-16 PK) for 5 minutes. The supernatants were collected into sterile eppendorf tubes and stored at -80°C, until assayed for MBP.

3.2.3.1 Eosinophil major basic protein detection assay

The samples collected for eosinophil degranulation were tested for MBP activity in the culture supernatant using a dot-blot assay as previously described by Hogan (1998). Briefly, polyvinylidene difluoride (PVDF) (Millipore, USA) membrane was soaked in 100% methanol (Scharlau) for 1 minute, followed by Tris-buffed saline (TBS) (Sigma, Australia) for 5 minutes. The membrane was placed onto a dot blot manifold (Schleicher & Schuell, Germany) to ensure uniform distribution of protein for each sample, and 100 µL of the culture supernatant was added to the appropriate wells on the apparatus. After incubation for 1 hour, each well was washed 3 times with 200 µL of TBS. followed by removal from the apparatus and transfer into a blocking solution, containing 1% BSA (Fraction V) (Sigma) in TBST (TBS supplemented with 0.1% Tween-20 (Sigma, Australia) overnight at 4°C. The membrane was then washed twice with TBST for 10 minutes followed by incubation with Rabbit anti-murine MBP monoclonal antibody (50 µg/L) (Santa Cruz Biotechnology, Australia) at a dilution of 1:2500 in 1% BSA (Fraction V) (Sigma) in TBST for 2 hours at room temperature. The membrane was washed three times with TBST for 15 minutes. Subsequently, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rat antibody at a dilution of 1:1000 in 1% BSA in TBST (Sigma, Australia) for 1 hour at room temperature. Finally, the

membrane was washed 3 times with TBST for 15 minutes each, followed by two 10 minutes TBS washes. The process was finalised with a 5 minute development of the blot by addition of the Western blue-stabilised substrate (Promega, USA). The reaction was stopped by rinsing the membrane in distilled water for 10 minutes. The membrane was placed onto paper towel and covered with aluminium foil where it was left to dry. The dot-blot (image available in Appendix 3) was digitised by densitometry and image analysed using Multi Gauge Version 2.2 (Fujifilm, Japan).

The following formulas were used to calculate percentage increase and decrease:

$$\text{Percentage increase} = 100 \text{ x } \frac{(\text{new number} - \text{original number})}{\text{original number}}$$

$$\text{Percentage decrease} = 100 \text{ x } \frac{(\text{original number} - \text{new number})}{\text{original number}}$$

Equation 3.2. Percentage increase and decrease formula

3.2.4 Eosinophil RSV kinetics

Purified eosinophils (3 x 10^5 cells/well) were treated with RSV at an MOI 1. The infection was set up in 24-well plates (Iwaki) and cells incubated at 37°C with 5% CO_2 for 3, 6, 12, 24 or 48 hours. Following the incubation, cells and the supernatant were collected and assayed for viral titres using the immunostaining RSV plaque assay (section 2.10).

3.2.5 Eosinophil activation – EPO release assay

To analyse eosinophil activation, 96-well plates (Iwaki) were prepared by coating each well with 60 µL of fibronectin (Sigma, Australia) solution (20 µg/mL in PBS) overnight at 4°C. Wells were washed twice with PBS (ANU) and all non-coated sites were blocked with 0.1% (w/v) BSA (Fraction V) (Sigma) for 60 minutes at 37°C with 5% CO_2. Plates were then washed twice with PBS (ANU) and quickly air-dried. Purified eosinophils (5×10^4 cells/well) were resuspended in Roswell Park Memorial Institute medium (RPMI)-1640 (Gibco, Australia) containing 4% HI-FCS (Sigma) and treated with Vero E6 cell lysate (negative control), UV-RSV (additional negative control), 0.1 mM (final concentration)

PMA (Sigma) (positive control) or RSV at a MOI of 1. Samples were prepared in five replicates and incubated for 90 minutes at 37°C with 5% CO_2. Following incubation, non-adhered cells were removed, and the remaining cells were washed twice with PBS (ANU) and lysed using 0.22% cetyl trimethylammonium bromide (CTAB) (Sigma, Australia) in 10 mM HEPES (pH 8) (Gibco, Australia). Cell lysates were serially diluted across an uncoated 96-well plate and 100µL/well of O-phenylenediamine dihydrochloride substrate (40 mg substrate dissolved in 32 mL 1mM HEPES, pH 8) (Sigma, Australia) was added, followed by for 30 minute incubation in dark at room temperature. The reaction was stopped by adding 50 µL/well of 4M H_2SO_4 (Univar) and the product analysed at 490 nm using a colorimetric plate reader (BioRad, USA). The number of adhered cells was calculated based on a standard curve generated by a known number of untreated eosinophils. Cells were lysed with 0.22% CTAB and processed in the same manner as the unknown samples. Readings from the negative control (eosinophils treated with Vero E6 cell lysate) were used as a baseline.

3.2.6 Eosinophil activation with an agonist

Eosinophil chemotaxis assay is based on the experimental set up by Olszewska *et al.* (1998) (Olszewska-Pazdrak *et al.*, 1998a). Approximately 2 x 10^5 HEp-2 cells/well was seeded in a 24-well plate (Iwaki). Cells were incubated with Vero E6 cell lysate, UV-RSV or RSV at an MOI of 1 for 24 hours at 37°C with 5% CO_2. RSV was allowed to adsorb for 2 hours, followed by change of RSV containing supernatant for fresh media. Following day, Costar transwell filters (Corning, USA) were coated with 50 ng/mL fibronectin for 2 hours at 37°C and rinsed twice with serum free RPMI-1640 (Gibco, USA). Freshly purified eosinophils were loaded in the top chamber of the transwell filter (5 x 10^4 cells/well) and allowed to adhere for 20 minutes at room temperature. Supernatant (agonist) collected from Vero E6 cell lysate treated HEp-2 cells (negative control), UV-RSV treated HEp-2 cells (additional negative control), 0.1 µM PMA (Sigma) with 100 ng/mL eotaxin (Invitrogen, Australia) (positive control) or RSV infected HEp-2 cells (at an MOI 1) was placed in the bottom chamber of the transwell plate. Eosinophils were allowed to migrate for 10 minutes towards the agonist at room temperature. Cells were fixed 4% paraformaldehyde (Sigma, Australia) for 10 minutes. Transwell filters were

excised and placed in the bottom of a 24-well plate (Iwaki) for immunohistochemistry staining of the primary eosinophils for cytoskeletal components (section 3.2.6.1).

3.2.6.1 Immunohistochemistry assay for cytoskeletal components in primary eosinophils

Fixed cells were rinsed three times for 5 minutes with 0.1% Triton X-100 (Sigma, Australia) pH 7.4 in PBS. Cells were permeabilised for 10 minutes with 0.5% Triton X-100 (Sigma) pH 7.4 in PBS and rinsed again three times for 5 minutes with 0.1% Triton X-100 (Sigma) pH 7.4 in PBS. Cells were then incubated for 2 hours at room temperature with blocking solution, 2% goat serum (Life technologies, Australia) in PBS (ANU) to eliminate any non-specific binding. This was followed by incubation with primary antibody, anti-F-actin or anti-α-tubulin at 1-10 µg/mL (Invitrogen, Australia) at 4°C overnight in a sealed plate or 1 hour at room temperature. Slides were rinsed five times for 5 minutes with 0.1% TritonX-100 (Sigma) in PBS (ANU) at room temperature. Secondary antibody, Texas red-phalloidin or boron-dipyrromethene (BODIPY)-FL (Invitrogen, Australia)) was prepared at 1:1000 in the blocking solution. Slides were incubated in the secondary antibody solution at 4°C overnight. Slides were rinsed five times for 5 minutes with 0.1% TritonX-100 (Sigma) in PBS (ANU) at room temperature. Slides were mounted using a small drop of 4',6-diamidino-2-phenylindole (DAPI) antifade (Invitrogen, Australia). The filter (containing adhered eosinophils) from the bottom of the 24-well plate (Iwaki) was harvested and inverted onto a drop of DAPI antifade placed on a coverslip and sealed with nail polish. Slides were analysed for actin polymerisation and α-tubulin activation using a confocal microscope (Nikon Eclipse Ti).

3.3 RESULTS

3.3.1 RSV-induced changes to eosinophil morphology

As eosinophils are found to play an important role in RSV infection, electron microscopy analysis of eosinophils was used to determine the extent of cellular activation following RSV treatment (Figure 3.1). Eosinophils showed signs of the granule protein release, mostly via a process of PMD (Figure 3.1). As seen in Figure 3.1 A, following 3 hour incubation untreated eosinophils (negative control) contained primarily type I granules with highly electron-dense crystals (core intact) and vesicles (matrix intact) (arrow and inset in A) with only 20% of type II granule (thick arrows) present. As seen in Figure 3.1 B-D, the number of type II granules present in untreated eosinophils (negative control) increased over time (6-18 hours), from 30 to 50%, determined by a progressive increase in a number of electron-lucent crystals and accompanied by electron-lucent vesicles. Moreover, at 24 hours post incubation untreated eosinophils displayed signs of type III granules (inset in E) (thin arrows), with the overall cellular structure still intact (Figure 3.1 E).

In contrast, RSV-treated eosinophils incubated for 3 hours (Figure 3.1 F) contained a greater proportion of activated eosinophils undergoing PMD, with 50% of type II granules present (indicative of loss of core content, thick arrows; inset in F). At 6 hours of incubation, RSV-treated eosinophils contained equal parts of type II and type III granules, approximately 50% each; while at 12 and 18 hours post incubation, RSV-treated eosinophils contained close to 90% of granules that had lost their core, matrix or both granule constituents (Figure 3.1 H and I). Following a 24 hour incubation period, the secondary granules of RSV-treated eosinophils were surrounded by small empty vesicles, type IV granules (arrow head), showing signs of apoptotic nucleus, cellular degradation (loss of membrane) and the release of the cellular contents into the surrounding (Figure 3.1 J).

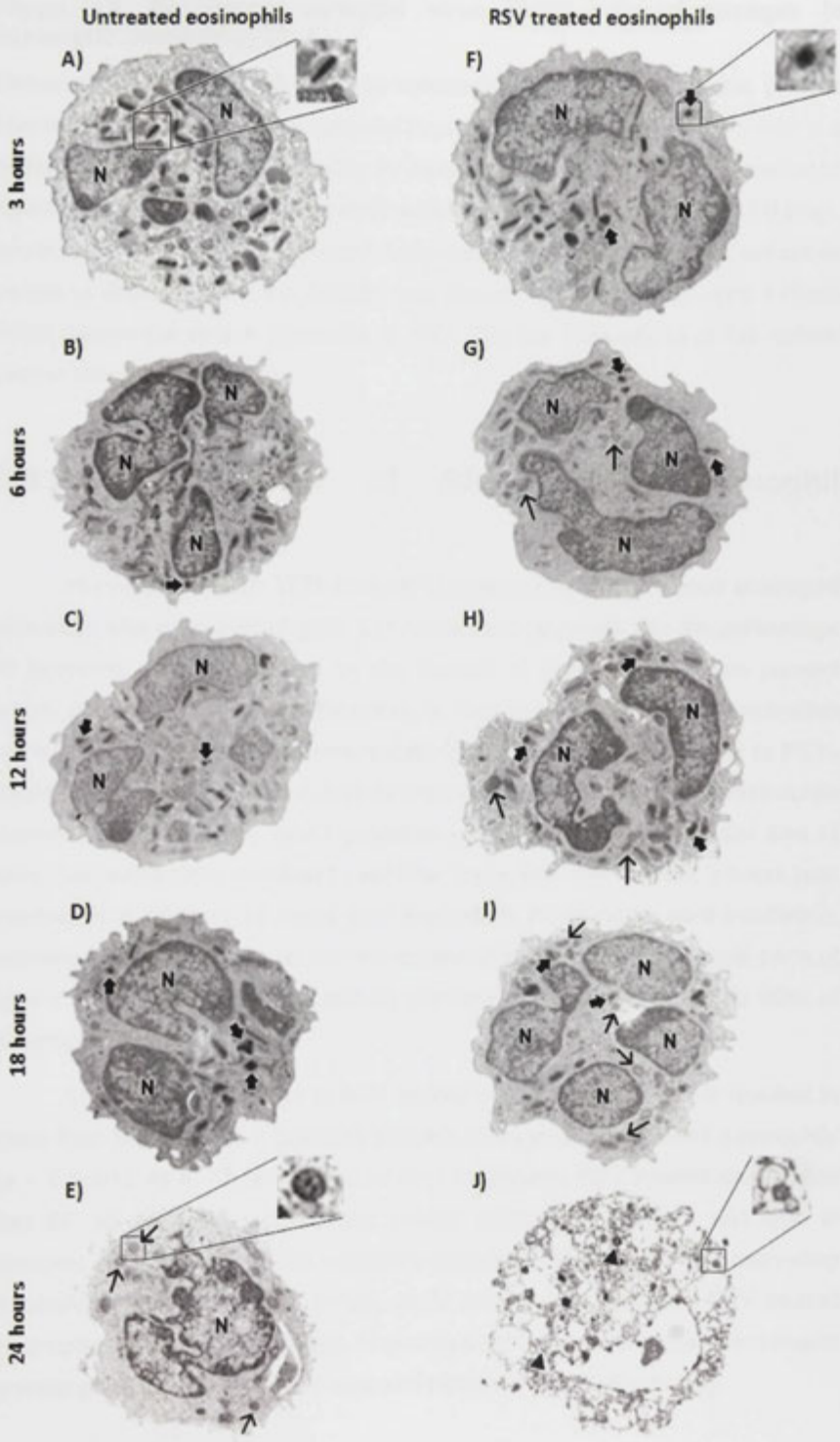

Untreated eosinophils
RSV treated eosinophils
3 hours
6 hours
12 hours
18 hours
24 hours
A)
B)
C)
D)
E)
F)
G)
H)
I)
J)
N

Figure 3.1. Respiratory syncytial virus (RSV) induced changes to eosinophil morphology (A-J)

Electron photomicrographs (EM) of mouse eosinophils following 3, 6, 12, 18 and 24 hours treatment with Vero E6 cell lysate (untreated eosinophils; negative control) (A-E) or RSV at a multiplicity of infection of 1 (F-J). Following the incubation, cells were collected and processed for transmission electron microscopy. Eosinophil multilobed nucleus is labelled N on each EM image. Arrows indicate different signs of eosinophil degranulation (loss of core and/or matrix) and cellular integrity as described in the text. Samples were analysed and photographed using a Hitachi H7000 transmission electron microscope at 75kV. Data are representative of five replicate samples taken for each time point.

3.3.2 Quantification of RSV-induced eosinophil activation

In conjunction with TEM analysis of eosinophils, RSV-induced eosinophil activation was quantified (Figure 3.2) by transforming data into the percentage of activated cells, determined by the number of type II-IV granules present within eosinophils. Distinct differences in the degree of eosinophil activation were observed for untreated eosinophils (negative control) compared to RSV-treated eosinophils. Following 3-18 hours post incubation, untreated eosinophils contained predominantly type I granules, with type II granules (partial loss of core) becoming more prominent over time, increasing from 20% at 3 hours post incubation to 55% by 18 hours post incubation. At 24 hours post incubation, untreated eosinophil granule content appeared to be made up of equal parts of type II and III (partial loss of matrix) granules, making approximately 90% of eosinophil granule content.

In contrast, incubation of RSV treated eosinophils for 3 hours resulted in more than 50% of type II granules present, compared to untreated eosinophils ($p < 0.0001$). At 6, 12 and 18 hours post incubation, RSV treated eosinophils had 28, 38 and 36% respectively, greater proportion of type II and type III granules present compared to untreated eosinophil control at the corresponding incubation times ($p < 0.005$). Finally, at 24 hours post incubation, RSV treated eosinophils contained 100% type IV granules with a complete loss of eosinophil granule protein content (loss of core and matrix) and cellular integrity.

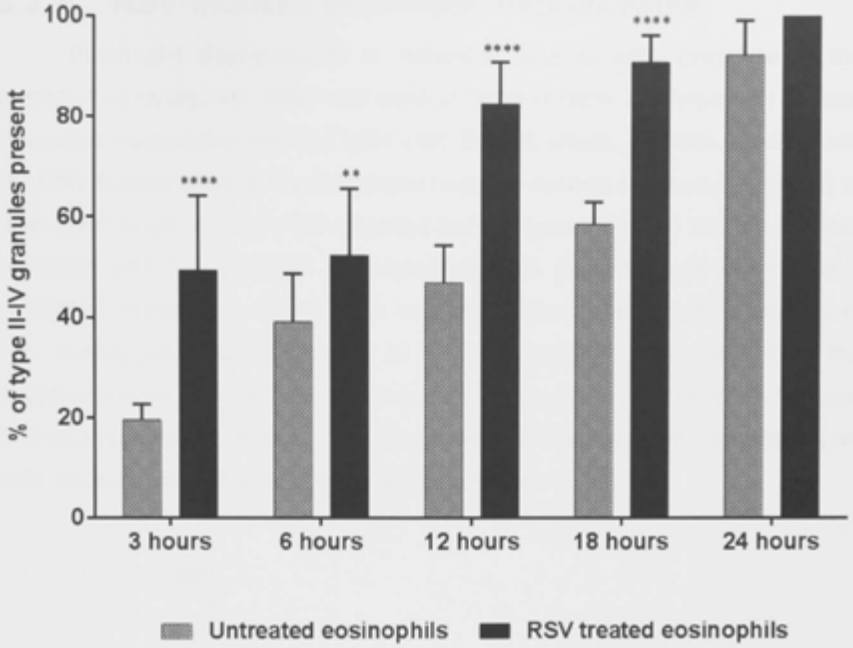


Figure 3.2. Quantification of respiratory syncytial virus (RSV) induced eosinophil activation

Freshly isolated eosinophils from interleukin-5 transgenic BALB/c mice were incubated with Vero E6 cell lysate (untreated eosinophils; negative control) or RSV at a multiplicity of infection of 1 and the percentage of degranulating cells was determined using transmission electron microscopy assay following 3, 6, 12, 18 and 24 hours incubation. The data is represented as the mean percentage of type II-IV granules present in eosinophils (n = 10) ± standard deviation. Significant differences as determined by TWO-way ANOVA with Sidak's multiple comparison test (GraphPad Prism version X7), were seen in RSV treated samples compared to the corresponding untreated eosinophil samples (**, $p < 0.005$ and ****, $p < 0.0001$).

3.3.3 RSV-induced eosinophil degranulation

Eosinophil degranulation is indicative of eosinophil activation in the presence of an agonist. MBP was used as an eosinophil degranulation marker. Untreated eosinophils (treated with Vero E6 cell lysate; negative control) and UV-RSV treated eosinophils (additional negative control) displayed low levels of eosinophil degranulation (100 density/pixel^2) (Figure 3.3). On the other hand, treatment with 0.1 µM (final concentration) PMA (positive control) induced a significant increase ($p < 0.0001$) in eosinophil degranulation with a reading of 165 density/pixel^2, corresponding to a 75% increase as compared to the negative control (Figure 3.3). However, RSV treatment of eosinophils induced degranulation with a reading of approximately 185 density/pixel^2 suggesting an 80% increase compared to the negative control ($p < 0.0001$).

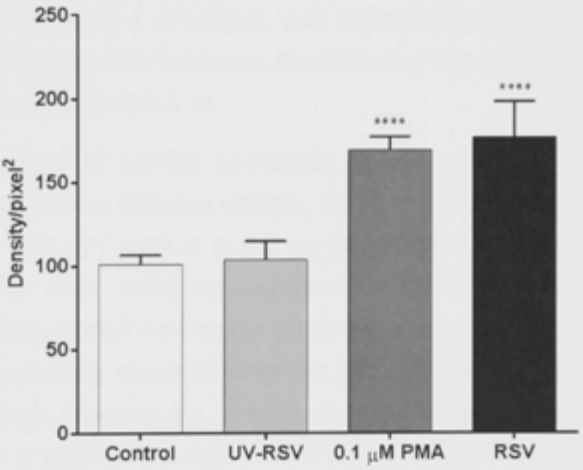


Figure 3.3. Respiratory syncytial virus (RSV) induced eosinophil degranulation

Freshly isolated eosinophils, from interleukin-5 transgenic BALB/c mice were incubated with Vero E6 cell lysate (untreated eosinophils; negative control – white bar), ultraviolet-inactivated RSV (UV-RSV) (additional negative control – light grey bar), 0.1 µM phorbol myristate acetate (PMA) (positive control – dark grey bar) or RSV (black bar) at multiplicity of infection of 1 for 4 hours. Samples were assessed for eosinophil degranulation using a major basic protein as a degranulation marker. Data is represented as the mean density/pixel^2 (n = 5) ± standard deviation. Data was analysed using ONE-way ANOVA with Dunnet multiple comparison test (GraphPad Prism version X7) (****, $p < 0.0001$ compared to the negative control).

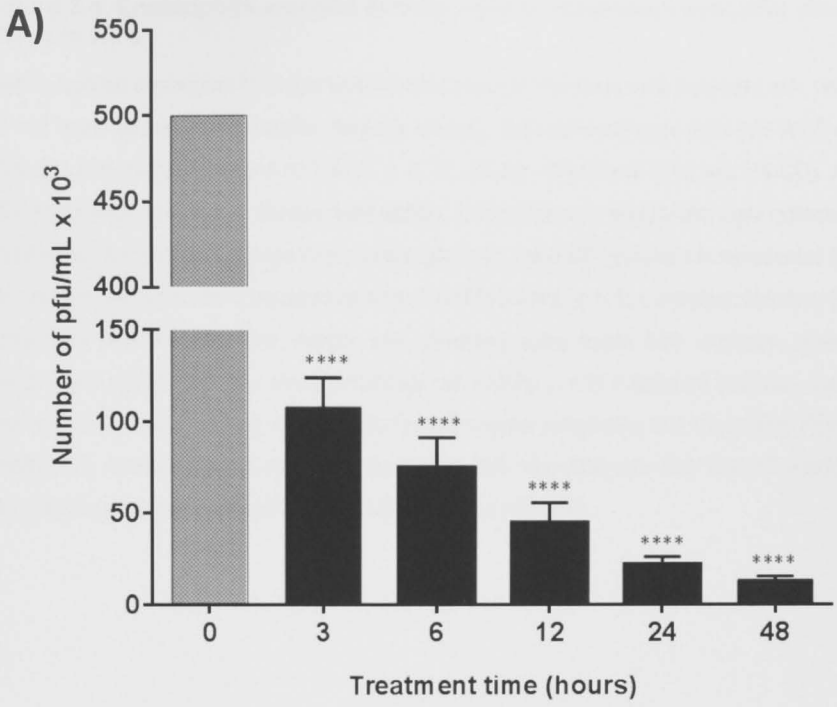
3.3.4 Eosinophil RSV antiviral activity

To investigate eosinophil antiviral activity, purified eosinophils isolated from IL-5 Tg BALB/c mice were treated with Vero E6 cell lysate (untreated eosinophils; negative control), UV-RSV (additional negative control) or RSV at an MOI of 1 for 0, 3, 6, 12, 18, 24 and 48 hours (Figure 3.4 A). Samples were collected and assayed for RSV titres. No viral titres were recovered from untreated (negative control) or UV-RSV (additional negative control) treated eosinophils.

At 0 hours post incubation, RSV treated eosinophils received approximately 500 x 10^3 pfu of RSV per sample. By 3 hours post incubation, the viral titre had decreased significantly ($p < 0.0001$) to 100 x 10^3 pfu/mL. The viral titres continued to decrease over time, from 75 x 10^3 pfu/mL at 6 hours post RSV treatment, down to 45 x 10^3 pfu/mL at 12 hours. At 24 hours post RSV treatment a count of 25 x 10^3 pfu/mL was observed. At the experimental end point of 48 hours post RSV treatment, the viral titres had significantly decreased to 15 x 10^3 pfu/mL (Figure 3.4 A).

Cell viability of purified eosinophils treated with Vero E6 cell lysate (untreated eosinophils; negative control), UV-RSV (additional negative control) or RSV at an MOI of 1 for 0, 3, 6, 12, 18, 24 and 48 hours was also assessed (Figure 3.4 B). Both, untreated (negative control) and UV-RSV (additional negative control) treated eosinophils induced a similar cell viability trend, with cell viability remaining above 85% for the first 6 hours of incubation for both treatment groups. However, by 12 hours post incubation, cell viability dropped to 38%, with a further decrease to 15% and 5% by 24 and 48 hours post incubation respectively for both, untreated and UV-RSV treated eosinophil groups.

RSV treatment of eosinophils resulted in a significant reduction in cell viability over time, compared to the negative controls ($p < 0.05$). Within the first 6 hours of incubation, RSV treated eosinophil cell viability decreased from 97% to 60% ($p < 0.05$). Only 20% of the RSV treated eosinophils were viable at 12 hours post incubation ($p < 0.0001$) with continued decline to 10% and 0% by 24 and 48 hours post incubation, respectively.


A)
550
500
450
400
150
100
50
0
Number of pfu/mL x 10^3
****
****
****
****
****
0
3
6
12
24
48
Treatment time (hours)

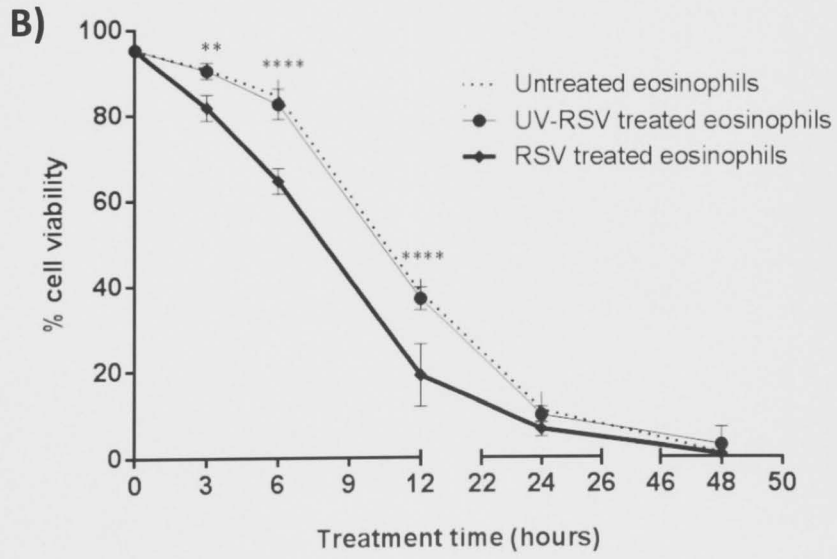

B)
100
80
60
40
20
0
% cell viability
**
****
****
Untreated eosinophils
UV-RSV treated eosinophils
RSV treated eosinophils
0
3
6
9
12
22
24
26
46
48
50
Treatment time (hours)

Figure 3.4. Eosinophils antiviral activity against respiratory syncytial virus (RSV) *in vitro*

Freshly isolated eosinophils from interleukin-5 transgenic BALB/c mice were incubated with Vero E6 cell lysate (untreated eosinophils; negative control), ultraviolet-inactivated RSV (UV-RSV) or RSV at a multiplicity of infection of 1 for 0, 3, 6, 12, 24 and 48 hours at 37ºC with 5% CO_2. **A) RSV replication kinetics in mouse eosinophils.** Eosinophils and supernatant were collected and assayed for viral titres (plaque forming units (pfu)/mL). Initial RSV inoculum is represented by the grey bar. Samples are represented as a mean of RSV pfu/mL (n = 5) ± standard deviation. **B) Eosinophil cell viability.** Cell viability was measured using trypan blue exclusion assay. Samples are represented as a mean percentage cell viability (n = 5) ± standard deviation. Data was analysed using ONE-way ANOVA with Dunnet multiple comparison test (GraphPad Prism version X7). Asterisks denote significant decrease in RSV viral titres over time and cell viability compared to untreated eosinophils (*, $p < 0.05$ and ****, $p < 0.0001$).

3.3.5 Eosinophil migration

3.3.5.1 Eosinophil activation – EPO release

EPO was used as an eosinophil activation marker (Figure 3.5). Untreated eosinophils (treated with Vero E6 cell lysate; negative control) and UV-RSV treated eosinophils (additional negative control) induced low levels of eosinophil activation (0.5 optical density (OD)) (Figure 3.5). Eosinophil treatment with 0.1 µM PMA (positive control) resulted in an increase in eosinophil activation with a reading of approximately 2 OD obtained ($p < 0.001$) (Figure 3.5) as well as 300% increase in eosinophil activation compared to the negative control. However, RSV treatment of eosinophils induced eosinophil activation with a reading of approximately 3 OD indicating a 500% increase in eosinophil activation compared to the negative control ($p < 0.001$).

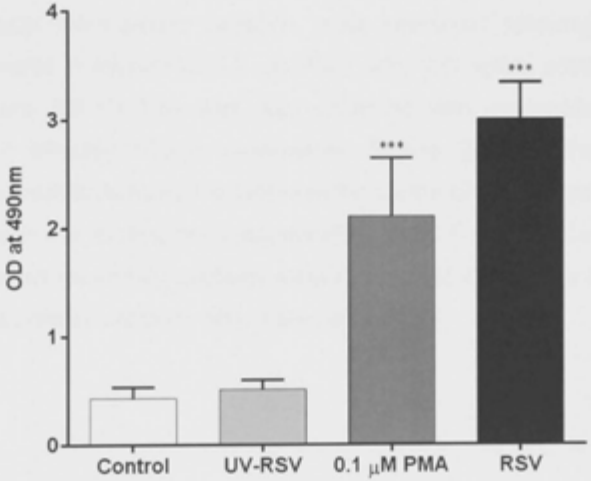


Figure 3.5. Respiratory syncytial virus (RSV) induced eosinophil activation

Freshly isolated eosinophils, from interleukin-5 transgenic BALB/c mice were incubated with Vero E6 cell lysate (untreated eosinophils; negative control – white bar), ultraviolet-inactivated RSV (UV-RSV) (additional negative control – light grey bar), 0.1 µM phorbol myristate acetate (PMA) (positive control – dark grey bar) or RSV (black bar) at a multiplicity of infection of 1 for 90 minutes. Samples were assessed for eosinophil activation using an eosinophil peroxidase as an activation marker. Data is represented as the mean optical density (OD) (n = 5) ± standard deviation. Data was analysed using ONE-way ANOVA with Dunnet multiple comparison test (GraphPad Prism version X7) (***, $p < 0.001$ compared to the negative control).

3.3.5.2 *Activation of alpha-tubulin*

Freshly sorted eosinophils, from IL-5 Tg BALB/c mice were allowed to migrate for 10 minutes towards Vero E6 cell lysate treated HEp-2 cells supernatant (untreated eosinophils; negative control) (Figure 3.6 A and B), UV-RSV treated HEp-2 cells supernatant (additional negative control) (Figure 3.6 C and D), 0.1 μM (final concentration) PMA with 100 ng/mL of eotaxin (positive control) (Figure 3.6 E and F) or supernatant collected following 24 hour RSV treatment of HEp-2 cells at an MOI of 1 (Figure 3.6 G and H).

Untreated eosinophils migrating towards either Vero E6 cell lysate treated HEp-2 cells supernatant (negative control) or UV-RSV treated HEp-2 cells supernatant (additional negative control) had punctate and non-polymerised α-tubulin localised radially from the microtubules organising centre (MTOC) and around the peripheral cell membrane (Figure 3.6 B and C). The addition of an agonist induced rapid α-tubulin polymerisation and a formation of a distinct radial microtubular network, most prominent following eosinophil migration towards a mixture of 0.1 μM PMA with 100 ng/mL eotaxin (positive control) (Figure 3.6 F) This was also observed with eosinophils migrating towards RSV infected HEp-2 supernatant (Figure 3.6 H). The MTOC in migrating eosinophils remained localised at the centre of the elongated nucleus, anterior towards the leading pseudopodia (Figure 3.6 F and H). Controls of the BODIPY-labelled secondary antibody were also run to ensure the specificity of binding to the primary antibody only (data not shown).

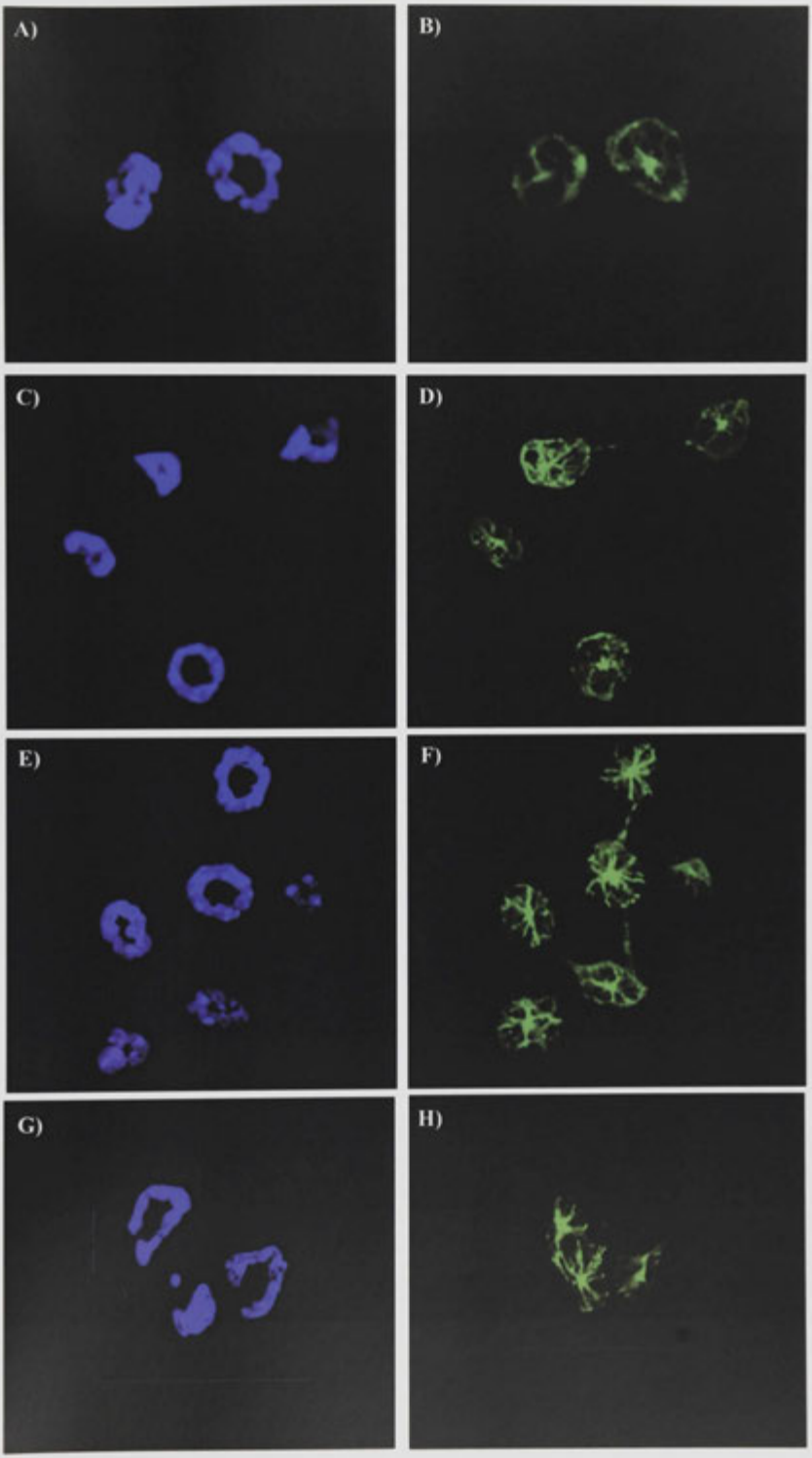

A)
B)
C)
D)
E)
F)
G)
H)

Figure 3.6. Respiratory syncytial virus (RSV) activation of eosinophil α-tubulin

Eosinophils migrating towards Vero E6 cell lysate treated HEp-2 cells supernatant (untreated eosinophils; negative control) (A and B), ultraviolet-inactivated (RSV) (UV-RSV) treated HEp-2 cells supernatant (additional negative control) (C and D), 0.1 μM phorbol myristate acetate (PMA) with 100 ng/mL of eotaxin (positive control) (E and F) or supernatant collected following 24 hour RSV treatment of HEp-2 cells at a multiplicity of infection of 1 (G and H) were stained with DAPI (A, C, E and G) and anti-α-tubulin and BODIPY-labelled secondary antibody (B, D, F and H) for detection of the microtubular network. Images were taken using the Nikon Eclipse Ti confocal microscope. Images are representative of two separate experiments with samples prepared in triplicates.

3.3.5.3 *Activation of F-actin*

Freshly sorted eosinophils, from IL-5 Tg BALB/c mice were allowed to migrate for 10 minutes towards Vero E6 cell lysate treated HEp-2 cell supernatant (untreated eosinophils; negative control) (Figure 3.7 A and B), UV-RSV) treated HEp-2 cell supernatant (additional negative control) (Figure 3.7 C and D), 0.1 μM (final concentration) PMA with 100 ng/mL of eotaxin (positive control) (Figure 3.7 E and F) or supernatant collected following 24 hour RSV treatment of HEp-2 cells at a multiplicity of infection of 1 (Figure 3.7 G and H).

Untreated eosinophils either migrating towards Vero E6 cell lysate treated HEp-2 cells supernatant (negative control) or towards UV-RSV treated HEp-2 cells supernatant (additional negative control) had F-actin primarily localised and adjacent to the peripheral membrane (Figure 3.7 B and C). The addition of an agonist induced cell polarisation and accumulation of F-actin towards the leading pseudopod observed in eosinophils migrating towards a mix of 0.1 μM PMA with 100 ng/mL eotaxin (positive control) (Figure 3.7 F). Similar observations were made for eosinophil migration towards RSV infected HEp-2 supernatant (Figure 3.7 H). Thick arrows indicate leading edge formation, while thin arrows point towards direction of eosinophil migration (Figure 3.7 F and H). Controls of the Texas red anti-phalloidin-labelled secondary antibody were also run to ensure the specificity of binding to the primary antibody only (data not shown).

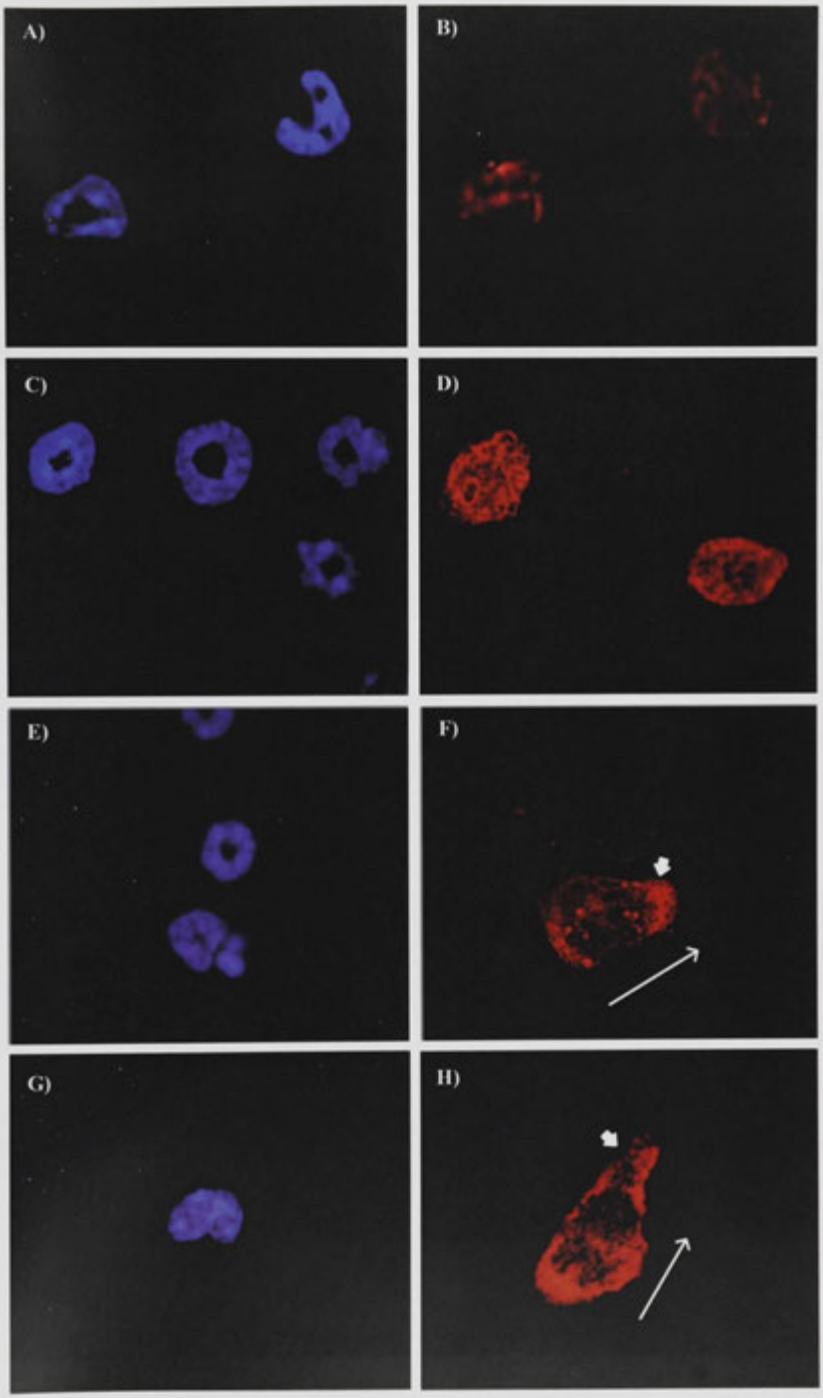

A)
B)
C)
D)
E)
F)
G)
H)

Figure 3.7. Respiratory syncytial virus (RSV) activation of eosinophil F-actin

Eosinophils migrating towards Vero E6 cell lysate treated HEp-2 cells supernatant (untreated eosinophils; negative control) (A and B), ultraviolet-inactivated RSV (UV-RSV) treated HEp-2 cells supernatant (additional negative control) (C and D), 0.1 μM phorbol myristate acetate (PMA) with 100 ng/mL of eotaxin (positive control) (E and F) or supernatant collected following 24 hour RSV treatment of HEp-2 cells at a multiplicity of infection of 1 (G and H)were stained with DAPI (A, C, E and G) and anti-F-actin and Texas red anti- phalloidin for the detection of F-actin (B, D, F and H). Images were taken using the Nikon Eclipse Ti confocal microscope. Thick arrows depict leading edge formation, while thin arrows indicate direction of eosinophil migration. Images are representative of two separate experiments with samples prepared in triplicates.

3.4 DISCUSSION

Since the unsuccessful RSV vaccine trial in 1956 (Morris *et al.*, 1956) numerous studies have tried to understand why the vaccine trial failed (Becker, 2006; Olson *et al.*, 2008; Waris *et al.*, 1996). The consensus has been that eosinophilia, described by an influx of eosinophils into the lung tissue, contributed to RSV disease pathology and therefore, became the hallmark of RSV disease (Rosenberg and Domachowske, 2001). Consequently, eosinophils were characterised as leukocytes contributing to RSV disease pathology and their presence during RSV infection or therapeutic interventions was deemed undesirable (Openshaw *et al.*, 2003).

Although some studies have concentrated on elucidating the role of eosinophils in viral infection (Adamko *et al.*, 1999; Domachowske *et al.*, 1998a; Phipps *et al.*, 2007; Rosenberg and Domachowske, 2001), there are limitations to the knowledge and understanding that these studies offer. One of the major limitations of the previous electron microscopy analysis is that ssRNA was used to stimulate eosinophils (Phipps *et al.*, 2007). As such, this provides a very limited view of RSV effect on eosinophil morphology. These findings could be expanded further by including a wider range of time points as well. It would enable a better understanding of RSV-induced changes to eosinophil morphology while characterising the eosinophil degranulation kinetics over time. In addition, previous eosinophil degranulation studies have only focussed on levels of ECP in tissue culture and nasopharyngeal secretions from human infants (Olszewska-Pazdrak *et al.*, 1998b; Saito *et al.*, 1997). However, these studies have failed to note that, as ECP is located in the matrix of the eosinophil granule its release is preceded by the release of MBP from the core. In addition, there is a difference in opinion whether RSV can infect eosinophils (Dyer *et al.*, 2009; Kimpen, 2001a) and therefore promote antiviral host defence. This controversy is under explored and requires further investigation. Finally, previous studies have only examined eosinophil activation using EPO, as the activation marker however expanding on these findings would enable us to generate a bigger picture of RSV-induced activation by also examining eosinophil cytoskeleton involvement in subsequent processes such as migration.

In vitro results from the electron microscopy analysis revealed a significant ($p < 0.005$) RSV-induced effect on eosinophil morphology over time (Figure 3.1 and Figure 3.2). TEM results indicate RSV-induced eosinophil activation as demonstrated by the release of eosinophil granule content into the external cell environment. The release was mainly characterised by partial and complete loss of eosinophil granule core contents following the RSV treatment of eosinophil for 6, 12 and 18 hours (Figure 3.1 G, H and I). RSV-induced changes to eosinophil morphology became even more evident following 24 hours post RSV incubation of eosinophils. At this time, eosinophil granules were completely devoid of core and matrix content and apoptotic nuclei became the hallmark of RSV treatment (Figure 3.1 J). These findings for the first time demonstrate clearly RSV-induced changes to eosinophil morphology over time as a result of RSV treatment of eosinophils. Previous findings by Phipps *et al.* (2007) support described TEM findings in this chapter, as they have also showed that a ten minute treatment of eosinophils with ssRNA results in a loss of the eosinophil crystal core content as demonstrated by TEM analysis (Phipps *et al.*, 2007). The study conducted by Clark *et al.* (2004) also revealed similar changes to eosinophil morphology observed in an allergic asthma model using TEM (Clark *et al.*, 2004). Together, these novel findings demonstrate how RSV treatment of eosinophils has detrimental effect on eosinophil morphology and integrity over time, resulting in granule content and subsequent cytotoxic protein release via process of PMD and concluding in a loss of cellular structure and cell apoptosis. It is important to note that the use of eosinophil survival factor was not required in the experiment examining RSV-induced changes to eosinophil morphology as appropriate eosinophil controls (untreated eosinophils) were used at each time point. Trypan blue viability test was the only test performed assessing the cell health status. Although this may be perceived as a limitation of this study, it is consistent with other research groups who have not used eosinophil survival factors in experiments similar to the one described in this study (Phipps *et al.*, 2007; Rosenberg and Domachowske, 2001).

The current studies suggest for the first time that presence of MBP may not be a by-product of cellular degenerative processes but rather result directly in response to RSV-mediated eosinophil activation. Earlier studies examining RSV-induced eosinophil degranulation, found high levels of ECP present in the

lung epithelial tissue culture (Olszewska-Pazdrak *et al.*, 1998b) and nasopharyngeal secretions of human infants (Saito *et al.*, 1997). However, the importance of eosinophil degranulation in response to respiratory viral infection has yet to be fully elucidated, with studies yet to examine early signs of eosinophil degranulation. The degranulation study in this chapter examined early signs of eosinophil degranulation using MBP as the degranulation marker. MBP is located in the core of eosinophil granule and is the first granular protein to be released following eosinophil activation. Results showed a significant increase ($p < 0.0001$) in eosinophil degranulation following RSV treatment of eosinophils (Figure 3.3). It is important to highlight the potential of RSV-mediated eosinophil activation as these results indicate that eosinophils have the potential to generate a targeted response to RSV infection.

Together, these findings are suggestive of eosinophil ability to play a role in antiviral activity. These observations were supported further by the *in vitro* study showing a significant decline ($p < 0.0001$) in RSV titres over time, following 3, 6, 12, 18 and 24 hours RSV treatment of eosinophils compared to the initial RSV inoculum (time 0 hours) (Figure 3.4 A). These results are in agreement with previous studies demonstrating eosinophil antiviral activity following RSV infection of the epithelial cells *in vitro* (Domachowske *et al.*, 1998b; Soukup and Becker, 2003). In addition, the current antiviral study implies RSV inability to infect murine eosinophils and to establish active replication within, as indicated by a reduction in viral titres recovered. Similarity, TEM analysis of RSV treated eosinophils also revealed no RSV viral particles present inside the eosinophils following RSV treatment, suggesting that RSV may be unable to infect murine eosinophils. Taking into account previous studies, which showed that RSV could infect human eosinophils and that PVM can infect mouse eosinophils (Dyer *et al.*, 2009; Kimpen *et al.*, 1996), these results may be a result of incompatibility between RSV as a human respiratory pathogen and the mouse purified eosinophils used in the *in vitro* studies. Despite similar morphology, human and mouse eosinophils differ from one another, and cannot be presumed to function identically in all circumstances (Lee *et al.*, 2012a). The mechanism that offers protection of mouse eosinophils against RSV infection may offer important insights into preventing human infection as well. It is therefore important to explore this mechanism further.

The current studies found that eosinophils were significantly less viable following the RSV treatment ($p < 0.05$), supporting the concurrent findings that demonstrated RSV-induced eosinophil activation and subsequent degranulation followed by the loss of cellular structure and cell apoptosis. However, these results contradict previous studies with PVM which showed that virus infection has no effect on the cell viability of eosinophils in cell culture (Dyer *et al.*, 2012). The difference in findings between PVM versus RSV treatment and eosinophil viability could only be due to the interaction between the human pathogen, RSV and murine eosinophils as the experimental set up was similar to PVM infection of murine eosinophils. Nevertheless, these findings are the first to show that RSV has detrimental effect on cell viability resulting in, accelerated cell apoptosis. Finally, RSV treatment of eosinophils leads to eosinophil activation, cell migration and degranulation; with RSV treated eosinophils showing significantly greater number of activated cells present compared to the negative control ($p < 0.001$) (Figure 3.5) as demonstrated by EPO release. These findings are in agreement with previous finding by Phipps *et al.* (2007) who also showed an increase in EPO as a result of ssRNA activation of eosinophils (Phipps *et al.*, 2007). Furthermore, Olszewska-Pazdrak *et al.* (1998) study found that RSV infection results in the secretion of the proinflammatory C-C chemokines RANTES, MCP-1 and MIP-1α from epithelial cells of the human lower respiratory tract. They also showed the chemokines produced by RSV-infected epithelial cells have biological activity relevant to the pathogenesis of allergic inflammation, as they exhibit chemotaxis for blood eosinophils (Olszewska-Pazdrak *et al.*, 1998a). In our study, we have expanded on Olszewska-Pazdrak *et al.* (1998) findings by examining which cytoskeletal components are activated as a result of eosinophil chemotaxis towards chemokine containing supernatant, collected from RSV infected HEp-2 cells. Eosinophil migration study revealed that eosinophil migration towards RSV infected HEp-2 cell supernatant resulted in activation of the microtubular network (Figure 3.6) and high polymerisation of F-actin (Figure 3.7). F-actin polymerisation led to formation of leading edge pseudopod as well as star-like microtubular formation of α-tubulin, indicative of a cell in its migration state. Migration study, in addition to eosinophil activation findings, is the first study to demonstrate RSV-induced effect on eosinophil cytoskeletal components, indicating that eosinophil migration may be a result of targeted response as

oppose to nonspecific response, which may be crucial in ensuring an effective viral clearance.

In summary, the chapter 3 findings suggest that eosinophils have a potential to play a positive role in viral clearance, demonstrated by, what appears to be RSV inability to infect murine eosinophils. As previously stated, the protection that murine eosinophils enjoy in response to RSV infection is worth further exploration. This study also demonstrated RSV-induced changes to eosinophil morphology, eosinophil degranulation, activation and migration as well as activation of eosinophil cytoskeletal components. Together, these findings indicate that eosinophil activation following RSV treatment may be a result of targeted response as oppose to nonspecific immune response. Although pulmonary eosinophilia is a hallmark response to FI-RSV (Percopo *et al.*, 2009), there is no clear indication that eosinophils actually contribute to the negative sequel of RSV disease. In addition, while severe primary RSV infection is associated with pulmonary eosinophilia and progression to asthma, the mechanism by which these two features are linked has not been elucidated clearly (Rosenberg *et al.*, 2009). Among the possibilities that have yet to be explored, eosinophil function may be less dependent on the number of recruited cells and related more closely to the quality and extent of the immune cell milieu. These are all issues that are worthy of further consideration. In conclusion, it is important to elucidate the role of eosinophils in viral infection, both antiviral and pathogenic, as they may be involved in immune compensation with assistance from other innate/adaptive immune cells.

Chapter 4. THE ROLE OF INNATE AND ADAPTIVE IMMUNITY IN RESPIRATORY SYNCYTIAL VIRUS INFECTION AND RE-INFECTION

4.1 INTRODUCTION

Expanding on the *in vitro* results from Chapter 3, the role of eosinophils in RSV infection was examined in the *in vivo* model. Previous studies examining human and murine models revealed that inefficient immune system regulation escalates respiratory disease pathology following RSV infection, and can also lead to less optimal viral clearance (Culley *et al.*, 2002; Tasker *et al.*, 2008; Welliver *et al.*, 2008). Therefore, in order to clear pathogens from the host, the immune system must induce balanced release of whatever is required to effectively activate and clear the pathogen. Consequently, immune system must induce self-regulated responses to prevent unbalanced immunity and successive collateral damage of the host tissue.

As innate immunity is the first line of defence (Goldsby *et al.*, 2003), initially its response involves a release of effector molecules and phagocytic cells to the site of infection. However, as innate immunity lacks immunological memory (McNamara and Smyth, 2002), it induces the same sequence of immune cell responses over and over again. Furthermore, RSV infection has been associated with skewing of the immune system away from an antiviral Th1 response towards a Th2 response, which is believed to be undesirable immune response leading to disease pathology instead of effective viral clearance (Welliver *et al.*, 2008). Nevertheless, important cells of the innate immunity involved in response to RSV are DCs, macrophages, neutrophils, eosinophils and NK cells as well as the lung epithelial cells.

DCs, as the primary APCs, are the first to encounter RSV, subsequently inducing activation of the naïve virus-specific T cells (Braciale, 2005). Macrophages play an important role in controlling the immune response to viral infection (Kimpen, 2001b) with murine studies showing that macrophages provide an immediate response of pro-inflammatory cytokines following RSV infection (Pribul *et al.*, 2008). Furthermore, macrophages are a major producer of type I IFN (Kumagai *et al.*, 2007) and appear to be important in both, restricting the virus and clearing cellular debris later in infection. Without clearance, cellular debris can lead to further damage and inflammation (Reed *et al.*, 2008). Neutrophils are the predominant airway leukocytes in RSV bronchiolitis and they are activated in the presence of infection (Lukens *et al.*, 2010).

Eosinophils have been shown to play a specialised role in the innate host defence against RSV as the potential exists to mediate direct killing via the release of cytotoxic proteins and the eosinophil RNases (Dyer *et al.*, 2009). As mentioned in Chapter 3, several studies have found that eosinophils have the means to promote antiviral host defence *in vitro* (Domachowske *et al.*, 1998a; Soukup and Becker, 2003). Also, Phipps *et al.* (2007) study revealed accelerated clearance of RSV from the lungs of IL-5 Tg BALB/c mice (Phipps *et al.*, 2007) demonstrating eosinophil antiviral role *in vivo* as well. Furthermore, NK cells are recruited locally during the initial phases of virus infection (Hussell and Openshaw, 1998) and accumulate in the lung during the first few days of RSV infection, producing T cell recruiting cytokines and chemokines, such as IL-8, MIP-1α and RANTES (Fauriat *et al.*, 2010; Hussel *et al.*, 1997a; Hussell and Openshaw, 1998). In addition, NK cells are responsible for the early production of IFN-γ and contributing to inhibition of lung eosinophilia (Hussel *et al.*, 1997a; Hussell and Openshaw, 1998). The main function of NK cells is to recognise and destroy virus-infected cells on the basis of alterations that occur on the surface proteins of the normal cell (Message and Johnston, 2001). Apart from being effector cells, NK cells have a regulatory role during an immune response by affecting DCs, macrophages, and mast cells (Moretta *et al.*, 2005). Recently, the notion that NK cells are truly innate cells has been called into question because specific subsets of mouse liver NK cells have been described to have the adaptive immunity property of lasting memory against specific viral antigens (Gillard *et al.*, 2011; Vivier *et al.*, 2011). In addition, recently discovered ILC2 cells, play a very important role in the innate immunity. In adult mice, ILC2 cells are a rare population that expands in response to IL-25 or IL-33 or during helminthic infection (Neill et al., 2010). They represent a chief source of IL-13, a pleiotropic cytokine that mediates a variety of effects, including suppression of cytokine production by monocytes (Minty *et al.*, 1993), mucus production by goblet cells and the recruitment of eosinophils by epithelial cells (Cohn *et al.*, 1999; Grunig *et al.*, 1998; Pope *et al.*, 2001). ILC2 cells can also produce IL-5, which has been linked to eosinophil recruitment, as well as IL-9 (Wilhelm *et al.*, 2011), which is prominent during helminthic infections (Faulkner *et al.*, 1997; Richard *et al.*, 2000) and has been linked to atopic disease (Shimbara *et al.*, 2000). ILC2 cells have been shown to be involved in the immunity against helminths as well as in the allergic conditions such as

asthma and chronic sinusitis (Chang *et al.*, 2011; Mjosberg *et al.*, 2011; Moro *et al.*, 2010; Neill *et al.*, 2010) but are also probably important for maintaining tissue homeostasis (Monticelli *et al.*, 2011). Finally, epithelial cells provide a link to the initial innate immune response assisting in an adaptive immune system activation following RSV infection (Lotz and Peebles, 2012).

As RSV has been found to re-infect the same individual several times throughout its life, understanding the adaptive immune response to RSV re-infection is imperative to providing long lasting protection against RSV. RSV re-infection is characterised by an initial influx of NK cells followed by recruitment of helper $CD4^+$ and cytotoxic $CD8^+$ lymphocytes to the site of infection (Olson *et al.*, 2008; Olson and Varga, 2007; Stevens *et al.*, 2009). A mouse study found that the $CD4^+$ and $CD8^+$ T lymphocyte subsets contribute individually to clearing of the RSV infection however, both can also contribute to disease pathogenesis, especially $CD8^+$ T cells (Graham *et al.*, 1991). Although RSV-specific $CD8^+$ T cells can provide protection against infection, the effect is short lived (Connors *et al.*, 1991; Kulkarni *et al.*, 1995). Nevertheless, there are also a number of risk factors that have been found to lead to severe RSV disease early in life, including prematurity, low titres of maternal antibodies, lack of previous RSV infection (Berkovich, 1964; Cunningham *et al.*, 1991), underlying cardiopulmonary disease (Groothuis *et al.*, 1988; MacDonald *et al.*, 1982), immunosuppression (especially T cell deficiencies) (Falsey, 2007; Falsey *et al.*, 2005) (Whimbey and Ghosh, 2000) as well as immunodeficiency disorders (Fishaut *et al.*, 1980; McIntosh *et al.*, 1984). RSV re-infection may also be aided by features that help RSV evade host defences. Several factors that allow RSV to evade host defence include the secreted form of the G protein (Bukreyev *et al.*, 2008), the glycoprotein sheath of the G protein, and the presence of two antigenic subgroups (Waris, 1991). Other factors of the immune evasion include the tropism of the virus for the superficial layer of the respiratory epithelium and its relatively non-invasive nature (Lotz and Peebles, 2012). This may delay and reduce the exposure of viral antigen to the host immune system. In addition, the lumen of the respiratory tract is poorly accessed by serum antibodies, therefore reducing their ability to restrict viral replication. In addition, recent murine studies indicate that $CD8^+$ cytotoxic T lymphocytes are functionally down-regulated in the lung, which may be a host mechanism to reduce tissue damage however this also would result in reduced immune protection (DiNapoli *et al.*,

2008; Vallbracht *et al.*, 2006). Thus, both, host and viral factors are believed to contribute to reduced efficacy of RSV immune control.

Previous research into RSV-induced innate and adaptive immunity has highlighted that RSV infection skews the immune system towards an undesirable Th2 response (Welliver *et al.*, 2008). Also, until recently eosinophils were believed to contribute to RSV disease pathology (Haynes *et al.*, 2003) and that RSV infection does not induce a long memory response (Connors *et al.*, 1991; Kulkarni *et al.*, 1995). With recent findings by Phipps *et al.* (2007) demonstrating the positive role of the eosinophil in RSV clearance *in vivo*, it is important to expand on this finding further.

The aim of this chapter was to evaluate eosinophil antiviral activity in four distinct mouse models following RSV infection and re-infection. These BALB/c mice were WT, IL-5 Tg and/or Rag $2^{-/-}$ animals. As such, the history of these genetic modifications is important to be discussed, including the development of the humanised models and their application.

Decades ago, IL-5 transgenic mice were produced in a way so the transcription of the IL-5 gene is under the influence of the dominant control region (DCR) of the gene encoding human CD2 (a T cell surface antigen) (Lang et al., 1988). The use of the CD2 DCR ensures constitutive expression by T cells (Gleich and Adolphson, 1986; Lang et al., 1988; Sanderson et al., 1988). It seems remarkable that these animals with such high levels of potentially damaging leukocytes remain normal; suggesting that eosinophils require other factors for degranulation and subsequent tissue damage. As eosinophils play a role in allergic disease, these animals provide a model for testing modifiers of eosinophil production (Dent et al., 1990). Furthermore, Rag $2^{-/-}$ mice were designed through the deletion of Rag 2 recombination activating gene, resulting in the arrest of rearrangement of the B (immunoglobulin production) and the T cell receptors; and the absence of the T and B cell differentiation. These mice have high levels of NK-cell activity but do not show Ig leakiness and they are less sensitive to radiation compared to SCID mice. The main applications of Rag $2^{-/-}$ mice are in the investigation of lymphocyte gene function in differentiation, HIV and other immune deficiency diseases, the immune system's role in tumourgenesis and metastasis, and autoimmune and infectious diseases (Shinkai et al., 1992; Shultz et al., 2007). Loss of Rag 2 function *in*

vivo results in total inability to initiate V(D)J rearrangement with Rag 2 function and V(D)J recombinase activity found to be only required by no other cells than lymphocytes (Shinkai *et al.*, 1992). As IL-5 Tg Rag 2-/- BALB/c was a new mouse model, recently developed by our group, it was novel to examine: if each of these genetic mutations continue to function as intended or if they would inhibit each other's function in some way.

Therefore, the hypothesis that eosinophils may have the capacity to provide immune compensation in the absence of the mature T ($CD4^+$ and $CD8^+$) and B cells following RSV re-infection was assessed; as well as eosinophil RSV antiviral potential to inhibit RSV infection across all four BALB/c strains, especially the eosinophil enriched ones. Furthermore, as $CD8^+$ cells have been shown to contribute to disease pathology, $CD4^+$ cell role in viral clearance and lung pathology was examined.

4.2 METHODS

4.2.1 Mice strains

Male BALB/c mice aged 6 to 8 weeks were obtained from the pathogen free facility at the University of Canberra (UC) Animal facility. Animals were housed in approved containment facilities and treated in accordance with UC animal experimentation guidelines. BALB/c mouse strains (section 2.12) used were WT, IL-5 Tg, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c. Genotype and phenotype differences are outlined in the Table 4.1 below.

Table 4.1. BALB/c strains genotypes and phenotypes

BALB/c strains	Innate immunity	Adaptive immunity	Gene Mutation	BALB/c strains
WT	complete	Complete	N/A	N/A
IL-5 Tg	complete	Complete	homozygous IL-5 gene knock-in	eosinophil overexpression
Rag $2^{-/-}$	complete	Incomplete	homozygous Rag 2 knock-out	fail to produce mature T and B cells
IL-5 Tg Rag $2^{-/-}$	complete	Incomplete	homozygous IL-5 gene knock-in and Rag 2 knock-out	eosinophil overexpression with failure to produce mature T and B cells

4.2.2 RSV infection of mouse strains

On day 0, mice were lightly anesthetised with isofluorane (PharmaChem) (section 2.12.4) and infected with RSV by intranasal inoculation (50 µL of 1 x 10^7 pfu/mL = 5 x 10^5pfu/mouse). On day 5, mice were euthanised by CO_2 asphyxiation (section 2.12.1) and samples collected for further processing (sections 2.13).

4.2.3 RSV infection of BALB/c mutants followed by RSV re-infection

On days 0 and 12 mice were lightly anesthetised with isofluorane (PharmaChem) (section 2.12.4) and infected with RSV by intranasal inoculation (50 µL of 1 x 10^7 pfu/mL = 2 x 10^6 pfu/mouse). On day 17, mice were

euthanised by CO_2 asphyxiation (section 2.12.1) and samples collected for further processing (sections 2.13).

4.2.4 Adoptive transfer of CD4⁺ cells

CD4⁺ T cells were harvested from spleens of WT BALB/c mice, and transferred into IL-5 Tg Rag $2^{-/-}$ BALB/c mice as described previously (Plotnicky-Gilquin *et al.*, 2002). Briefly, spleens of WT BALB/c mice were removed, mashed and filtered through a cell strainer (BD, Australia). Red blood cells were removed using lysing buffer (BD Pharmingen). CD4⁺ cells were purified from the resulting lymphocyte population using MagCellect Mouse Naïve CD4⁺ T cell isolation kit (R&D Systems, USA) according to the manufacturer's protocol. IL-5 Tg Rag $2^{-/-}$ BALB/c mice received 3 x 10^6 CD4⁺ cells/ mouse via tail-vein injection 4 days prior to the first RSV inoculation (Figure 4.1).

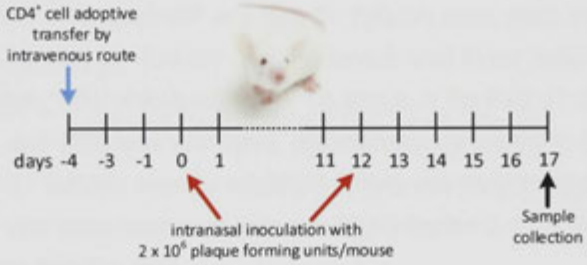


Figure 4.1. Experimental timeline of CD4⁺ T cell adoptive transfer followed by respiratory syncytial virus (RSV) re-infection of interleukin-5 transgenic Rag 2 knockout (IL-5 Tg Rag $2^{-/-}$) BALB/c

Purified CD4⁺ T cell were adoptively transferred into IL-5 Tg Rag $2^{-/-}$ mice intravenously 4 days prior to RSV primary infection on day 0 (intranasal inoculation with 5 x 10^5 plaque forming units (pfu)/mouse). Mice were re-infected with RSV (5 x 10^5 pfu/mouse) on day 12 and samples collected on day 17.

4.2.5 Statistical analysis

Data are presented as mean ± standard deviation. A ONE-way or TWO-way ANOVA was used to determine statistical significance. Data were analysed using GraphPad Prism version X7. A p value of less than 0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 Primary RSV infection in BALB/c mice

Primary RSV infection was set up in four different BALB/c mouse strains with their genotype and phenotype make up contributing to the variations in the innate immune response. The outcome of primary RSV infection *in vivo* was assessed by examination of lung viral titres, BAL and blood leukocyte population infiltrates as well as lung histopathology.

4.3.1.1 Viral titres in BALB/c mice following primary RSV infection

No viral titres were recovered from the lung samples of Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculated WT, IL-5 Tg, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice, following immunostaining RSV plaque assay (data not shown). Highest viral titres were recovered from the lung of RSV inoculated WT and Rag $2^{-/-}$ BALB/c mice. RSV inoculated IL-5 Tg and IL-5 Tg Rag $2^{-/-}$ BALB/c had the lowest viral loads recovered (Figure 4.2). In addition, RSV infection of IL-5 Tg and IL-5 Tg Rag $2^{-/-}$ BALB/c mice yielded 90% and 75% less viral titres, respectively, compared to RSV infected WT and Rag $2^{-/-}$ BALB/c mice ($p < 0.0001$). There was no statistically significant difference in viral titres recovered between RSV infected IL-5 Tg BALB/c and to IL-5 Tg Rag $2^{-/-}$ BALB/c mice.

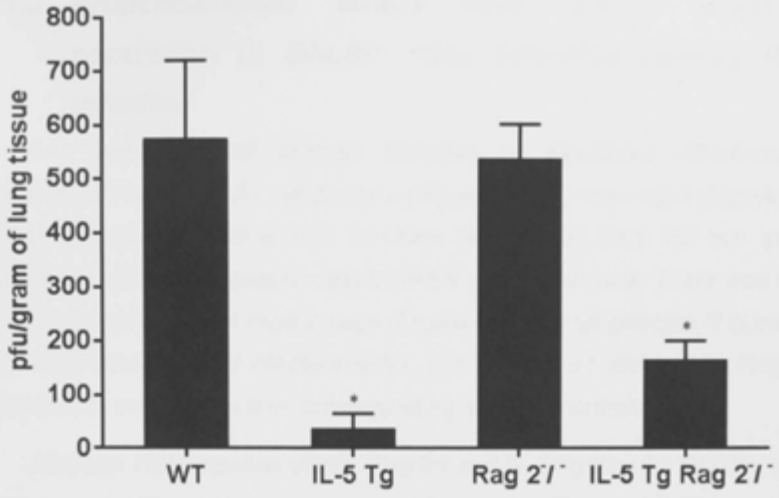


Figure 4.2. Eosinophil overproduction leads to accelerated viral clearance in the lung of the BALB/c mice

Wild type (WT), interleukin-5 transgenic (IL-5 Tg), Rag 2 knockout (Rag 2-/-) and IL-5 Tg Rag 2-/- BALB/c mice were infected with Vero E6 cell lysate, ultraviolet-inactivated respiratory syncytial virus (UV-RSV) or RSV at 5 x 10^5 plaque forming units (pfu)/mouse on day 0, by intranasal inoculation. Lungs were harvested 5 days after infection and viral titres enumerated using a RSV immunohistochemistry viral titre assay. Titres are represented as pfu/gram of lung tissue. Data is representative of mean (n = 5) ± standard deviation. Data was analysed using a ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism version X7) with *, $p < 0.0001$ for IL-5 Tg mice compared to WT and Rag 2-/- ; also for IL-5 Tg Rag 2-/- compared to WT and Rag 2-/- mice.

4.3.1.2 *Bronchoalveolar lavage fluid (BALF) leukocyte population in BALB/c mice following primary RSV infection*

BAL leukocyte cell analysis revealed no significant difference in macrophage (Figure 4.3 A), lymphocyte (Figure 4.3 B), eosinophil (Figure 4.3 C) and neutrophil (Figure 4.3 D) numbers between all Vero E6 cell lysate (negative control) and corresponding UV-RSV inoculated mice. There was also no significant difference in macrophage (Figure 4.3 A) or lymphocyte (Figure 4.3 B) numbers following RSV infection of WT, IL-5 Tg, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice compared to their corresponding negative controls.

Although RSV infection of WT, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice revealed no significant change in eosinophil numbers (Figure 4.3 C) compared to their corresponding negative controls, RSV infection of IL-5 Tg did induce a significant decrease in the number of eosinophils compared to the IL-5 Tg negative control ($p < 0.0001$). There was no statistical difference in the number of eosinophils recovered from BAL between IL-5 Tg and IL-5 Tg Rag $2^{-/-}$ mice infected with RSV. However, there was a significant decrease in the number of eosinophil infiltrates following RSV infection of IL-5 Tg and IL-5 Tg Rag $2^{-/-}$ mice compared to Rag $2^{-/-}$ mice ($p < 0.005$) (Figure 4.3 C).

Neutrophil numbers (Figure 4.3 D) decreased slightly following RSV infection of WT BALB/c mice compared to WT negative control while a slight increase in numbers was observed following RSV infection of IL-5 Tg and IL-5 Tg Rag $2^{-/-}$ BALB/c mice compared to their corresponding negative control. Neutrophil numbers were significantly higher ($p < 0.001$) following RSV infections of Rag $2^{-/-}$ BALB/c mice compared to the negative control. In addition, neutrophil numbers were significantly higher for RSV infected Rag $2^{-/-}$ BALB/c mice compared to RSV inoculated IL-5 Tg and IL-5 Tg Rag $2^{-/-}$ BALB/c mice ($p < 0.005$).

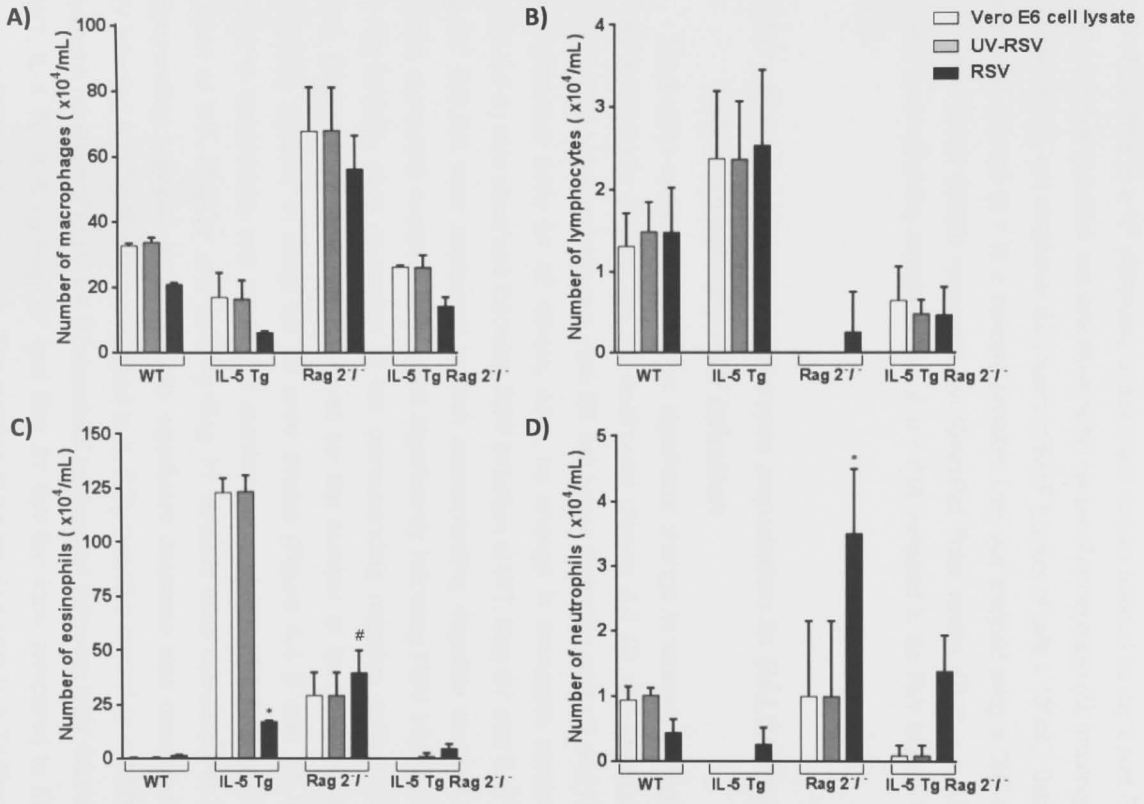

A)
Number of macrophages (x10⁴/mL)
100
80
60
40
20
0
WT
IL-5 Tg
Rag 2⁻/⁻
IL-5 Tg Rag 2⁻/⁻
B)
Number of lymphocytes (x10⁴/mL)
4
3
2
1
0
Vero E6 cell lysate
UV-RSV
RSV
C)
Number of eosinophils (x10⁴/mL)
150
125
100
75
50
25
0
*
#
D)
Number of neutrophils (x10⁴/mL)
5
4
3
2
1
0
*

Figure 4.3. Leukocyte population in bronchoalveolar lavage fluid (BALF) following primary respiratory syncytial virus (RSV) infection *in vivo*

Wild type (WT), interleukin-5 transgenic (IL-5 Tg), Rag 2 knockout (Rag $2^{-/-}$) and IL-5 Tg Rag $2^{-/-}$ BALB/c mice were inoculated with Vero E6 cell lysate (negative control), ultraviolet-inactivated RSV (UV-RSV) RSV (5×10^5 pfu/mouse) on day 0 and samples collected on day 5 post RSV infection. Leukocyte population was determined by the number of macrophages (A), lymphocytes (B), eosinophils (C) and neutrophils (D) present in BALF (number of cells x 10^4/mL). Data is representative of mean (n = 5) ± standard deviation. Data was analysed using a ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism version X7) (*, $p < 0.005$ compared to corresponding negative control, #, $p < 0.05$ compared to the RSV infected WT BALB/c).

4.3.1.3 Peripheral blood leukocyte population in BALB/c mice following primary RSV infection

Leukocyte analysis revealed no significant change in monocyte (Figure 4.4 A), lymphocyte (Figure 4.4 B), eosinophil (Figure 4.4 C) and neutrophil (Figure 4.4 D) numbers between Vero E6 cell lysate (negative control) and UV-RSV inoculated mice for all strains. Also, no change in monocyte numbers (Figure 4.4 A) was observed following RSV infection of WT, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice compared to their corresponding negative controls. In contrast, monocyte numbers decreased significantly following RSV infection of IL-5 Tg BALB/c mice compared to the corresponding negative control ($p < 0.001$). Similar trend was also observed for the number of lymphocytes and eosinophils present in blood for all mice strains (Figure 4.4 B and C). No change in lymphocyte and eosinophil numbers was observed following RSV infection of WT, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice compared to the corresponding negative controls; while significant decrease was observed in RSV infected IL-5 Tg BALB/c compared to IL-5 Tg negative control ($p < 0.005$). Neutrophil numbers (Figure 4.4 D) remained unchanged following RSV infection of WT, IL-5 Tg, IL-5 Tg Rag $2^{-/-}$ and Rag $2^{-/-}$ BALB/c mice compared to their corresponding negative controls. The number of neutrophils from IL-5 Tg Rag $2^{-/-}$ BALB/c mice was significantly higher compared to RSV infected Rag $2^{-/-}$ BALB/c mice ($p < 0.0001$).

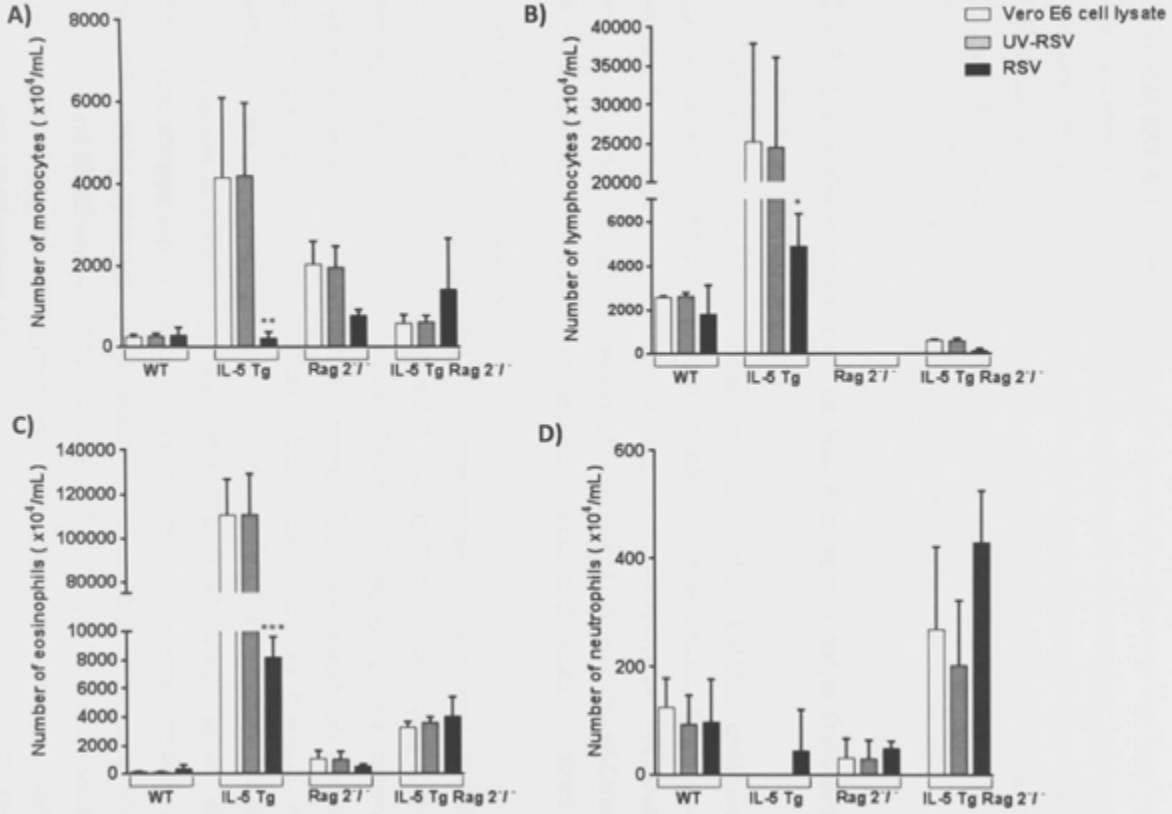

A)
Number of monocytes (x10⁴/mL)
8000
6000
4000
2000
0
**
WT
IL-5 Tg
Rag 2⁻/⁻
IL-5 Tg Rag 2⁻/⁻
B)
Number of lymphocytes (x10⁴/mL)
40000
35000
30000
25000
20000
6000
4000
2000
0
*
Vero E6 cell lysate
UV-RSV
RSV
C)
Number of eosinophils (x10⁴/mL)
140000
120000
100000
80000
10000
8000
6000
4000
2000
0
***
D)
Number of neutrophils (x10⁴/mL)
600
400
200
0

Figure 4.4. Leukocyte population in blood following primary respiratory syncytial virus (RSV) infection *in vivo*

Wild type (WT), interleukin-5 transgenic (IL-5 Tg), Rag 2 knockout (Rag 2$^{-/-}$) and IL-5 Tg Rag 2$^{-/-}$ BALB/c mice were inoculated with Vero E6 cell lysate (negative control), ultraviolet-inactivated RSV (UV-RSV) RSV (5 x 10^5 pfu/mouse) on day 0 and samples collected on day 5 post RSV infection. Leukocyte population was determined by the number of macrophages (A), lymphocytes (B), eosinophils (C) and neutrophils (D) present in blood (number of cells x 10^4/mL). Data is representative of mean (n = 5) ± standard deviation. Data was analysed using a ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism version X7) (*, $p < 0.005$, **, $p < 0.001$ and ***, $p < 0.0001$ compared to corresponding negative control).

4.3.1.4 Lung histopathology in BALB/c strain mice following primary RSV infection

Following a Vero E6 cell lysate (negative control), UV-RSV (additional negative control) or RSV inoculation of BALB/c mice strains, lungs were assessed for airways mucus occlusion, degree of parenchymal pneumonia, peribronchial infiltrates, quality of peribronchial infiltrates, perivascular infiltrates, histopathological score, number of goblet cells and eosinophil tissue infiltrates.

4.3.1.4.1 Descriptive analysis

Lung pathology of, Vero E6 cell lysate (negative control) and UV-RSV (additional negative control) inoculated WT, IL-5 Tg, Rag 2$^{-/-}$ and IL-5 Tg Rag 2$^{-/-}$ BALB/c mice, show clear alveoli (Figure 4.5 squares) and minimal inflammatory infiltrates present around bronchioles (thin arrows) as well as blood vessels (thick arrows) indicating the absence of viral infection. An inflammatory response was observed on day 5 post RSV infection in all BALB/c mice. The extent of inflammation was different for each BALB/c strain. WT BALB/c mice showed signs of moderate parenchymal pneumonia, presence of goblet cells (circle), moderate airway occlusion and leukocyte infiltration around bronchioles and blood vessels. RSV infection of IL-5 Tg BALB/c mice resulted in mild signs of parenchymal pneumonia and leukocyte infiltrates around bronchioles and blood vessels while RSV infection of Rag 2$^{-/-}$ and IL-5 Tg Rag 2$^{-/-}$ BALB/c resulted in the same pathological signs as WT BALB/c however with increased goblet cells present (Figure 4.5).

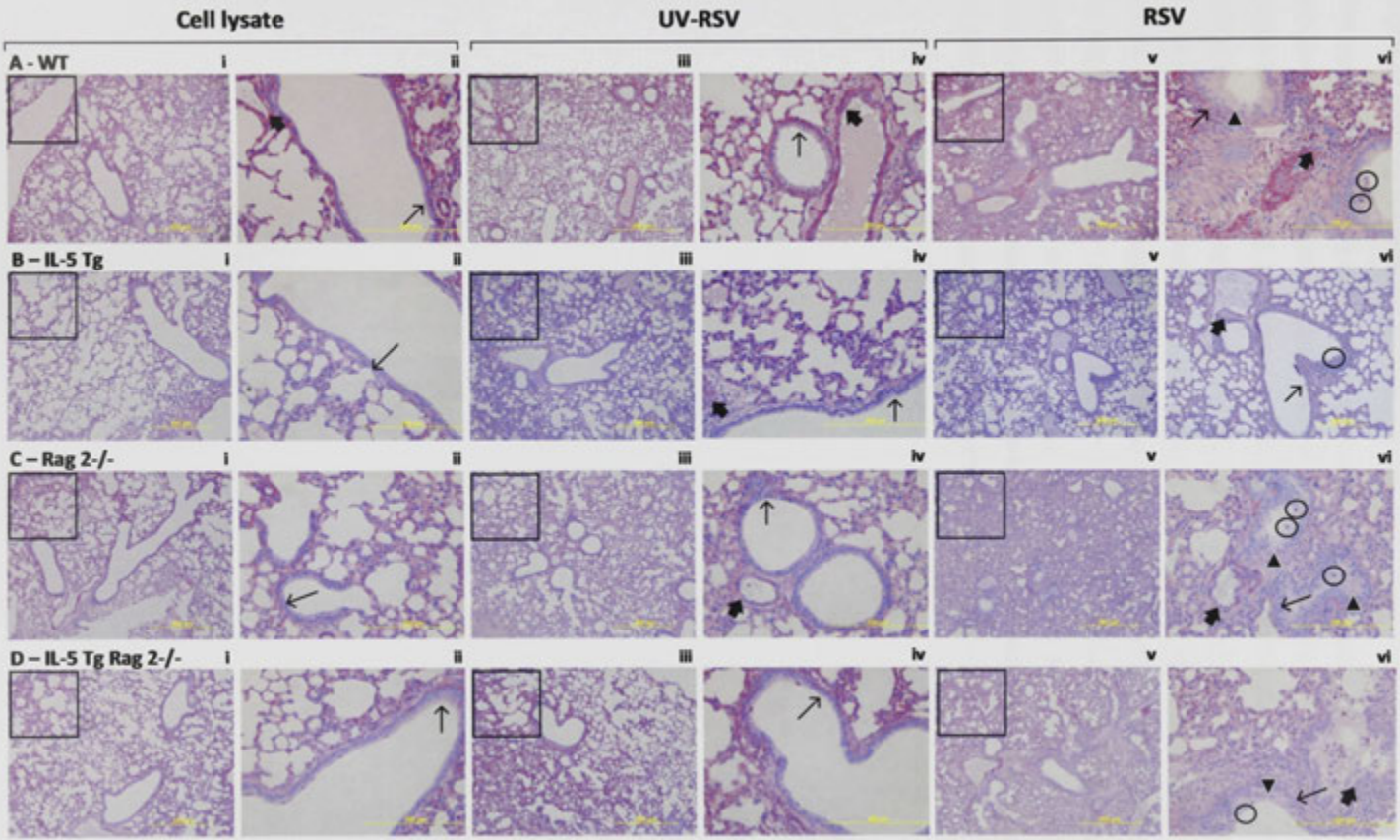

Cell lysate
UV-RSV
RSV
A - WT
B – IL-5 Tg
C – Rag 2-/-
D – IL-5 Tg Rag 2-/-
i
ii
iii
iv
v
vi

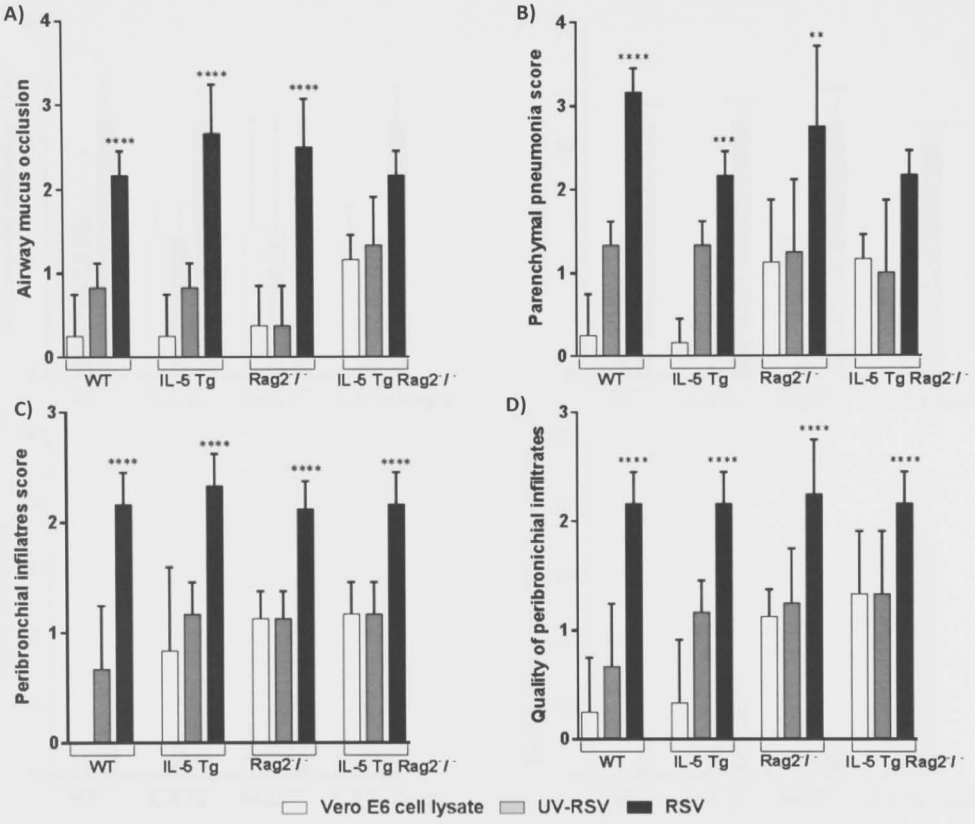
Figure 4.5. Hypereosinophilic (IL-5 Tg) BALB/c mice alleviate pathological signs of primary respiratory syncytial virus (RSV) infection

Wild type (WT), IL-5 Tg, Rag 2 knockout (-/-) and IL-5 Rag 2-/- BALB/c were infected intranasally with Vero E6 cell lysate (negative control), ultraviolet-inactivated RSV (UV-RSV) or RSV (5×10^5 plaque forming units/mouse). A) WT negative control (i and ii), WT UV-RSV (iii and iv) and WT RSV (v and vi). B) IL-5 Tg negative control (i and ii), IL-5 Tg UV-RSV (iii and iv) and IL-5 Tg RSV (v and vi). C) Rag 2-/- negative control (i and ii), Rag 2-/- UV-RSV (iii and iv) and Rag 2-/- RSV (v and vi). D) IL-5 Tg Rag 2-/- negative control (i and ii), IL-5 Tg Rag 2-/- UV-RSV (iii and iv) and IL-5 Tg Rag 2-/- RSV (v and vi). Lungs were collected on day 5 and processed for histology using periodic acid Schiff (PAS) stain. Arrows, squares and circles indicate the different signs of inflammation in mice as described in the text. Magnification (i, iii and v) was 100X and (ii, iv and vi) 400X including the eyepiece. Images are representative of each group (n = 5).

4.3.1.4.2 Numerical (score) analysis

Vero E6 cell lysate (negative control) compared to UV-RSV (additional negative control) inoculated groups for all BALB/c strains showed no statistical differences across all pathological scores. RSV infection of WT, IL-5 Tg, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c, has resulted in a significant increase in airway mucus occlusion (Figure 4.6 A), parenchymal pneumonia (except for IL-5Tg Rag $2^{-/-}$) (Figure 4.6 B), peribronchial infiltrates (Figure 4.6 C) and quality of peribronchial infiltrates (Figure 4.6 D) compared to the corresponding Vero E6 cell lysate and UV-RSV inoculated BALB/c strains ($p < 0.05$). Perivascular infiltration analysis revealed statistically significant increase in leukocyte infiltration around blood vessels for RSV infection of WT BALB/c mice only ($p < 0.05$) (Figure 4.6 E). Combined HPS analysis revealed a significant increase in HPS for all RSV infected BALB/c strains with HPS ranging between 9-11 out of 17 compared to the corresponding Vero E6 cell lysate and UV-RSV controls with HPS ranging between 1-5 out of 17 ($p < 0.0001$) (Figure 4.6 F).

Additionally, RSV infection of WT and IL-5 Tg BALB/c mice compared to their Vero E6 cell lysate and UV-RSV groups, has resulted in no significant increase in goblet cell numbers; while RSV infection of Rag $2^{-/-}$ and IL-5 Tg Rag 2-/- induced a significant increase in the number of goblet cells recovered compared to Vero E6 cell lysate and UV-RSV controls (Figure 4.6 G). Primary RSV infection of IL-5 Tg and IL-5 Tg Rag $2^{-/-}$ mice induced an increase in the number of eosinophils present in the lung tissue ($p < 0.005$) compared to negative controls, while no significant change was observed for RSV infected WT and Rag $2^{-/-}$ mice compared to their negative controls (Figure 4.6 H).


A)
Airway mucus occlusion
B)
Parenchymal pneumonia score
C)
Peribronchial infilatres score
D)
Quality of peribronichial infiltrates
WT
IL-5 Tg
Rag2-/-
IL-5 Tg Rag2-/-
Vero E6 cell lysate
UV-RSV
RSV

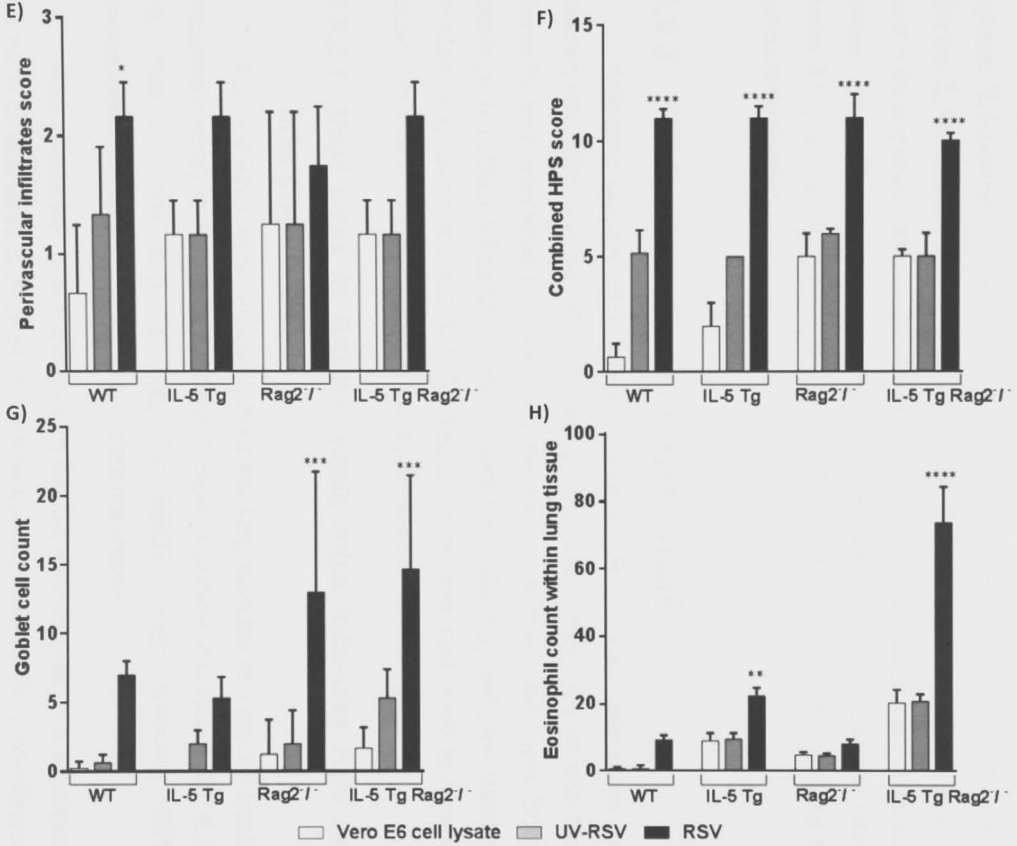

E)
Perivascular infiltrates score
3
2
1
0
*
WT
IL-5 Tg
Rag2-/-
IL-5 Tg Rag2-/-
F)
Combined HPS score
15
10
5
0
****
G)
Goblet cell count
25
20
15
10
5
0
***
H)
Eosinophil count within lung tissue
100
80
60
40
20
0
**
****
Vero E6 cell lysate
UV-RSV
RSV

Figure 4.6. Histopathological score (HPS) assessment of mouse lungs following

Wild type (WT), IL-5 Tg, Rag 2 knockout ($^{-/-}$) and IL-5 Rag 2$^{-/-}$ BALB/c were infected intranasally with Vero E6 cell lysate (negative control), ultraviolet-inactivated respiratory syncytial virus (UV-RSV) or RSV (5 x 10^5 plaque forming units /mL final concentration) on day 0 and lungs tissue collected and processed for histopathology. Lungs were analysed for: A) Airways mucus occlusion, B) Parenchymal pneumonia, C) Peribronchial infiltrates, D) Quality of peribronchial infiltrates, E) Perivascular infiltrates, F) Combined HPS, G) Goblet cell count and H) Eosinophil count in the lung tissue. Data is representative of mean (n = 5) ± standard deviation. Data was analysed using ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism version X7) (*, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.001$ and ****, $p < 0.0001$ compared to corresponding negative control).

4.3.2 RSV re-infection of BALB/c mice

Following primary RSV infection, four BALB/c mouse strains we re-infected with RSV; and the effect of their genotype and phenotype on adaptive immunity was assessed by examining the lung viral titres, BAL and blood leukocyte population infiltrates, as well as lung histopathology. Adoptive transfer of CD4$^+$ T lymphocytes into IL-5 Tg Rag 2$^{-/-}$ mice was performed to assess aspects of innate and/or adaptive immunity involved in RSV re-infection.

4.3.2.1 Viral titres in BALB/c strain mice following RSV re-infection in vivo

No viral titres were recovered from the lung samples of Vero E6 lysate (negative control) and UV-RSV (additional negative control) inoculated WT, IL-5 Tg, Rag 2$^{-/-}$ and IL-5 Tg Rag 2$^{-/-}$ BALB/c mice, following RSV immunohistochemistry viral titres assay (data not shown).

RSV re-infection of WT and IL-5 Tg BALB/c mice resulted in a significant reduction of viral titres compared to titres recovered following RSV re-infection of the Rag 2$^{-/-}$ and IL-5 Tg Rag 2$^{-/-}$ mice ($p < 0.0001$) (Figure 4.7). RSV re-infection of WT and IL-5 Tg mice resulted in 70 and 83% lower viral titres, respectively, compared to Rag 2$^{-/-}$ mice and IL-5 Tg Rag 2$^{-/-}$ ($p < 0.0001$). IL-5 Tg Rag 2$^{-/-}$ and Rag 2$^{-/-}$ resulted in a similar number of viral titres recovered

with no significant difference observed between the two BALB/c strains following RSV re-infection.

CD4$^+$ adoptive transfer into RSV re-infected IL-5 Tg Rag 2$^{-/-}$ mice resulted in a 75% reduction in viral titre compared to IL-5 Tg Rag 2$^{-/-}$ and Rag 2$^{-/-}$ BALB/c RSV re-infected controls. CD4$^+$ adoptive transfer into IL-5 Tg Rag 2$^{-/-}$ reduced viral titres to the same levels observed following RSV re-infection of the WT and IL-5 Tg BALB/c mice.

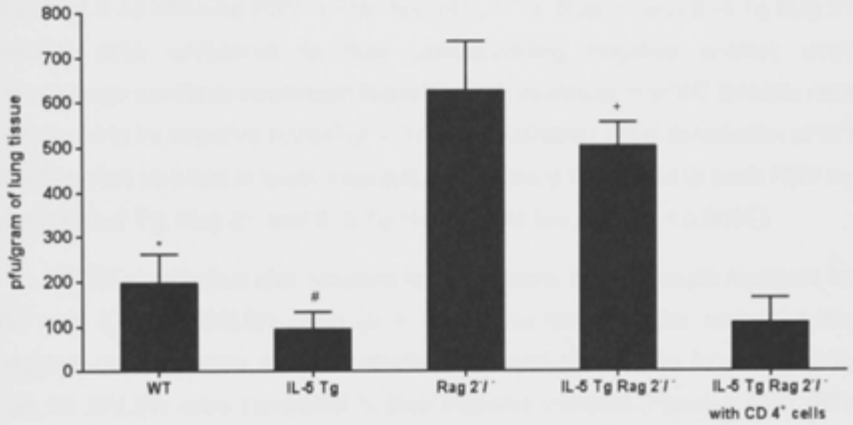


Figure 4.7. CD4$^+$ cells adoptive accelerates viral clearance following respiratory syncytial virus (RSV) re-infection *in vivo*

Wild type (WT), interleukin-5 transgenic (IL-5 Tg), Rag 2 knockout (Rag 2$^{-/-}$) and IL-5 Tg Rag 2$^{-/-}$ BALB/c mice were infected intranasally with Vero E6 cell lysate (negative control), ultraviolet-inactivated RSV (UV-RSV) or RSV (5 x 10^5 plaque forming units (pfu)/mouse) on day 0 and re-infected with Vero E6 cell lysate (negative control), ultraviolet-inactivated RSV (UV-RSV) or RSV (5 x 10^5 pfu/mouse) on day 12. CD4$^+$ cells (3 x 10^6/ mouse) were isolated from WT mice spleen and adoptively transferred intravenously into IL-5 Tg Rag 2$^{-/-}$ mice 4 days prior to day 0 inoculation. Lungs were harvested 17 days post infection, and viral titres enumerated using a RSV immunohistochemistry viral titre assay. Titres are represented as pfu/gram of lung tissue. Data is representative of mean (n = 5) ± standard deviation. Data was analysed using a ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism version X7) (*, $p < 0.0001$ compared to Rag 2$^{-/-}$ and IL-5 Tg Rag 2$^{-/-}$; #, $p < 0.0001$, compared to Rag 2$^{-/-}$ and IL-5 Tg Rag 2$^{-/-}$; +, $p < 0.0001$, compared to IL-5 Tg Rag 2$^{-/-}$ with CD4$^+$ cells adoptive transfer).

4.3.2.2 *Analysis of BALF leukocyte population in BALB/c strain mice following RSV re-infection in vivo*

Following re-infection with RSV there was no significant difference in macrophage (Figure 4.8 A), lymphocyte (Figure 4.8 B), eosinophil (Figure 4.8 C) and neutrophil (Figure 4.8 D) numbers between the corresponding Vero E6 cell lysate (negative control) and UV-RSV (additional negative control) inoculated BALB/c mice.

BAL leukocyte cell analysis revealed no change in macrophage numbers (Figure 4.8 A) following RSV re-infection of IL-5 Tg, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice compared to their corresponding negative control; while macrophage numbers decreased following RSV re-infection of WT BALB/c mice compared to its negative control ($p < 0.001$). In addition, RSV re-infection of WT BALB/c mice resulted in lower macrophage numbers compared to each RSV re-infected IL-5 Tg, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice ($p < 0.0001$).

RSV re-infection also resulted in an increase in lymphocyte numbers for WT and IL-5 Tg BALB/c mice ($p < 0.001$) compared to the corresponding negative controls while numbers remained unchanged for Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice compared to their negative controls (Figure 4.8 B). RSV re-infection of WT and IL-5 Tg BALB/c mice induced an increase in lymphocyte numbers compared to all other RSV re-infected BALB/c strains ($p < 0.0001$).

RSV re-infection of IL-5 Tg BALB/c mice resulted in a decrease in eosinophil numbers compared to its negative control ($p < 0.001$) while eosinophil numbers remained unchanged following RSV re-infection of WT, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice compared to the corresponding negative controls (Figure 4.8 C). RSV re-infection of WT BALB/c mice resulted in significantly lower number of eosinophils compared to RSV re-infected IL-5 Tg and Rag $2^{-/-}$ BALB/c mice ($p < 0.0001$). Neutrophil numbers (Figure 4.8 D) remained unchanged for all RSV re-infected BALB/c mice strains compared to their corresponding negative controls.

CD4+ adoptive transfer into IL-5 Tg Rag $2^{-/-}$ BALB/c mice followed by RSV re-infection resulted in no change in macrophage numbers, significant increase in lymphocyte and neutrophil numbers as well as a decrease in eosinophil numbers compared to its negative control ($p < 0.05$). $CD4^+$ adoptive

transfer into IL-5 Tg Rag $2^{-/-}$ BALB/c mice followed by RSV re-infection has resulted in a significant increase in lymphocyte numbers compared to RSV re-infected Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice. In addition, $CD4^+$ adoptive transfer into IL-5 Tg Rag $2^{-/-}$ BALB/c mice followed by RSV re-infection has induced significant increase in eosinophil numbers compared to RSV re-infected WT and IL-5 Tg Rag $2^{-/-}$ BALB/c mice ($p < 0.0001$).

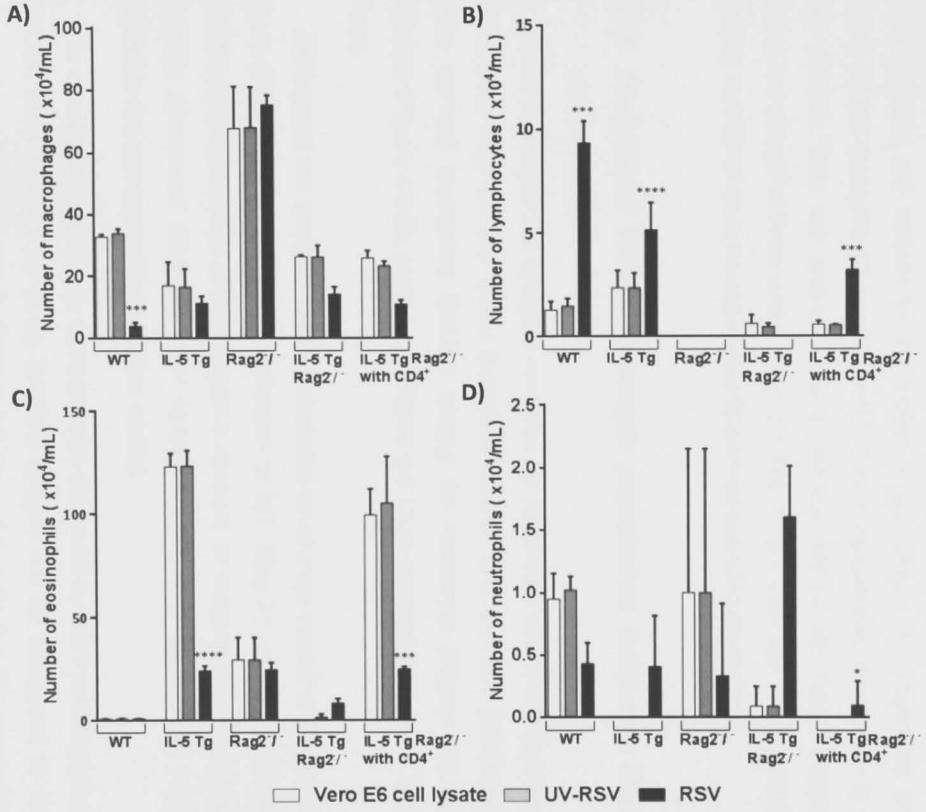

A)
Number of macrophages (x10⁴/mL)
100
80
60
40
20
0
***
WT
IL-5 Tg
Rag2⁻/⁻
IL-5 Tg Rag2⁻/⁻
IL-5 Tg Rag2⁻/⁻ with CD4⁺
B)
Number of lymphocytes (x10⁴/mL)
15
10
5
0
***
****
***
WT
IL-5 Tg
Rag2⁻/⁻
IL-5 Tg Rag2⁻/⁻
IL-5 Tg Rag2⁻/⁻ with CD4⁺
C)
Number of eosinophils (x10⁴/mL)
150
100
50
0
****
***
WT
IL-5 Tg
Rag2⁻/⁻
IL-5 Tg Rag2⁻/⁻
IL-5 Tg Rag2⁻/⁻ with CD4⁺
D)
Number of neutrophils (x10⁴/mL)
2.5
2.0
1.5
1.0
0.5
0.0
*
WT
IL-5 Tg
Rag2⁻/⁻
IL-5 Tg Rag2⁻/⁻
IL-5 Tg Rag2⁻/⁻ with CD4⁺
Vero E6 cell lysate
UV-RSV
RSV

Figure 4.8. Leukocyte population in bronchoalveolar lavage fluid (BALF) following respiratory syncytial virus (RSV) re-infection *in vivo*

Wild type (WT) , interleukin-5 transgenic (IL-5 Tg), Rag 2 knockout (Rag $2^{-/-}$), IL-5 Tg Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c with $CD4^+$ mice were infected by intranasal inoculation with RSV (5 x 10^5 plaque forming units (pfu)/mouse) on day 0 and re-infected with RSV (5 x 10^5 pfu/mouse) on day 12. $CD4^+$ cells (3 x 10^6 cells/mouse) were isolated from WT mice spleen and adoptively transferred intravenously into IL-5 Tg Rag $2^{-/-}$ mice 4 days prior to day 0 inoculation. On day 17, BALF was collected and analysed for the number of macrophages (A), lymphocytes (B), eosinophils (C) and neutrophils (D) present in BALF. Data is representative of mean (n = 5) ± standard deviation. Data was analysed using a ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism version X7) (*, $p < 0.05$, ***, $p < 0.001$ and ****, $p < 0.0001$ compared to Vero E6 cell lysate inoculated group for each corresponding mouse strain).

4.3.2.3 Peripheral blood leukocyte population following RSV re-infection

Blood leukocyte cell analysis revealed no significant difference between corresponding Vero E6 cell lysate (negative control) and UV-RSV (additional negative control) inoculated BALB/c strains for monocyte (Figure 4.9 A), lymphocyte (Figure 4.9 B), eosinophil (Figure 4.9 C) or neutrophil (Figure 4.9 D) numbers.

Differential cell analysis of blood revealed no change overall in monocyte numbers (Figure 4.9 A) following re-infection of WT, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice compared to their negative controls. In contrast, the number of monocytes decreased following RSV re-infection of IL-5 Tg BALB/c mice compared to the corresponding negative control.

No change in lymphocyte numbers was observed for RSV re-infection of all BALB/c mice (except for IL-Tg BALB/c) (Figure 4.9 B) compared to the corresponding negative controls. RSV re-infection of IL-5 Tg BALB/c resulted in significant increase in lymphocyte numbers compared to negative control ($p < 0.0001$). Additionally, RSV re-infection of IL-5 Tg BALB/c resulted in significantly greater eosinophil numbers compared to RSV re-infected WT, IL-5 Tg, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice ($p < 0.0001$).

No difference in eosinophil numbers was observed for any BALB/c strains re-infected with RSV (Figure 4.9 C) compared to their corresponding negative controls. However, re-infection of the IL-5 Tg BALB/c group with Vero E6 cell lysate, UV-RSV and RSV has resulted in significantly higher eosinophil numbers present in blood compared to all other BALB/s groups ($p < 0.0001$) re-infected with Vero E6 cell lysate, UV-RSV and RSV. No change in neutrophil numbers was observed for RSV re-infection of any BALB/c strains (Figure 4.9 C) compared to the corresponding negative controls.

CD4+ adoptive transfer into IL-5 Tg Rag $2^{-/-}$ BALB/c mice followed by RSV re-infection resulted in no change in monocyte, lymphocyte, eosinophil and neutrophil numbers compared to the corresponding negative controls. $CD4^+$ adoptive transfer into IL-5 Tg Rag $2^{-/-}$ BALB/c mice followed by RSV re-infection has resulted in a significant increase in monocyte numbers compared to RSV re-infected WT BALB/c, while monocyte numbers decreased compared to RSV re-infected IL-5 Tg BALB/c mice. In addition, $CD4^+$ adoptive transfer into IL-5 Tg Rag $2^{-/-}$ BALB/c mice followed by RSV re-infection has induced significant decrease in lymphocyte numbers compared to RSV re-infected IL-5 Tg BALB/c mice ($p < 0.0001$) as well as significant increase in eosinophil numbers compared to RSV re-infected WT and IL-5 Tg BALB/c mice ($p < 0.05$).

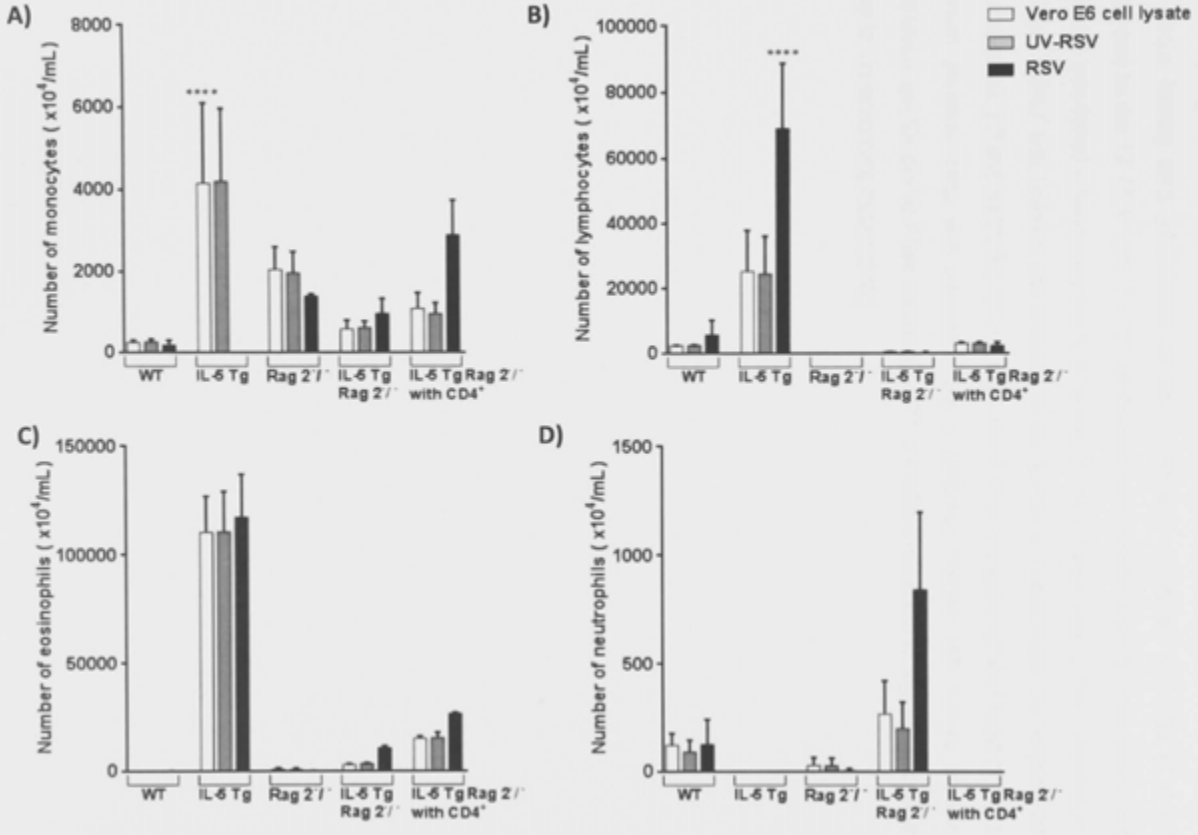

A)
Number of monocytes (x10⁴/mL)
8000
6000
4000
2000
0
****
WT
IL-6 Tg
Rag 2⁻/⁻
IL-6 Tg Rag 2⁻/⁻
IL-6 Tg Rag 2⁻/⁻ with CD4⁺
B)
Number of lymphocytes (x10⁴/mL)
100000
80000
60000
40000
20000
0
****
Vero E6 cell lysate
UV-RSV
RSV
C)
Number of eosinophils (x10⁴/mL)
150000
100000
50000
0
D)
Number of neutrophils (x10⁴/mL)
1500
1000
500
0

Figure 4.9. Leukocyte population in blood following respiratory syncytial virus (RSV) re-infection *in vivo*

Wild type (WT) , interleukin-5 transgenic (IL-5 Tg), Rag 2 knockout (Rag $2^{-/-}$) , IL-5 Tg Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c with $CD4^+$ mice were infected by intranasal inoculation with RSV (5 x 10^5 plaque forming units (pfu)/mouse) on day 0 and were re-infected with RSV (5 x 10^5 pfu/mouse) on day 12. $CD4^+$ cells (3 x 10^6 cells/mouse) were isolated from WT mice spleen and adoptively transferred intravenously into IL-5 Tg Rag $2^{-/-}$ mice 4 days prior to day 0 inoculation. On day 17, BALF was collected and analysed for the number of macrophages (A), lymphocytes (B), eosinophils (C) and neutrophils (D) present in blood. Data is representative of mean (n = 5) ± standard deviation. Data was analysed using a ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism version X7) (****, $p < 0.0001$ compared to negative inoculated group for corresponding mouse strain).

4.3.2.4 *Lung histopathology of BALB/c strain mice following RSV re-infection*

Following a Vero E6 cell lysate (negative control), UV-RSV (additional negative control) or RSV inoculation of BALB/c mice strains, lungs were assessed for airways mucus occlusion, degree of parenchymal pneumonia, peribronchial infiltrates, quality of peribronchial infiltrates, perivascular infiltrates, histopathological score, number of goblet cells and eosinophil tissue infiltrates.

4.3.2.4.1 Descriptive analysis

Lung pathology for Vero E6 cell lysate (negative control) and UV-RSV (additional negative control) inoculated WT, IL-5 Tg, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice demonstrates absence of viral infection as per clear alveoli (squares) and no/minimal inflammatory infiltrates present around bronchioles (thin arrows) or vessels (thick arrows) (Figure 4.10). RSV re-infection resulted in extensive inflammatory response characterised by macrophages, lymphocytes and eosinophils infiltrates. On day 17, RSV re-infection of WT and IL-5 Tg BALB/c mice induced mild to moderate parenchymal pneumonia and airway occlusion while macrophage and lymphocyte infiltration around bronchioles and blood vessels was moderate to severe. RSV re-infection of Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice resulted in moderate parenchymal pneumonia, airway occlusion with several goblet cells present as well as cellular infiltrates around bronchioles and blood vessels, dominated predominantly by eosinophils and macrophages. Pathology of lung collected from IL-5 Tg Rag $2^{-/-}$ with adoptive transfer of $CD4^{+}$ cells, shows very high levels of leukocyte infiltrates (mainly eosinophils and macrophages) in addition to severe parenchymal pneumonia and moderate airway occlusion.

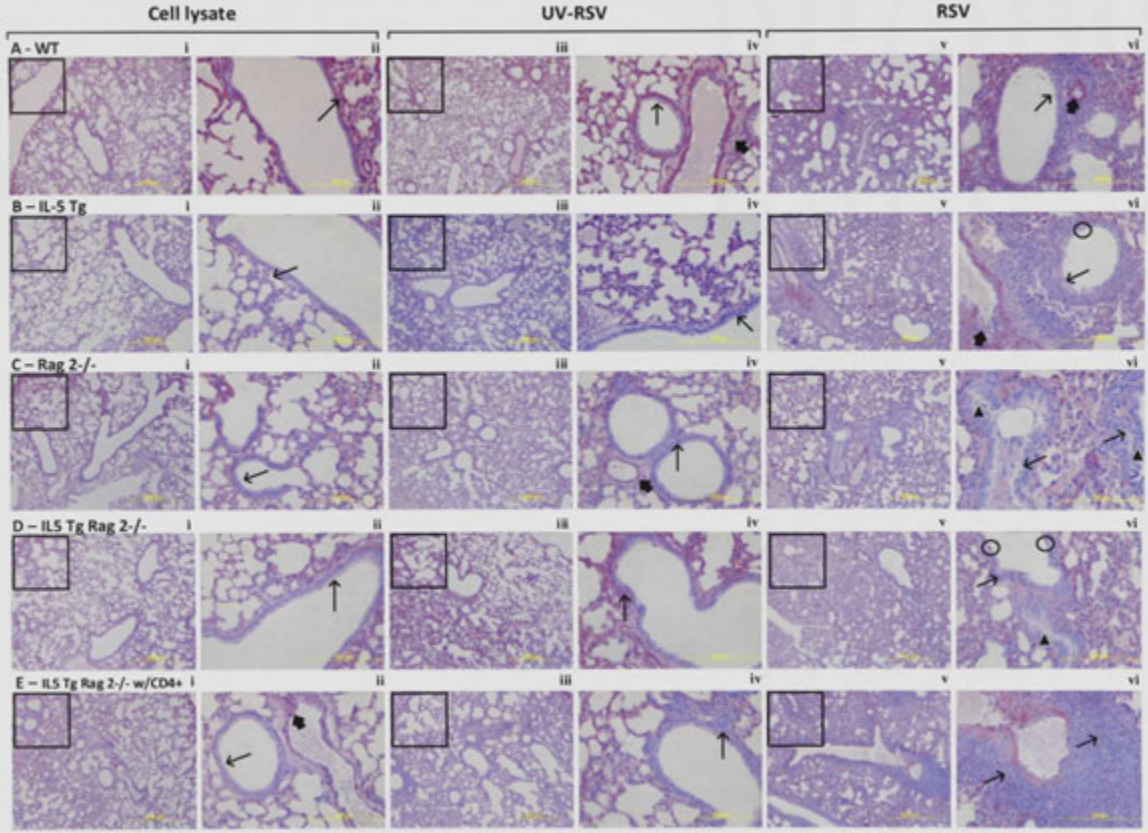

Cell lysate
UV-RSV
RSV
A - WT
B – IL-5 Tg
C – Rag 2-/-
D – IL5 Tg Rag 2-/-
E – IL5 Tg Rag 2-/- w/CD4+
i
ii
iii
iv
v
vi

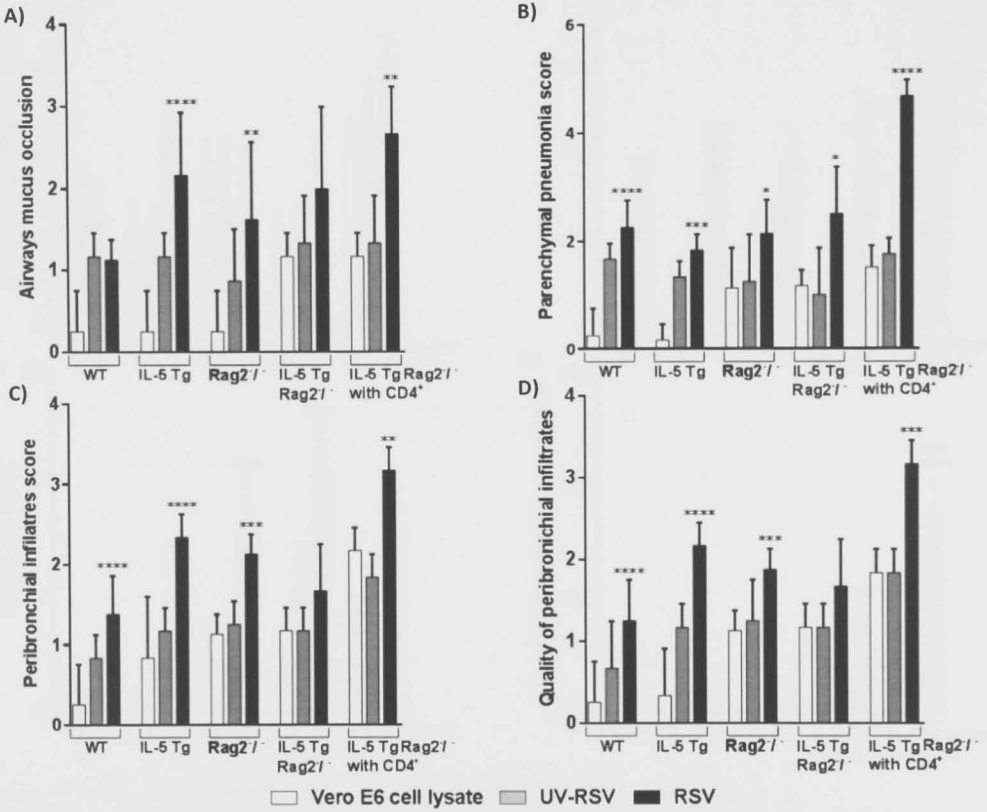
Figure 4.10. Importance of a functional adaptive immunity on BALB/c mice pathology following respiratory syncytial virus (RSV) re-infection

Wild type (WT), interleukin-5 transgenic (IL-5 Tg), Rag 2 knockout (Rag $2^{-/-}$), IL-5 Rag $2^{-/-}$ and were IL-5 Rag $2^{-/-}$ BALB/c with adoptive transfer of $CD4^+$ cells were infected intranasally with Vero E6 cell lysate (negative control), ultraviolet-inactivated RSV (UV-RSV) or RSV (5×10^5 plaque forming units/mouse). A) WT negative control (i and ii), WT UV-RSV (iii and iv) and WT RSV (v and vi). B) IL-5 Tg negative control (i and ii), IL-5 Tg UV-RSV (iii and iv) and IL-5 Tg RSV (v and vi). C) Rag $2^{-/-}$ negative control (i and ii), Rag $2^{-/-}$ UV-RSV (iii and iv) and Rag $2^{-/-}$ RSV (v and vi). D) IL-5 Tg Rag $2^{-/-}$ negative control (i and ii), IL-5 Tg Rag $2^{-/-}$ UV-RSV (iii and iv), IL-5 Tg Rag $2^{-/-}$ RSV (v and vi). E) IL-5 Rag $2^{-/-}$ with $CD4^+$ cells negative control (i and ii), IL-5 Rag $2^{-/-}$ with $CD4^+$ cells UV-RSV (iii and iv) and IL-5 Rag $2^{-/-}$ with $CD4^+$ cells RSV (v and vi). Lungs were collected on day 17 and processed for histology using periodic acid Schiff (PAS) stain. Arrows, squares and circles indicate the different signs of inflammation in mice as described in the text. Magnification (i, iii and v) was 100X and (ii, iv and vi) 400X including the eyepiece. Images are representative of each group (n = 5).

4.3.2.4.2 Numerical (score) analysis

Negative control compared to UV-RSV inoculated groups for all BALB/c strains showed no statistical differences across all histopathological analyses. However, RSV re-infection of WT, IL-5 Tg, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ resulted in a significant increase in airway mucus occlusion for all BALB/c strains ($p < 0.005$) except for WT BALB/c where no change was observed (Figure 4.11 A). Increase in parenchymal pneumonia was observed for all BALB/c strains with $p < 0.05$ (Figure 4.11 B). Peribronchial infiltrates and quality of those infiltrates mirrored the same trend across all RSV re-infected BALB/c strains, with an increase observed in peribronchial infiltrates compared to their negative controls ($p < 0.005$); except for RSV re-infected Rag $2^{-/-}$ BALB/c where no change was observed compared to its negative control (Figure 4.11 C and D). No change in perivascular infiltrates was observed for all RSV re-infected BALB/s strains (Figure 4.11 E). Combined HPS analysis revealed significant increase in HPS for all RSV infected and re-infected BALB/c mice with HPS ranging between 7-16 out of 17 compared to the corresponding negative and UV-RSV controls with HPS ranging between 1-7 out of 17 ($p < 0.05$) (Figure 4.11 F). Increase in a number of goblet cell was observed for IL-5 Tg, Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c only ($p < 0.005$) (Figure 4.11 G).

RSV re-infection of IL-5 Tg Rag $2^{-/-}$ mice and IL-5 Tg Rag $2^{-/-}$ mice with $CD4^+$ adoptive transfer induced an increase in a number of eosinophils present in the lung tissue ($p < 0.001$) compared to their negative controls while no significant change was observed for RSV re-infected WT, IL-5 Tg and Rag $2^{-/-}$ mice (Figure 4.11 H). The number of eosinophils was also significantly higher in IL-5 Tg Rag $2^{-/-}$ mice following $CD4^+$ adoptive transfer ($p < 0.0001$).


A)
Airways mucus occlusion
B)
Parenchymal pneumonia score
C)
Peribronchial infiltres score
D)
Quality of peribronichial infiltrates
WT
IL-5 Tg
Rag2-/-
IL-5 Tg Rag2-/-
IL-5 Tg Rag2-/- with CD4+
Vero E6 cell lysate
UV-RSV
RSV

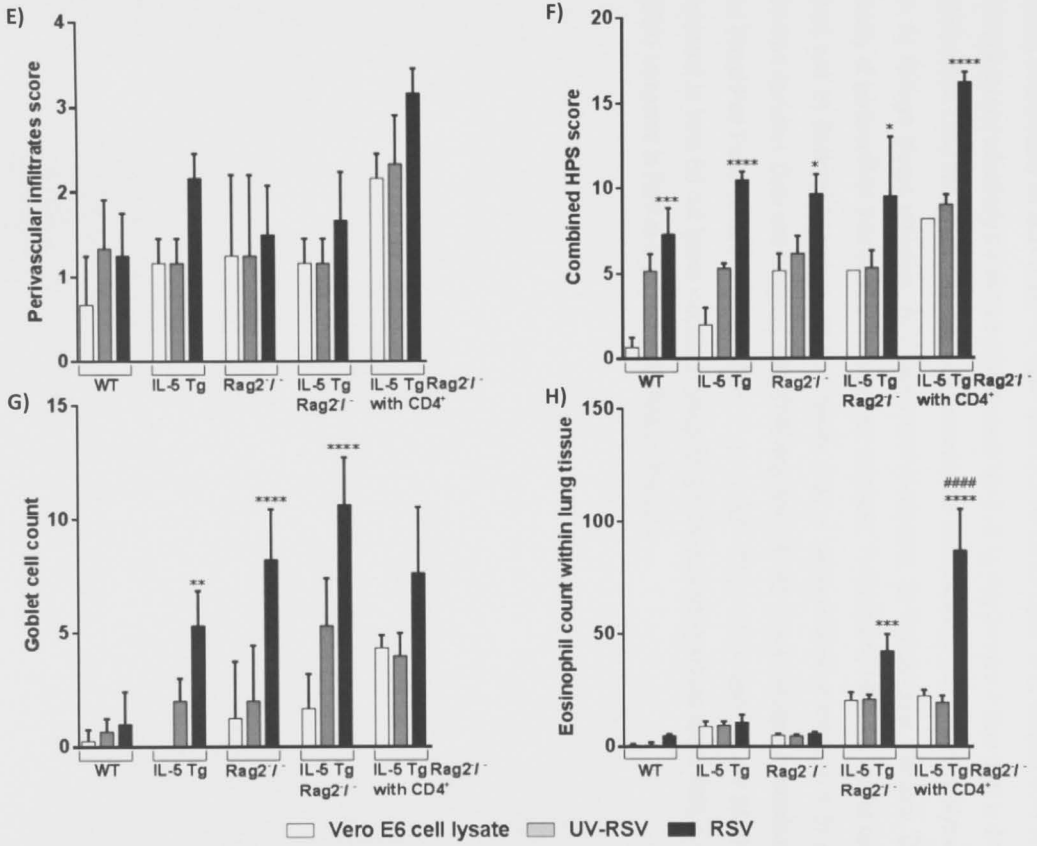

E)
Perivascular infiltrates score
0
1
2
3
4
WT
IL-5 Tg
Rag2-/-
IL-5 Tg Rag2-/-
IL-5 Tg Rag2-/- with CD4+
F)
Combined HPS score
0
5
10
15
20
***
****
*
*
****
WT
IL-5 Tg
Rag2-/-
IL-5 Tg Rag2-/-
IL-5 Tg Rag2-/- with CD4+
G)
Goblet cell count
0
5
10
15
**
****
****
WT
IL-5 Tg
Rag2-/-
IL-5 Tg Rag2-/-
IL-5 Tg Rag2-/- with CD4+
H)
Eosinophil count within lung tissue
0
50
100
150
***
####
****
WT
IL-5 Tg
Rag2-/-
IL-5 Tg Rag2-/-
IL-5 Tg Rag2-/- with CD4+
Vero E6 cell lysate
UV-RSV
RSV

Figure 4.11. Histopathological score (HPS) assessment of mice lungs following respiratory syncytial virus (RSV) re-infection *in vivo*

Wild type (WT), interleukin-5 transgenic (IL-5 Tg), Rag 2 knockout (Rag 2$^{-/-}$), IL-5 Rag 2$^{-/-}$ and were IL-5 Rag 2$^{-/-}$ BALB/c with adoptive transfer of CD4^{+} cells were infected intranasally with Vero E6 cell lysate (negative control), ultraviolet-inactivated RSV (UV-RSV) or RSV (5 x 10^{5} plaque forming units/mouse) on days 0 and 12. CD4^{+} cells (3 x 10^{6} cells/mouse) were isolated from WT mice spleen and adoptively transferred intravenously into IL-5 Tg Rag 2$^{-/-}$ mice 4 days prior to day 0 inoculation. Lung tissue was collected and processed for histopathology. Lungs were analysed for: A) Airways mucus occlusion, B) Parenchymal pneumonia, C) Peribronchial infiltrates, D) Quality of peribronchial infiltrates, E) Perivascular infiltrates, F) Combined HPS, G) Goblet cell count and H) Eosinophil count in the lung tissue. Data is representative of mean (n = 5) ± standard deviation. Data was analysed using ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism version X7) (*, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.001$ and ****, $p < 0.0001$ compared to Vero E6 cell lysate inoculated group for the corresponding mouse strain; ####, $p < 0.0001$ compared to RSV re-infected IL-5 Tg Rag 2$^{-/-}$ BALB/c).

4.4 Discussion

To understand the pleiotropic effects of RSV infection on the immune response, it is important to acknowledge that infections with different strains of RSV, that exhibit significant genetic variability, result in altered virulence and subsequent disease pathogenesis (Lotz and Peebles, 2012). The mechanisms by which RSV causes disease and is capable of repeated infection in humans remain to be determined. As many promising vaccine/therapeutic candidates in the animal model were found to be unsuccessful in human trials, it is important to continue on building our knowledge and understanding on how RSV interacts with humans to cause disease, evade innate immune response and inhibit the development of long-term protective immunity. More research is urgently needed to bridge this gap.

Previous studies suggest that RSV infection and re-infection result in skewed immunity towards an undesirable Th2 response (Welliver *et al.*, 2008) resulting in eosinophilia (Haynes *et al.*, 2003) and a short-term memory response (Connors *et al.*, 1991; Kulkarni *et al.*, 1995). In addition to *in vitro* findings (Domachowske *et al.*, 1998a; Soukup and Becker, 2003), murine study has shown that eosinophils play a positive role in RSV clearance *in vivo* (Phipps *et al.*, 2007). Consequently, the aim of this chapter was to evaluate eosinophil antiviral activity in four distinct mouse models following RSV infection and re-infection. The hypothesis that eosinophils may have the immune capacity to compensate for the absence of the mature T ($CD4^+$ and $CD8^+$) and B cells following RSV re-infection was assessed; as well as the potential of the eosinophil to inhibit RSV infection across all four BALB/c strains. Furthermore, as $CD8^+$ cells have been shown to contribute to disease pathology, the role of $CD4^+$ cells in viral clearance and lung pathology was also examined. A greater understanding of the role of the innate and adaptive immunity in RSV infection and re-infection could assist in the development of a successful RSV vaccine and/or therapeutic.

As previously mentioned innate immunity is the first line of defence in RSV infection (Goldsby *et al.*, 2003). Expanding on the study by Phipps *et al.* (2007), the antiviral role of the eosinophil in RSV infection was examined using a murine RSV model. The infection study results revealed a significant reduction ($p < 0.0001$) in viral titres following RSV infection of IL-5 Tg and IL-5

Tg Rag 2$^{-/-}$ BALB/c compared to RSV infected WT and Rag 2$^{-/-}$ BALB/c mice (Figure 4.2). Furthermore, it is important to note that WT and Rag 2$^{-/-}$ BALB/c mice had the same viral load present suggesting that adaptive immunity does not contribute to viral clearance in RSV primary infection. Together, these results are in agreement with findings by Phipps *et al.* (2007) who found that following ssRNA treatment of IL-5 Tg BALB/c, a significant reduction in viral infection was observed (Phipps *et al.*, 2007), demonstrating eosinophil driven viral inhibition in IL-5 Tg mice. In addition, eosinophil involvement in antiviral activity against RSV could also be responsible for observed reduction ($p < 0.001$) in eosinophil numbers present in the BALF and blood of IL-5 Tg BALB/c subsequent to RSV infection. Through a process of degranulation, eosinophils could become apoptotic, accounting for the reduction in eosinophil numbers observed. It is interesting that although, RSV inoculation of IL-5 Tg Rag 2$^{-/-}$ BALB/c induced 70% reduction in viral load compared to the RSV inoculated WT BALB/c; RSV inoculation of IL-5 Tg BALB/c induced 90% compared to the RSV inoculated WT BALB/c (Figure 4.2). This 20% difference in viral inhibition could be a result of the Rag 2 deletion in IL-5 Tg Rag 2$^{-/-}$ BALB/c and therefore a result of humanised model limitation. As the transcription of the IL-5 gene is under the influence of the DCR of the gene encoding CD2, expressed at the cell surface of T cells (Gleich and Adolphson, 1986; Lang *et al.*, 1988; Sanderson *et al.*, 1988), it is possible that Rag 2 deletion has an effect on eosinophil recruitment numbers or even the rate at which eosinophils are recruited. This is noted as the number of eosinophils present in BALF (Figure 4.3) and blood (Figure 4.4) following a primary infection of IL-5 Tg Rag 2$^{-/-}$ BALB/c did not reflect the same eosinophil response following a primary infection of IL-5 Tg BALB/c. In contrast, RSV infected IL-5 Tg Rag 2$^{-/-}$ BALB/c had significantly ($p < 0.0001$) higher number of eosinophils present in the lung tissue (Figure 4.6 H) compared to RSV infected IL-5 Tg BALB/c. These results suggest that Rag 2 deletion may affect the rate of eosinophil recruitment, resulting what appears to be a slightly delayed response in comparison to IL-5 Tg BALB/c.

Furthermore, these findings are supported by previous studies which show that eosinophils do not play a negative role in viral infections (Domachowske *et al.*, 1998a; Phipps *et al.*, 2007; Rosenberg *et al.*, 2009; Soukup and Becker, 2003) and do not contribute to viral pathology (Castilow *et al.*, 2008). In contrast, no eosinophil infiltration observed following RSV infection

of the WT and Rag 2$^{-/-}$ BALB/c can be correlated to the absence in viral load reduction Figure 4.2). RSV primary infection of WT and Rag 2$^{-/-}$ BALB/c also reveals that eosinophil immune response is dependant not only on the transcription of the IL-5 but also the presence of mature T cells to enable eosinophil recruitment. Taken together with the study by Phipps *et al.* (2007), these results demonstrate the importance of a prompt and effective eosinophil recruitment. Interestingly, in uninfected IL-5 Tg BALB/c mice, eosinophils normally contribute to over 30% of the leukocyte population in blood and BALF while in WT and Rag 2$^{-/-}$ BALB/c, eosinophils constitute for only about 1-3% of leukocyte population in blood and BALF. If eosinophils are the key to viral clearance, then the prophylactic treatment with immune modulatory therapeutics may facilitate eosinophil induced viral clearance necessary to clear RSV infection in WT and Rag 2$^{-/-}$ BALB/c mice. However, it is important to highlight that the key to eosinophil beneficial role most likely lies in the close regulation of the eosinophil recruitment.

RSV infection in the first two years of life is believed to lead to long-term deficiencies in protective immunity to RSV (Welliver, 2003). As such, the hypothesis that the eosinophil may be involved in the immune compensation in the absence of the mature T (CD4$^+$ and CD8$^+$) and B cells was investigated. Furthermore, as CD8$^+$ cells have been shown to contribute to disease pathology, the role of CD4$^+$ cells in viral clearance and disease pathology was also examined. RSV re-infection of IL-5 Tg BALB/c and WT BALB/c mice (Figure 4.7), has resulted in the highest reduction ($p < 0.0001$) of viral load compared to the RSV re-infected Rag 2$^{-/-}$ and IL-5 Tg Rag 2$^{-/-}$ BALB/c. BALB/c strains containing Rag 2 deletion exhibited the highest viral load present following RSV re-infection. These results demonstrate importance of the mature T and B cells in secondary viral infections. Furthermore, these results are also indicative of blurred line existing between innate and adaptive immunity especially in terms of their involvement in primary and secondary infections. This is especially important when examining the effect of RSV re-infection on the Rag 2$^{-/-}$ BALB/c strains. Therefore, as Rag 2$^{-/-}$ BALB/c have NK and ILC2 cells present despite the lack of mature T and B cells, it is important to reflect on how these cells could be involved in the immune response following RSV re-infection. Recently, the notion that NK cells are truly innate cells has been called into question because specific subsets of mouse liver NK cells have been

described to have the adaptive immunity property of lasting memory against specific viral antigens (Gillard *et al.*, 2011; Vivier *et al.*, 2011). Furthermore, ILC2 cells, present in Rag $2^{-/-}$ mice, are responsive to IL-25 and IL-33 during helminthic infection. However, their population numbers decrease soon after induction and these mice are unable to expel the worm burden effectively (Neill *et al.*, 2010). This suggests that T cells play a role in ILC2 cells maintenance, and in addition, boost the type 2 immune response by producing more type 2 cytokines. Respiratory infections with rhinovirus or respiratory syncytial virus are known to promote type 2 responses, and exacerbate allergic asthma. Chang *et al.* (2011) demonstrated that influenza virus-induced asthma is not mediated by adaptive immunity, but by IL-33-dependent ILC2 cells (Chang *et al.*, 2011). Together, these findings highlight the importance of chapter 4 findings. Although, Rag $2^{-/-}$ BABL/c strains do not express the mature T and B cells, results revealed some lymphocytes present in BALF and blood of IL-5 Tg Rag $2^{-/-}$ BABL/c. These lymphoid cells could be NK cells and/or even ILC2 cells as these are known to be present in Rag $2^{-/-}$ mice.

Previous depletion studies have demonstrated an importance of adaptive immunity in viral infection with studies revealing an increase in morbidity in $CD8^+$ and NK cell depleted mice following RSV infection (Bender *et al.*, 1992; Graham and Braciale, 1997; Topham and Doherty, 1998) while more recently, Lee *et al.* (2005) showed that mice lacking B cells succumbed to influenza H1N1 infection despite the infiltration of a larger number of $CD8^+$ cells (Lee *et al.*, 2005). These findings are in agreement with chapter 4 results, which indicate that intact adaptive immunity is very important for RSV inhibition. Despite its previously described inadequate response, each subsequent RSV infection results in an increase in protective immunity (Henderson *et al.*, 1979) and reduction in disease severity.

Although RSV-specific $CD8^+$ T cells have been shown to provide protection against infection, their effect is short-lived (Connors *et al.*, 1991; Kulkarni *et al.*, 1995). However, depletion of $CD4^+$ cells has been shown to result in delayed viral clearance and survival of infected mice following RSV re-infection (Allan *et al.*, 1990; Mozdzanowska *et al.*, 2000). Also, H1N1 infection of mice lacking $CD4^+$ cells resulted in mice recovering from infection in a similar fashion to the WT mice (Lee *et al.*, 2005). Therefore, the role of $CD4^+$ T cells

and their effect on viral clearance and disease pathology was examined in more detail. $CD4^+$ T lymphocytes adoptive transfer into IL-5 Rag $2^{-/-}$ BALB/c followed by RSV re-infection, resulted in over 80% viral titres reduction ($p < 0.0001$) (Figure 4.7) and an increase in eosinophil tissue infiltrates compared to RSV re-infected IL-5 Rag $2^{-/-}$ BALB/c ($p < 0.0001$) (Figure 4.11). These results mimic the trend of the immune response observed in BALF and blood following RSV re-infection of IL-5 Tg BALB/c mice. However, the main difference is in the HPS results, where $CD4^+$ T lymphocytes adoptive transfer into IL-5 Tg Rag $2^{-/-}$ BALB/c induced the highest HPS scores across the board. These results confirm the importance of $CD4^+$ T cells in viral inhibition which is supported by previous study showing that the $CD4^+$ and $CD8^+$ T lymphocyte contribute individually to clearing of the RSV infection (Graham *et al.*, 1991). The lack of eosinophil numbers present in BALF (Figure 4.8) and blood (Figure 4.9) following RSV re-infection of IL-5 Tg Rag $2^{-/-}$ BALB/c, demonstrates the importance of T cells role in a T cell-driven prompt eosinophil response to a viral infection. This result is similar to those following primary RSV infection of IL-5 Tg Rag $2^{-/-}$ BALB/c in terms of eosinophil recruitment to BALF and blood. As such, primary and secondary RSV infection of IL-5 Tg Rag $2^{-/-}$ BALB/c reveal significant increase ($p < 0.0001$) in eosinophil tissue infiltrates, suggesting that a delay in eosinophil immune response may be a result of T cell deficiency present in Rag $2^{-/-}$ mice. However, this issue appears to be overcome at a much slower rate potentially through the signalling the innate cells such as ILC2 cells. Furthermore, an increase ($p < 0.0001$) in eosinophil tissue infiltration (Figure 4.11 H) was also observed following the RSV re-infection of IL-5 Rag $2^{-/-}$ BALB/c with $CD4^+$ T cells adoptive transfer, supporting the hypothesis that T cells are very important in eosinophil recruitment process. Also, these findings demonstrate for the first time that accelerated viral clearance in IL-5 Tg BALB/c is dependent on a presence of at least one component of adaptive immunity (in this case $CD4^+$ T lymphocytes), hence indicating that eosinophil antiviral activity may be dependent on a degree of T cell response. Furthermore, $CD4^+$ T lymphocytes adoptive transfer into IL-5 Rag $2^{-/-}$ BALB/c has also resulted in an increase ($p < 0.0001$) in HPS scores compared to RSV re-infected IL-5 Tg Rag $2^{-/-}$ BALB/c (Figure 4.11). These findings suggest that although $CD4^+$ T cells have been shown to play an important role in viral clearance following RSV re-infection, they also contribute to RSV disease pathology. These findings are in

agreement with a previous study by Graham *et al.* (1991) which showed that both, $CD4^+$ and $CD8^+$ lymphocytes contribute to RSV disease pathology (Graham *et al.*, 1991). However, recent study by Lee *et al.* (2012) also found that vaccine-elicited effector anti-RSV $CD8^+$ T cells protected mice against RSV infection and pathogenesis (Lee *et al.*, 2012b). Based on the findings of chapter 4 study and previous studies by other research groups, it appears that more work is required to elucidate the role of T lymphocytes in RSV infection and disease pathology.

In summary, eosinophils have been shown to play a very positive role in viral clearance following RSV infection and re-infection. However, their antiviral activity appears to be modulated by the number of cells recruited to lungs in response to viral infection. This has been especially evident with Rag $2^{-/-}$ mice, which are lacking the T and B cells, as eosinophil recruitment has been shown to depend on IL-5 production; tightly regulated by the CD2 expressed on the T cell surface. This regulation appears to extend to eosinophil numbers present in BALF and blood. However, in the case of Rag $2^{-/-}$ mice, this issue appears somewhat overcome through the eosinophil regulation via most likely ILC2 cells IL-5 production. Recent study by Roediger *et al.* (2013) has shown that both NK and ILC2 cell express CD2, required for eosinophil recruitment (Roediger *et al.*, 2013). Furthermore, several studies have shown that RSV infection alters functionality of T lymphocytes (Fulton *et al.*, 2008; Gray *et al.*, 2005) resulting in inadequate immune response and low T lymphocyte numbers. Although this phenomenon has been observed in multiple viral models, the underlying mechanisms are still unknown. The impaired function of T lymphocytes in the lung during virus infection has been proposed to be the consequence of virus-induced changes in the lung environment (Gray *et al.*, 2005) as well as virus-induced immune evasion tactics (Lotz and Peebles, 2012; Moreau *et al.*, 2003). Future RSV immunisation strategies should strive to achieve a balanced immune response. In addition, to induce a more desirable immune response, a focus on blocking RSVs evasion tactics is recommended which could be achieved using therapeutics targeting virus itself or by immune modulation. This information would be invaluable in facilitating the design of safe and effective vaccines against what remains a major human pathogen.

Chapter 5. HEPARAN SULFATE TREATMENT OF RESPIRATORY SYNCYTIAL VIRUS INFECTION

5.1 INTRODUCTION

In Chapters 3 and 4, innate and adaptive immunity were examined in response to RSV infection (*in vitro* and *in vivo*) and re-infection (*in vivo*). The knowledge gained from the previous two chapters could be utilised in the development of much needed RSV therapeutics. In Chapter 1, it was shown that HS mimetics have a potential to become a therapeutic agent against RSV infection and that further *in vitro* and *in vivo* investigation is warranted.

As HSPGs consist of a core protein bearing GAG chains, they are composed of unbranched HS chains, structurally related to heparin (Bishop *et al.*, 2007). Heparin is a GAG, exclusively synthesised by mast cells and it has a crucial role as a depot for various mediators and for the morphology of the granules (Forsberg *et al.*, 1999; Humphries *et al.*, 1999). In contrast, HS is expressed on the cell surface and ECM of a wide range of cells of vertebrate and invertebrate tissues (Iozzo and San Antonio, 2001; Kjellen and Lindahl, 1991). However, both heparin and heparan sulfate play a very important role in host immune defence. In 1964, heparin was found to be the first GAG to affect virus replication and was shown to limit the growth of HSV (Nahmias and Kibrick, 1964). Other viruses have also been found to interact with GAGs, including dengue virus (Lee *et al.*, 2006), Sindbis virus (Byrnes and Griffin, 1998; Klimstra *et al.*, 1998), foot and mouth disease virus (O'Donnell *et al.*, 2008), human immunodeficiency virus type 1 (De Francesco *et al.*, 2011) and vaccinia virus (Ho *et al.*, 2005). At the molecular level, the negatively charged sulfated or carboxyl groups of HSPGs or heparin (Hallak *et al.*, 2000b) interact with a cluster of positively charged basic amino acids present within the linear HBD of RSV G protein (Feldman *et al.*, 1999). Interestingly, a similar putative HBD was also identified in RSV F protein (Feldman *et al.*, 2000), suggesting that the HSPG-HBD interaction is a common theme for RSV proteins that mediate infection, making it a preferred target for the development of antiviral compounds against RSV. It is not clear, however, what type of GAGs and GAG components are involved, whether the important GAGs are on the virus (Bourgeois *et al.*, 1998) or the cell (Krusat and Streckert, 1997), and the magnitude of their contribution to the infection (Hallak *et al.*, 2000a). It is apparent that they have an important role as demonstrated by previous studies

which have shown an 80% reduction in RSV infection using GAG deficient cell lines (Hallak *et al.*, 2000a).

Based on the findings of previous studies, the importance and efficacy of HS mimetics treatment on RSV infection was assessed. Viral attachment, cell surface interaction and inhibition of viral replication were tested *in vitro* in the presence of 53 HS mimetic compounds at concentrations of 5, 20 and 100 µg/mL. Following *in vitro* studies, six HS mimetics were tested *in vivo* for their efficacy against RSV infection and as a potential candidate for prophylactic treatment of RSV infection.

5.2 METHODS

5.2.1 HS mimetics

HS mimetics (Table 5.1) were generously supplied by Dr. C. Freeman (John Curtin School of Medical Research (JCSMR), Australian National University (ANU)). All HS compounds, in their powder form, were stored in a desiccated jar at 4°C. Compounds were diluted in sterile saline solution and aliquots kept frozen at -20°C until required.

Table 5.1. List of heparan sulfate mimetics

Group 1-Heparins and modified heparins (~12kDa)	
201	Mucosal heparin (12.5kDa)
204	Mucosal heparin glycol split (gc) periodate treated/$NaBH_4$ reduced
205	Mucosal heparin gc (pNAc) (part deNS/NAc)
207	Mucosal heparin gc de2S (periodate then lyophilise in 0.1M NaOH)
230	Desulfated mucosal heparin
384	Mucosal heparin gc Nac
448	MH-NAc ie deNS, then re acetylate ; loss of 1 anionic charge perdisaccharide
465	Mucosal heparin-gc butyl
466	Mucosal heparin-gc hexyl
481	MH-gc-NH
483	MH-NH ie deNS ie GlcNH+ ; loss of 2 anionic charges per disaccharide

Group 2-Low molecular weight heparins (LMWH) (5kDa and 3kDa) and enoxaparin with/without glycol split (gc)

217	Mucosal heparin 5kDa LMWH (Sigma)
218	Mucosal heparin 5kDa gc split
219	Mucosal heparin 5kDa gc split part deNS/NAc
228	Decarboxylated mucosal heparin
331	Mucosal heparin Sigma 3kDa
332	Mucosal heparin Sigma 3kDa gc
333	Mucosal heparin Sigma 3kDa de2S
394	Enoxaparin 3kDa
408	Enoxaparin-gc
416	Mucosal heparin3kDa gc-CHO + Benzhydrazide
418	Mucosal heparin3kDa gc-CHO + Anthranilic acid (NH_2-Q-COOH)
419	Mucosal heparin3kDa gc-CHO + ANTS (1,3,6 triSO_3-aminonaphthaline)
424	Mucosal heparin with glycol split 3kDa

Group 3-Fucoidans and modified fucoidan

259	Fucoidan (Sigma)
497	Fucoidan Marinova
498	Fucoidan 30min ascorbate/peroxide
499	Fucoidan 60min ascorbate/peroxide
500	Fucoidan 90min ascorbate/peroxide
501	Fucoidan 120min ascorbate/peroxide
502	Fucoidan 180min ascorbate/peroxide

Group 4-Carrageenans

266	Iota-carrageenan
267	Lambda-carrageenan 3S/disacc
494	Iota-carragenan Fe/Ascorbate
495	Lambda-carragenan Fe II
496	Lambda-carragenan Fe/ascorbate

Group 5-Other HS compounds

81	Cellobiose sulfate
106	Maltotetraose H -itol (reduced)
109	Lactose-CO-NH-CH_2-C_6H_4-CH_2-NH-CO-lactose (meta linked xylyl)
111	Lact-CO-NH-$(CH_2)_n$-NH-CO-lact n = 12
229	Pentosan polysulfate 5kDa Sigma
238	Chondroitin 4-sulfate
241	Dermatan sulfate
242	Chondroitin 6-sulfate
254	Dextran Sulfate 5kDa
486	Suledexide 5kDa
510	Polyvinyl sulfate Na+ (Sigma) charcoal treated
511 A	Polyvinyl sulfate Na+ (Sigma) polymerised A
511 B	Polyvinyl sulfate Na+ (Sigma) polymerised B
512 D	Polyvinyl sulfate Na+ (Sigma) polymerised D
512 E	Polyvinyl sulfate Na+ (Sigma) polymerised E
PI-88	A mixture of highly sulfated, monophosphorylated mannose oligosaccharides
Suramin	A polyanionic compound- contains eight benzene rings, four amide groups, one urea and six sulfonate groups

Table 5.1 legend:

The following legend is provided to explain abbreviations used in the table above. Abbreviations listed refer to chemical and/or structural modifications present in the corresponding HS mimetic compounds.

- gc = periodate treated (glycol split); opens ring; makes it more flexible; no anticoagulant activity
- de2S = 2 sulfate (S) groups removed; low/no anticoagulant activity
- gs/de2S = glycol split; then 2 sulfate (S) groups removed; it makes it less likely to bind to other proteins
- pNAc = part deNS, replace with NAc; even less sulfation and binding to other proteins
- CR = decarboxylated (not an anti-coagulant, reduces binding to many compounds)-COOH >> -CH_2OH

5.2.2 Mouse strain

Male WT BALB/c mice aged 6 to 8 weeks were obtained from the pathogen free animal facility at the University of Canberra (UC). Animals were housed in approved containment facilities and treated in accordance with UC animal experimentation guidelines.

5.2.3 HS cytotoxicity assay

HS mimetic compounds were evaluated for cell cytotoxicity *in vitro*. The AlamarBlue assay (Invitrogen, Australia) was used to assess cell health and viability following incubation with HS mimetics. The assay was set up in a 96-well plate. Vero E6 cells were prepared in Opti-MEM (Gibco, Australia) with 5% FCS (Sigma) and plated at 6000 cells/well. All samples were prepared in quadruplicates. Cells in the 96-well plate were allowed to incubate and adhere for 4 hours at 37°C and 5% CO_2. Following the 4 hour incubation, 100 µL of each HS mimetic at 100 µg/mL (final concentration) was added to appropriate set of quadruplicates. Plate was incubated for 24 hours at 37°C and 5% CO_2, following which, 10X AlamarBlue reagent (Invitrogen) was added to each well at 1X final concentration and the plate incubated for 4 hours at 37°C and 5% CO_2. Samples was analysed using a spectrophotometer (SPECTROstar Omega, BMG Labtech) at dual wavelength of 570 and 600 nm. Vero E6 cells (ATCC) were used as a standard for the cell proliferation assay. Standards were serially diluted 1 in 2 with concentrations ranging from 12000 to 94 cells per well. Standards were included in the assay to enable determination of cell concentration of unknown samples (Vero E6 cells incubated with HS mimetics).

5.2.4 HS mimetics treatment of RSV infection *in vitro*

The effect of HS mimetics on RSV infection was tested *in vitro*. All compounds were tested at 100 µg/mL, while a selected number of compounds were tested at 20 and 5 µg/mL concentration. All samples were processed for immunostaining RSV plaque assay (section 2.10). Compounds were tested under four different conditions and variations were as follows: **Condition A**- incubating Vero E6 cell lysate (negative control), UV-RSV (additional negative control) or RSV (200 pfu) with HS mimetics for 10 minutes at 37°C and 5% CO_2; **Condition B**- incubating Vero E6 monolayer with HS mimetics for 10 minutes at 37°C and 5% CO_2; **Condition C**- incubating Vero E6 cell lysate (negative

control), UV-RSV (additional negative control) or RSV (200 pfu) with the cell monolayer for 10 minutes (allowing virus to adhere) at 37°C and 5% CO_2; and **Condition D**- incubating Vero E6 cell lysate (negative control), UV-RSV (additional negative control) or RSV (200 pfu) with the cell monolayer for 1 hour (allowing virus to adhere) at 37°C and 5% CO_2. The experimental design is detailed in Figure 5.1.

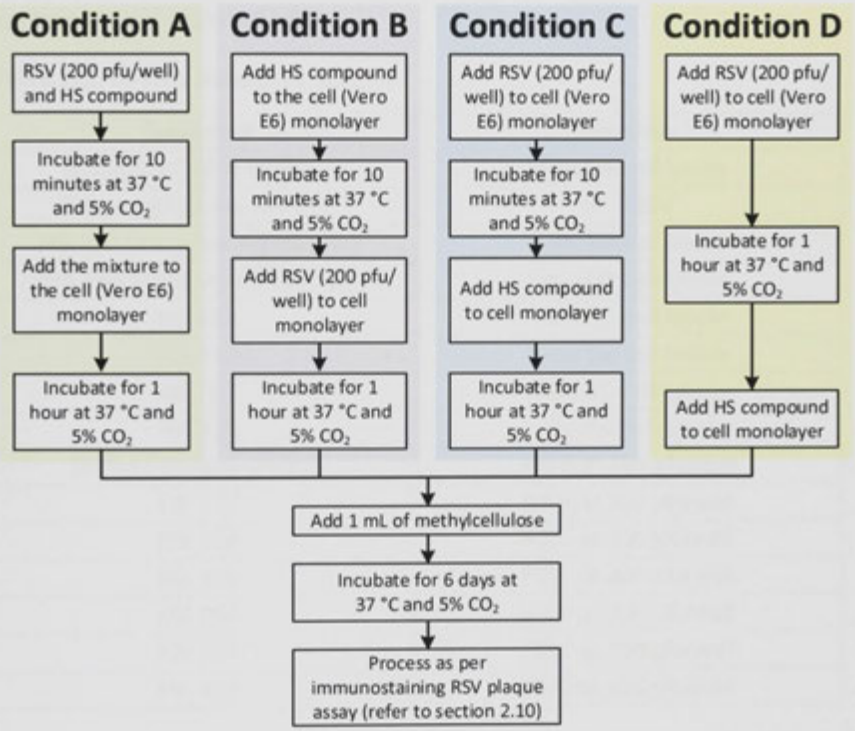


Figure 5.1. Heparan sulfate (HS) mimetics *in vitro* experimental design

HS treatment of respiratory syncytial virus (RSV) (200 plaque forming (pfu)/well) infection under four conditions: **Condition A**- incubating RSV with HS for 10 minutes at 37°C and 5% CO_2; **Condition B**- incubating Vero E6 monolayer with HS mimetics for 10 minutes at 37°C and 5% CO_2; **Condition C**- incubating RSV (allowing virus to adhere) with the cell monolayer for 10 minutes at 37°C and 5% CO_2; and **Condition D**- incubating RSV (allowing virus to adhere) with the cell monolayer for 1 hour at 37°C and 5% CO_2. Viral titres were determined by immunostaining RSV plaque assay.

5.2.5 HS mimetics treatment of RSV infection *in vivo*

Following *in vitro* studies, the best performing HS compounds were selected for *in vivo* studies. The selected HS mimetics were: HS 228 (carboxyl reduced mucosal heparin), HS 259 (fucoidan-sulfated polysaccharide), HS 267 (lambda carrageenan (200kDa)) and HS 424 (mucosal heparin with glycol split (3kDa)). HS 230 (desulfated heparin) and HS 254 (dextran sulfate (5kDa)) were included as HS negative and positive controls, respectively. Treatment groups are listed in the Table 5.2 below.

Table 5.2. *In vivo* treatment groups

Treatment	Inoculum
Saline (negative control)	Vero E6 cell lysate
Saline (additional control)	UV-RSV
HS 230 (negative HS control)	Vero E6 cell lysate
HS 254 (positive HS control)	Vero E6 cell lysate
HS 228	Vero E6 cell lysate
HS 259	Vero E6 cell lysate
HS 267	Vero E6 cell lysate
HS 424	Vero E6 cell lysate
Saline (positive control)	RSV at 200 pfu/well
HS 230	RSV at 200 pfu/well
HS 254	RSV at 200 pfu/well
HS 228	RSV at 200 pfu/well
HS 259	RSV at 200 pfu/well
HS 267	RSV at 200 pfu/well
HS 424	RSV at 200 pfu/well

Selected HS mimetics were tested for the RSV inhibition under following two conditions: HS mimetics treatment of RSV infection and prophylactic HS treatment of RSV infection.

The effect of HS mimetics on RSV infection, *in vitro* and *in vivo* was assessed by calculating the percent inhibition of viral infection. This was done using the formula below:

Inhibition = 100 x (Original Number – New Number) / Original Number

Equation 3.1. Formula for the percent inhibition of RSV infection

5.2.5.1 HS mimetics treatment of RSV infection

On day 0 WT BALB/c mice were lightly anesthetised with isofluorane (PharmaChem) (section 2.12.4) and intranasally inoculated with either Vero E6 cell lysate, UV-RSV or RSV (50 µL of 1 x 10^7 pfu/mL = 5 x 10^5 pfu/mouse). On days 1-4, mice were treated with selected HS mimetics at 10 mg/mL/kg (100 µL intraperitoneal injections) (Figure 5.2). On day 5 mice were euthanised by CO_2 asphyxiation (section 2.12.1) and samples collected (sections 2.13).

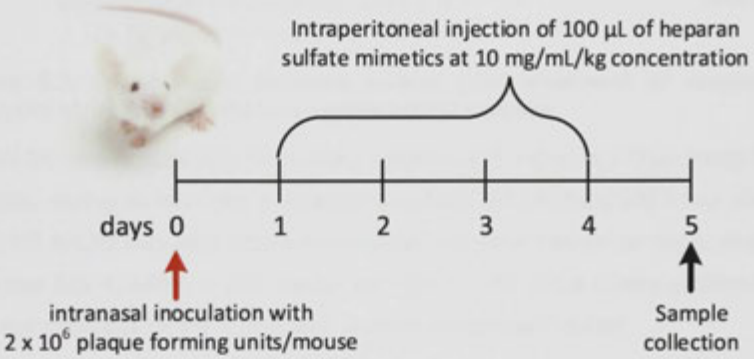


Figure 5.2. Heparan sulfate (HS) mimetic treatment of respiratory syncytial virus (RSV) infection experimental timeline

On day 0, wild type BALB/c mice were inoculated with RSV and receiving daily HS mimetics treatment at 10 mg/mL/kg on days 1-4. On day 5, mice were sacrificed and samples collected.

5.2.5.2 Prophylactic HS treatment of RSV infection

WT BALB/c mice received daily HS mimetics treatment at 10 mg/mL/kg (100 µL intraperitoneal injections), starting 48 hours prior to intranasal inoculation and continuing until 4 days after the inoculation. On day 0 WT BALB/c mice were intranasally inoculated with either Vero E6 cell lysate, UV-RSV or RSV (50 µL of 1 x 10^7 pfu/mL= 5 x 10^5 pfu/mouse) (Figure 5.3). On day 5 mice were euthanised by CO_2 asphyxiation (section 2.12.1) and samples collected (sections 2.13).

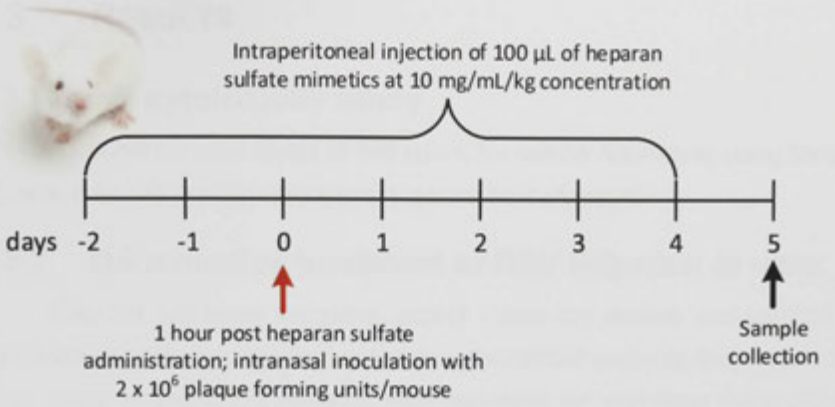


Figure 5.3. Prophylactic heparan sulfate (HS) treatment of respiratory syncytial virus (RSV) infection experimental timeline

WT BALB/c mice received daily HS mimetics treatment at 10 mg/mL/kg (100µL intraperitoneal injections), starting 48 hours prior to intranasal inoculation and continuing until 4 days after. On day 0, WT BALB/c mice were intranasally inoculated with either Vero E6 cell lysate, ultraviolet-inactivated RSV (UV-RSV) or RSV infected with RSV (5 x 10^5 plaque forming units/mouse) by intranasal inoculation. On day 5, mice were sacrificed and samples collected.

5.2.6 Statistical analysis

Data are presented as mean ± standard deviation. A ONE-way or TWO-way ANOVA was used to determine statistical significance. Data were analysed using GraphPad Prism version X7. A p value of less than 0.05 was considered statistically significant.

5.3 RESULTS

5.3.1 HS cytotoxicity study

HS mimetics were tested at 100 µg/mL for cellular cytotoxicity using Vero E6 cells. It was found that HS mimetics are not toxic (Appendix 4).

5.3.2 HS mimetics treatment of RSV infection *in vitro*

Vero E6 cell lysate (negative control - data not shown) and UV-RSV (additional negative control-data not shown) inoculated samples that received either saline or tested HS mimetics were assessed for viral titres using RSV immunohistochemistry plaque following all four testing conditions and no viral titres were recovered. In addition, *in vitro* results of only 27 out of 53 tested HS mimetics are represented in this result chapter. These compounds were selected based on their antiviral activity, chemical and structural variations; representing an average HS mimetics performance, at three different concentrations and under four different conditions, for the group that they are representing. Graphs of other compounds can be found in Appendix 5.

5.3.2.1 HS induced RSV inhibition in vitro

5.3.2.1.1 Condition A

HS 230 (negative HS control) showed no reduction of RSV infection while HS 254 (positive HS control) induced 90 and 50% inhibition of RSV infection ($p < 0.001$) at 100 and 5µg/mL, respectively. Overall, group 1 HS mimetics, the heparins and modified heparins (~12kDa), induced a 60-80% reduction in RSV plaques (Figure 5.4 A). HS 201 was found to inhibit RSV 30% better ($p < 0.001$) at 100 µg/mL concentration compared to 5 µg/mL. HS 204, HS 205 and HS 207 did not inhibit RSV at any of the three tested HS concentrations.

Group 2 (low molecular weight heparins (5kDa and 3kDa) and enoxaparin with/without glycol split) HS mimetics showed more dose dependant RSV inhibition (Figure 5.4 B). HS 218 and HS 219 induced 80% RSV inhibition at 5 and 100 µg/mL concentrations resulting in almost 20% greater ($p < 0.005$) inhibition compared to 20 µg/mL dose. HS 331 and HS 332 RSV inhibition increased from 60-90% following an increase in HS mimetics concentration

from 5 to 100 µg/mL. In contrast, HS 416 and HS 424 RSV inhibition was improved from 65-85% ($p < 0.005$) by reducing HS mimetics concentration from 100 to 5 µg/mL. HS 217 and HS 333 achieved 80% and 40% RSV inhibition, respectively, and were unaffected by change in HS mimetic concentration.

HS 259, belonging to group 3 (fucoidans and modified fucoidans) HS mimetics, showed almost 100% RSV inhibition at 5, 20 and 100 µg/mL concentrations (Figure 5.4 C). All Group 4 (carrageenans) HS mimetics were found to induce 100% RSV inhibition in at least one of the concentrations examined. HS 266 induced 100% RSV inhibition ($p < 0.0001$) at 100 and 20 µg/mL while only 60% inhibition at 5 µ/mL ($p < 0.0001$). HS 267 inhibited RSV 100% ($p < 0.0001$) irrespective of the HS mimetics concentration (Figure 5.4 D).

Several group 5 (other) HS mimetics were found to perform better at lower concentrations (Figure 5.4 E). HS 111, HS 238, HS 241, HS 242, PI-88 and suramin showed a significant RSV inhibition (60-80%) ($p < 0.005$) at 5 and/or 20 µg/mL while RSV inhibition was reduced to 0% at 100 µg/mL concentration. HS 81, HS 106 and HS 109 induced 60%, 90% and 70% RSV inhibition ($p < 0.005$) respectively, irrespective of the HS mimetic concentration.

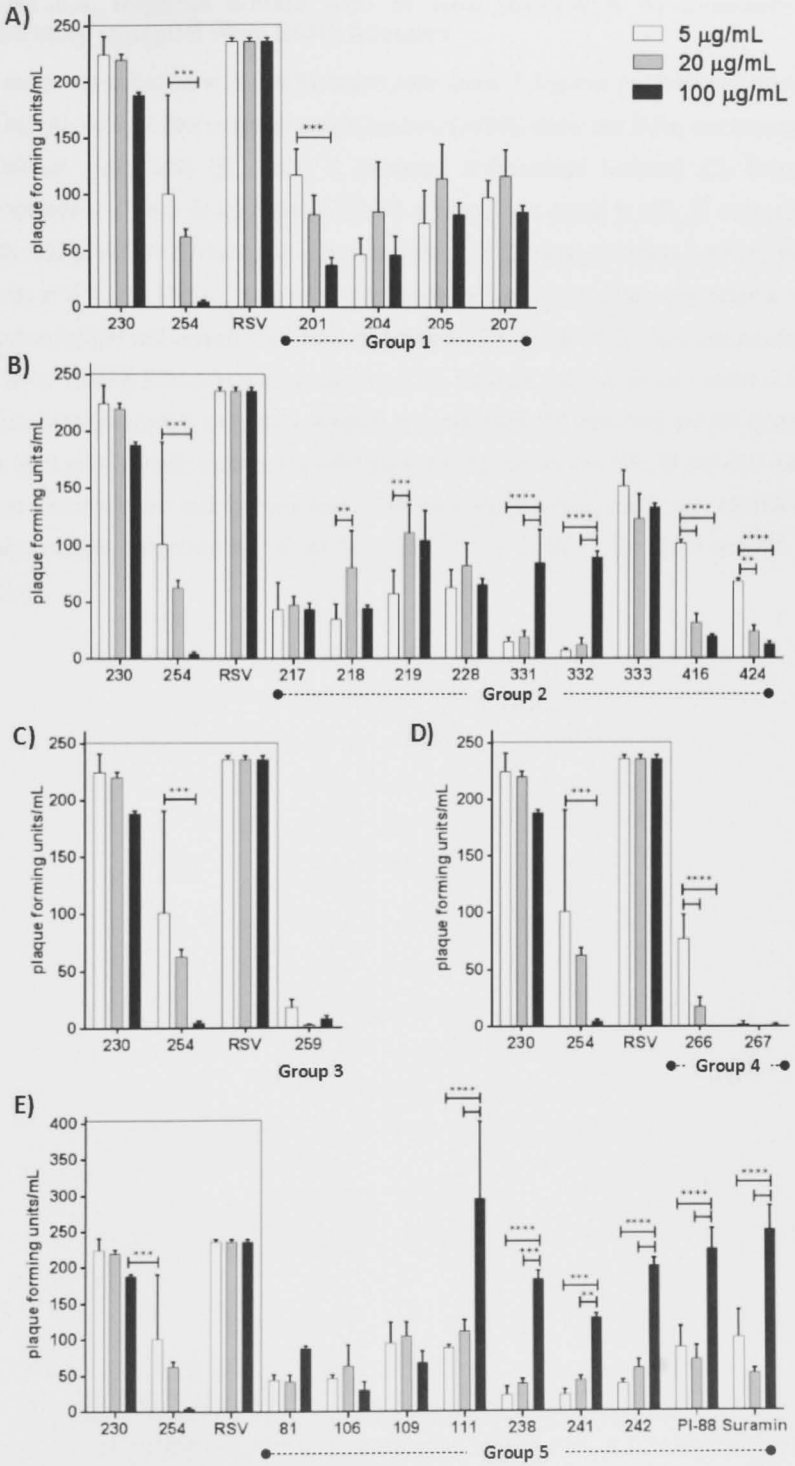

A)
5 µg/mL
20 µg/mL
100 µg/mL
plaque forming units/mL
250
200
150
100
50
0
***
***
230
254
RSV
201
204
205
207
Group 1
B)
plaque forming units/mL
250
200
150
100
50
0
***
**
***
****
****
****
****
230
254
RSV
217
218
219
228
331
332
333
416
424
Group 2
C)
plaque forming units/mL
250
200
150
100
50
0
***
230
254
RSV
259
Group 3
D)
plaque forming units/mL
250
200
150
100
50
0
***
****
230
254
RSV
266
267
Group 4
E)
plaque forming units/mL
400
350
300
250
200
150
100
50
0
***
****
****
***
****
****
****
230
254
RSV
81
106
109
111
238
241
242
PI-88
Suramin
Group 5

Figure 5.4. Heparan sulfate (HS) *in vitro* (Condition A) treatment of respiratory syncytial virus (RSV) infection

The assay was carried out in 24-well flat bottom plate. Group 1 (heparins and modified heparins (~12kDa)) (A), Group 2 (low molecular weight heparins (LMWH) (5kDa and 3kDa) and enoxaparin with/without glycol split) (B), Group 3 (fucoidans and modified fucoidan) (C), Group 4 (carrageenans) (D) and Group 5 (other) (E) HS mimetics were tested at 100, 20 or 5 µg/mL. Briefly, 100 µl/well of HS mimetics was incubated with RSV (200 plaque forming units/well) for 10 minutes at 37°C and 5% CO_2. Following the incubation, HS with RSV mixture was added to Vero E6 cell monolayer and allowed to incubate for 1 hour at 37°C and 5% CO_2. Plate was processed by immunostaining RSV plaque assay (section 2.10). Data are represented as a mean of RSV plaques recovered (n = 4) ± standard deviation. HS compounds 230 (negative) and 254 (positive) were used as HS mimetic controls. For each graph, the controls HS 230, HS 254 and RSV results are presented in boxed area for comparison. Data were analysed using a ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (**, $p < 0.005$, ***, $p < 0.001$ and ****, $p < 0.0001$).

5.3.2.1.2 Condition B

The hypothesis that HS mimetics may bind to the negatively charged sulfate and carboxyl groups of the glycosaminoglycan chains of HS on the cell surface of Vero E6 cells was tested. HS 230 (negative HS control) did not induce RSV inhibition while HS 254 (positive HS control) exhibited approximately 75% inhibition at all three tested HS concentrations. Treatment with group 1 HS mimetics (heparins and modified heparins (~12kDa)) (Figure 5.5 A) resulted in a 30-80% reduction in RSV plaques recovered. HS 201 was found to be almost 40% more effective against RSV at the 20 and 100 µg/mL concentrations than at 5 µg/mL ($p < 0.0001$). HS 204, 205 and 207 achieved 65, 30% and 45% inhibition, respectively, and were unaffected by HS concentrations (Figure 5.5 B).

The Group 2 (low molecular weight heparins (5kDa and 3kDa) and enoxaparin with/without glycol split) HS mimetics 217, HS 218 and HS 228 induced approximately 80% RSV inhibition and their efficacy was found to be unaffected by change in HS concentration (Figure 5.5 C). HS 219, HS 331 and HS 332 induced 75-80% RSV inhibition at 5 and 20 µg/mL compared to only 35-45% inhibition at 100 µg/mL ($p < 0.05$). HS 416 and HS 424 showed the opposite trend with an increase in RSV inhibition from 65-90% as the concentration increased from 5-100 µg/mL. HS 333 induce the lowest level of RSV inhibition, only 30%, at all three tested HS concentrations.

Group 3 (fucoidans and modified fucoidans) HS 259 showed an average of 90% RSV inhibition at 5, 20 and 100 µg/mL concentrations (Figure 5.5 C). In contrast, Group 4 (carrageenans) HS mimetics RSV inhibition decreased with reduction in HS mimetic concentrations. HS 266 induced 100%, 90% and 60% RSV inhibition at 100, 20 and 5 µg/m, respectively ($p < 0.0001$). HS 267 induced 100% RSV inhibition at 10 and 20 µg/mL while only 70% at 5 µg/mL (Figure 5.5 D).

Most of the group 5 (other) HS mimetics performed best at 5 and/or 20 µg/mL concentration (Figure 5.5 E). HS 81, HS 111, HS 238, HS 241, HS 242, PI-88 and suramin showed a significant increase (65-90%) in RSV inhibition at 5 and/or 20 µg/mL compared to only 0-20% at 100 µg/mL ($p < 0.05$). HS 106 and HS 109 induced close to 85% and 65% RSV inhibition respectively, irrespective of the HS mimetic concentrations.

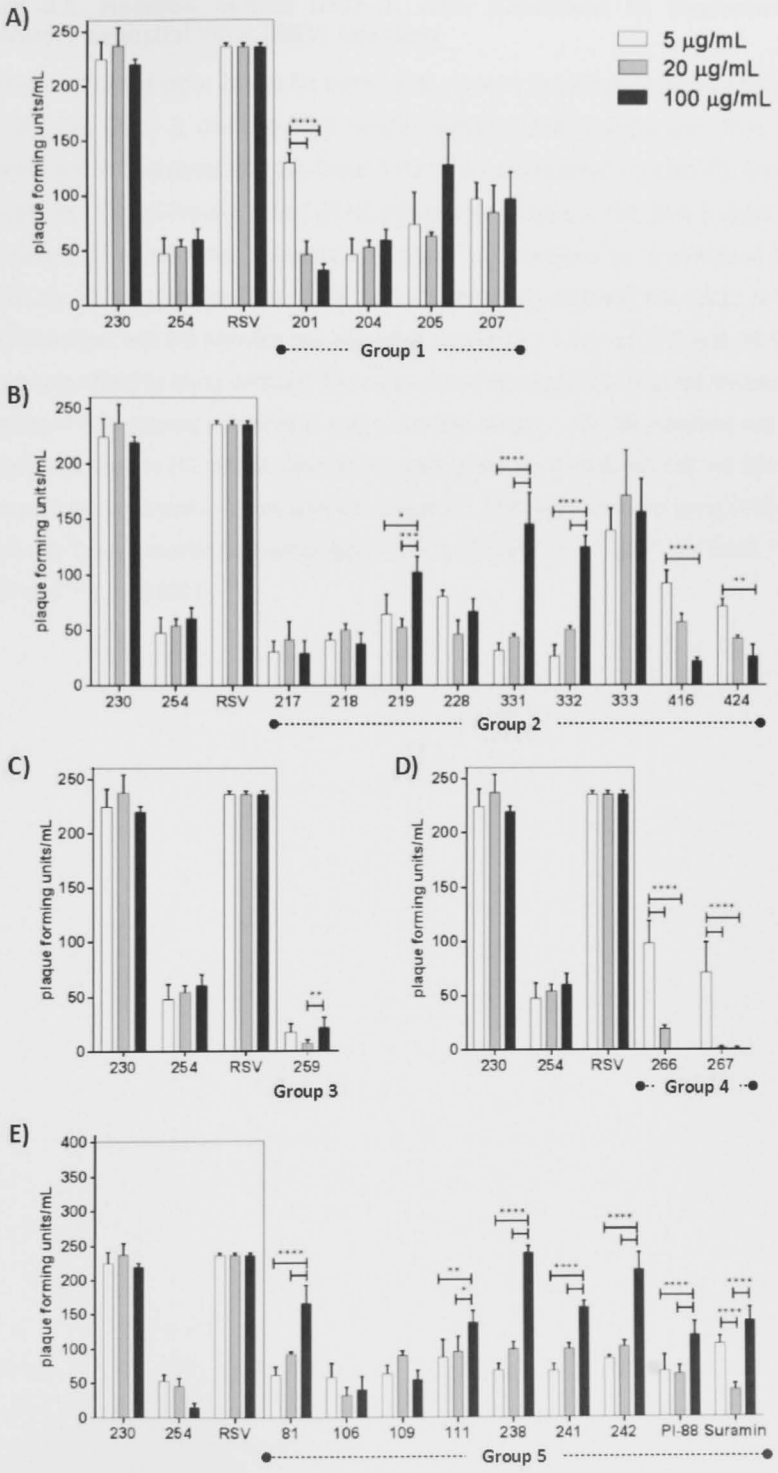

A)
5 µg/mL
20 µg/mL
100 µg/mL
plaque forming units/mL
230
254
RSV
201
204
205
207
Group 1
B)
plaque forming units/mL
230
254
RSV
217
218
219
228
331
332
333
416
424
Group 2
C)
plaque forming units/mL
230
254
RSV
259
Group 3
D)
plaque forming units/mL
230
254
RSV
266
267
Group 4
E)
plaque forming units/mL
230
254
RSV
81
106
109
111
238
241
242
PI-88
Suramin
Group 5

Figure 5.5. Heparan sulfate (HS) *in vitro* (Condition B) treatment of respiratory syncytial virus (RSV) infection

The assay was carried out in 24-well flat bottom plate. Group 1 (heparins and modified heparins (~12kDa)) (A), Group 2 (low molecular weight heparins (LMWH) (5kDa and 3kDa) and enoxaparin with/without glycol split) (B), Group 3 (fucoidans and modified fucoidan) (C), Group 4 (carrageenans) (D) and Group 5 (other) (E) HS mimetics were tested at 100, 20 or 5 µg/mL and 100 µl/well of HS mimetics was incubated with Vero E6 cell monolayer for 10 minutes at 37°C and 5% CO_2. Following the incubation, RSV (200 plaque forming units/well) was added to Vero E6 cell monolayer with HS mimetics and allowed to incubate for 1 hour at 37°C and 5% CO_2. Plate was processed by immunostaining RSV plaque assay (section 2.10.). Data are represented as a mean of RSV plaques recovered (n = 4) ± standard deviation. HS 230 (negative) and 254 (positive) were used as HS mimetic controls. For each graph, the controls HS 230, HS 254 and RSV results are presented in boxed area for comparison. Data were analysed using ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (*, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.001$ and ****, $p < 0.0001$).

5.3.2.1.3 Condition C

The ability of the HS mimetics to compete with RSV for the cell surface HS binding site, following the RSV incubation with Vero E6 cells for 10 minutes at 37°C and 5% CO_2, was assessed under condition C. HS 230 (negative HS control) did not inhibit RSV infection while HS 254 (positive HS control) treatment inhibited RSV by 80% and 60% at 100 and 5 µg/mL, respectively ($p < 0.05$). Group 1 HS mimetics (heparins and modified heparins (~12kDa)) induced an increase in RSV inhibition from 50% to 80% by increasing HS concentration from 5-100 µg/mL ($p < 0.05$) (Figure 5.6 A). HS 204, HS 205 and HS 207 performed the best at 5 µg/mL concentration achieving 70%, 85% and 80% RSV inhibition, respectively ($p < 0.05$).

The group 2 (low molecular weight heparins (5kDa and 3kDa) and enoxaparin with/without glycol split) HS mimetics exhibited a more pronounced dose dependant effect on RSV inhibition (Figure 5.6 B). HS 217 performed the best at 5 and 100 µg/mL inducing over 80% RSV inhibition compared to only 60% at 20 µg/mL (100 µg/mL versus (vs.) 20 µg/mL, $p < 0.05$). HS 218 and HS 219 showed similar inhibition trends achieving 80%, 50% and 55% at 100, 20 and 5 µg/mL, respectively (100 µg/mL vs. 20 µg/mL, $p < 0.05$). HS 228 performed best at 5 and 100 µg/mL inducing over 80% RSV inhibition compared to only 45% at 20 µg/mL (100 µg/mL vs. 20 µg/mL; 100 µg/mL vs. 5 µg/mL, $p < 0.0001$). HS 331, HS 332 and HS 424 inhibited RSV at 80%, 70% and 80% respectively at all three tested HS concentrations. HS 333 and HS 416 inhibited RSV by 70% at 20 µg/mL.

The group 3 (fucoidans and modified fucoidans) HS 259 inhibited RSV by 90% at 5, 20 and 100 µg/mL concentrations (Figure 5.6 C). The Group 4 (carrageenans) HS mimetic, 266, inhibited RSV by 80% at 5 and 20 µg/mL in contrast to only 65% at the higher dose of 100 µg/mL ($p < 0.001$). HS 267 induced 90% RSV inhibition at 5 and 100 µg/mL while only 70% at 20 µg/mL ($p < 0.0001$) (Figure 5.6 D).

The group 5 (other) HS mimetics HS 81, HS 238, HS 241 and HS 242 induced approximately 70% inhibition of RSV irrespective of the HS mimetic concentration. HS 106, HS 111, PI-88 and suramin performed best at 5 and 100 µg/mL inhibiting RSV by 75-90% compared to 20-45% at 20 µg/mL ($p < 0.05$) (Figure 5.6 E).

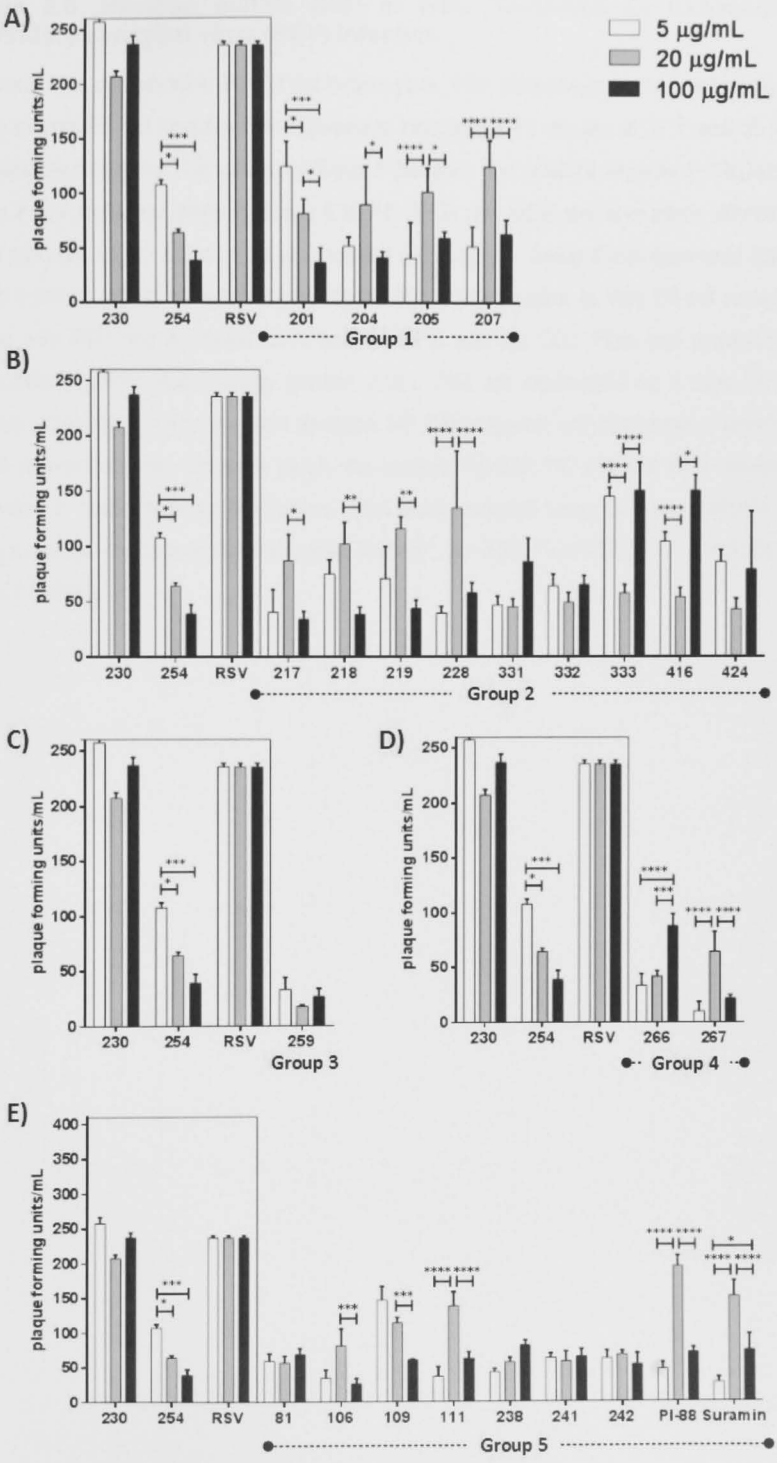

A)
5 µg/mL
20 µg/mL
100 µg/mL
plaque forming units/mL
230
254
RSV
201
204
205
207
Group 1
B)
plaque forming units/mL
230
254
RSV
217
218
219
228
331
332
333
416
424
Group 2
C)
plaque forming units/mL
230
254
RSV
259
Group 3
D)
plaque forming units/mL
230
254
RSV
266
267
Group 4
E)
plaque forming units/mL
230
254
RSV
81
106
109
111
238
241
242
PI-88
Suramin
Group 5

Figure 5.6. Heparan sulfate (HS) *in vitro* (Condition C) treatment of respiratory syncytial virus (RSV) infection

The assay was carried out in 24-well flat bottom plate. RSV (200 plaque forming units/well) was added to Vero E6 cell monolayer and allowed to incubate for 10 minutes at 37°C and 5% CO_2. Following the incubation, 100 µl/well of Group 1 (heparins and modified heparins (~12kDa)) (A), Group 2 (low molecular weight heparins (LMWH) (5kDa and 3kDa) and enoxaparin with/without glycol split) (B), Group 3 (fucoidans and modified fucoidan) (C), Group 4 (carrageenans) (D) and Group 5 (other) (E) HS mimetics were added at 100, 20 or 5 µg/mL to Vero E6 cell monolayer treated with RSV and incubated for 1 hour at 37°C and 5% CO_2. Plate was processed by immunostaining RSV plaque assay (section 2.10.). Data are represented as a mean of RSV plaques recovered (n = 4) ± standard deviation. HS 230 (negative) and 254 (positive) were used as HS mimetic controls. For each graph, the controls HS 230, HS 254 and RSV results are presented in boxed area for comparison. Data were analysed using ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (*, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.001$ and ****, $p < 0.0001$).

5.3.2.1.4 Condition D

The HS mimetics were also assessed for their ability to compete with RSV for the cell surface HS binding site following RSV incubation with Vero E6 cells for 1 hour at 37°C and 5% CO_2 and HS for potentially inhibiting the cell-to-cell spread of RSV under condition D. This condition represents the most clinically relevant *in vitro* experimental set up.

HS 230 (negative HS control) showed no viral inhibition while HS 254 (positive HS control) inhibited RSV by 80% at 100 µg/mL ($p < 0.005$). The group 1 HS mimetics (heparins and modified heparins (~12kDa)) induced 40-65% reduction in RSV plaques at 100 µg/mL while less than 10% at 5 and 20 µg/mL (Figure 5.7 A).

The group 2 (low molecular weight heparins (5kDa and 3kDa) and enoxaparin with/without glycol split) HS mimetics induced dose dependant RSV inhibition with HS 217, HS 218, HS 219 and HS 228 inhibiting RSV by 60-65% at 100 µg/mL while at 5 and 20 µg/mL these HS mimetics had minimal effect on RSV inhibition (Figure 5.7B). HS 331 induced 40% RSV inhibition at all three HS mimetics concentrations while treatment with HS 332 resulted in 40 and 50% RSV inhibition at 20 and 5 µg/mL, respectively. HS 333, HS 416 and HS 424 RSV inhibition improved with an increase in HS concentrations, resulting in 20-80% inhibition ($p < 0.0001$).

The group 3 (fucoidans and modified fucoidans) HS mimetic HS 259 inhibited RSV by 30% at all three concentration, 100, 20 and 5 µg/mL (Figure 5.7 C). Group 4 (carrageenans) HS mimetic, HS 266, inhibited RSV by 20% at 5 and 20 µg/mL and by 40% at the higher dose of 100 µg/mL (Figure 5.7D). HS 267 induced 55% and 40% RSV inhibition at 5 and 20 µg/mL and 60% at 100 µg/mL.

Group 5 (other) HS mimetics HS 81, HS 106, HS 109, HS 238, HS 241 and HS 242 induced approximately 35-40% inhibition of RSV and was unaffected by the HS mimetic concentration. HS 111, PI-88 and suramin performed best at 20 and 100 µg/mL inhibiting RSV by 35% compared to less than 10% at 5 µg/mL ($p < 0.0001$) (Figure 5.7E).

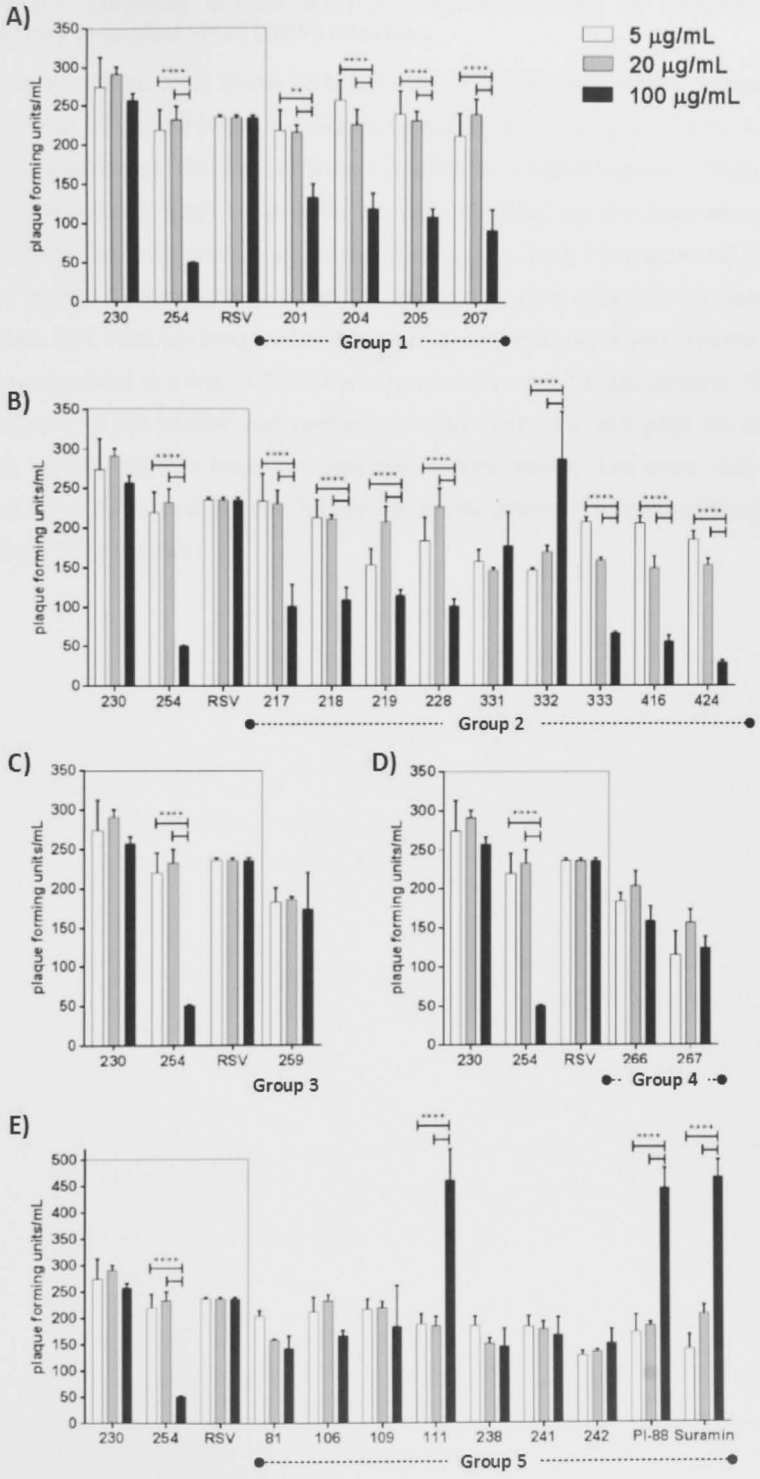

A)
5 µg/mL
20 µg/mL
100 µg/mL
plaque forming units/mL
350
300
250
200
150
100
50
0
230
254
RSV
201
204
205
207
Group 1
B)
plaque forming units/mL
230
254
RSV
217
218
219
228
331
332
333
416
424
Group 2
C)
plaque forming units/mL
230
254
RSV
259
Group 3
D)
plaque forming units/mL
230
254
RSV
266
267
Group 4
E)
plaque forming units/mL
500
450
400
350
300
250
200
150
100
50
0
230
254
RSV
81
106
109
111
238
241
242
PI-88
Suramin
Group 5

Figure 5.7. Heparan sulfate (HS) *in vitro* (Condition D) treatment of respiratory syncytial virus (RSV) infection

The assay was carried out in 24-well flat bottom plate. RSV (200 plaque forming units/well) as added to Vero E6 cell monolayer and allowed to incubate for 1 hour at 37°C and 5% CO_2. Following the incubation, 100 µl/well of Group 1 (heparins and modified heparins (~12kDa)) (A), Group 2 (low molecular weight heparins (LMWH) (5kDa and 3kDa) and enoxaparin with/without glycol split) (B), Group 3 (fucoidans and modified fucoidan) (C), Group 4 (carrageenans) (D) and Group 5 (other) (E) HS mimetics were added at 100, 20 or 5 µg/mL to Vero E6 cell monolayer treated with RSV. Plate was processed as per immunostaining RSV plaque assay (Section 2.7.). Data are represented as a mean of RSV plaques recovered (n = 4) ± standard deviation. HS 230 (negative) and HS 254 (positive) were used as HS mimetic control. For each graph, the controls HS 230, HS 254 and RSV results are presented in boxed area for comparison. Data were analysed using ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (**, $p < 0.005$ and ****, $p < 0.0001$).

5.3.3 HS mimetics treatment of RSV infection *in vivo*

Following *in vitro* HS screening, the four best performing compounds across all four conditions A, B, C and D, in addition to the positive and negative HS controls, were tested for their antiviral ability *in vivo*. The selected compounds were: HS 228 (carboxyl reduced mucosal heparin), HS 259 (fucoidan - sulfated polysaccharide), HS 267 (lambda carrageenan (200kDa) and HS 424 (mucosal heparin with glycol split (3kDa) with the negative HS control, HS 230 (desulfated heparin) and the positive HS control, HS 254 (dextran sulfate (5kDa). It is important to note that preliminary *in vivo* results (data not shown) revealed that HS mimetic treatment could not be delivered intranasally to the RSV-inoculated mice. Intranasal HS mimetic treatment evoked an excessive immune response in the mucosal surfaces of the nose, throat and lungs of RSV-inoculated mice, resulting in death within the 24-48 hours of the HS mimetic treatment. Consequently, through another pilot study, an i.p. delivery of HS mimetics to RSV-inoculated mice for both, post and prophylactic treatment of RSV-infection, resulted in an effective immune response and successful recovery from the RSV infection. As a result, HS treatment was delivered via i.p. route to all RSV-inoculated mice involved in the *in vivo* studies discussed in this chapter. It is important to note that i.p. delivery route was also used by several studies treating HSV and dengue infections, and proteinuria with HS mimetics (Lee et al., 2006; Levidiotis et al., 2004; Nyberg et al., 2004).

5.3.3.1 Weight and RSV clinical scores

There were no significant changes in weights of mice for Vero E6 cell lysate (negative control), UV-RSV (additional negative control) or RSV inoculated conditions (data not shown). RSV inoculated mice showed signs of RSV infection as described in the section 2.12.2, RSV clinical scores reaching a maximum score of 4. There was no statistically significant difference in the clinical scores recorded for Vero E6 cell lysate, UV-RSV or RSV inoculated HS treated groups (data not shown).

5.3.3.2 Viral titres

RSV immunohistochemistry plaque assay analysis of Vero E6 cell lysate (negative control - data not shown) and UV-RSV (additional negative control-

data not shown) inoculated groups following a treatment with saline, revealed no viral titres present (data not shown). Treatment of RSV inoculated groups with HS 230 (negative HS control) and 254 (positive HS control) did not have any effect of RSV infection compared to treatment of RSV inoculated group with saline (positive control). Only compounds HS 228 (carboxyl reduced mucosal heparin) and HS 259 (fucoidan) induced a statistically significant reduction ($p < 0.05$) in RSV titres compared to the positive control (Figure 5.8). HS 228 and HS 259 RSV inhibition was also significant ($p < 0.05$) compared to the treatment of RSV inoculated groups with HS 230 (negative HS control) and HS 254 (positive HS control) (Figure 5.8). HS 267 and HS 424 appeared to inhibited RSV infection marginally however the reduction was not statistically significant.

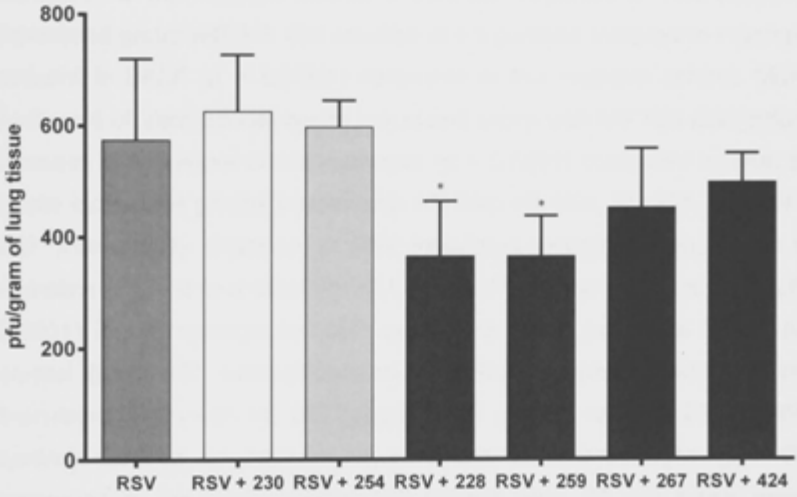


Figure 5.8. Post respiratory syncytial virus (RSV) infection treatment with heparan sulfate (HS) mimetics induced viral titres reduction *in vivo*

On day 0, wild type BALB/c mice were infected by intranasal inoculation with RSV (dark grey bar) (5×10^5 plaque forming units (pfu)/mouse). On days 1-4, mice received daily HS mimetics (228, 230, 254, 259, 267 and 424) treatment at 10 mg/mL/kg concentration by intraperitoneal injections (100 µL). On day 5, mice were euthanised and samples collected. Titres are represented as pfu/gram of lung tissue. Data are represented as mean (n = 5) ± standard deviation. HS 230 (negative) (white bar) and 254 (positive) (light grey bar) were used as HS mimetic controls. Data were analysed using a ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (* $p < 0.05$ compared to saline treatment of RSV inoculated group).

5.3.3.3 *Leukocyte population in a BALF*

Analysis of the leukocyte population present in BALF revealed a significantly lower (7.5 x 10^4 macrophages/mL of BALF) number of macrophages present, following the Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculation of saline treated groups compared to treatment of RSV inoculated group with saline (positive control) (21 x 10^4 macrophages/mL of BALF) ($p < 0.0001$) (Figure 5.9 A). Treatment of Vero E6 cell lysate inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) resulted in a significant reduction ($p < 0.0001$) of macrophages present in BALF compared to the negative control. Reduction in macrophages numbers present in BALF was also observed following treatment of Vero E6 cell lysate inoculated groups with HS 259 or HS 267 ($p < 0.0001$) compared to the negative control. In contrast, treatment of Vero E6 cell lysate inoculated group with HS 424 resulted in a significant increase in macrophages present in BALF ($p < 0.0001$) compared to the negative control. Moreover, treatment of Vero E6 cell lysate inoculated group with HS 424 also induced an increase in a number of macrophages ($p < 0.0001$) compared to Vero E6 cell lysate inoculated groups treated with HS 230, HS 254, HS 228, HS 259 or HS 267. Interestingly, treatment of RSV inoculated groups with any of the six HS mimetics resulted in a reduction (2.5-7.5 x 10^4 macrophages/mL of BALF) ($p < 0.0001$) in macrophage numbers present in BALF compared to the positive control (21 x 10^4 macrophages/mL of BALF). However, treatment of RSV inoculated group with HS 230 (negative HS control) and HS 254 (positive HS control) induced an increase in a number of macrophages ($p < 0.0001$) compared to the corresponding HS treated Vero E6 cell lysate inoculated groups. In contrast, treatment of RSV inoculated groups with HS 228, HS 267 or HS 424 resulted in a decrease ($p < 0.0001$) in a number of macrophages compared to the corresponding HS treatment of Vero E6 cell lysate inoculated groups.

Lymphocyte BALF analysis revealed a significantly lower (0.25-0.5 x 10^4 lymphocytes/mL of BALF) number of lymphocytes present in the BALF following Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculation of saline treated groups compared to the positive control (1.5 x 10^4 lymphocytes/mL of BALF) ($p < 0.0001$) (Figure 5.9 B). Treatment of Vero E6 cell

lysate inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) resulted in no change in lymphocyte numbers compared to the negative control. In addition, treatment of Vero E6 cell lysate inoculated groups with HS 228, HS 259, HS 267 or HS 424 also induced no change to lymphocyte numbers compared to the negative control. Similarly, treatment with HS 230 (negative HS control), HS 254 (positive HS control) or HS 228 of RSV inoculated group has resulted in no change in lymphocyte numbers compared to the lymphocyte numbers from the positive control group. However, treatment with HS 259, HS 267 and HS 424 following RSV inoculation resulted in a reduction ($p < 0.0001$) in lymphocytes present in BALF compared to the lymphocyte numbers recovered from the positive control. In addition, treatment of RSV inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) resulted in an increase ($p < 0.05$) in the number of lymphocytes compared to Vero E6 cell lysate inoculated groups treated with the corresponding HS mimetic. Treatment of RSV inoculated groups with HS 228 induced an increase ($p < 0.05$) in the number of lymphocytes compared to Vero E6 cell lysate inoculated groups treated with HS 228. Finally, treatment of RSV inoculated groups with HS 259, HS 267 or HS 424 resulted in no change in lymphocyte numbers compared to Vero E6 cell lysate inoculated groups treated with the corresponding HS mimetic.

No significant difference in eosinophil numbers was observed between saline treatments of Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculated groups (Figure 5.9 C). In addition, treatment of Vero E6 or UV-RSV inoculated group with HS mimetics (applies for all six tested HS mimetics) showed no significant difference in eosinophil numbers between groups. RSV inoculation of the saline treated group (positive control) induced a significant trafficking of eosinophils into BALF ($p < 0.0001$) compared to the negative control. Treatment of RSV inoculated groups with HS 230 (negative HS control) and HS 254 (positive HS control) resulted in no change in eosinophil numbers compared to the positive control (Figure 5.9 C). In contrast, treatment of the RSV inoculated groups with HS 228, HS 259 or HS 424 has resulted in lower ($p < 0.0001$) eosinophil numbers compared to the eosinophil numbers present in BALF of the positive control. Treatment of the RSV inoculated group with HS 230 (negative HS control) resulted in a significant increase ($p < 0.0001$) in a number of eosinophils in BALF compared to the

group treated with HS 230 following Vero E6 cell lysate inoculation. However, eosinophil increase following treatment of the RSV inoculated groups with HS 254 (positive HS control), HS 228, HS 259 or HS 424 was not statistically significant compared to Vero E6 cell lysate inoculated groups treated with corresponding HS mimetics. In the RSV inoculated group treated with HS 267, a significant increase ($p < 0.0001$) in a number of eosinophils in BALF was observed compared to the group treated with HS 267 following Vero E6 cell lysate inoculation.

Finally, no significant difference in neutrophil numbers was observed between saline treatments of, Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculated groups (Figure 5.9 D). In addition, treatment of Vero E6 or UV-RSV inoculated group with HS mimetics (applies for all six tested HS mimetics) showed no significant difference in neutrophil numbers between groups. RSV inoculation of the saline treated group (positive control) resulted in no change in neutrophil numbers present in BALF compared to the negative control. Treatment of Vero E6 cell lysate inoculated groups with HS 230 (negative HS control), HS 254 (positive HS control), HS 228, HS 259, HS 267 or HS 424 resulted in no change in neutrophil numbers compared to negative control (Figure 5.9 D). In addition, treatment of RSV inoculated groups with HS 230 (negative HS control), HS 254 (positive HS control), HS 228, HS 259, HS 267 or HS 424 resulted in no change in neutrophil numbers compared to positive control (Figure 5.9 D).

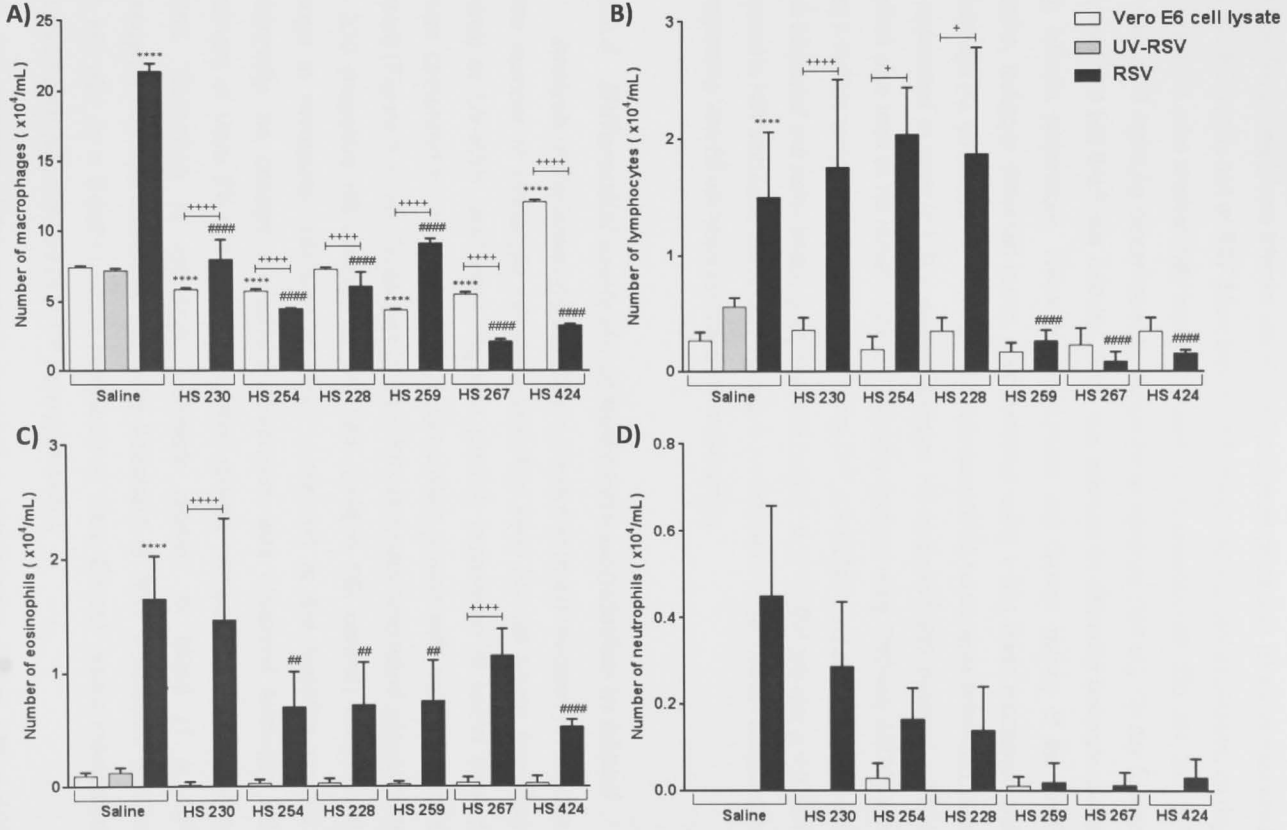

A)
Number of macrophages (x10⁴/mL)
25
20
15
10
5
0
Saline
HS 230
HS 254
HS 228
HS 259
HS 267
HS 424
B)
Vero E6 cell lysate
UV-RSV
RSV
Number of lymphocytes (x10⁴/mL)
3
2
1
0
C)
Number of eosinophils (x10⁴/mL)
D)
Number of neutrophils (x10⁴/mL)
0.8
0.6
0.4
0.2
0.0

Figure 5.9. Differential leukocyte count in bronchoalveolar lavage fluid (BALF) following HS treatment of respiratory syncytial virus (RSV) infection *in vivo*

Wild type BALB/c mice were infected with Vero E6 cell lysate (white bar), ultraviolet-inactivated RSV (UV-RSV) (grey bar) or RSV (black bar) (5×10^5 plaque forming units (pfu)/mouse) on day 0. On days 1-4, mice received daily treatment of saline or HS mimetics (228, 230, 254, 259, 267 and 424) at 10 mg/mL/kg concentration by intraperitoneal injections (100 µL). On day 5, mice were sacrificed and BALF was collected. BALF was assessed for differential leukocyte counts using cytospin preparations. Following May-Grunwald and Giemsa staining of the BALF cytospins, leukocyte differential counts were performed using a light Nikon microscope and macrophage (A), lymphocyte (B), eosinophil (C) and neutrophil (D) counts were determined. Data are represented as mean (n = 5) ± standard deviation. HS compounds 230 (negative) and 254 (positive) were used as HS mimetic controls. Data were analysed using TWO-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (****, $p < 0.0001$ compared to Vero E6 cell lysate inoculated and saline treated group (negative control); ##, $p < 0.005$ and ####, $p < 0.0001$ compared to RSV inoculated, saline treated group; +, $p < 0.05$ and ++++, $p < 0.0001$ compared to corresponding Vero E6 cell lysate inoculated HS treated group).

5.3.3.4 Differential analysis of leukocyte population in blood

Analysis of the leukocyte population present in blood revealed no change in the number of monocyte infiltrates following Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculation of saline treated groups compared to treatment of RSV inoculated groups with saline (positive control) (Figure 5.10 A). Treatment of Vero E6 cell lysate inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) induced no change in monocyte infiltrates in blood compared to the negative control. Additionally, no change in monocyte numbers was observed following HS treatment of Vero E6 cell lysate inoculated groups compared to the negative control. Reduction in monocyte numbers present in blood (1×10^4 monocytes/mL) was observed following treatment of RSV inoculated groups with HS 424 ($p < 0.0001$) when compared to Vero E6 cell lysate inoculated groups treated with HS 424 (5×10^6 monocytes/mL).

Lymphocyte blood analysis revealed a significantly higher (23×10^6 lymphocytes/mL) number of lymphocytes following Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculation of saline treated

groups compared to the positive control (10 x 10^6 lymphocytes/mL) ($p < 0.0001$) (Figure 5.10 B). Treatment of Vero E6 cell lysate inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) resulted in an increase ($p < 0.0001$) in lymphocyte numbers compared to the negative control. Moreover, Vero E6 cell lysate inoculated groups treated with HS 228, HS 259, HS 267 or HS 424 also showed an increase ($p < 0.0001$) in lymphocyte numbers compared to the negative control. Treatment with HS 230 (negative HS control), HS 254 (positive HS control), HS 228, HS 259, HS 267 or HS 424 following RSV inoculation resulted in an increase ($p < 0.0001$) in lymphocytes present in blood compared to the lymphocyte numbers present in the positive control. However, treatment of RSV inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) resulted in a reduction ($p < 0.0001$) in the number of lymphocytes compared to treatment with corresponding HS mimetics in Vero E6 cell lysate inoculated groups. In addition, HS 228 and HS 424 treatment of RSV inoculated groups induced a reduction ($p < 0.0001$) in the number of lymphocytes compared to the Vero E6 cell lysate inoculated groups treated with corresponding HS mimetics. Treatment of RSV inoculated groups with HS 259 resulted in no change in lymphocyte numbers compared to Vero E6 cell lysate inoculated groups treated with HS 259. However, treatment of RSV inoculated groups with HS 267 resulted in an increase ($p < 0.005$) in lymphocyte numbers compared to Vero E6 cell lysate inoculated groups treated with HS 267.

No significant difference in eosinophil numbers was observed between Vero E6 (negative control) and UV-RSV (additional negative control) inoculated groups treated with either, saline or HS mimetics. RSV inoculation of saline treated groups also resulted in no change in eosinophil trafficking compared to the negative control. Similarly, treatment of the RSV inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) resulted in no change in the number of eosinophils compared to the positive control. Treatment of RSV inoculated groups with HS 228 or HS 424 also resulted in no change in a number of eosinophils. In contrast, treatment of the RSV inoculated groups with HS 259 or HS 267 induced an increase ($p < 0.0001$) in eosinophil numbers compared to the positive control (Figure 5.10 C). Treatment of the RSV inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) resulted in no change in the number of eosinophils present in blood

compared to Vero E6 cell lysate groups treated with HS 230 or HS 254. In addition, treatment of RSV inoculated groups with HS 228 or HS 424 induced no change to the number of eosinophils present in blood compared to treatment of Vero E6 cell lysate inoculated group with corresponding HS mimetics. On the contrary, treatment of RSV inoculated groups with HS 259 or HS 267 resulted in higher ($p < 0.0001$) eosinophil numbers compared to Vero E6 cell lysate inoculated groups treated with HS 259 or HS 267.

In addition, no significant difference in neutrophil numbers was observed between saline treatments of, Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculated groups (Figure 5.10 D). In addition, treatment of Vero E6 or UV-RSV inoculated group with HS mimetics (applies for all six tested HS mimetics) showed no significant difference in neutrophil numbers between groups. Positive control resulted in no change in neutrophil numbers present in blood compared to the negative control. Treatment of Vero E6 cell lysate inoculated groups with HS 230 (negative HS control), HS 254 (positive HS control), HS 228, HS 259, HS 267 or HS 424 resulted in no change in neutrophil numbers compared to negative control (Figure 5.10 D). In addition, treatment of RSV inoculated groups with HS 230 (negative HS control), HS 254 (positive HS control), HS 228, HS 259, HS 267 or HS 424 resulted in no change in neutrophil numbers compared to positive control (Figure 5.10 D).

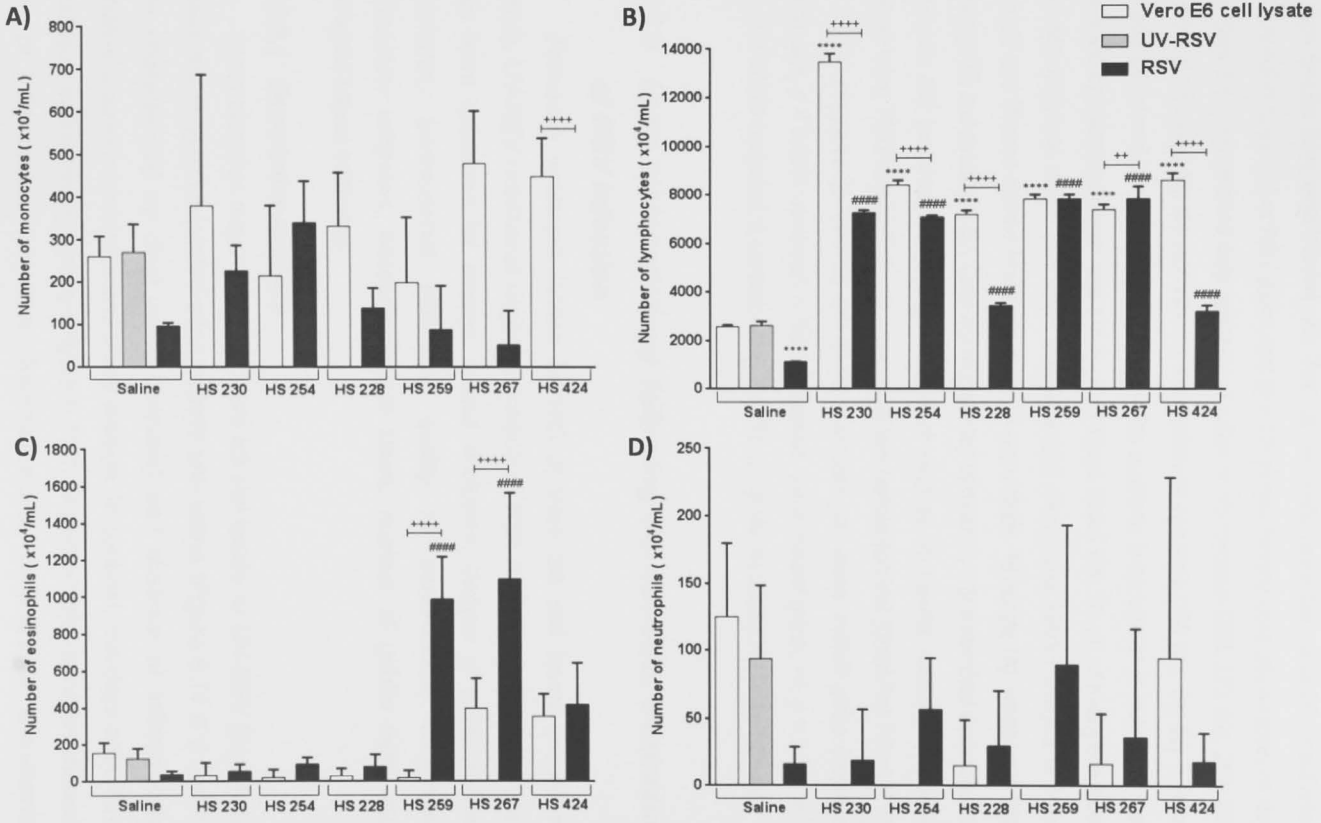

A)
Number of monocytes (x10⁴/mL)
800
700
600
500
400
300
200
100
0
Saline
HS 230
HS 254
HS 228
HS 259
HS 267
HS 424
++++
B)
Vero E6 cell lysate
UV-RSV
RSV
Number of lymphocytes (x10⁴/mL)
14000
12000
10000
8000
6000
4000
2000
0
****
++++
####
++
C)
Number of eosinophils (x10⁴/mL)
1800
1600
1400
1200
1000
800
600
400
200
0
D)
Number of neutrophils (x10⁴/mL)
250
200
150
100
50
0

Figure 5.10. Differential leukocyte count in blood following heparan sulfate (HS) treatment of respiratory syncytial virus (RSV) infection *in vivo*

Wild type BALB/c mice were infected with Vero E6 cell lysate (white bar), ultraviolet-inactivated RSV (UV-RSV) (grey bar) or RSV (black bar) (5 x 10^5 plaque forming units (pfu)/mouse) on day 0. On days 1-4, mice received daily treatment of saline or HS mimetics (228, 230, 254, 259, 267 and 424) at 10 mg/mL/kg concentration by intraperitoneal injections (100 µL). On day 5, mice were euthanised and blood was collected by cardiac puncture. Total number of leukocytes was determined by haemocytometer count of 5 µL of blood mixed with 95 µL of methylene blue. Differential leukocyte counts was done through a light microscope (Nikon) analysis of May-Grunwald and Giemsa stained blood smears on a glass slides. Monocyte (A), lymphocyte (B), eosinophil (C) and neutrophil (D). Data are represented as mean (n = 5) ± standard deviation. HS compounds 230 (negative) and 254 (positive) were used as HS mimetic controls. Data were analysed using TWO-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (****, $p < 0.0001$ compared to Vero E6 cell lysate inoculated and saline treated group (negative control); ####, $p < 0.0001$ compared to RSV inoculated, saline treated group; ++, $p < 0.005$ and ++++, $p < 0.0001$ compared to corresponding Vero E6 cell lysate inoculated HS treated group).

5.3.3.5 Lung histopathology following HS mimetics treatment of RSV infection

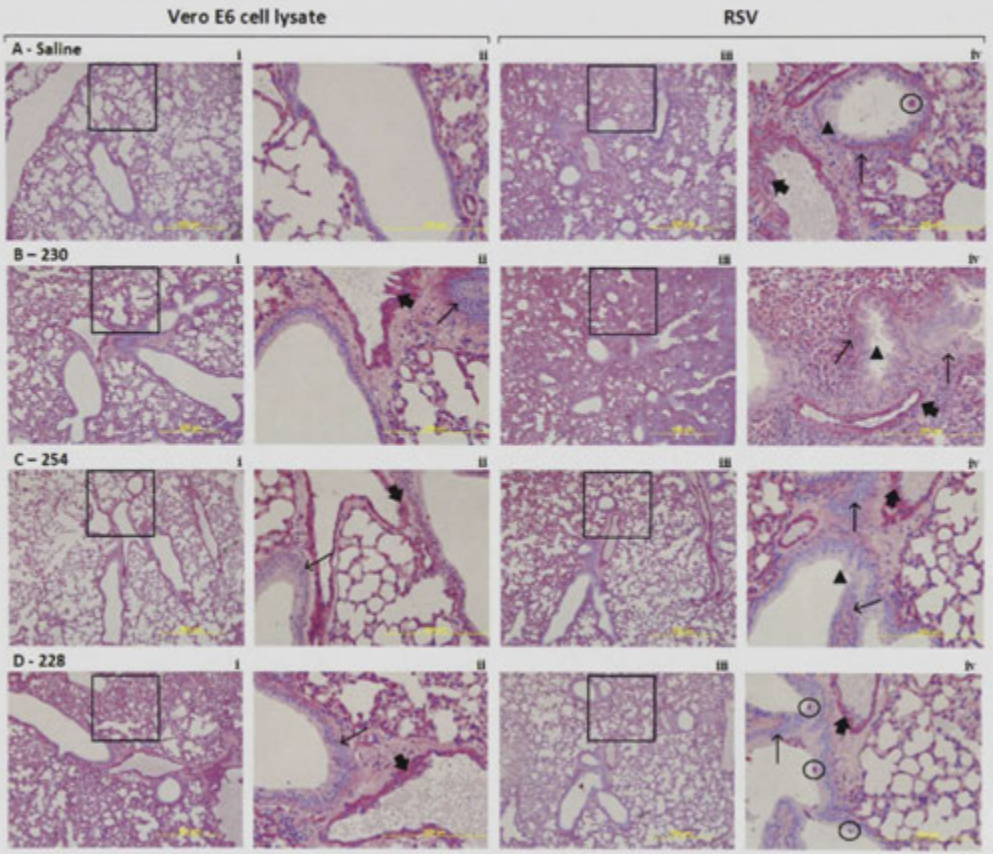
Following treatment (saline or HS) of Vero E6 cell lysate (negative control), UV-RSV (additional negative control) or RSV inoculated BALB/c mice, lungs were assessed for airways mucus occlusion, degree of parenchymal pneumonia, peribronchial infiltrates, quality of peribronchial infiltrates, perivascular infiltrates, histopathological score, number of goblet cells and eosinophil tissue infiltrates.

5.3.3.5.1 Descriptive analysis

Lung samples recovered from Vero E6 cell lysate or UV-RSV (histology images not shown) inoculated mice treated with saline (Figure 5.11 A (i and ii) were characterised by clear alveoli (square) and absence of inflammatory infiltrates around bronchioles and blood vessels. In contrast, five days post RSV inoculation saline treated group (Figure 5.11 A (iii and iv) lung samples showed signs of inflammatory response characterised by parenchymal pneumonia (square), airway mucus occlusion (arrow head) and extensive infiltration dominated by an excess of lymphocytes and macrophages surrounding both

bronchioles (thin arrow) and pulmonary blood vessels (thick arrow). The arrow heads indicate airways occlusion and the circles show the presence of goblet cells within bronchi. HS 230 (negative HS control) treatment of Vero E6 cell lysate inoculated mice resulted in minimal parenchymal pneumonia (square) and mild inflammatory infiltrates present around bronchioles (thin arrows) and blood vessels (thick arrow). However, treatment with HS 254 (positive HS control) of Vero E6 cell lysate inoculated mice showed no signs of parenchymal pneumonia (square) or inflammatory infiltrates around bronchioles (thin arrows) or blood vessels (thick arrow). Treatment with HS compounds, HS 228, HS 259, HS 267 and HS 424 of Vero E6 cell lysate inoculated mice was characterised by minimal parenchymal pneumonia (square) and mild infiltration dominated by lymphocytes and macrophages surrounding both, bronchioles (thin arrows) and pulmonary blood vessels (thick arrows) (Figure 5.11 D, E, F and G (i and ii)).

Treatment with compound HS 230 (negative HS control) of RSV inoculated mice (Figure 5.11 B (iii and iv)) induced medium parenchymal pneumonia (square) and airway occlusion (arrow head) with goblet cells present within bronchioles (circle). However, treatment of RSV inoculated mice with compound 254 (positive HS control) was characterised by mild-medium parenchyma, mild airway mucus occlusion, moderate perivascular (thick arrows) and peribronchiolar infiltrates (thin arrows) (Figure 5.11 C (iii and iv)). Treatment of RSV inoculated mice with compound 228 was also characterised by mild-moderate parenchyma (square), mild airway mucus occlusion (arrow head) with the presence of several goblet cells (circle), moderate perivascular infiltrates (thick arrows) and moderate peribronchiolar infiltrates (thin arrows) (Figure 5.11 D (iii and iv)). Finally, treatment with compounds HS 259, HS 267 and HS 424 of RSV inoculated mice (Figure 5.11 B (iii and iv)) resulted in moderate parenchymal pneumonia (square) and airway occlusion (arrow head) with goblet cells present within bronchioles (circle) and moderate perivascular (thick arrows) and peribronchiolar infiltrates (thin arrows).


Vero E6 cell lysate
RSV
A - Saline
B – 230
C – 254
D - 228
i
ii
iii
iv

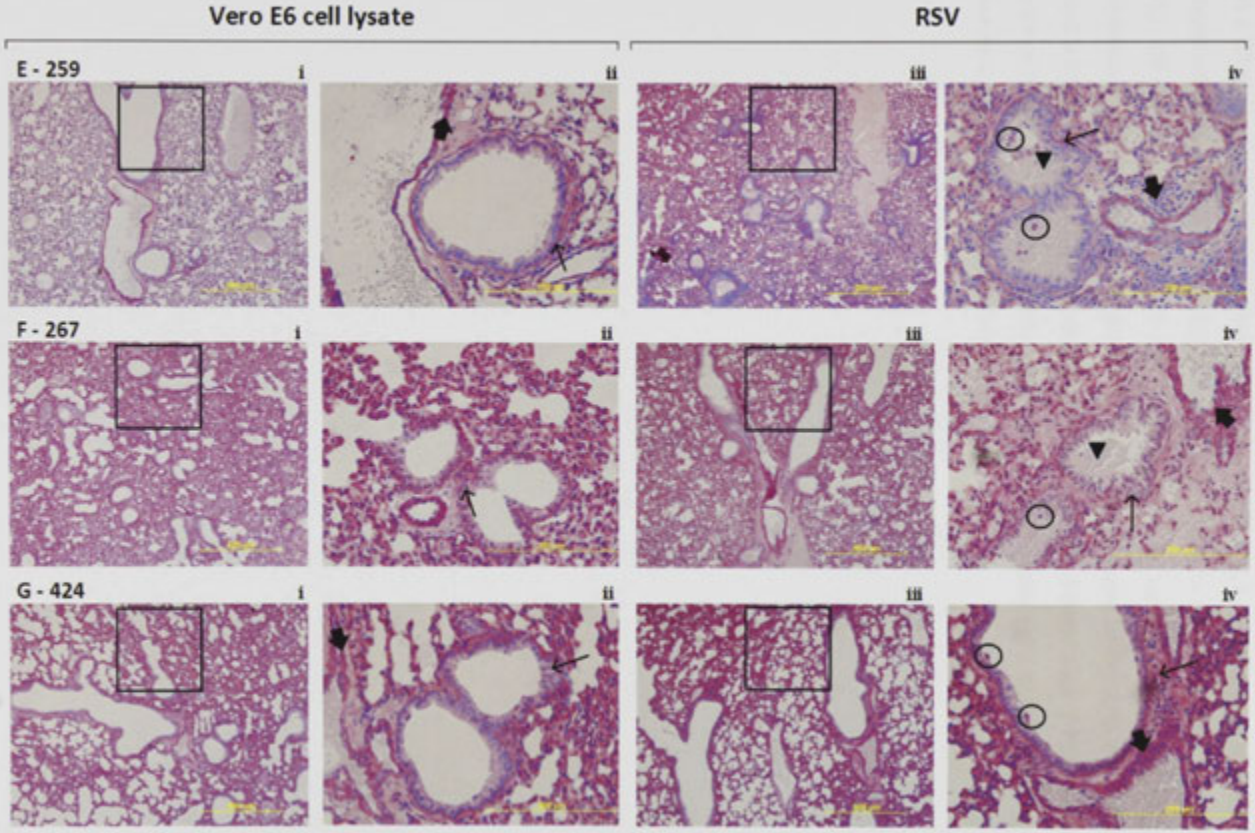

Vero E6 cell lysate
RSV
E - 259
i
ii
iii
iv
F - 267
i
ii
iii
iv
G - 424
i
ii
iii
iv

Figure 5.11. Effect of heparan sulfate (HS) treatment on respiratory syncytial virus (RSV) infected mice lung histology

Wild type (WT) BALB/c mice were infected intranasally with Vero E6 cell lysate (i and ii) or RSV (5×10^5 plaque forming units (pfu)/mouse) on day 0 (iii and iv). On days 1-4, mice received daily treatment of saline (A) or HS mimetics: 230 (negative HS control) (B), 254 (positive HS control) (C), 228 (D), 259 (E), 267 (F) and 424 (G) at 10 mg/mL/kg concentration by intraperitoneal injections (100 µL). Lungs were collected on day 5 and processed for histology using periodic acid Schiff (PAS) stain. Arrows, squares and circles indicate the different signs of inflammation in mice as described in the text. Magnification (i and ii) was 100X and (iii and iv) 400X including the eyepiece. Images are representative of each group (n = 5).

5.3.3.5.2 Numerical (score) analysis

No significant difference was observed between negative control and UV-RSV (histology images not shown) inoculated groups treated with saline (additional negative control) (Figure 5.12), in terms of scores allocated to airway mucus occlusion (A), peribronchial infiltrates (C), quality of peribronchial infiltrates (D), perivascular infiltrates (E), goblet cell count (G) and eosinophil tissue infiltrates (H). The only difference found between these two groups was that UV-RSV inoculated groups treated with saline had greater parenchymal pneumonia ($p < 0.05$) and combined HPS score ($p < 0.0001$) compared to Vero E6 cell lysate inoculated group treated with saline (Figure 5.12 B and F).

Saline treatment of RSV inoculated groups resulted in a significant increase in scores ($p < 0.05$) allocated to all eight assessed measurements (Figure 5.12, A-H) compared to the negative control. Similarly, treatment of Vero E6 cell lysate inoculated group with HS 230 (negative HS control), induced a significant increase ($p < 0.005$) in airway occlusion (A), parenchymal pneumonia (B) and combined HPS score (F) when compared to the negative control, however, no change was observed for peribronchial infiltrates (C), quality of peribronchial infiltrates (D), perivascular infiltrates (E), goblet cell count (G) and eosinophil tissue infiltrates (H) (Figure 5.12). In addition, treatment of Vero E6 cell lysate inoculated group with HS 254 (positive HS control), has induced significant increase ($p < 0.05$) in airway occlusion (A), peribronchial infiltrates (C), quality of peribronchial infiltrates (D) and combined HPS score (F) compared to the negative control; while no change was observed for parenchymal pneumonia (B), perivascular infiltrates (E), goblet cell count (G) and eosinophil tissue infiltrates (H) following HS 254 treatment of the Vero E6 cell lysate inoculated group compared to negative control (Figure 5.12).

Furthermore, treatment of Vero E6 cell lysate inoculated groups with HS 259, HS 267 or HS 424 resulted in an increase ($p < 0.05$) in airway occlusion (A), parenchymal pneumonia (B) (for HS 228 as well), peribronchial infiltrates (C) and quality of peribronchial infiltrates (D) compared to the negative control (Figure 5.12). Treatment with HS 228 of Vero E6 cell lysate inoculated groups showed no difference in airway occlusion (A), peribronchial infiltrates (C) and quality of peribronchial infiltrates (D) compared to the negative control. Also, treatment with compounds HS 259, HS 267 or HS 424 of Vero E6 cell lysate

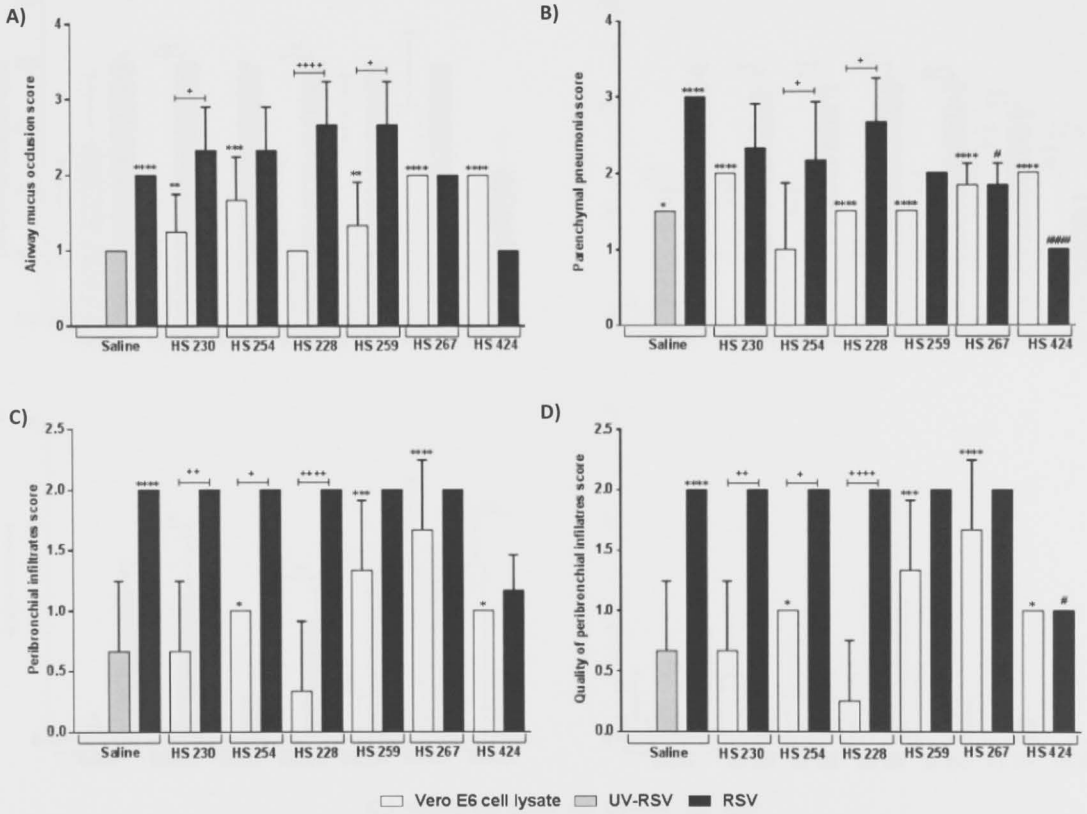
inoculated groups showed greater ($p < 0.0001$) combined HPS (5-7 out of 17) compared to the negative control (1 out of 17) (Figure 5.12 F). Treatment with of Vero E6 cell lysate inoculated groups with HS 228 showed no difference in combined HPS (score out of 17) compared to the negative control. There was no significant difference in goblet cell count following the HS (applies to all six HS mimetics) treatment of Vero E6 cell lysate compared to the negative control. Treatment with HS 267 and HS 424 of Vero E6 cell lysate inoculated groups induced an increase ($p < 0.05$) in eosinophil tissue infiltrates compared to the negative control (Figure 5.12 H).

Treatment of RSV inoculated groups with HS 230 (negative HS control) resulted in an increase ($p < 0.05$) in airway mucus occlusion (A), peribronchial infiltrates (C), quality of peribronchial infiltrates (D) and perivascular infiltrates (E) compared to HS 230 treated Vero E6 cell lysate inoculated groups (Figure 5.12). Parenchymal pneumonia score remained the same following treatment of RSV inoculated groups with HS 230 (negative HS control) compared to HS 230 treated Vero E6 cell lysate inoculated group (Figure 5.12 B). Treatment of RSV inoculated group with HS 254 induced no change in airway mucus occlusion compared to HS 254 treated Vero E6 cell lysate inoculated groups; while parenchymal pneumonia (B), peribronchial infiltrates (C), quality of peribronchial infiltrates (D) and perivascular infiltrates (E) scores increased ($p < 0.05$) following treatment of RSV inoculated groups with HS 254 (positive HS control) compared to HS 254 treated Vero E6 cell lysate inoculated group (Figure 5.12).

In contrast, treatment of RSV inoculated groups HS 228 and HS 259 has resulted in an increase ($p < 0.05$) in airway mucus occlusion compared to the corresponding HS treated Vero E6 cell lysate inoculated groups (Figure 5.12 A). However, treatment with HS 267 and HS 424 induced no change in airway mucus occlusion compared to the corresponding HS treated Vero E6 cell lysate inoculated groups. Parenchymal pneumonia score (B), peribronchial infiltrates (C), quality of peribronchial infiltrates (D) and perivascular infiltrates (E) only increased ($p < 0.05$) following the treatment of RSV inoculated groups with HS 228 while these remained unchanged following the treatment of RSV inoculated groups with HS 259, HS 267 and HS 424 compared to the corresponding HS treated Vero E6 cell lysate inoculated groups (Figure 5.12). HS treatment of (except for HS 424) RSV inoculated groups induced an increase ($p < 0.0001$) in

the HPS combined score compared to the corresponding HS treated Vero E6 cell lysate inoculated group. In contrast, treatment with HS 424 of RSV inoculated groups had resulted in a reduced ($p < 0.0001$) combined HPS score compare to the HS 424 treated Vero E6 cell lysate inoculated group (Figure 5.12 F). Goblet cell count increased ($p < 0.05$) following the treatment of RSV inoculated groups with HS 228 and HS 259 compared to corresponding HS treated Vero E6 cell lysate inoculated groups (Figure 5.12 G). However, goblet cell count remained unchanged following the treatment of RSV inoculated groups with HS 267 compared to HS 267 treated Vero E6 cell lysate inoculated group. Treatment of RSV inoculated group with HS 424 has resulted in a decrease ($p < 0.05$) in a number of goblet cells compared to its corresponding HS treated Vero E6 cell lysate inoculated group (Figure 5.12 G). Treatment of RSV inoculated group with HS mimetics had no effect on eosinophil tissue infiltration compared to the corresponding HS treated Vero E6 cell lysate inoculated group (Figure 5.12 H).

Treatment with HS 230 of RSV inoculated group resulted in no effect on any of the eight pathological measurements compared to the positive control. Treatment of RSV inoculated group with HS 424 resulted in a decrease ($p < 0.05$) in parenchymal pneumonia (B), quality of peribronchial infiltrates (D) and combined HPS score (F) compared to the positive control (Figure 5.12). Treatment with compounds HS 254 (positive HS control), HS 259, HS 267 or HS 424 of RSV inoculated groups showed lower ($p < 0.0001$ combined histopathological score (5-9 out of 17) compared to saline treated RSV inoculated control (positive control) (11 out of 17). In contrast, treatment of RSV inoculated group with HS 228 did not reduce combined HPS score compared to the positive control (11 out of 17). Additionally, treatment of RSV inoculated group with HS 254 (positive HS control) resulted in a reduction ($p < 0.05$) of goblet cell numbers compared to the positive control.


A)
Airway mucus occlusion score
Saline
HS 230
HS 254
HS 228
HS 259
HS 267
HS 424
B)
Parenchymal pneumonia score
C)
Peribronchial infiltrates score
D)
Quality of peribronchial infiltrates score
Vero E6 cell lysate
UV-RSV
RSV

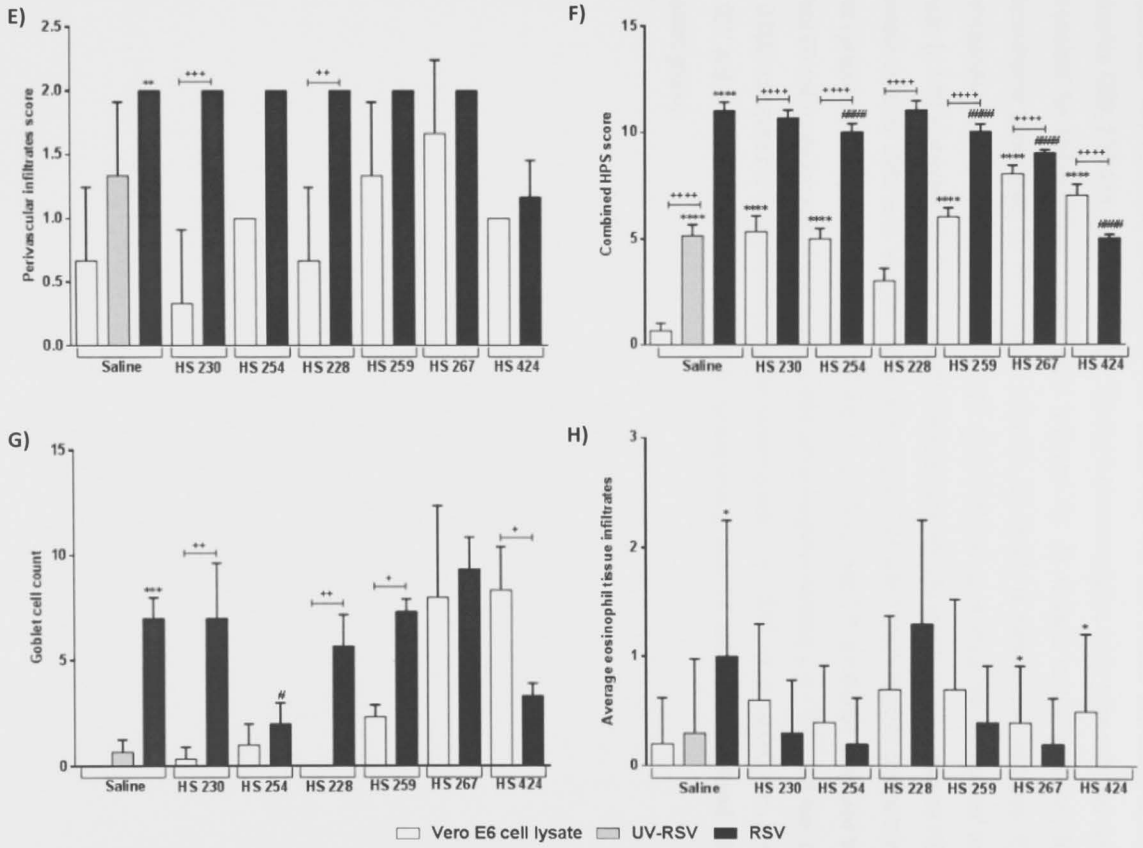

E)
Perivascular infiltrates score
2.5
2.0
1.5
1.0
0.5
0.0
Saline
HS 230
HS 254
HS 228
HS 259
HS 267
HS 424
F)
Combined HPS score
15
10
5
0
G)
Goblet cell count
H)
Average eosinophil tissue infiltrates
3
2
1
Vero E6 cell lysate
UV-RSV
RSV

Figure 5.12. Histopathological score (HPS) assessment of mice lungs following heparan sulfate (HS) mimetics treatment of respiratory syncytial virus (RSV) infection

Wild type BALB/c mice were inoculated with either Vero E6 lysate (white bar), ultraviolet-inactivated RSV (UV-RSV) (grey bar) or RSV (black bar) and then treated with saline or HS mimetics (228, 230, 254, 259, 267 or 424). On day 5 post inoculation, lungs were collected and processed for histopathology. Lungs were analysed for: A) Airways mucus occlusion, B) Parenchymal pneumonia, C) Peribronchial infiltrates, D) Quality of peribronchial infiltrates, E) Perivascular infiltrates, F) Combined HPS and G) Goblet cell count. Data are represented as mean (n = 5) ± standard deviation. HS 230 (negative) and HS 254 (positive) were used as HS mimetic controls. Data were analysed using ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (*, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.001$ and ****, $p < 0.0001$ compared to Vero E6 cell lysate inoculated and saline treated group (negative control); #, $p < 0.05$ and ####, $p < 0.0001$ compared to RSV inoculated, saline treated group; +, $p < 0.05$, ++, $p < 0.005$, +++, $p < 0.001$ and ++++, $p < 0.0001$ compared to corresponding Vero E6 cell lysate inoculated HS treated group).

5.3.4 Prophylactic HS treatment of RSV infection *in vivo*

5.3.4.1 *Weight and RSV clinical scores*

There were no significant changes in weights for Vero E6 cell lysate (negative control), UV-RSV (additional negative control) or RSV inoculated mice (data not shown). RSV inoculated mice showed signs of RSV infection as described in the section 2.12.2 (RSV clinical scores); reaching a maximum score of 4. There was no significant difference in the clinical scores recorded for Vero E6 cell lysate, UV-RSV or RSV inoculated HS treated groups (data not shown).

5.3.4.2 *Effect of HS prophylactic treatment on viral titres following RSV infection in vivo*

No viral titres were recovered following the RSV immunohistochemistry plaque assay analysis of Vero E6 cell lysate (negative control-data not shown) or UV-RSV (additional negative control-data not shown) inoculated lung samples treated with saline. Treatment of RSV inoculated groups with HS 230 (negative HS control) and 254 (positive HS control) did not inhibit RSV infection compared to saline treatment of RSV inoculated groups (positive control) (Figure 5.13). Prophylactic treatment of the RSV inoculated group with HS 228 (carboxyl reduced mucosal heparin), HS 259 (fucoidan), HS 267 (lambda carrageenan (200kDa) or HS 424 (mucosal heparin with glycol split (3kDa) induced a significant reduction ($p < 0.05$) in RSV titres compared to the positive control (Figure 5.13). Treatment of RSV inoculated group with compounds HS 228, HS 259, HS 267 and HS 424 also inhibited RSV infection significantly ($p < 0.05$) compared to the treatment of RSV inoculated group with HS 230 (negative HS control).

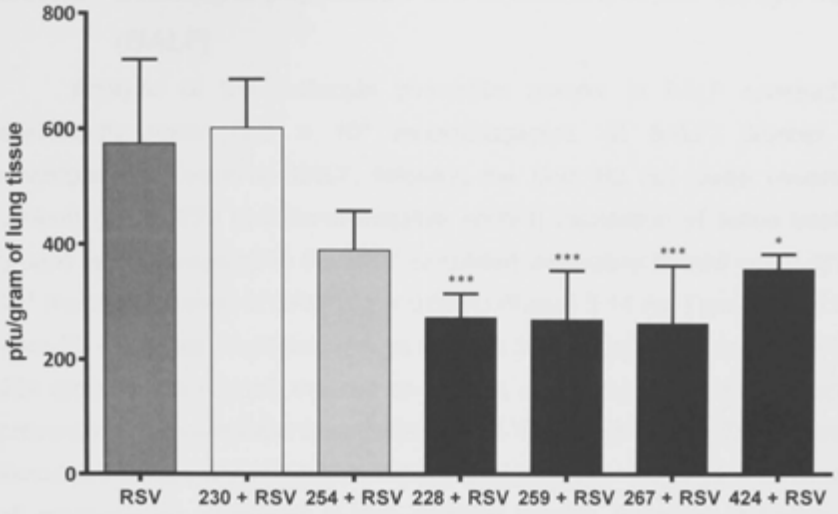


Figure 5.13. Prophylactic heparan sulfate (HS) treatment of respiratory syncytial virus (RSV) infection induced reduction in titres *in vivo*

Starting 48 hours prior to intranasal inoculation and continuing daily for 4 days after, wild type BALB/c mice received treatment with saline or HS mimetics (228, 230, 254, 259, 267 and 424) at 10 mg/mL/kg concentration by intraperitoneal injections (100 µL). On day 0, mice were infected by intranasal inoculation with RSV (black bar) (5×10^5 plaque forming units (pfu)/mouse). On day 5, mice were euthanised and samples collected. Titres are represented as pfu/gram of lung tissue. Data are represented as mean (n = 5) ± standard deviation. HS 230 (negative) (white bar) and 254 (positive) (light grey bar) used HS mimetic controls. Data were analysed using a ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism).

5.3.4.3 *Leukocyte population in a bronchoalveolar lavage fluid (BALF)*

Analysis of the leukocyte population present in BALF revealed a significantly lower (7.5 x 10^4 macrophages/mL of BALF) number of macrophages present in BALF, following the Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculation of saline treated groups when compared to the RSV inoculated and saline treated group (21 x 10^4 macrophages/mL of BALF) ($p < 0.0001$) (Figure 5.14 A). Treatment of the Vero E6 cell lysate inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) induced no change in the number of macrophages present in BALF compared to negative control. Treatment of Vero E6 cell lysate inoculated groups with HS 228 or HS 267 also induced no change in a number of macrophages compared to the negative control. However, reduction in macrophage numbers present in BALF was observed following treatment of Vero E6 cell lysate inoculated groups with HS 259 ($p < 0.0001$) compared to negative control. In contrast, treatment of Vero E6 cell lysate inoculated group with HS 424 resulted in significant increase in macrophages present in BALF ($p < 0.0001$) compared to negative control (Figure 5.14 A). Interestingly, treatment of RSV inoculated group with HS 230 (negative HS control) or HS 254 (positive HS control) induced an increase in a number of macrophages ($p < 0.001$) compared to the corresponding HS treated Vero E6 cell lysate inoculated groups (Figure 5.14 A). Also, treatment of RSV inoculated groups with HS 228 or HS 267 resulted in an increase ($p < 0.0001$) in the number of macrophages compared to the corresponding HS treated Vero E6 cell lysate inoculated groups. In contrast, treatment of RSV inoculated group with HS 259 did not induce a change in macrophage numbers while treatment of RSV inoculated group with HS 424 induced a decrease ($p < 0.0001$) in macrophage numbers in BALF compared to corresponding Vero E6 cell lysate inoculated controls (Figure 5.14 A). HS treatment of RSV inoculated group resulted in no change in macrophage numbers present in BALF compared to positive control.

Lymphocyte BALF analysis revealed no difference in a number of lymphocytes present following Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculation of saline treated groups compared to the RSV inoculated and saline treated group (positive control) (Figure 5.14 B).

In addition, treatment of Vero E6 cell lysate inoculated groups with any of the HS compounds: HS 230 (negative HS control), HS 254 (positive HS control), HS 228, HS 259, HS 267 or HS 424 resulted in no change in lymphocyte numbers compared to the negative control. Additionally, treatment of RSV inoculated groups with HS 230 (negative HS control), HS 254 (positive HS control), HS 259, HS 267 and HS 424 induced no change in lymphocyte numbers compared to both, positive control and corresponding HS treated Vero E6 cell lysate inoculated groups (Figure 5.14 B). Treatment of RSV inoculated groups with HS 228 induced an increase ($p < 0.0001$) in the number of lymphocyte infiltrates compared to both, positive control and corresponding HS 228 treated Vero E6 cell lysate inoculated group (Figure 5.14 B).

Saline treatment of Vero E6 (negative control) and UV-RSV (additional negative control) inoculation resulted in no change in eosinophil numbers compared to the saline treatment of RSV inoculated group (positive control) (Figure 5.14 C). Treatment of Vero E6 cell lysate inoculated groups with any HS mimetic compound (HS 230 (negative HS control), HS 254 (positive HS control), HS 228, HS 259, HS 267 or HS 424) did not induce change in eosinophil numbers present in BALF compared to negative controls. Treatment with HS 230 (negative HS control) of RSV inoculated groups also resulted in no change in the number of eosinophils in BALF compared to HS 230 treated group following Vero E6 cell lysate inoculation. In contrast, treatment of the RSV inoculated group with HS 254 (positive HS control) resulted in an increase ($p < 0.005$) in eosinophil numbers compared to the HS 254 treatment of the Vero E6 cell lysate inoculated group (Figure 5.14 C). Treatment of RSV inoculated groups with HS 228 and HS 424 resulted in a significant increase ($p < 0.0001$) in eosinophil numbers compared to both, corresponding HS treatment of RSV inoculated groups and positive controls (Figure 5.14 C). Treatment of RSV inoculated groups with HS 267 also induced an eosinophil influx ($p < 0.05$) compared to HS 267 treatment of RSV inoculated groups (Figure 5.14 C).Treatment of RSV inoculated group with 259 did not induce any change to eosinophil numbers compared to positive controls (Figure 5.14 C).

Furthermore, no significant difference in neutrophil numbers was observed between saline treatments of, Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculated groups (Figure 5.14 D). In

addition, treatment of Vero E6 or UV-RSV inoculated group with HS mimetics (applies for all six tested HS mimetics) showed no significant difference in neutrophil numbers between groups. Positive control resulted in no change in neutrophil numbers present in BALF compared to the negative control. Treatment of Vero E6 cell lysate inoculated groups with HS 230 (negative HS control), HS 254 (positive HS control), HS 228, HS 259, HS 267 or HS 424 resulted in no change in neutrophil numbers compared to negative control. In addition, treatment of RSV inoculated groups with HS 230 (negative HS control), HS 254 (positive HS control), HS 228, HS 259, HS 267 or HS 424 resulted in no change in neutrophil numbers compared to positive control.

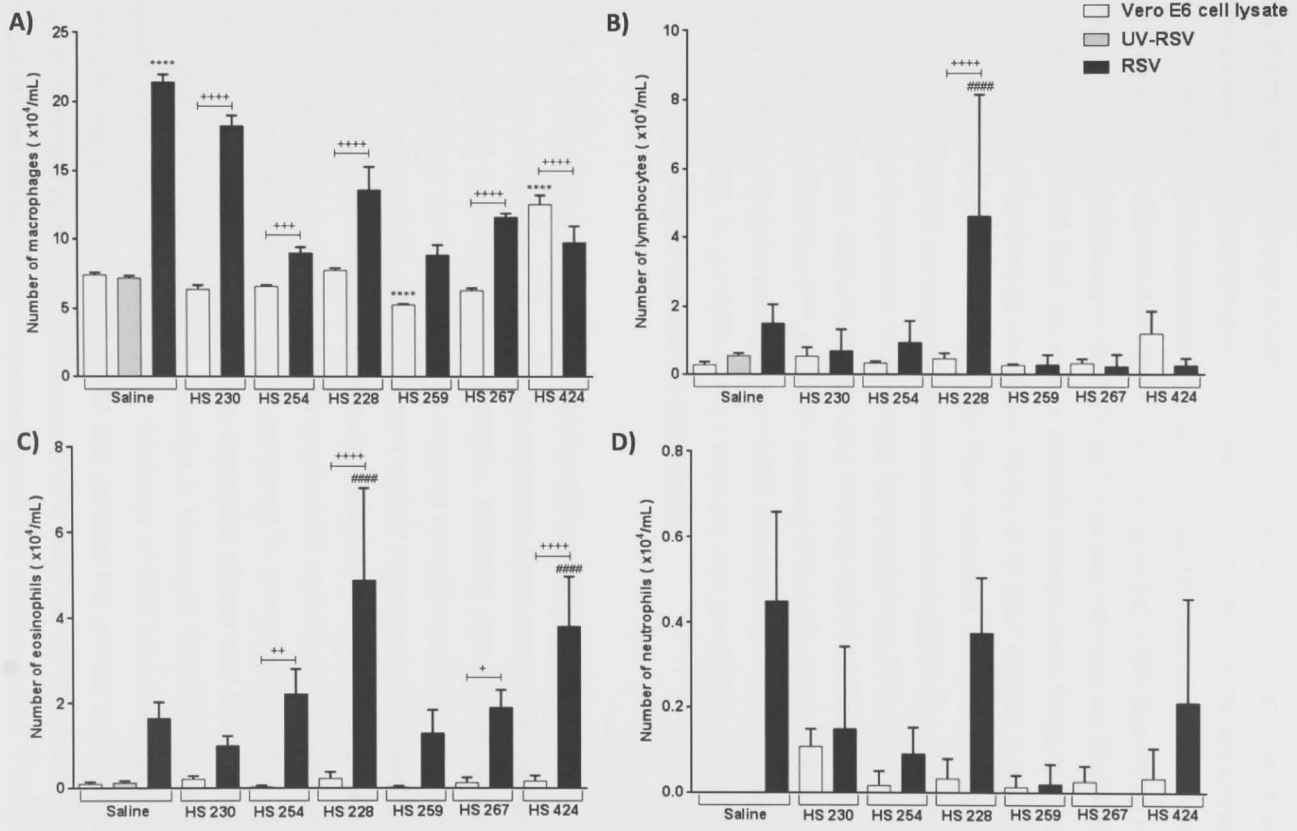

A)
B)
C)
D)
Vero E6 cell lysate
UV-RSV
RSV
Number of macrophages (x10⁴/mL)
Number of lymphocytes (x10⁴/mL)
Number of eosinophils (x10⁴/mL)
Number of neutrophils (x10⁴/mL)
Saline
HS 230
HS 254
HS 228
HS 259
HS 267
HS 424

Figure 5.14. Differential leukocyte count in bronchoalveolar lavage fluid (BALF) following heparan sulfate (HS) prophylactic treatment of respiratory syncytial virus (RSV) infection *in vivo*

Starting 48 hours prior to intranasal inoculation and continuing daily for 4 days after, wild type BALB/c mice received a treatment of saline or HS mimetics (228, 230, 254, 259, 267 and 424) at 10 mg/mL/kg concentration by intraperitoneal injections (100 µL). Mice were infected with Vero E6 cell lysate (white bar), ultraviolet-inactivated RSV (UV-RSV) (grey bar) or RSV (black bar) (5 x 10^5 plaque forming units (pfu)/mouse) on day 0. On day 5, mice were euthanised and BALF was collected by lavage with PBS. BALF was used for differential leukocyte counts though cytospin preparations. Following May-Grunwald and Giemsa staining of the BALF cytospins, leukocyte differential counts were performed using a light Nikon microscope and macrophage (A), lymphocyte (B), eosinophil (C) and neutrophil (D) counts were determined. Data are represented as mean (n = 5) ± standard deviation. HS compounds 230 (negative) and 254 (positive) were used as HS mimetic controls. Data were analysed using TWO-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (****, $p < 0.0001$ compared to Vero E6 cell lysate inoculated and saline treated group (negative control); ####, $p < 0.0001$ compared to RSV inoculated, saline treated group; +, $p < 0.05$, ++, $p < 0.005$, +++, $p < 0.001$ and ++++, $p < 0.0001$ compared to corresponding Vero E6 cell lysate inoculated HS treated group).

5.3.4.4 Differential analysis of leukocyte population in blood

Analysis of the leukocyte population present in blood revealed no change in the number of monocyte infiltrates present, following Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculation of saline treated groups compared to saline treatment of RSV inoculated group (positive control) (Figure 5.15 A). Treatment of Vero E6 cell lysate inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) also induced no change in monocyte infiltrates in blood compared to the negative control. In addition, treatment of Vero E6 cell lysate inoculated groups with HS 228, HS 259, HS 267 or HS 424 induced no change in monocyte infiltrates in blood compared to the negative control. Also, treatment of RSV inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) induced no change in monocyte infiltrates in blood compared to both, the corresponding HS treatment of Vero E6 cell lysate inoculated groups and positive control. Finally, treatment of RSV inoculated groups with HS 228, HS 259, HS 267 or HS 424 also induced no change in monocyte infiltrates in blood compared to both, the

corresponding HS treatment of Vero E6 cell lysate inoculated groups and positive control.

Lymphocyte blood analysis revealed no significant change in lymphocyte numbers following Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculation of saline treated groups compared to the positive control (Figure 5.15 B). However, treatment of Vero E6 cell lysate inoculated groups with HS 230 (negative HS control) or HS 254 (positive HS control) resulted in an increase ($p < 0.0001$) in lymphocyte numbers compared to the negative control. In addition, treatment of Vero E6 cell lysate inoculated groups with HS 228, HS 259, HS 267 and HS 424 induced an increase ($p < 0.0001$) in a number of lymphocytes compared to the negative control. On the contrary, treatment of RSV inoculated group with HS 230 (negative HS control) resulted in a decrease ($p < 0.0001$) in lymphocyte numbers compared to HS 230 treatment of Vero E6 cell lysate inoculated groups. Treatment of RSV inoculated group with HS 254 (positive HS control) did not induce any change to lymphocyte numbers present in blood compared HS 254 treatment of Vero E6 cell lysate inoculated group. In contrast, treatment of RSV inoculated group with HS 228 resulted in a decrease ($p < 0.0001$) in lymphocyte numbers compared to HS 228 treatment of Vero E6 cell lysate inoculated groups. In addition, treatment of RSV inoculated group with HS 267 has resulted in an increase ($p < 0.0001$) in lymphocyte numbers compared to the Vero E6 cell lysate inoculated group treated with HS 267. Treatment of RSV inoculated groups with HS 259 or HS 424 did not induce any change in lymphocyte numbers present in blood compared to the corresponding HS treatment of Vero E6 cell lysate inoculated groups (Figure 5.15 C). Finally, treatment with HS mimetics (HS 259, HS 267 and HS 424) following RSV inoculation has resulted in an increase ($p < 0.0001$) in lymphocytes present in blood compared to the lymphocyte numbers recovered from the positive control samples.

Vero E6 (negative control) and UV-RSV (additional negative control) inoculation of saline treated groups did not have any effect on eosinophil infiltrates into blood compared to the positive control (Figure 5.15 C). No change in eosinophil numbers were observed following HS 230 (negative HS control) or HS 254 (positive HS control) treatment of the Vero E6 cell lysate inoculated groups compared to the negative control. Treatment with all HS

compounds (except HS 267) of Vero E6 cell lysate inoculated groups did not induce a change in eosinophil numbers compared to the negative control. In contrast, treatment of the Vero E6 cell lysate inoculated group with HS 267 has resulted in an increase ($p < 0.0001$) in eosinophil numbers compared to the negative control. HS 230 (negative HS control) or HS 254 (positive HS control) treatment of RSV inoculated groups did not have any effect on eosinophil numbers in blood compared to both, the corresponding HS treatment of Vero E6 cell lysate inoculated groups and positive control. Additionally, treatment with all HS compounds (except HS 267) of RSV inoculated groups did not induce a change in eosinophil numbers compared to both, corresponding HS treatment of Vero E6 cell lysate inoculated groups and positive control. Also, treatment of the RSV inoculated group with HS 267 resulted in higher ($p < 0.0001$) eosinophil numbers compared to both, Vero E6 cell lysate inoculated group treated with HS 267 and positive control (Figure 5.15 C).

Finally, no significant difference in neutrophil numbers was observed between saline treatments of, Vero E6 cell lysate (negative control) or UV-RSV (additional negative control) inoculated groups (Figure 5.15 D). In addition, treatment of Vero E6 or UV-RSV inoculated group with HS mimetics (applies for all six tested HS mimetics) showed no significant difference in neutrophil numbers between groups. Positive control resulted in no change in neutrophil numbers present in blood compared to the negative control. Treatment of Vero E6 cell lysate inoculated groups with HS 230 (negative HS control), HS 254 (positive HS control), HS 228, HS 259, HS 267 or HS 424 resulted in no change in neutrophil numbers compared to negative control. In addition, treatment of RSV inoculated groups with HS 230 (negative HS control), HS 254 (positive HS control), HS 228, HS 259, HS 267 or HS 424 resulted in no change in neutrophil numbers compared to positive control.

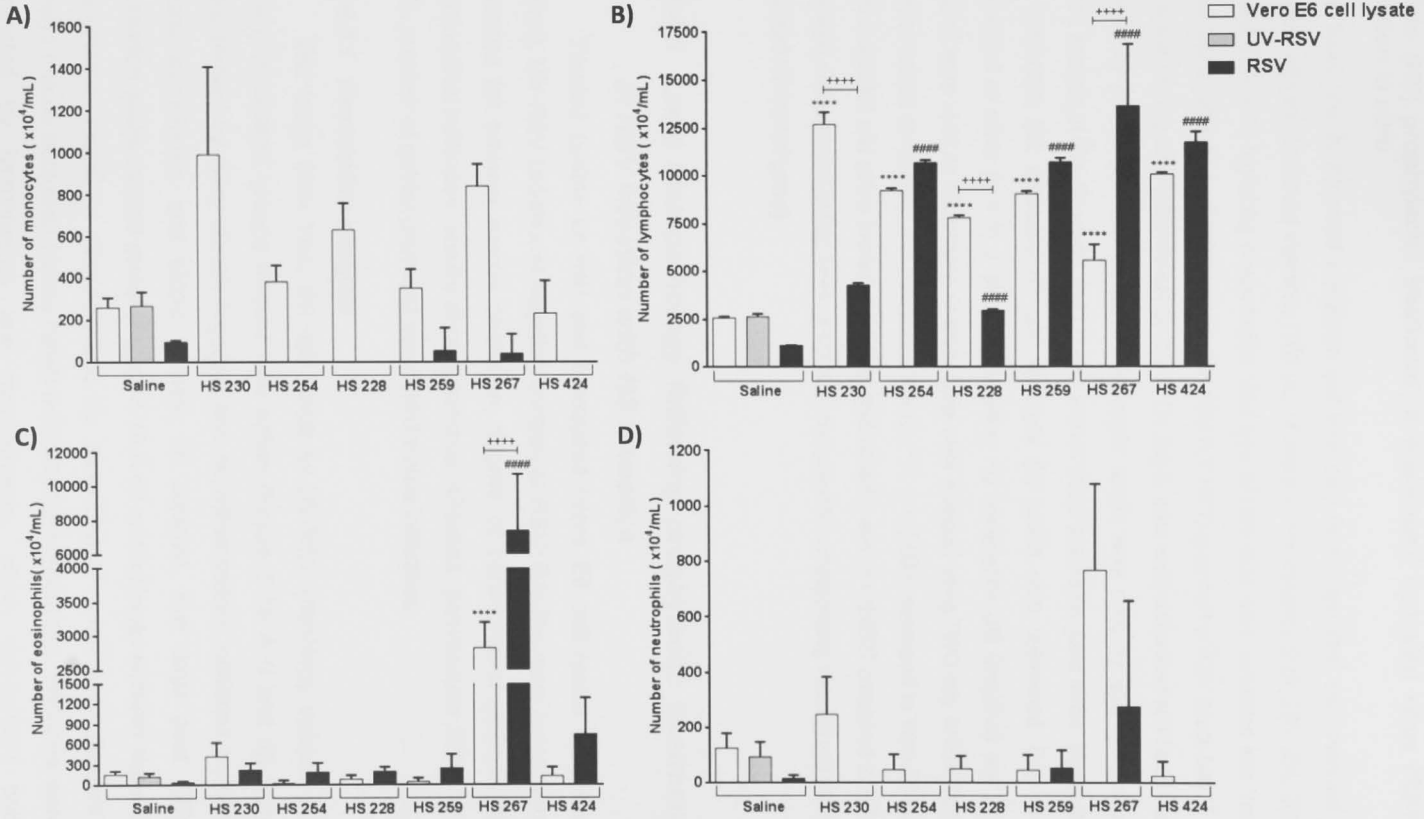

A)
Number of monocytes (x10⁴/mL)
1600
1400
1200
1000
800
600
400
200
0
Saline
HS 230
HS 254
HS 228
HS 259
HS 267
HS 424
B)
Number of lymphocytes (x10⁴/mL)
17500
15000
12500
10000
7500
5000
2500
0
++++
****
####
Vero E6 cell lysate
UV-RSV
RSV
C)
Number of eosinophils(x10⁴/mL)
12000
10000
8000
6000
4000
3500
3000
2500
1500
1200
900
600
300
0
D)
Number of neutrophils (x10⁴/mL)
1200
1000
800
600
400
200
0

Figure 5.15. Differential leukocyte count in blood following heparan sulfate (HS) prophylactic treatment of respiratory syncytial virus (RSV) infection *in vitro*

At 48 hours prior to intranasal inoculation and then daily for 4 days after, mice received a treatment with intraperitoneal injections (100 µL) of saline or HS mimetics (228, 230, 254, 259, 267 and 424) at 10 mg/mL/kg concentration. Wild type BALB/c mice were inoculated with Vero E6 cell lysate (white bar), ultraviolet-inactivated RSV (UV-RSV) (grey bar) or RSV (black bar) (5 x 10^5 plaque forming units (pfu)/mouse) on day 0. On day 5, mice were euthanised and blood was collected by cardiac puncture. Differential leukocyte counts were done by light microscope (Nikon) analysis of May-Grunwald and Giemsa stained blood smears on glass slides. Monocyte (A), lymphocyte (B), eosinophil (C) and neutrophil (D) counts were determined. Data are represented as mean (n = 5) ± standard deviation. HS compounds 230 (negative) and 254 (positive) were used as HS mimetic controls. Data were analysed using TWO-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (****, $p < 0.0001$ compared to Vero E6 cell lysate inoculated and saline treated group (negative control); ####, $p < 0.0001$ compared to RSV inoculated, saline treated group; ++++, $p < 0.0001$ compared to corresponding Vero E6 cell lysate inoculated HS treated group).

5.3.4.5 Lung histopathology following prophylactic treatment of RSV infection with HS mimetics

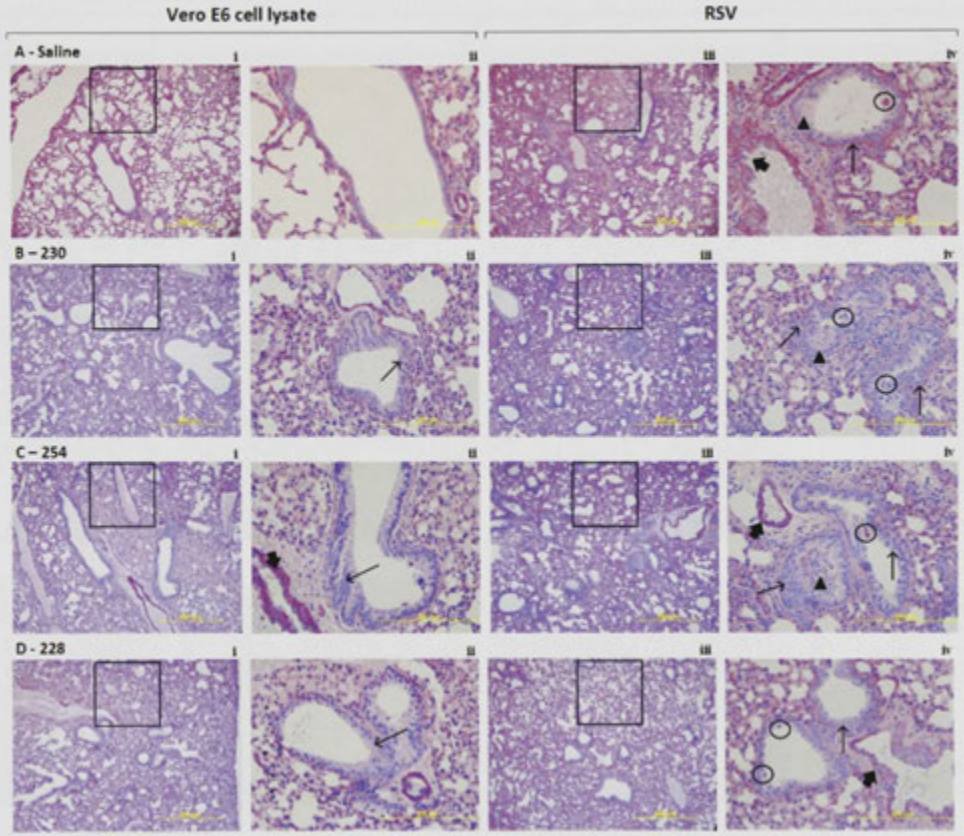
Treated (saline or HS) and inoculated (Vero E6 cell lysate (negative control), UV-RSV (additional negative control) or RSV) BALB/c mice lungs were assessed for airways mucus occlusion, degree of parenchymal pneumonia, peribronchial infiltrates, quality of peribronchial infiltrates, perivascular infiltrates, HPS, number of goblet cells and eosinophil tissue infiltrates.

5.3.4.5.1 Descriptive analysis

The lungs from Vero E6 cell lysate or UV-RSV (histology images not shown) inoculated groups treated with saline (Figure 5.16 A (i and ii)) were characterised by clear alveoli (square) and no inflammatory infiltrates present around bronchioles and blood vessels. In contrast, five days post RSV inoculation saline treated group (Figure 5.16 A (iii and iv) lungs showed signs of inflammatory response characterised by parenchymal pneumonia (square), airways mucus occlusion (arrow head) and extensive infiltration. Infiltrates were dominated by lymphocytes and macrophages, which surrounded both

bronchioles (thin arrow) and pulmonary blood vessels (thick arrow). The arrow heads indicates airway occlusion and the circles show the presence of goblet cells within bronchi. HS 230 (negative HS control) treatment of Vero E6 cell lysate inoculated groups resulted in minimal parenchymal pneumonia (square) and mild inflammatory infiltrates present around bronchioles (thin arrows) and blood vessels (thick arrow) (Figure 5.16 B (i and ii)). Treatment with HS 254 of Vero E6 cell lysate inoculated groups showed no signs of parenchymal pneumonia (square), or inflammatory infiltrates around bronchioles (thin arrows) and blood vessels (thick arrow) (Figure 5.16 C (i and ii)). Treatment with HS compounds, HS 228, HS 259, HS 267 and HS 424 of Vero E6 cell lysate inoculated mice was characterised by minimal parenchymal pneumonia (square) and mild infiltration dominated by lymphocytes and macrophages surrounding both, bronchioles (thin arrows) and pulmonary blood vessel infiltrates (thick arrows) (Figure 5.16 D, E, F and G (i and ii)).

Treatment with compound HS 230 (negative HS control) of RSV inoculated groups (Figure 5.16 B (iii and iv)) induced moderate parenchymal pneumonia (square) and airways occlusion (arrow head) with goblet cells present within bronchioles (circle). Treatment with compound 254 (positive HS control) of RSV inoculated group was characterised by mild-moderate parenchyma, mild airway mucus occlusion, moderate perivascular (thick arrows) and peribronchial infiltrates (thin arrows) (Figure 5.16 C (iii and iv)). Treatment with compound HS 228 of RSV inoculated group was also characterised by mild-moderate parenchyma (square), mild airway mucus occlusion (arrow head) with several goblet cells present (circle), moderate perivascular (thick arrows) and peribronchial infiltrates (thin arrows) (Figure 5.16 D (iii and iv)). Finally, treatment with compounds HS 259, HS 267 and HS 424 of RSV inoculated group (Figure 5.16 B (iii and iv)) resulted in moderate parenchymal pneumonia (square) and airway occlusion (arrow head) with goblet cells present within bronchioles (circle), as well as, moderate perivascular (thick arrows) and peribronchial infiltrates (thin arrows).


Vero E6 cell lysate
RSV
A - Saline
B – 230
C – 254
D - 228
i
ii
iii
iv

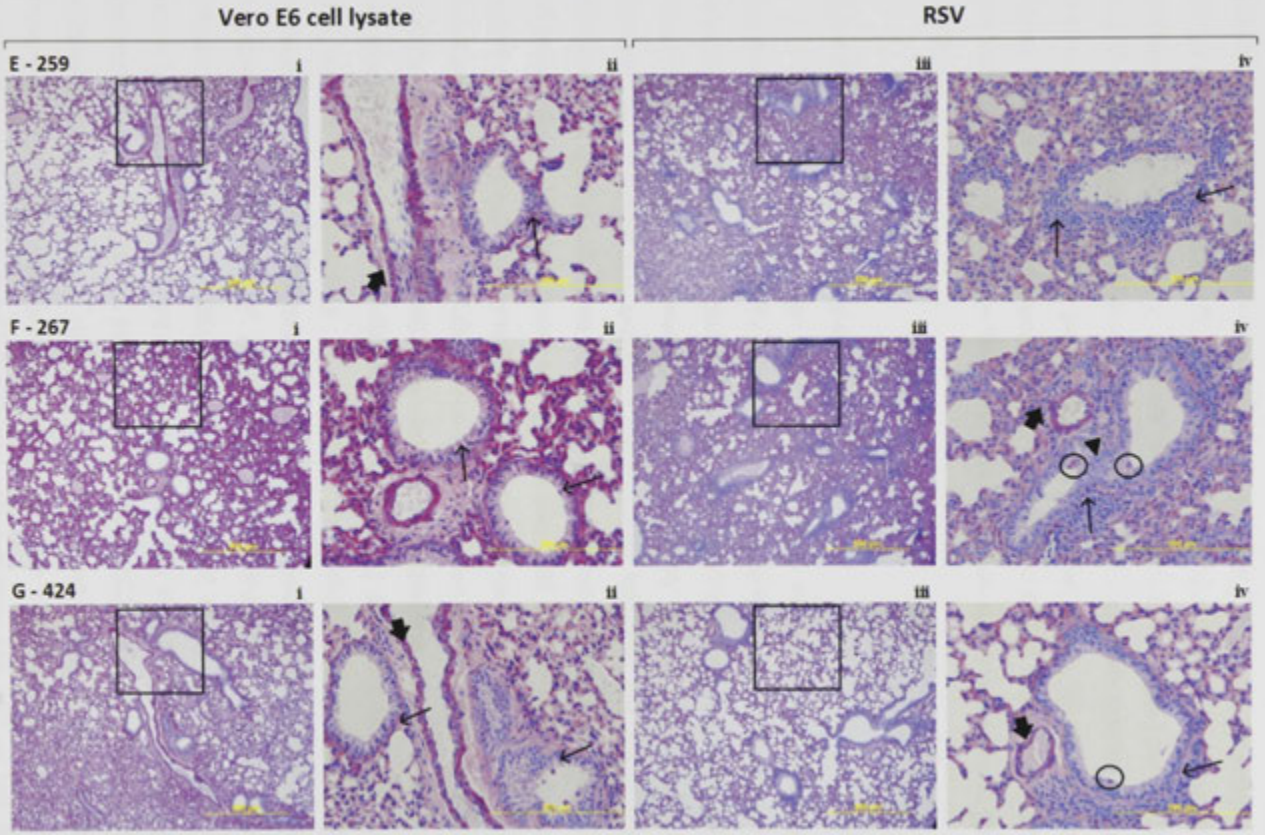

Vero E6 cell lysate
RSV
E - 259
F - 267
G - 424
i
ii
iii
iv

Figure 5.16. Effect of prophylactic heparan sulfate (HS) treatment on respiratory syncytial virus (RSV) infected mouse lung histology

Starting 48 hours prior to intranasal inoculation and continuing daily for 4 days after, wild type BALB/c mice received a treatment of saline (A) or HS mimetics: 230 (negative HS control) (B), 254 (positive HS control) (C), 228 (D), 259 (E), 267 (F) and 424 (G) at 10 mg/mL/kg concentration by intraperitoneal injections (100 µL). Mice were infected with Vero E6 cell lysate (i and ii) or RSV (5×10^5 plaque forming units (pfu)/mouse) on day 0 (iii and iv). Lungs were collected on day 5 and processed for histology using periodic acid Schiff (PAS) stain. Arrows, squares and circles indicate the different signs of inflammation in mice as described in the text. Magnification (i and ii) was 100X and (iii and iv) 400X including the eyepiece. Images are representative of each group (n = 5).

5.3.4.5.2 Numerical (score) analysis

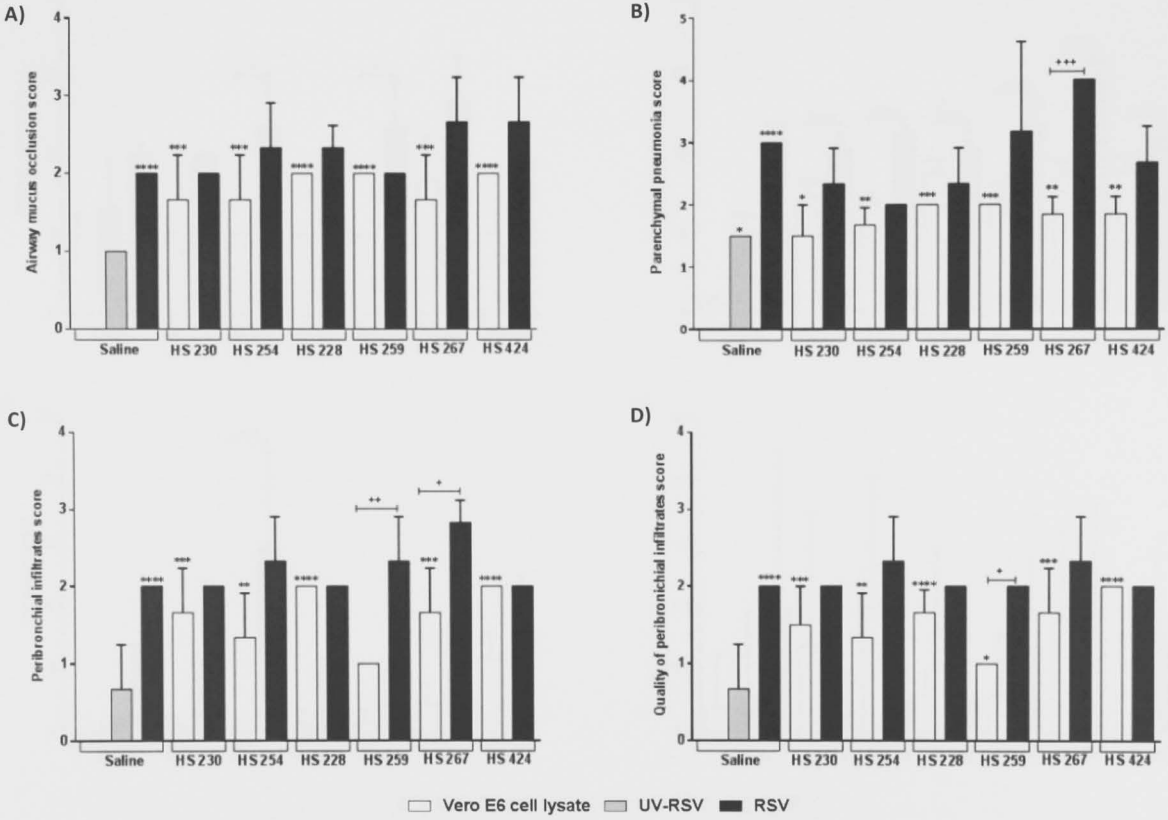
No difference between Vero E6 cell lysate inoculated saline treated (negative control) and UV-RSV (additional negative control-histology images not shown) saline treated (additional negative control) (Figure 5.17) groups were observed in terms of scores allocated to airway mucus occlusion (A), peribronchial infiltrates (C), quality of peribronchial infiltrates (D), perivascular infiltrates (E), goblet cell count (G) or eosinophil tissue infiltrates (H). The only difference found between these two groups was that the UV-RSV inoculated saline treated group had greater parenchymal pneumonia ($p < 0.05$) and combined HPS score ($p < 0.0001$) compared negative controls (Figure 5.17 B and F). Saline treatment of RSV inoculated groups (positive control) induced a significant increase in ($p < 0.05$) airway occlusion (A), parenchymal pneumonia (B), peribronchial infiltrates (C), quality of peribronchial infiltrates (D), perivascular infiltrates (E) and combined HPS score (F) compared to the negative controls (Figure 5.17, A-F). In addition, treatment of Vero E6 cell lysate inoculated group with HS 230 (negative HS control) or HS 254 (positive HS control) induced a significant increase ($p < 0.05$) in airway occlusion (A), parenchymal pneumonia (B), peribronchial infiltrates (C), quality of peribronchial infiltrates (D) and combined HPS score (F) compared to the negative control. However, no change was observed for perivascular infiltrates (E), goblet cell count (G) and eosinophil tissue infiltrates (H) following HS 230 or HS 254 treatment of the Vero E6 cell lysate inoculated group compared to the negative control (Figure 5.17).

Treatment of Vero E6 cell lysate inoculated groups with HS 228, HS 259, HS 267 or HS 424 resulted in a significant increase ($p < 0.001$) in airway mucus occlusion compared to the negative control (Figure 5.17 A). Treatment of Vero E6 cell lysate inoculated groups with HS 228, HS 259, HS 267 and HS 424 resulted in a significant increase ($p < 0.005$) in parenchymal pneumonia score compared to negative control (Figure 5.17 B). Additionally, treatment of Vero E6 cell lysate inoculated groups with HS 228, HS 267 and HS 424 resulted in an increase ($p < 0.005$) in peribronchial infiltrates compared to negative controls (Figure 5.17 C). Treatment of Vero E6 cell lysate inoculated groups with HS 228, HS 259, HS 267 and HS 424 resulted in an increase ($p < 0.05$) in quality of peribronchial infiltrates compared to negative control (Figure 5.17 D). In contrast, treatment of Vero E6 cell lysate inoculated groups with HS 228, HS 259, HS 267 and HS 424 did not induce any change to perivascular infiltrates compared to negative controls (Figure 5.17 E). Treatment with compounds HS 228, HS 259, HS 267 and HS 424 of Vero E6 cell lysate inoculated groups had greater ($p < 0.0001$) combined HPS (score out of 17) compared to negative control (Figure 5.17 F). There was no change in goblet cell count and eosinophil tissue infiltrates following HS treatment of Vero E6 cell lysate inoculated groups compared to negative control.

Treatment of RSV inoculated groups with HS 230 (negative HS control) or 254 (positive HS control) did not induce change to any of the eight histopathological measurements assessed in this study compared to the corresponding HS treatment of Vero E6 cell lysate inoculated groups. Additionally, RSV inoculation of HS (HS 228, HS 259, HS 267 or HS 424) treated groups did not have any effect on airway mucus occlusion compared to the corresponding HS treatment of Vero E6 cell lysate inoculated groups. RSV inoculation of HS 228, HS 259 and HS 424 treated groups also did not have any effect on parenchymal pneumonia compared to the corresponding HS treatment of Vero E6 cell lysate inoculated groups. However, RSV inoculation of HS 267 treated groups has exacerbated ($p < 0.001$) parenchymal pneumonia compared to HS 267 treatment of Vero E6 cell lysate inoculated group. RSV inoculation of HS 228 and HS 424 treated groups did not have any effect on peribronchial infiltration compared to the corresponding HS treatment of Vero E6 cell lysate inoculated groups. However, RSV inoculation of HS 259 and HS 267 treated groups has induced an increase ($p < 0.05$) in peribronchial infiltrate numbers

compared to the corresponding HS treatment of Vero E6 cell lysate inoculated groups. RSV inoculation of HS 228, HS 267 and HS 424 treated groups did not have any additional effect on quality of peribronchial infiltration compared to the corresponding HS treatment of Vero E6 cell lysate inoculated groups. However, RSV inoculation of HS 259 treated groups has resulted in an increase ($p < 0.05$) in quality of peribronchial infiltration compared to the corresponding HS treatment of Vero E6 cell lysate inoculated groups. RSV inoculation of HS 228, HS 267 and HS 424 treated groups did not have any effect on perivascular infiltration compared to the corresponding HS treatment of Vero E6 cell lysate inoculated groups. However, RSV inoculation of HS 259 treated groups has induced ($p < 0.05$) perivascular infiltration compared to the corresponding HS treatment of Vero E6 cell lysate inoculated groups. Also, treatment with compounds HS 254, HS 259, HS 267 and HS 424 of RSV inoculated groups induced an increase ($p < 0.0001$) in combined HPS compared to the corresponding HS treated Vero E6 cell lysate inoculated groups. However, there was no change in goblet cell count following HS treatment (except HS 254) of RSV inoculated groups compared to corresponding HS treatment of Vero E6 cell lysate inoculated groups. Treatment of RSV inoculated group with HS 254 (positive HS control) resulted in an increase ($p < 0.0001$) in goblet cell count compared to the HS 254 treated Vero E6 cell lysate inoculated group (Figure 5.17 G). However, there was no change in eosinophil tissue influx following HS treatment of RSV inoculated groups compared to corresponding HS treatment of Vero E6 cell lysate inoculated groups.

Finally, treatment of RSV inoculated groups with HS mimetics (HS 228, HS 259, HS 267 or HS 424) did not have any additional effect on airway mucus occlusion (A), parenchymal pneumonia (B), peribronchial infiltration (C), quality of peribronchial infiltrates (D), perivascular infiltrates (E), goblet cell count (G) and eosinophil tissue infiltrates (H) compared to the positive control. All HS treated (except for HS 267) RSV inoculated groups showed no change in HPS combined score compared to the saline treatment of RSV inoculated group (positive control). In contrast, treatment with HS 267 of RSV inoculated groups resulted in an increase ($p < 0.0001$) in combined HPS score compare to the positive control (Figure 5.17 F).


A)
Airway mucus occlusion score
Saline
HS 230
HS 254
HS 228
HS 259
HS 267
HS 424
B)
Parenchymal pneumonia score
C)
Peribronchial infiltrates score
D)
Quality of peribronichial infiltrates score
Vero E6 cell lysate
UV-RSV
RSV

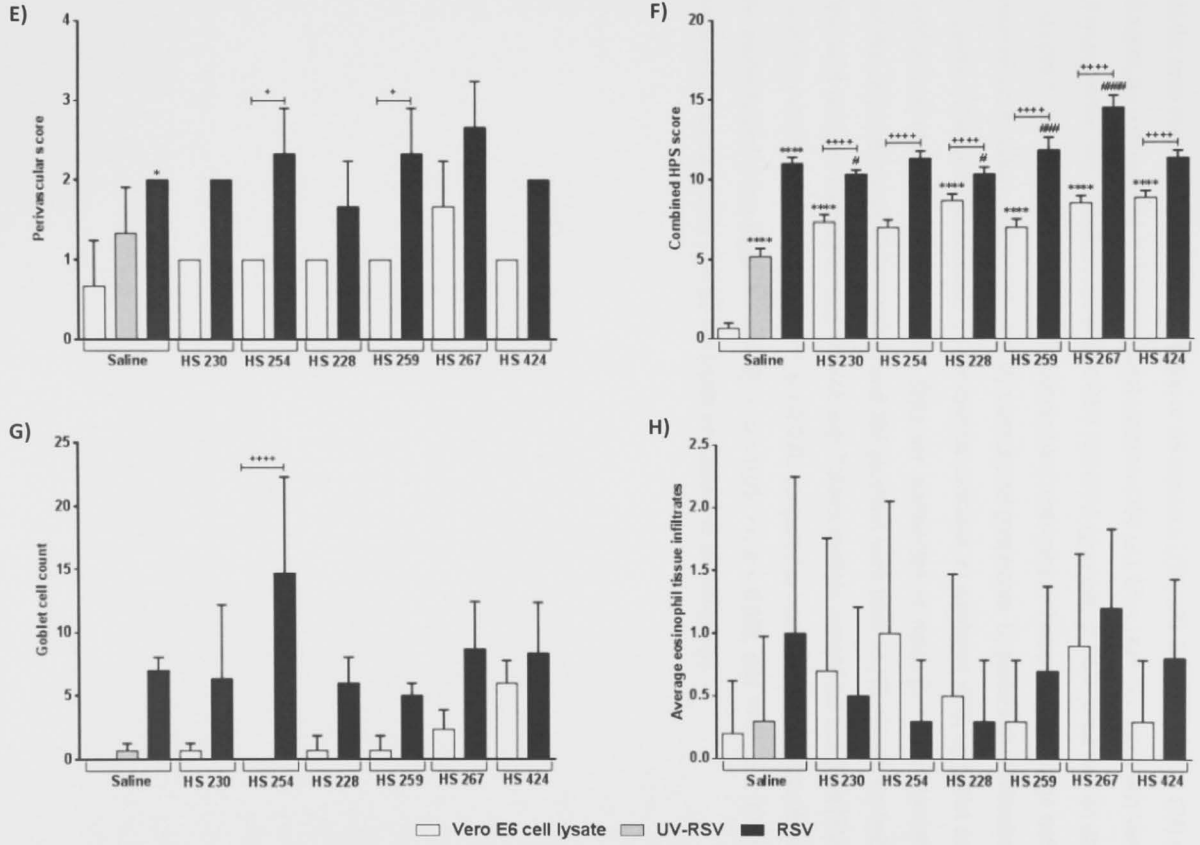

E)
Perivascular score
Saline
HS 230
HS 254
HS 228
HS 259
HS 267
HS 424
F)
Combined HPS score
G)
Goblet cell count
H)
Average eosinophil tissue infiltrates
Vero E6 cell lysate
UV-RSV
RSV

Figure 5.17. Histopathological score (HPS) assessment of mice lungs following prophylactic heparan sulfate (HS) mimetics treatment of respiratory syncytial virus (RSV) infection

Starting 48 hours prior to intranasal inoculation and continuing daily for 4 days after, wild type BALB/c mice received a treatment with saline or HS mimetics (228, 230, 254, 259, 267 or 424) at 10 mg/mL/kg concentration by intraperitoneal injections (100 µL). Mice were inoculated with Vero E6 lysate (white bar), ultraviolet-inactivated RSV (UV-RSV) (grey bar) or RSV (black bar) on day 0. On day 5 post inoculation, lungs were collected and processed for histopathology. Lungs were analysed for: A) airway mucus occlusion, B) parenchymal pneumonia, C) peribronchial infiltrates, D) quality of peribronchial infiltrates, E) perivascular infiltrates, F) combined HPS, G) goblet cell count and H) eosinophil tissue infiltrates. Data are represented as mean (n = 5) ± standard deviation. HS compounds 230 (negative) and 254 (positive) were used as HS mimetic controls. Data were analysed using ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (*, $p < 0.05$, ***, $p < 0.001$ and ****, $p < 0.0001$ compared to Vero E6 cell lysate inoculated and saline treated group (negative control); +, $p < 0.05$, ++, $p < 0.005$, and ++++, $p < 0.0001$ compared to corresponding Vero E6 cell lysate inoculated HS treated group).

5.4 Discussion

While there is an ample of evidence demonstrating that the binding of RSV to cultured cells involves an interaction between the viral envelope glycoproteins (G and F) and the cell surface HSPGs (Bourgeois *et al.*, 1998; Escribano-Romero *et al.*, 2004; Feldman *et al.*, 2000; Feldman *et al.*, 1999; Hallak *et al.*, 2000a; Harris and Werling, 2003; Karger *et al.*, 2001; Krusat and Streckert, 1997; Martinez and Melero, 2000; Techaarpornkul *et al.*, 2002), only recently has the cellular protein nucleolin been identified as a specific receptor for RSV (Tayyari *et al.*, 2011). Tayyari *et al.* (2001) proposed that RSV binds to the cell surface proteoglycans to allow the RSV fusion protein to interact with nucleolin (Tayyari *et al.*, 2011). The interactions between the RSV and the cell surface HSPGs are required for the RSV attachment and entry into the host cell, and therefore, represent a valid target for the inhibition of RSV infection.

An effective and inexpensive treatment against RSV is urgently required, especially since motavizumab, a variant of palivizumab, which was expected to replace palivizumab for the prevention of RSV infection in infants, was recently denied approval by the U.S. Food and Drug Administration (FDA) due to concerns about safety and allergic reactions (Donalisio *et al.*, 2012). Currently, no vaccine for RSV is available as previous vaccine attempts have failed to elicit a long-lasting protective immune response (Castilow *et al.*, 2008) and the approval of a new RSV vaccine is not expected before 2020 (Donalisio *et al.*, 2012). Considering the high burden of RSV disease worldwide on both the population and healthcare systems, further antiviral research and development is critical (Storey, 2010).

A key limitation of previous studies examining the RSV interaction with GAGs is that all studies to date have been performed *in vitro* only. Therefore, a key focus of this chapter was to elucidate the role of HS mimetics in viral inhibition *in vitro* and *in vivo*, testing the anti-viral activity of 53 HS mimetics *in vitro* while facilitating the selection of 6 HS mimetics for *in vivo* testing. This extensive HS mimetics assessment included compounds from five, chemically and structurally different groups: group 1- heparins and modified heparins (12kDa), group 2- low molecular weight heparins (3kDa and 5kDa) and enoxaparin with/without glycol split, group 3- fucoidans and modified fucoidan, group 4- carrageenans and group 5- other HS compounds.

It is important to note that HS compounds cytotoxicity on the Vero E6 cells was the only cytotoxicity assay performed. As no cytotoxicity was detected following the HS mimetic incubation with Vero E6 cells, it was concluded that any changes observed in terms of viral load recovery would likely be due to HS effect on RSV viral load as opposed to the cell death in the presence of HS mimetics and RSV. In addition, positive infection control (RSV infected Vero E6 cells with saline treatment) was used in all of the Chapter 5 experimental work. Therefore, all HS treated and RSV infected Vero E6 results were compared to the positive infection control. Furthermore, if there was any cell death due to RSV infection, it was taken into account by using the positive infection control and comparing all of RSV infected and HS treated samples to it.

Following the incubation of RSV with HS mimetics *in vitro* for 10 minutes (condition A), 20 out of 53 tested HS mimetic compounds were found to induce 80-100% RSV inhibition ($p < 0.005$); with 11 out of those 20 showing over 90% RSV inhibition (Figure 5.4). Findings from this study demonstrate HS mimetics antiviral potential against RSV. The best performing compound in this study was HS 267 (lambda carrageenan), achieving 100% ($p < 0.0001$) RSV inhibition at all three HS mimetic concentrations. These results are in agreement with previous studies reporting that pre-incubation of RSV with certain GAGs (e.g. heparin) inhibited virus infectivity (Krusat and Streckert, 1997; Martinez and Melero, 2000). The mechanism behind the viral inhibition exhibited by tested HS compounds could involve an interaction of HS mimetics with RSV glycoproteins (G or F) and the formation of an RSV glycoprotein-HS complex. This complex could inhibit RSV binding to the HS at the cell surface, resulting in a reduction of RSV cell infectivity. The proposed mechanism requires further investigation to elucidate the interaction between HS mimetics and RSV, providing a better understanding, which could be utilised in vaccine design.

Furthermore, the hypothesis that HS mimetics may bind to the negatively charged sulfate and carboxyl groups of the GAG chains on the cell surface was tested by pre-incubating HS mimetics with Vero E6 cell monolayer for 10 minutes (condition B). Findings indicate that 20 out of 53 tested HS mimetic compounds inhibited RSV infection at a rate of 80-100% ($p < 0.0001$) (Figure 5.5). Moreover, six out of these 20 HS mimetics induced over 90% RSV inhibition following the pre-incubation of HS mimetics with the Vero E6 cell

monolayer. The best performing compound under this testing condition was again HS 267 (lambda carrageenan), achieving 100% ($p < 0.0001$) RSV inhibition at all three HS mimetic concentrations. Previous studies examining the effect of HEp-2 cells pre-treatment with GAGs (condition B), prior to RSV inoculation, have also shown that this treatment approach results in reduced binding efficiency of RSV infection (Donalisio *et al.*, 2012; Donalisio *et al.*, 2010; Hallak *et al.*, 2000b). The efficacy of this treatment condition suggests the potential for the development of a specific and targeted RSV inhibiting therapeutic while rendering the GAGs unavailable for RSV glycoprotein binding.

A short incubation of RSV with the Vero E6 cells (condition C), resulted in 21 out of 53 tested HS mimetic compounds inducing 80-95% RSV inhibition (Figure 5.6). Ten out of these 21 HS mimetics showed over 90% RSV inhibition. Results from this study suggest that HS mimetics may be able to compete with RSV for the HS interaction on the cell surface of the Vero E6 cells as long as RSV pre-incubation with the cell monolayer is kept to a very short period (e.g. 10 minutes). A longer incubation period (1 hour - condition D) had detrimental effect on viral inhibition. Nevertheless, the best performing compound under condition C was HS 267 (lambda carrageenan), achieving 95% ($p < 0.0001$) RSV inhibition at 5 µg/mL. Previous studies have shown that sulfated polysaccharides can mimic HS chains and interact with viral glycoproteins, blocking viral attachment to cell surface HS through competitive inhibition (Rusnati and Urbinati, 2009; Rusnati *et al.*, 2009). Similarly, studies have shown SB105-A10 (peptide-derivatised dentridimer) exerts antiviral activity by competing with glycoprotein G or F for binding to cell surface HS (Donalisio *et al.*, 2012). Other studies have also shown that pre-incubation of RSV with heparin inhibited virus infectivity (Krusat and Streckert, 1997; Martinez and Melero, 2000).

Under the final testing condition, pre-incubation of RSV with the Vero E6 cell monolayer for 1 hour (clinically the most relevant condition), resulted in 15 out of 53 HS mimetic compounds inducing 50-90% RSV inhibition at 100 µg/mL (Figure 5.7). These results suggest the HS mimetics have an ability to compete with RSV for interaction with HS on the cell surface as well as reduce RSV replication by inhibiting cell-to-cell spread. The best performing compound under this condition was HS 424 (mucosal heparin with glycol split 3kDa),

achieving 65% ($p < 0.0001$) RSV inhibition at the highest HS concentration of 100 µg/mL. As sulfated polysaccharides have been studied extensively as broad-range antiviral compounds, some of them [e.g. heparin (Hallak *et al.*, 2000b), chondroitin sulfate (Hallak *et al.*, 2000a), and dextran sulfate (Kimura *et al.*, 2004)] have already been shown to inhibit RSV successfully. To elucidate the mechanism underlying conditions C and D, more work is required to gain a better understanding of resulting competitive inhibition as well as potential ways to enhance HS efficacy while reducing cell-to-cell spread of the virus. Broader analysis should involve assessment of HS-RSV glycoprotein and HS-cell surface interactions, as these are part of an overarching HS driven RSV inhibition mechanism of competitive inhibition.

Taken together, the *in vitro* HS mimetic studies have generated a list of compounds with great antiviral potential. The best performing compounds across all four conditions are outlined in Table 5.3 demonstrating compounds that achieved viral inhibition between 50-100%, depending on a testing condition. Furthermore, *in vitro* results demonstrate significant RSV inhibition achieved following a pre-treatment of the virus with HS mimetics, pre-treatment of the cells with HS mimetics and HS treatment of RSV infection. Results from chapter 5 *in vitro* studies are in an agreement with those of Hallak *et al.* (2000) who reported that RSV infection was reduced significantly by pre-treatment of the virus with soluble GAGs, pre-treatment of the cells with GAG-binding molecules, pre-treatment of the cells with GAG-destroying enzymes or in cells genetically deficient in GAGs (Hallak *et al.*, 2000a). Krusat and Streckert (1997) also showed that pre-incubation of virus with heparin, or enzymatic digestion of cell surface GAGs, inhibited virus infectivity.

Table 5.3. List of the best performing heparan sulfate (HS) mimetics *in vitro* under four testing conditions, arranged in descending order, from most viral inhibition to least viral inhibition

	Condition A	Condition B	Condition C	Condition D
90-100%	465, 466, 494, 495, 496, 497, 502, 267, 500, 229, 266, 499, 501, 259, 511A, 510, 424, 498, 511B, 512D, 416	466, 495, 497, 500, 267, 494, 499, 266, 511A, 496, 229, 465, 498, 502, 510, 259, 416, 512D, 501	266	
80-90%	394, 419, 418, 408, 106, 486, 448, 384, 201, 483, 217, 481, 218, 254	511B, 424, 419, 217, 486, 201	259, 424	
70-80%	204, 228, 109	106, 218, 408, 384, 448, 394, 418, 512E, 481, 254	254, 408, 267, 416, 481, 494, 242, 511A, 448, 512E, 418, 511B, 384, 106, 419, 483, 241, 510, 333, 332	
60-70%	512E, 331, PI-88, 205, 207, 81, 332	109, 204, 228, PI-88, 483	81, 486, 238, 502, 394, 217, 496, 495, 465, 331, 51D, 201	424
50-60%	Suramin, 219, 111	111, Suramin	218, 497, 501, 204, 230, 229, 500, 219, 498, 466	207, 228, 267, 394

The best performing compounds list across four testing conditions: A (respiratory syncytial virus (RSV) 10 minute pre-incubation with HS), B (HS mimetic 10 minute pre-incubation with cell surface), C (short RSV pre-incubation with cell surface) and D (1 hour RSV pre-incubation with cell surface). Colours denote different degrees of inhibition, in 10% increments, from 50-100%.

An analysis of the large data set generated from the *in vitro* HS mimetic revealed a novel trend emerging. *In vitro* studies showed that all group 3 (fucoidans and modified fucoidan) and group 4 (carrageenans) HS mimetic compounds induced over 80% RSV inhibition under conditions A (RSV 10 minute pre-incubation with HS) and B (HS mimetic 10 minute pre-incubation with cell surface) (Table 5.4), demonstrating a potential involvement of these compounds at earlier stages of viral infection, e.g. attachment. These findings suggest that group 3 (fucoidans and modified fucoidan) and group 4 (carrageenans) may be good candidates for prophylactic RSV treatment. In addition, as demonstrated previously, there was a significant difference in viral inhibition observed following a short and long RSV incubation with the cell monolayer. Keeping these results in mind, the most effective HS mimetic group under condition D (1 hour RSV pre-incubation with cell surface; the most clinically relevant condition) was group 2 (low molecular weight heparins (3kDa and 5kDa) and enoxaparin with/without glycol split) followed by group 1 (heparins and modified heparins (12kDa). This finding indicates the importance of heparin modified compounds in RSV competitive inhibition while also highlighting the potential importance of HS mimetic size (3 or 12kDa) in successful viral inhibition. Although, almost all HS mimetics in group 3 and group 4 performed the best under conditions, A, B and C, these groups performed poorly under condition D. Results suggest that groups 1 and 2 are the most suited for competitive RSV inhibition and cell-to-cell spread, while groups 3 and 4 are the best suited to inhibit RSV by interacting with the cell surface HS and RSV glycoproteins. The proposed and arising trends should be investigated further with some HS compounds showing a great potential for prophylactic treatment while others appear to be the best suited for post RSV infection treatment.

Table 5.4. The average respiratory syncytial virus (RSV) inhibition across five heparan sulfate (HS) mimetic groups

	Percentage of HS compounds that achieved an average RSV inhibition levels under specific testing condition			
HS mimetic groups	Condition A > 80%	Condition B > 80%	Condition C > 50%	Condition D > 50%
Group 1	63.6	27.3	81.8	36.4
Group 2	61.5	38.5	92.3	53.8
Group 3	100.0	100.0	85.7	14.3
Group 4	100.0	100.0	100.0	20.0
Group 5	47.1	41.2	76.5	23.5

Five HS mimetic groups: group 1- heparins and modified heparins (12kDa), group 2- low molecular weight heparins (3kDa and 5kDa) and enoxaparin with/without glycol split, group 3- fucoidans and modified fucoidan, group 4- carrageenans, and group 5-other HS compounds. Four testing condition *in vitro*: Conditions A (RSV 10 minute pre-incubation with HS), B (HS mimetic 10 minute pre-incubation with cell surface), C (short RSV pre-incubation with cell surface) and D (1 hour RSV pre-incubation with cell surface). Data is represented as an average RSV inhibition and expressed in percentages. Average RSV inhibition differed across the four test conditions; hence, data presented for condition A is above 80%, condition B above 80%, condition C above 50% and condition D above 50%.

It is important to note that some HS compounds performed best at the highest (100 µg/mL) and/or lowest (5 µg/mL) HS concentrations, whilst other were unaffected by the change in HS concentration (Figures 5.4-5.7). Unexpectedly, *in vitro* study analysis revealed that 10 out of 53 tested HS compounds achieved an average of 65-90% RSV inhibition across the four test conditions at the lowest, 5 µg/mL, HS concentration. Similar findings were also observed following amantadine antiviral treatment of influenza A virus, which blocks the influenza A M2 protein ion channels (Jefferson *et al.*, 2004). The proton channel formed by M2 protein, transports protons from the endosome to the inside of the influenza A virion (Hay *et al.*, 1985; Pinto *et al.*, 1992; Wang *et al.*, 1993). As a weakly basic substance, amantadine binds protons in the endosome itself, thereby making them impossible to flow inside the virion. This way, both the interaction of the matrix M1 protein and release of the virion nucleus into the host cell environment, are prevented (Hay *et al.*, 1985; Pinto *et al.*, 1992; Wang *et al.*, 1993). This process has been found to be the most

effective at lower concentrations of amantadine (e.g. 1 µg/mL) (Nguyen *et al.*, 2009). In contrast, high concentrations of amantadine are believed to contribute to enhanced viral replication, with amantadine, itself, becoming a source of protons, leading to an increase in proton flow towards the virion while enabling viral uncoating and viral replication (Nguyen *et al.*, 2009; Nguyen *et al.*, 2012).

A similar mechanism may be behind HS-induced RSV inhibition at low HS concentrations. However, until recently, none of the RSV glycoproteins were known as a proton-selective ion channel proteins; which is a main characteristic of influenza A M2 protein. The role played by SH protein during RSV infection is still unknown and SH ion channel potential was not investigated until few years ago. Gan *et al.* (2008) have provided experimental evidence that the transmembrane domain of SH protein forms pentameric α-helical bundles that form cation-selective ion channels in planar lipid bilayers (Gan *et al.*, 2008). This suggests that SH may act as a proton-selective ion channel protein, transporting protons from the outside to the inside of the RSV virion. At low concentrations, HS mimetics could bind protons while inside the SH-proton channel, thereby making it impossible for protons to flow inside the virion. As a result, RSV uncoating would be blocked and viral replication would be inhibited (Figure 5.18). In contrast, in some instances high concentrations of HS mimetics have resulted in enhanced viral replication *in vitro*. This finding could be a result of HS mimetics acting as protons themselves at high concentrations, with an increase in proton flow towards the virion enabling viral uncoating and viral replication. This study highlights the importance of dose dependant viral inhibition and the need to evaluate a range of concentrations, including lower concentrations. Drugs performing well at lower concentrations have the potential to control tissue toxicity and any drug related side effects; much desired characteristics of any effective therapeutic.

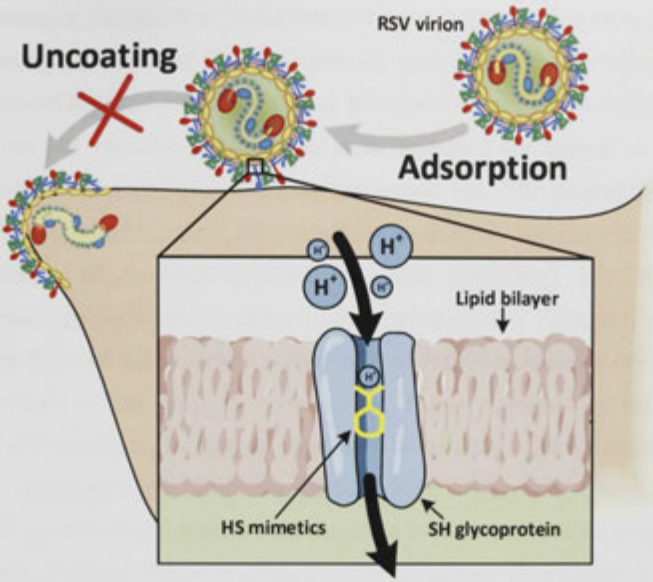


Figure 5.18. Proposed mechanism of heparan sulfate (HS) mimetics induced inhibition of respiratory syncytial virus (RSV) replication at low HS concentration

RSV small hydrophobic (SH) protein acts as a proton-selective ion channel protein, transporting protons (H^+) to the inside of the RSV virion. At low concentrations HS mimetics bind protons while inside the SH-proton channel, thereby blocking the protons from entering the virion, inhibiting uncoating and release of free ribonucleoproteins into the cytoplasm. Adapted from (Focosi, 2001-2013).

Previous studies have shown that mucosal heparin, dextran sulfate, fucoidan and/or lambda carrageenan have good antiviral activity against RSV, hepatitis C, human T-lymphotropic virus type-1, HIV, influenza A and Newcastle disease virus (Araya *et al.*, 2011; Crim *et al.*, 2007; Elizondo-Gonzalez *et al.*, 2012; Hayashi *et al.*, 2013; Ito *et al.*, 1987; Malhotra *et al.*, 2003; Mori *et al.*, 2012). Furthermore, studies have found that fucoidans and carrageenans can exhibit the antioxidant activity and that this is related to the molecular weight and sulfate content of fucoidan. The *in vitro* model suggested that the ratio of sulfate content/fucose was an effective indicator to antioxidant activity of the samples (Rocha de Souza *et al.*, 2007; Wang *et al.*, 2008). As antioxidants are the compounds, which terminate the attack of reactive species and reduce the

risk of diseases, the fact that fucoidans exhibit this activity is a very positive role in decreasing the effect of RSV infection on lung pathology. In conjunction with *in vitro* results detailed in table 5.5 and the current literature on relevant *in vitro* studies, six HS mimetic compounds were selected for testing in an *in vivo* mouse model. The compounds selected were: HS 230 (desulfated mucosal heparin-negative HS control), HS 254 (dextran sulfate- positive HS control), HS 228 (variation of mucosal heparin), HS 259 (fucoidan), HS 267 (lambda carrageenan) and HS 424 (variation of mucosal heparin). Table 5.5 summarises the *in vitro* RSV inhibition levels across all four tested conditions and at all three concentrations of the selected compounds. These compounds, excluding the negative and positive HS control, represent a selection of four chemically (e.g. sulfation, carboxylation) and structurally (e.g. open or closed ring, size difference (3 or 200kDa) different compounds which may provide insight into the importance of these characteristics in viral inhibition. This study is the first to assess HS mimetics antiviral potential against RSV *in vivo*.

Table 5.5. *In vitro* inhibition levels of selected HS mimetics for *in vivo* study across all four conditions

	Average RSV inhibition levels (%)						
Test conditions	**Heparan sulfate (HS) concentrations**	**HS 230**	**HS 254**	**HS 228**	**HS 259**	**HS 267**	**HS 424**
Condition A	5 µg/mL	8	59	74	91	100	93
	20 µg/mL	8.5	74	63	99	100	89
	100 µg/mL	22	97	70	98	100	74
Condition B	5 µg/mL	2	80	65	96	67	72
	20 µg/mL	0	78	78	98	100	78
	100 µg/mL	4	76	76	96	100	87
Condition C	5 µg/mL	-8.7	57	83	85	97	67
	20 µg/mL	13	76	39	91	72	79
	100 µg/mL	0	83	74	87	92	65
Condition D	5 µg/mL	-19.5	2.1	24	22	53	22
	20 µg/mL	-24	0	0	22	35	30
	100 µg/mL	-11	83	63	25	52	65

Greyed out areas denote the best RSV inhibition levels for the corresponding HS compounds at the specified concentration under the specified test condition.

In vivo post (therapeutic) RSV infection treatment with HS 228 (carboxyl reduced mucosal heparin) and HS 259 (fucoidan) has resulted in a statistically significant reduction ($p < 0.05$) in RSV titres compared to the RSV inoculated saline treated group (positive control) (Figure 5.8). These results demonstrate HS 228 and HS 259 antiviral activity against RSV and potential involvement in competitive as well as cell-to-cell spread inhibition. It is hypothesised that these compounds could be used as therapeutic candidates utilising a proposed mechanism of competitive inhibition previously described. These results are in agreement with earlier *in vitro* studies which have demonstrated the importance of HS compounds in RSV inhibition, with compounds such as heparan sulfate, dextran sulfate, heparin and chondroitin sulfate B inducing effective RSV inhibition (Hallak *et al.*, 2000a; Hallak *et al.*, 2007; Hallak *et al.*, 2000b; Karger *et al.*, 2001). In addition, a study using dengue virus and flaviviral encephalitis mouse model found that PI-88 (a mixture of highly sulfated, monophosphorylated mannose oligosaccharides) had a significant and beneficial effect in disease outcome (Lee *et al.*, 2006).

Although, post RSV infection treatment with HS mimetics resulted in only two HS compounds effectively reducing RSV infection, their performance *in vivo* is in agreement with previously suggested trend extrapolated from the HS *in vitro* studies (previously described in Tables 5.4 and 5.5). HS 228 (decarboxylated mucosal heparin) belongs to group 2 of HS mimetics tested *in vitro*. Group 2 HS mimetics were found to perform, on average, better under condition D *in vitro* (RSV pre-incubation with a cell monolayer for 1 hour) which is closely related to post RSV infection HS treatment *in vivo*, in terms of experimental set up. The *in vitro* proposed trend translated well to *in vivo* study showing that HS 228 (group 2 HS mimetic) induced a reduction in RSV titres following post RSV infection treatment with HS 228. However, HS 259 (fucoidan) belongs to group 3 of HS mimetics tested *in vitro*. Group 3 HS mimetics were proposed to perform the best under conditions A (RSV 10 minute pre-incubation with HS mimetics) and B (HS mimetic 10 minute pre-incubation with cell surface), while not so well under C (short RSV pre-incubation with cell surface) and D (1 hour RSV pre-incubation with cell surface). *In vivo* HS 259 performed well, inducing statistically significant reduction in viral tires following post RSV infection HS 259 treatment; however this unexpected result, demonstrates that some HS compounds individual antiviral effect may deviate

from the group classification proposed by *in vitro* findings. HS 254 (group 5), HS 267 (group 4) and HS 424 (group 2) did not induce statistically significant reduction in viral titres. Based on the *in vitro* study proposed trend, this was an expected result for HS 254 and HS 267, however it was unexpected finding for HS 424 which belongs to group 2 of HS mimetics and was predicted to be more effective. A plausible explanation for this finding could be related to HS 424 chemical structure. HS 424 has a glycol split and there is a lot to be noted about a glycol split modification on any compound. Firstly, most of the attempts to modulate protein binding and biological properties of heparin have been made by the modulation of the sulfation patterns of the GAG backbone and enhance of chain flexibility of the GAG by the glycol splitting of C2-C3 bonds of nonsulfated GlcA and IdoA residues. Both the degree of sulfation (charge density) and the appropriate distribution of N-sulfate and N-acetyl groups (charge distribution) along the heparin molecule is determinant in its biological properties (Vlodavsky *et al.*, 2007). For example, the strongest protein binding was observed for 'fully sulfated' heparin and extra-sulfate groups potentiated this interaction. IdoA-containing sequences are considered to facilitate the appropriate orientation of substituents for the suitable interaction (Casu *et al.*, 2002a; Casu *et al.*, 2002b). Additional local flexibility was obtained by glycol splitting that act as flexible joints along the heparin chain were conformation changes can be induced by the protein interaction. Chain flexibility is thought to play a key role in heparin-protein interactions (Ritchie *et al.*, 2011). The most important result of glycol split modification is that glycol split of heparin drastically reduces the anticoagulant activity normally associated with heparin. (Conrad and Guo, 1992; Vlodavsky *et al.*, 2007). Taking all of these findings together, the benefits of glycol split are apparent and maybe HS 424 inactivity is simply a result of HS 424 inability to act as a treatment for RSV infection. However, HS 424 structure may be more favourable for prophylactic treatment.

On the other hand, prophylactic HS treatment of the RSV inoculated group, with HS 228 (carboxyl reduced mucosal heparin), HS 259 (fucoidan), HS 267 (lambda carrageenan (200kDa) or HS 424 (mucosal heparin with glycol split (3kDa) induced a statistically significant reduction ($p < 0.05$) of RSV viral titres compared to the positive control (Figure 5.13). Prophylactic treatment with HS mimetics resulted in all tested compounds demonstrating their antiviral potential by, either, HS mimetic interaction with the HS at the cell surface or HS

mimetics interaction with RSV glycoproteins. Characterising the proposed mechanisms could help determine which of the tested HS mimetic has the potential to become prophylactic RSV infection treatment candidate. These results are supported by chapter 5 *in vitro* findings, with each of the tested HS mimetics also performing very well *in vivo*. Condition B (HS mimetic 10 minute pre-incubation with cell surface) experimental set up is equivalent to the one for prophylactic HS treatment of RSV infection. Enhanced RSV inhibition achieved following prophylactic treatment of RSV infection is possibly due to the HS mimetics interaction with the cell surface HS where HS mimetics were not required to compete for the binding site, it was available to them prior to RSV infection. Structurally, HS 259 and HS 267 belong to groups 3 and 4, respectively, and as proposed by *in vitro* results, these are the best suited for prophylactic treatment. HS 254 belongs to group 5 of HS mimetics, which remained uncharacterised; however, in the *in vivo* model, HS 254 appears to be promising prophylactic treatment candidate. Finally, HS 228 and HS 424 belong to group 2 of HS mimetics, which was found to perform the best as a therapeutic for RSV infection during an *in vitro* study, however, *in vivo*, HS 228 and HS 424 were found to perform well as prophylactic candidates against RSV infection. As suggested earlier HS 424 efficacy as prophylactic compound may be due to its open ring structure, however HS 228 has proven to be HS compound performing well as a therapeutic treatment following RSV infection and as prophylactic treatment of RSV infection. HS 228 should be tested further *in vivo* at different concentrations to elucidate its antiviral ability against RSV.

Table 5.6 compares the *in vitro* and *in vivo* findings. It outlines RSV inhibition levels induced by HS 230, HS 254, HS 228, HS 259, HS 267 and HS 424 following their *in vivo* treatment of RSV infection compared to condition D *in vitro* and prophylactic *in vivo* HS treatment of RSV infection compared to condition B *in vitro*. The comparison of *in vitro* and *in vivo* results is possible as the *in vitro* concentration of 100 µg/mL is equivalent to the 10 mg/mL/kg HS concentration used *in vivo*. The result analysis revealed an emerging trend from the *in vitro* study allowing prediction of HS mimetic inhibition of RSV *in vivo* by taking a 50% of its *in vitro* inhibition value. This trend could be used to determine the most suited compound for *in vivo* study based on its *in vitro* results, hence reducing the number of compounds required for *in vivo* testing. In contrast, *in vitro* anti-flaviviral effectiveness of the HS mimetics did not reliably

predict their *in vivo* therapeutic activity (Lee *et al.*, 2006) suggesting that caution must be exercised when eliminating compounds at the *in vitro* stage.

Table 5.6. Comparison of *in vitro* and *in vivo* RSV inhibition

		Average RSV inhibition levels (%)					
Test conditions	**Heparan sulfate (HS) concentrations**	**HS 230**	**HS 254**	**HS 228**	**HS 259**	**HS 267**	**HS 424**
Post RSV infection HS treatment/ ***Condition D***	10 mg/mL/kg	-1.7	0	37	38	24	19
	100 µg/mL	-11	83	63	25	46	87
Prophylactic HS treatment of RSV infection/ ***Condition B***	10 mg/mL/kg	0	37	54	52	52	48
	100 µg/mL	4	76	76	96	100	87

Post RSV infection treatment with HS mimetics resulted in measured immune response in BALF (Figure 5.9) and blood (Figure 5.10), leading to reduction in combined HPS (Figure 5.12). The measured immune response observed here, suggests HS mimetics immunomodulatory effect on RSV viral load while preventing immune overreaction. These results are supported by findings that sulfated polysaccharides, have been shown to possess immunomodulatory activities that may be of potential application in stimulating the immune response or in controlling immune cell activity to mitigate associated negative effects such as inflammation (Chen *et al.*, 2008; Parish *et al.*, 2001). Fucoidans and carrageenans specifically have been found to be involved in immune modulation, playing a very positive role in inflammation (Blondin *et al.*, 1994; Senni *et al.*, 2006). Therefore, further investigation into the mechanism behind this immune modulation is required to gain better understanding of, the HS effect on the immune response and targeted immune response using HS mimetics.

Prophylactic treatment of RSV infection demonstrates for the first time that prophylactic treatment with HS mimetics results in a similar immune response as observed following the RSV re-infection in the absence of HS mimetics (chapter 4). Consequently, it can be hypothesised that prophylactic HS treatment induces innate immune response, which is followed by adaptive immune activation once RSV inoculation is administered. This study has not

characterised which adaptive immune cells are present following the prophylactic HS treatment of RSV infection; as such, this hypothesis is solely based on an increase in lymphocyte numbers, in BALF and blood. These results also differ from those observed in a previous section on post RSV infection HS treatment, where a decrease in lymphocyte numbers present in BALF was observed (Figure 5.14). Finally, prophylactic treatment of RSV infection with HS 259 and HS 267 resulted in significant increase ($p < 0.0001$) in combined HPS compared to the positive control; while HS 288 prophylactic treatment of RSV infection resulted in a decrease ($p < 0.05$) in combined HPS compared to the positive control (Figure 5.17). This finding contributes to ever growing evidence that an increase in eosinophil infiltration in BALF does not contribute to combined HPS. HS 228 prophylactic treatment of RSV infection resulted in an increase in eosinophil numbers present in BALF while it reduced combined HPS. This is a novel finding demonstrating HS mimetics effect on RSV infection *in vivo* and immune modulation.

In addition, *in vivo* findings indicate for the first time that HS may have a direct effect on immune response even in the absence of viral infection. A pre-primed immune response may lead to more efficient viral clearance and may be a useful treatment for infants and elderly who may be highly susceptible to viral infection and could benefit from already activated immunity. Also, groups like HIV patients, medical staff working in infectious disease units and immunodeficient patients (e.g. lacking IgA) could benefit from this treatment, as it can be administered as a preventative measure. Currently, some therapies are already in use as prophylactic treatments for asthma, multiple sclerosis and other diseases. XOLAIR (omalizumab) is a monoclonal antibody targeting circulating IgE, preventing them from making a contact with its receptor on the effector cell. XOLAIR is recommended for people with allergic asthma who still have asthma symptoms even though they are taking inhaled steroids and it seems to work well (Norman *et al.*, 2013). Also, the therapy of relapsing-remitting multiple sclerosis is also based on the use of immune-modulating treatments with drugs such as, IFN-β, glatiramer acetate, natalizumab etc. (Clerico *et al.*, 2008). As demonstrated, immune modulating treatment is widely used and HS mimetics pose as potential immune modulating candidate to be used in RSV infection while HS production costs are kept low.

It has been reported that efficient RSV infection requires the intact G protein (Kwilas *et al.*, 2009). However, most laboratory-derived RSV stock, including the stock used in this study, is propagated through Vero cells which, according to Kwilas *et al.* (2009), results in the formation of truncated G protein (Kwilas *et al.*, 2009). The truncated form of the G protein is believed to be dysfunctional in terms of viral attachment to the cell surface HS based on the *in vitro* findings (Kwilas *et al.*, 2009). This loss of attachment function is believed to also result in poor infection initiation *in vivo*, potentially negatively impacting on both animal experiments and attenuated-vaccine studies with volunteers (Kwilas *et al.*, 2009). This limitation was not directly addressed in this present study, however it should be noted that RSV infection in the mouse model was achieved and this was consistent with other studies using a laboratory derived viral stock (Tripp *et al.*, 2000; Waris *et al.*, 1996). Currently there are no *in vivo* studies that have compared truncated and non-truncated virus forms, therefore, repeating the *in vivo* studies using a virus with a non-truncated form of G protein, would be required to assess the effect on viral attachment and RSV interaction with GAGs.

In conclusion, this is the first HS mimetic study that has examined and reported on 53 HS mimetic compounds antiviral activity against RSV *in vitro*. In addition, this study is the first to perform *in vivo* studies with HS mimetics using RSV mouse model, and compare post RSV infection HS treatment with prophylactic HS treatment of RSV infection. Together, findings from *in vitro* and *in vivo* studies demonstrate the antiviral potential of HS mimetics against RSV. HS 228 was found to be the best performing compound, both, as post RSV infection HS treatment and prophylactic treatment of RSV infection, while reducing RSV disease pathology. In addition, HS mimetics were found to activate an immune response even in the absence of viral infection, suggesting their potential in pre-emptive immune activation and immune modulation. The development of an effective and safe RSV vaccine has remained elusive to date, making HS mimetics true contenders in this race.

Chapter 6. General Discussion and Conclusions

6.1 INTRODUCTION

In 2010, RSV was found to be responsible for the majority of 2.8 million infantile lower respiratory tract infections (Lozano *et al.*, 2012). The virus infects nearly all children by three years of age and is a leading cause of infant hospitalisation and childhood wheezing (Hall *et al.*, 2009; Shay *et al.*, 1999). Globally, RSV accounts for 6.7% of deaths among infants, one month to one year old, which is more than any other single pathogen except malaria (Lozano *et al.*, 2012). The only intervention currently available is passive administration of the licensed monoclonal antibody palivizumab (Beeler and van Wyke Coelingh, 1989; Johnson *et al.*, 1997). However, this treatment is very expensive and is reserved for high risk patients only. Therefore, controlling RSV has remained a formidable challenge and more work is urgently required, to understand the role of innate and adaptive immunity in RSV infection and in the development of new therapeutics.

6.2 RESEARCH OUTCOMES

6.2.1 Effect of RSV infection on eosinophil biology

Since, the first FI-RSV vaccine trial yielded disastrous results with young recipients, pulmonary eosinophilia elicited in response to primary RSV infection became the hallmark of RSV disease (Chin *et al.*, 1969; Fulginiti *et al.*, 1969; Rosenberg *et al.*, 2009; van Hagen *et al.*, 1999). Subsequent FI-RSV studies have led to the conclusion that eosinophils contribute to the pathological cascade of RSV disease and their presence was deemed undesirable (Graham *et al.*, 2000; Hancock *et al.*, 1996; Openshaw *et al.*, 2001; Tripp *et al.*, 2000; van Hagen *et al.*, 1999; Varga *et al.*, 2001). In the late 1990s, several studies have focused on elucidating the beneficial role of eosinophils in viral infection (Adamko *et al.*, 1999; Domachowske *et al.*, 1998a; Phipps *et al.*, 2007; Rosenberg and Domachowske, 2001) which was further elucidated in the chapter 3 *in vitro* study.

Eosinophil treatment with RSV has resulted in eosinophil activation as evaluated by degranulation, activation and migration assays. The eosinophil degranulation study revealed RSV-induced increase in MBP release (Figure 3.3) suggesting that MBP release results from the targeted activation of

eosinophils as opposed to a cellular degeneration process. Moreover, RSV treatment of eosinophils also resulted in increased eosinophil activation (Figure 3.5) and activation of the eosinophil cytoskeleton, as illustrated by α-tubulin and F-actin (Figure 3.6 and 3.7). These results suggest that eosinophil migration and recruitment may also result from a targeted response as opposed to a nonspecific immune response. Together these findings contribute to the hypothesis that an effective viral clearance may be determined by the activation of targeted cellular response when and where required. This approach would yield controlled immune response with a very specific purpose - viral clearance. Earlier studies are in agreement with chapter 3 degranulation findings as high levels of ECP were also found present in the lung epithelial tissue culture (Olszewska-Pazdrak *et al.*, 1998b) and nasopharyngeal secretions of human infants (Saito *et al.*, 1997). Similarly, RSV-induced eosinophil activation results confirm finding by Phipps *et al.* (2007) who also showed an increase in EPO as a result of RSV activation of eosinophils (Phipps *et al.*, 2007), demonstrating RSV-induced eosinophil activation. The current migration study is the first to report on the RSV-induced activation of the eosinophil cytoskeleton.

The RSV kinetics study revealed the potential for eosinophils to induce viral inhibition, with a reduction in viral titres observed over 0-24 hours period (Figure 3.4). In contrast, previous studies have revealed that RSV can infect human eosinophils while PVM was shown to infect murine eosinophils (Dyer *et al.*, 2009; Kimpen *et al.*, 1996); however, this was not the case following a treatment of murine eosinophils with RSV. This contradictory *in vitro* finding could be a result of incompatibility between RSV as a human respiratory pathogen and murine eosinophils. However, the mechanism that provides protection for mouse eosinophils against RSV infection, may offer a novel insight into prevention of human infection and as a result, it should be explored further. Finally, RSV treatment of eosinophils has demonstrated for the first time that RSV has detrimental effect on eosinophil morphology over time (0, 3, 6, 12, 18 and 24 hours) (Figure 3.1 and Figure 3.2). This was confirmed by the continuous release of cytotoxic proteins and loss of cellular integrity over the 24 hour incubation period. Previous findings by Phipps *et al.* (2007) are in agreement with described RSV-induced eosinophil morphological changes, as Phipps *et al.* (2007) have also showed that a ten minute treatment of eosinophils with ssRNA results in eosinophil morphological changes as

demonstrated by a loss of eosinophil crystal core content (Phipps *et al.*, 2007). Current study demonstrates for the first time that RSV treatment has an increasingly detrimental effect on eosinophil morphology over time, with apoptosis becoming a hallmark of RSV-induced effect on eosinophil structure at 24 hours post incubation. This finding supports chapter 3 results highlighting that eosinophil response to RSV is a targeted response to viral infection, with focus on viral clearance through the release of eosinophil cytotoxic proteins.

6.2.2 The role of innate and adaptive immunity in RSV infection and re-infection

RSV infection in the first two years of life is believed to lead to long-term deficiencies in protective immunity to RSV (Welliver, 2003). Inadequate immunity in response to RSV re-infection leads to limited viral clearance (Welliver *et al.*, 2008) characterised by the infiltration of NK cells and subsequent recruitment of helper $CD4^+$ and cytotoxic $CD8^+$ lymphocytes to the site of infection (Olson *et al.*, 2008; Olson and Varga, 2007; Stevens *et al.*, 2009). Although RSV-specific $CD8^+$ T cells can provide protection against infection, the effect is short-lived (Connors *et al.*, 1991; Kulkarni *et al.*, 1995). Therefore, further investigation into long lasting RSV adaptive immune response is urgently required, leading to more effective vaccine development.

Primary infection and re-infection study results confirm eosinophil involvement in RSV clearance as demonstrated by a reduction in viral titres following RSV infection of eosinophil overproducing mice (IL-5 Tg BALB/c) (Figures 4.2 and 4.7). Previous studies by Phipps *et al.* (2007) had also demonstrated accelerated RSV clearance from the lungs of the IL-5 Tg BALB/c (Phipps *et al.*, 2007). Our current studies imply that eosinophil antiviral activity may be modulated through the availability of T cells and/or T cell counterparts such as ILC2 cells in the immune system. This was especially evident with Rag $2^{-/-}$ mice, lacking T and B cells, where eosinophil recruitment was regulated by the CD2 expressed on the T cell surface. The absence of T cells in the Rag $2^{-/-}$ humanised model has resulted in a reduced eosinophil recruitment into the BALF and blood following RSV infection and re-infection. In addition, this issue appears somewhat overcome in the IL-5 Tg Rag $2^{-/-}$ mice, through the eosinophil regulation via most likely, ILC2 cells IL-5 production. Recent study by

Roediger *et al.* (2013) has shown that both NK and ILC2 cell express CD2, required for eosinophil recruitment (Roediger *et al.*, 2013). Adoptive transfer of $CD4^+$ cell into adaptive immunity deficient mice induced a reduction in RSV infection (Figure 4.7). An increase in eosinophil tissue infiltration was observed following $CD4^+$ adoptive cell transfer, correlating to the viral clearance observed (Figure 4.11). These results suggest that $CD4^+$ cells are required for eosinophil recruitment following RSV re-infection, demonstrating eosinophil potential to assist in viral clearance. Although the adoptive transfer of $CD4^+$ cells assisted in RSV re-infection viral titres reduction, their presence was found to exacerbate RSV disease as demonstrated by an increase in combined HPS. RSV disease pathology observed appears to be due to $CD4^+$ cell transfer as oppose to eosinophil tissue infiltration. This is in agreement with previous antibody depletion study of mouse lymphocytes which revealed that the $CD4^+$ and $CD8^+$ T lymphocyte contribute individually to clearing of the RSV infection, but that both also contribute to disease, especially $CD8^+$ T cells (Graham *et al.*, 1991). Furthermore, several studies have shown that RSV infection alters functionality of T lymphocytes (Fulton *et al.*, 2008; Gray *et al.*, 2005) resulting in inadequate immune response and low T lymphocyte numbers. The impaired function of T lymphocytes in the lung during virus infection has been proposed to be the consequence of virus-induced changes in the lung environment (Gray *et al.*, 2005) as well as virus- induced immune evasion tactics (Lotz and Peebles, 2012; Moreau *et al.*, 2003). Future RSV immunisation strategies should strive to achieve a balanced immune response.

6.2.3 HS treatment of RSV infection

It is well documented that the interaction between RSV and cell surface HSPGs are required for RSV attachment and entry into host cells, consequently it is a preferential target for the development of antiviral compounds against RSV (Feldman *et al.*, 2000). It is not clear, however, what type of GAGs and GAG components are involved, whether the important GAGs are on the virus (Bourgeois *et al.*, 1998) or the target cell (Krusat and Streckert, 1997), and the magnitude of their contribution to the infection (Hallak *et al.*, 2000a). Nevertheless, an effective and inexpensive treatment against RSV is urgently required. In 2012, motavizumab, a variant of palivizumab, was denied approval by the U.S. FDA due to concerns about safety and allergic reactions (Donalisio

et al., 2012). Currently, no vaccine for RSV is available. Considering the high burden of RSV disease worldwide on both the population and healthcare systems, further antiviral research and development is critical (Storey, 2010). HS mimetics have a great potential as antiviral candidates. Unlike previously tested drugs, which were found to be unsafe and very expensive, HS mimetics are highly effective against RSV as demonstrated by current study findings and with no/low cellular toxicity and the low cost of production. Therefore, HS mimetics could be the antiviral therapeutics that we have been waiting for the last six decades.

The incubation of RSV with HS mimetics *in vitro* for 10 minutes (condition A), pre-incubation of HS mimetics with Vero E6 cell monolayer for 10 minutes (condition B) and a short incubation of RSV with the Vero E6 cells (condition C) have resulted in 20 out of 53 tested HS mimetics inducing 80-100% RSV inhibition ($p < 0.005$) (Figures 5.4-5.6). The best performing compound under conditions A, B and C was HS 267 (lambda carrageenan), inducing 90-100% RSV inhibition. These results are in agreement with previous studies who reported that pre-incubation of RSV with certain GAGs (e.g. heparin) inhibited virus infectivity (Krusat and Streckert, 1997; Martinez and Melero, 2000) as well as that HEp-2 cells pre-treatment with GAGs, prior to RSV inoculation, results in reduced efficiency of RSV infection (Donalisio *et al.*, 2012; Donalisio *et al.*, 2010; Hallak *et al.*, 2000b). These results suggest that there may be a mechanism behind the interaction of HS mimetics with RSV glycoproteins (G or F) with formation of RSV glycoprotein - HS complex resulting in reduction of RSV cell infectivity. In addition, HS mimetics interaction with HS on the cell surface may involve HS mimetic binding to the HS on the cell surface. Consequently the cell surface HS would become unavailable for RSV glycoprotein interaction resulting in RSV inhibition. The proposed mechanisms require further research to elucidate the exact interaction between HS mimetics and RSV as well as HS mimetics and HS on the cell surface; enabling a more specific and targeted RSV inhibition and production of therapeutic and/vaccine candidates with much specified role. Under final testing condition, pre-incubation of RSV with the Vero E6 cell monolayer for 1 hour (clinically the most relevant condition, condition D), resulted in 15 out of 53 HS mimetic compounds inducing 50-90% RSV inhibition (Figure 5.7). The best performing compound under condition D was HS 424 (mucosal heparin with glycol split 3kDa), which

induced 90% RSV inhibition. Previous studies have shown that sulfated polysaccharides can mimic HS chains and interact with viral glycoproteins, blocking viral attachment to cell surface HS through competitive inhibition (Rusnati and Urbinati, 2009; Rusnati *et al.*, 2009). Other studies have also shown that pre-incubation of RSV with heparin inhibited virus infectivity (Krusat and Streckert, 1997; Martinez and Melero, 2000). These results demonstrate HS mimetic antiviral potential, specifically in competitive RSV inhibition. Characterisation of the best performing compounds is essential for the elucidation of HS mimetics with competitive inhibition potential are fully elucidated *in vivo* followed by clinical trials if found to be successful as RSV antiviral therapeutics.

It is important to note that some HS compounds performed best at the highest (100 µg/mL) and/or lowest (5 µg/mL) HS concentrations, whilst other were unaffected by change in HS concentration (Figures 5.4-5.7). *In vitro* study analysis revealed that 10 out of 53 tested HS compounds achieved an average of 65-90% RSV inhibition across the four test conditions at the lowest, 5 µg/mL, HS concentration. It was proposed that a mechanism behind RSV inhibition following a HS treatment at low HS concentrations involved a proton-selective ion channel protein. In 2008, Gan *et al.* (2008) provided experimental evidence that the transmembrane domain of SH protein forms pentameric α-helical bundles that form cation-selective ion channels in planar lipid bilayers (Gan *et al.*, 2008). This suggests that SH may act as a proton-selective ion channel protein, transporting protons from the outside to the inside of the RSV virion. At low concentrations, HS mimetics could bind protons while inside the SH-proton channel, thereby making it impossible f0or protons to flow inside the virion. As a result, RSV uncoating would be blocked and viral replication would be inhibited (Figure 5.18). In contrast, in some instances high concentrations of HS mimetics have resulted in enhanced viral replication *in vitro*. This finding is hypothesised to be a result of HS mimetics acting as protons themselves at high concentrations, with an increase in proton flow towards the virion enabling viral uncoating and viral replication. The current study highlights the importance of dose dependant viral inhibition and the need to evaluate a range of concentrations, including lower concentrations essential in development of any therapeutic. Figure 6.1 summarises proposed mechanisms of RSV inhibition discussed so far.

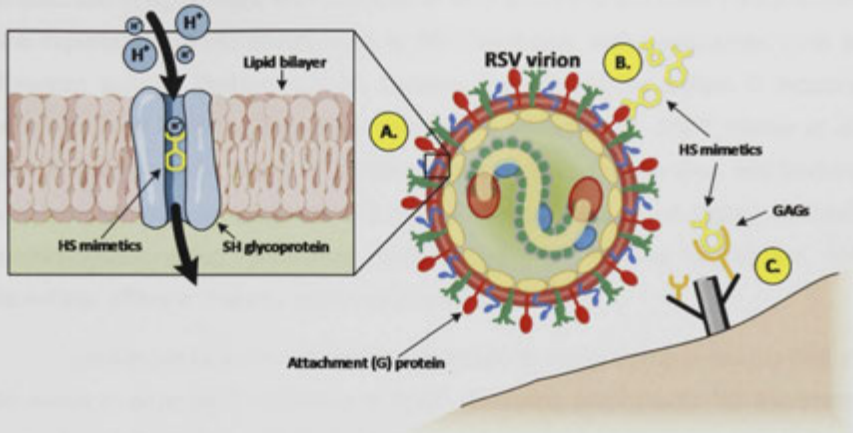


Figure 6.1. Heparan sulfate (HS) mimetics inhibition of respiratory syncytial virus (RSV) infection initiation

A) HS mimetics block the influx of protons (H^+ ions) through the SH (small hydrophobic protein) - proton channel, inhibiting uncoating and release of free ribonucleoproteins into the cytoplasm; B) HS mimetics interaction with the RSV G attachment protein, preventing virus binding to the glycosaminoglycans (GAGs) on the cell surface; and C) HS mimetics bind to GAGs on the cell surface blocking the virus interaction with GAGs on the cell surface.

In vivo post (therapeutic) RSV infection treatment with HS 228 (carboxyl reduced mucosal heparin) and HS 259 (fucoidan) resulted in a statistically significant reduction ($p < 0.05$) in RSV titres compared to the RSV inoculated saline treated group (positive control) (Figure 5.8). These results demonstrate HS 228 and HS 259 antiviral role in RSV infection with potential involvement in competitive and cell-to-cell spread inhibition. Prophylactic HS treatment of RSV inoculated group, with HS 228 (carboxyl reduced mucosal heparin), HS 259 (fucoidan), HS 267 (lambda carrageenan (200 kDa)) or HS 424 (mucosal heparin with glycol split (3kDa)) induced a statistically significant reduction ($p < 0.05$) of RSV viral titres compared to the positive control (Figure 5.13). This finding demonstrates HS mimetic antiviral potential again and it could be a result of either, HS mimetic interaction with the HS at the cell surface or the interaction of HS mimetics with RSV glycoproteins. Characterising the proposed mechanisms could help determine which of the tested HS mimetics have the potential to become prophylactic RSV infection treatment candidates. These

results are in agreement with previous *in vitro* studies which have demonstrated the importance of HS compounds in RSV inhibition, with compounds such as heparan sulfate, dextran sulfate, heparin and chondroitin sulfate B inducing effective RSV inhibition (Hallak *et al.*, 2000a; Hallak *et al.*, 2007; Hallak *et al.*, 2000b; Karger *et al.*, 2001). In addition, a study using dengue virus and flaviviral encephalitis mouse model found that PI-88 (a mixture of highly sulfated, monophosphorylated mannose oligosaccharides) had a significant and beneficial effect in disease outcome (Lee *et al.*, 2006).

Leukocyte recruitment results contribute to the growing evidence that an increase in eosinophil infiltration in BALF (following prophylactic HS treatment) or in blood (following post RSV infection HS treatment) does not contribute to the combined HPS. Post RSV infection HS treatment with HS 259, HS 267 and HS 424 has resulted in a reduced ($p < 0.0001$) combined HPS compared to the positive control (Figure 5.12) while HS 228 prophylactic treatment of RSV infection resulted in a reduced combined HPS. This novel finding supports the antiviral role of HS mimetics in RSV infection *in vivo*, as well as highlighting their potential role in immune modulation. A pre-primed immune response may contribute to more effective viral clearance in high-risk patients such as infants and the elderly. Also, HIV patients, medical staff working in infectious disease units and immune-deficient patients (eg. lacking IgA) could benefit from this treatment, as HS mimetics could be administered as a preventative measure. Currently, there are some therapies that are used as prophylactic treatments (for asthma, multiple sclerosis etc.). XOLAIR (omalizumab) is a monoclonal antibody targeting circulating IgE, preventing IgE from making a contact with its receptor on the effector cell. XOLAIR is recommended for people with allergic asthma who have atopic symptoms even though they are taking inhaled steroids (Norman *et al.*, 2013). As demonstrated by the current studies, the use of HS mimetics as prophylactic therapeutics may provide a low cost and high efficacy treatment of RSV infection as well as prevention of subsequent airways disease.

In conclusion, this is the first HS mimetic study that has examined and reported on the anti-viral activity of 53 HS mimetic compounds *in vitro* against RSV. In addition, this study is the first to perform *in vivo* studies with HS mimetics using RSV mouse model, and compare post RSV infection HS

treatment with prophylactic HS treatment of RSV infection. Together, findings from *in vitro* and *in vivo* studies demonstrate the anti-viral potential of HS mimetics against RSV with HS 228 characterised as the best performing compound for post RSV infection HS treatment and prophylactic treatment of RSV infection, while reducing RSV disease pathology. In addition, HS mimetics were found to activate immune response even in the absence of viral infection, suggesting their potential in pre-emptive immune activation and immune modulation.

6.2.4 Vaccine candidates and therapeutic agents on the edge of success

Major barriers to vaccine development to date have included: the early age of primary RSV infection; the capacity of RSV to evade innate immunity; the failure of RSV-induced adaptive immunity to prevent re-infection; the history of RSV vaccine-enhanced disease; and the lack of an animal model fully permissive to human RSV infection (Graham, 2011). These biological challenges, safety concerns, and practical issues, have significantly impeded RSV vaccine development progress (Graham, 2011). Nevertheless, recently the pre-fusion state of RSV F protein was identified as a target of most RSV-neutralising activity in human sera; however, its metastability has hindered characterisation. To overcome this obstacle, a study has identified prefusion-specific antibodies that were substantially more potent than the prophylactic antibody palivizumab (McLellan *et al.*, 2013). This finding could potentially lead to successful vaccine development in the near future. In addition, a clinical-stage biopharmaceutical company, Okairos, has evaluated its RSV vaccine candidate in well-established preclinical models and found that it stimulates both a strong neutralising antibody and a robust T-cell response (Okairos, 2014). Okairos' vaccine candidate has also been shown to provide complete protection against RSV infection in challenge experiments in both cotton rats and neonatal calves, as well as having an excellent safety profile, both before and after challenge (Okairos, 2014).

Unfortunately, all of these vaccine candidates are antibody based and just like previously mentioned XOLAIR (omalizumab), their application may be limited to a certain age. Currently XOLAIR can be used only in children over 12

years of age, which is beyond the age of the high risk RSV patients (1 month- 2 years). Also, the production of these therapeutics remains costly as previously seen with palivizumab and therefore their application is likely to be highly restrictive and out of reach of the general community.

In contrast, the therapeutic use of HS mimetics has the potential to overcome the issues faced by previous monoclonal antibody-based vaccines. Firstly, HS mimetics are cheap and easy to produce. Results from this thesis demonstrate that HS mimetics induce viral clearance, *in vitro* and *in vivo*. In addition, HS mimetic have been shown to induce an immune response even in the absence of viral infection, suggesting that they may have a role in immune modulation. The fact that HS mimetics have the potential to be used in antiviral treatment is supported by patent submitted in 2013 for iota-carrageenan HS compound. In early 2013 a patent titled "Antiviral composition comprising a sulfated polysaccharide: iota-carrageenan" was submitted by Marinomed Biotechnologie GmbH and published on 27th of May 2013. In their patent Marinomed Biotechnologie GmbH suggest that iota-carrageenan can be used as an active antiviral ingredient in medication for the prophylactic or therapeutic treatment of a symptom, condition or disease caused by or associated with an infection by a respiratory virus selected from the group consisting of paramyxovirus (human parainfluenza virus (HPV) type 1, HPV type 2, HPV type 3, HPV type 4 and RSV), human influenza A virus, and adenovirus of subtype B. Their results show that iota-carrageenan reduces plaque formation of parainfluenza virus 3 in Hep-2 cells by 75% inhibition at 100µg/ml. Also, iota-carrageenan pre-treatment of HeLa cells results in 65% reduction of plaque formation of parainfluenza virus 3 at 400 µg/ml. Finally, they also show that iota-, kappa-, lambda-carrageenan and fucoidan induce reduce plaque formation of influenza A H5N1 virus in epithelial cells by 80% inhibition at 400 µg/ml (IFI CLAIMS Patent Services, 2012). These findings are supported by chapter 5 *in vitro* results where lambda- and iota-carrageenans were found to perform very well across all four testing conditions with approximately 60-100% RSV inhibition achieved. The *in vivo* analysis provided by the current studies further illustrates the anti-viral potential of HS mimetics. In summary, the *in vivo* results revealed HS 228 (carboxyl reduced mucosal heparin) as the best performing compound *in vivo* following by HS 424 (mucosal heparin with glycol split

(3kDa)). Further *in vivo* analysis of HS mimetics is required to elucidate their vaccine and therapeutic potential.

6.3 CONCLUSIONS

RSV is an important viral pathogen that causes significant morbidity, utilisation of health care resources, and impact on secondary disease processes. While there are significant biological and historical barriers that have delayed vaccine development, continued evaluation of new vaccine and therapeutic candidates, such as HS mimetics, indicate the potential for a safe and efficacious therapeutic treatment to reduce the disease burden induced by RSV. Research to-date highlights that the success of any therapeutic will hinge on the generation of an appropriate immune response within the patient, hence, continued research towards understanding the innate and adaptive immune responses involved in RSV infection and re-infection is essential.

Findings from this thesis have advanced current understanding and knowledge regarding RSV infection specifically in terms of the role of eosinophils in RSV infection, innate and adaptive immune responses to RSV as well as potential therapeutic candidates against RSV. The role of eosinophils in RSV infection has been expanded on with study findings suggesting that RSV is unable to infect mouse eosinophils and that RSV infection induces eosinophil activation resulting in eosinophil-mediated and targeted immune response. Innate and adaptive immune studies of RSV infection and re-infection advanced current knowledge on eosinophil antiviral potential under both experimental conditions. Furthermore, eosinophil recruitment is believed to be regulated through the expression of CD2 on the T cell surface. Limited eosinophil recruitment in the Rag $2^{-/-}$ model following RSV infection and re-infection is suggestive of T cell regulation. However, this regulation does not seem to be limited to T cells only, as recent study by Roediger *et al.* (2013) has shown that both NK and ILC2 cell express CD2, required for eosinophil recruitment (Roediger *et al.*, 2013). As both of these cells are present in the Rag $2^{-/-}$ model, it is possible that the delay in the eosinophil recruitment observed following RSV infection and re-infection of IL-5 Tg Rag $2^{-/-}$ mice, is a result of NK and ILC2 regulation of eosinophil recruitment in the absence of T cells. This novel finding could be immune cell regulation, instrumental in the early stages of life when dealing with underdeveloped immunity. *In vitro* and *in vivo* studies using HS

mimetics reported for the first time data from over 53 HS mimetic compounds demonstrating HS treatment results in RSV inhibition following a pre-incubation of RSV with HS mimetics, pre-incubation of HS mimetics with cell surface or pre-incubation of RSV with cell surface. HS mimetics also showed antiviral potential *in vivo* as a therapeutic and prophylactic treatment of RSV infection. Also, a mechanism for RSV inhibition at low HS mimetic concentrations was proposed to utilise interaction between using RSV SH protein and HS mimetics. Finally, novel findings that HS mimetics induce immune response in the absence of viral infection suggest their potential in immune modulation.

In conclusion, novel HS mimetics have been identified as potential prophylactic or post-infection therapeutic candidates for RSV infection. HS mimetics have been shown to have the potential to inhibit viral attachment directly or to induce an immune modulation response, further inhibiting the viral infection. A modulated immune response could be used to induce a targeted antiviral response with eosinophils demonstrating a therapeutic role in RSV infection and re-infection. However, in the absence of effective immunisation against RSV, the focus needs to remain on reducing prenatal and environmental risk factors, including prematurity, smoking and improved hygiene practices. In the meantime, search for RSV vaccine and effective therapeutic agent continues.

6.4 FUTURE WORK

Following the investigations described in this thesis, there are a number experiments that should be undertaken as part of the future work. As RSV, human respiratory pathogen was found to be unable to infect murine eosinophils, it was suggested that a mechanism that offers protection for mouse eosinophils against RSV infection should be examined further. Although, mouse and human eosinophils are similar, these cells generate different responses to RSV infection. To elucidate this mechanism, a FACS analysis of the eosinophil (human and mouse) cell surface receptors following the RSV treatment should be undertaken. This may identify a cell surface receptor(s) present and used by one type of eosinophil in eosinophil-RSV interaction that enables RSV infection.

Moreover, the contribution of eosinophils to RSV clearance appears to be of high importance and the modulation of eosinophil recruitment may be the

key to RSV inhibition. HS mimetics, in the absence of viral infection, have been found to induce immune modulation. Several experiments could assess this finding and fine-tune the immune response. Initially, an *in vitro* migration study can be done to assess the effect of HS mimetics on leukocyte chemotaxis. In addition, real time migration study can be performed using a TAXIScan machine examining eosinophil migration towards HS mimetics as an agonist. Furthermore, a dose dependant HS mimetics study could be performed to characterise the ability of HS mimetics to induce greater viral inhibition at lower concentrations as observed in *in vitro* studies.

As $CD4^+$ adoptive transfer into IL-5 Tg Rag $2^{-/-}$ BALB/c mice has resulted in the reduction of viral load, it would be useful to assess: if $CD4^+$ adoptive transfer into Rag 2-/- BALB/c; and $CD8^+$ adoptive transfer into Rag $2^{-/-}$ and IL-5 Tg Rag $2^{-/-}$ BALB/c mice would also, result in the same viral titre reduction. In addition, $CD4^+$ and $CD8^+$ adoptive transfer effect on lung pathology in response to RSV infection. This would be of interest as $CD8^+$ cells are believed to contribute to more RSV disease pathology compared to the $CD4^+$ cells. Furthermore, as eosinophils have been found to be regulated by T cells and potentially T cell innate counterparts, the focus of future studies should be on elucidating the cell population involved in the regulation process. Furthermore, any future work should also utilise assays described in chapter 3 (degranulation, activation and chemotaxis) which could assist in elucidating the difference in eosinophil response observed between primary and secondary RSV infection.

As mentioned in chapter 5, it is imperative to understand the interaction between RSV, HS mimetics and/ or HS at the cell surface, so this knowledge can be used and applied to vaccine and therapeutic designs. Therefore, wild type RSV could be assessed for its ability to utilise HS-mediated viral replication by performing an *in vitro* attachment experiments. These experiments would involve characterising RSV G or F protein interaction with HS at the cell surface by removing RSV receptors one at the time using WT BALB/c model and blocking RSV receptors. If results were promising from this study the next step should involve investing into developing genetically modified BALB/c mice lacking specific RSV receptors. Similar approach could be applied while characterising HS mimetics and cell surface HS interaction using WT BALB/c

model and blocking the cell surface receptors one at a time *in vivo*. These experiments would facilitate a better understanding of RSV attachment and fusion requirements, and hence, enable the development and characterisation of novel HS mimetics to inhibit RSV to target cell binding. In parallel, expanding on previous studies by Gan *et al.* (2008) through a series of structural studies aimed to characterise SH potential as cation-transport channel protein, would be worthwhile in elucidating the role of SH protein in RSV infection as well as RSV and HS interaction.

The HS mimetic studies, *in vitro* and *in vivo,* have generated a number of potential therapeutic/vaccine candidates. It was beyond the scope of this study to test all the candidates *in vivo;* however, future work should focus on characterising more HS mimetic compounds, specifically group 1 – modified mucosal heparins (~12kDa) with a glycol split (HS 204, HS 205), group 2 – modified mucosal heparin (3kDa) (HS 331, HS 332) and group 5 – other HS compounds (HS 238, HS 241, HS 242, PI-88 and Suramin) as these were found to be the most efficient at lower concentrations of 5 and/or 20 µg/mL across all four testing conditions. Therefore, these compounds should be tested further *in vitro* at 1 µg/mL and 0.1 µg/mL concentrations to assess if their RSV antiviral efficacy can be improved at these concentrations. These compounds should be tested in an *in vivo* model, as many of them are promising anti-RSV candidates. Furthermore, as HS mimetics were delivered i.p. in the *in vivo* studies, it is important to assess how much of the HS dose would actually make it to its destination, lungs. There appears to be several experimental approaches available and future work should focus on answering this crucial question arising from the chapter 5 findings. Experiments proposed could entail monoclonal antibody detection of HS present in lungs (David *et al.*, 1992), fluorophore labelling (Skidmore *et al.*, 2009) and/or radioactive isotope labelling. All of these would enable quantification of HS present in lungs.

Finally, it is worth repeating some of the studies presented in this thesis using RSV clinical isolates, to address the importance of truncated G protein. Clinical isolates express full length G protein and studies using these isolates could help alleviate any doubt inferred by G protein truncation following the RSV derivation in the laboratory.

APPENDIX 1

Buffered Distilled Water

Sorensons Buffer

- **Solution A:** 0.2 M sodium di-hydrogen orthophosphate (MW = 156 g/mol) =3.12 g in 100 ml distilled water
- **Solution B:** 0.2 M di-sodium hydrogen orthophosphate (anhydrous) (MW 142) = 2.83 g in 100 ml distilled water

pH 6.8 Stock Solution: 25.5 ml Solution A and 24.5 ml Solution B

Make up to 100ml with distilled water.

Working Solution: 50 ml Stock pH 6.8 diluted to 1 litre with distilled water.

APPENDIX 2

Transmission Electron Microscopy Protocol

1. Primary fixation: 2% glutaraldehyde in 0.1M sodium cacodylate for 2 hours
2. Buffer washes: 2 x 0.1M Sodium cacodylate for 15 minutes each
3. Secondary fixation: 1% Osmium textroxide for 1.5 hours
4. Buffer washes: 2 x 0.1M Sodium cacodylate for 15 minutes each
5. Samples were rinsed in distilled water twice for 5 minutes each
6. Dehydration in acetone
    - 15 minutes each in 30, 50, 70, 90, 95% acetone and then 3 x 100% acetone
7. Embedded in Spurr's resin as follows: 2 hours in 50:50 - resin: acetone, 2 hours in 100% resin, overnight in 100% resin, 2 hours in 100% resin, overnight in fresh 100% resin to embed at 70°C
8. Sections approximately 80nm thick were cut
    - Sections were stained with 2% uranyl acetate and Reynold's lead citrate
9. Samples were photographed using a Hitachi H7000 transmission electron microscope at 75kV

Appendix 3

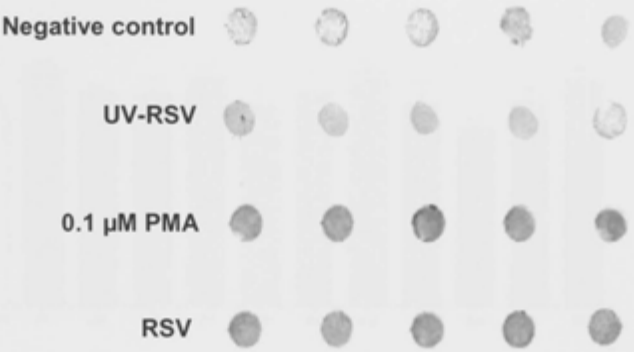
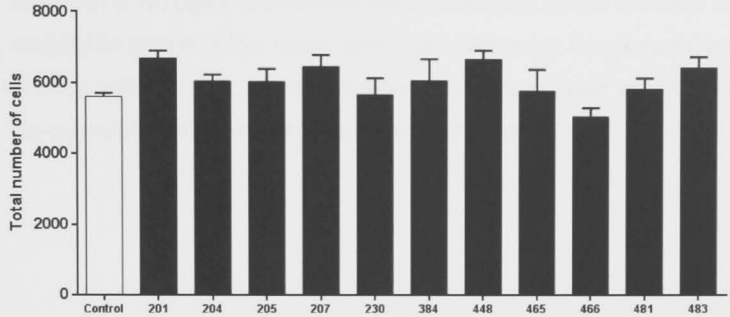


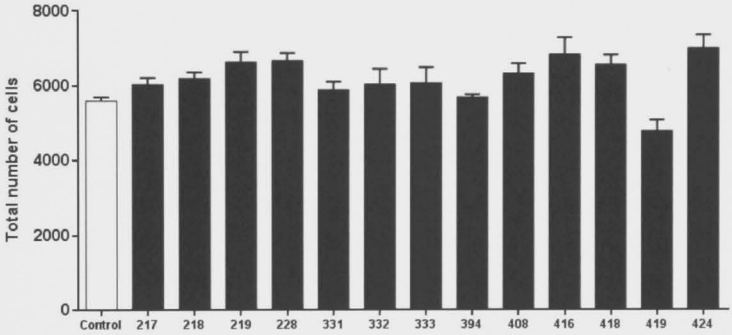
Figure A.1. Respiratory syncytial virus (RSV) induced eosinophil degranulation – dot blot membrane

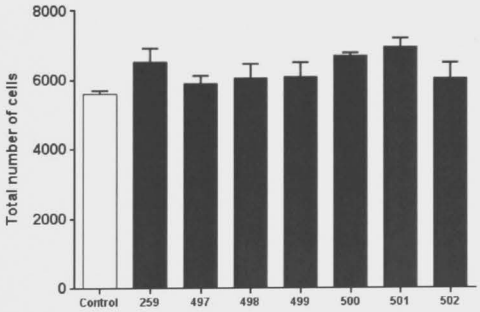
Freshly isolated eosinophils from interleukin-5 transgenic BALB/c mice were incubated with Vero E6 cell lysate (untreated eosinophils; negative control), ultraviolet-inactivated RSV (UV-RSV) (additional negative control), 0.1 μM phorbol myristate acetate (PMA) (positive control) or RSV at multiplicity of infection of 1 for 4 hours and assessed for eosinophil degranulation using major basic protein as a degranulation marker. Dots represent five individual samples tested for each sample type.

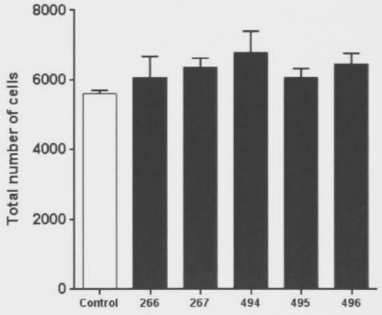
APPENDIX 4

Heparan sulfate cytotoxicity study









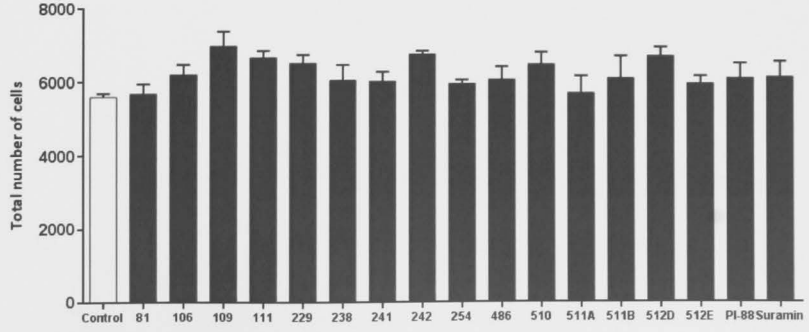


Figure A.2. Heparan sulfate (HS) treatment results in no cellular toxicity

Assay was prepared in 96-well plate with 6000 Vero E6 cells seeded per well. Once adhered to the plate, Vero E6 cell were treated with saline (negative control- white bar) or HS mimetics (black bar) at 100 µg/mL final concentration. Following the 24 hour incubation at 37°C, cells were analysed for signs of cellular toxicity using AlamarBlue assay. Samples are represented as a total of viable cells (n = 4) ± standard deviation. Data was analysed using ONE-way ANOVA with Dunnet multiple comparison test (GraphPad Prism version X7).

Appendix 5

Condition A

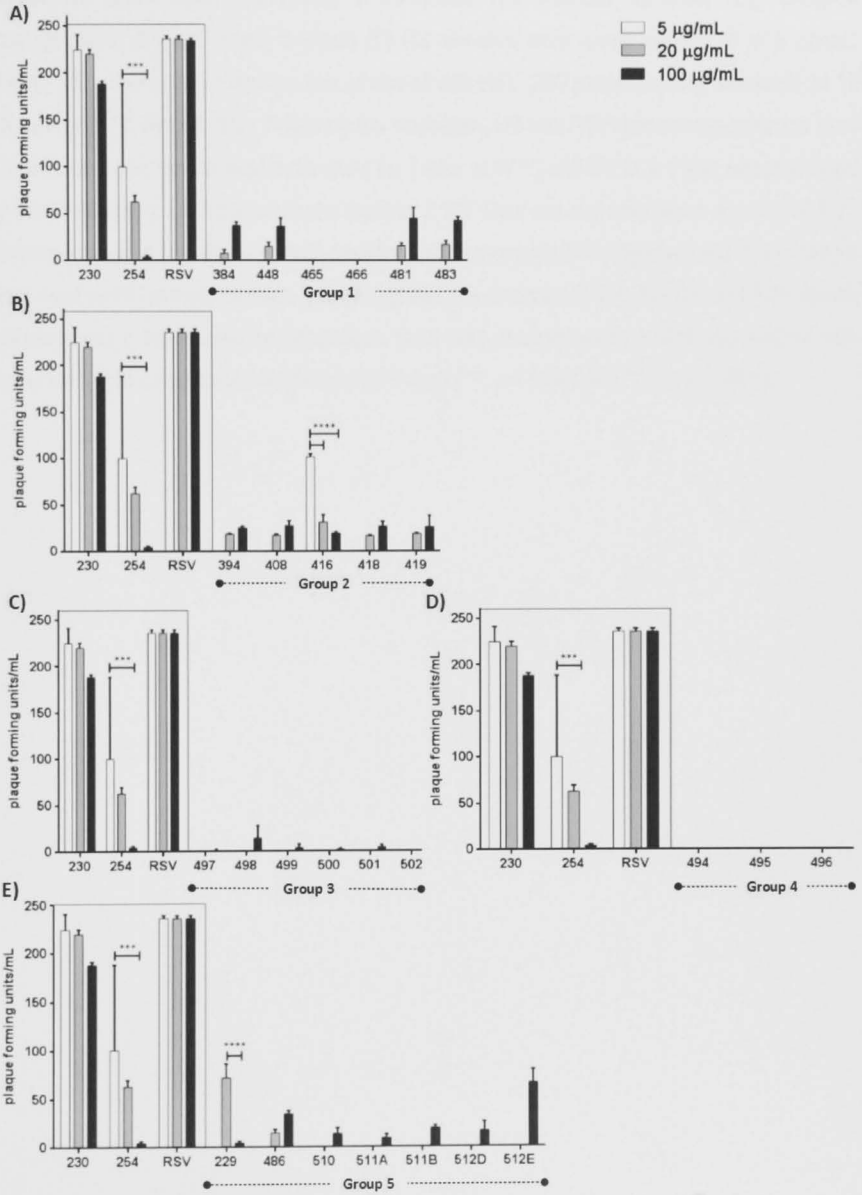


Figure A.3. Heparan sulfate (HS) *in vitro* (Condition A) treatment of respiratory syncytial virus (RSV) infection

The assay was carried out in 24-well flat bottom plate. Group 1 (heparins and modified heparins (~ 12kDa)) (A), Group 2 (low molecular weight heparins (LMWH) (5kDa and 3kDa) and enoxaparin with/without glycol split) (B), Group 3 (fucoidans and modified fucoidan) (C), Group 4 (carrageenans) (D) and Group 5 (other) (E) HS mimetics were tested at 100, 20 or 5 µg/mL. Briefly, 100 µl/well of HS mimetics was incubated with RSV (200 plaque forming units/well) for 10 minutes at 37°C and 5% CO_2. Following the incubation, HS with RSV mixture was added to Vero E6 cell monolayer and allowed to incubate for 1 hour at 37°C and 5% CO_2. Plate was processed by immunostaining RSV plaque assay (section 2.10). Data are represented as a mean of RSV plaques recovered (n = 4) ± standard deviation. HS compounds 230 (negative) and 254 (positive) were used as HS mimetic controls. For each graph, the controls HS 230, HS 254 and RSV results are presented in boxed area for comparison. Data were analysed using a ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (***, $p < 0.001$ and ****, $p < 0.0001$).

Condition B

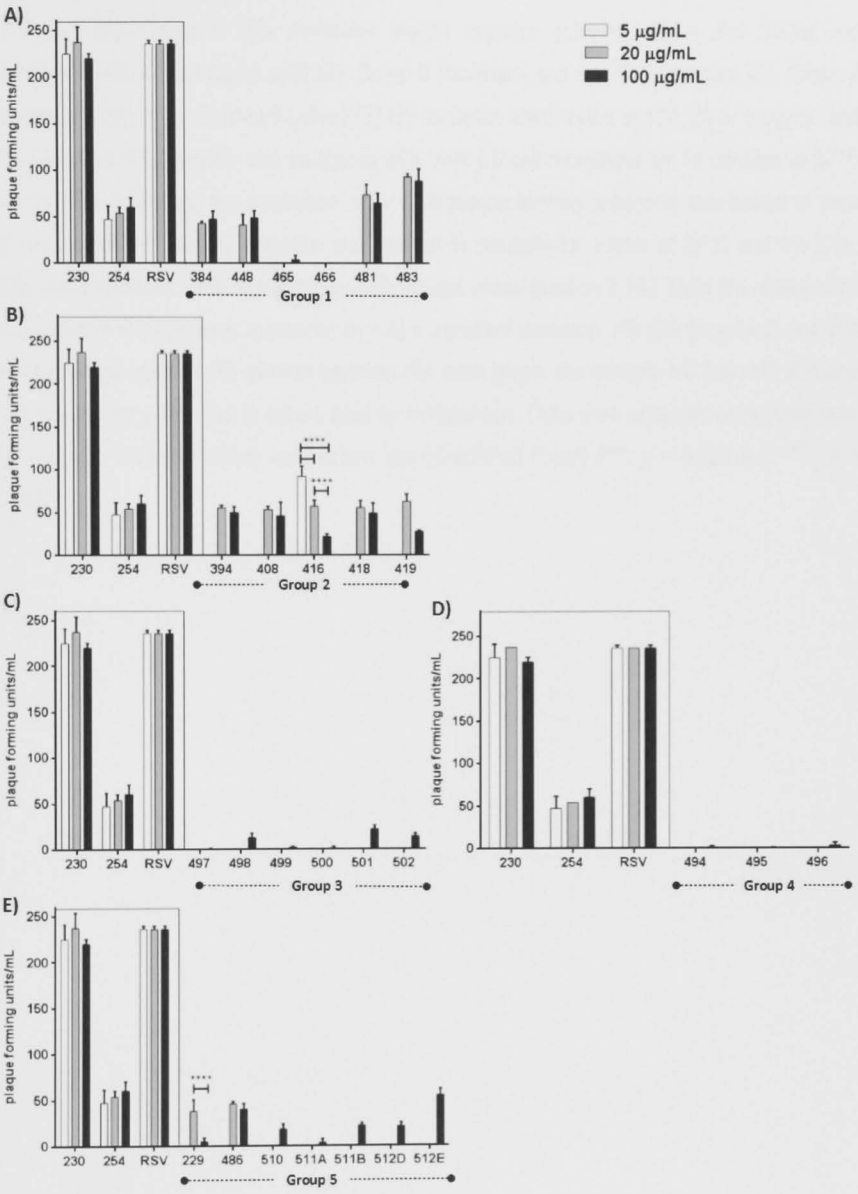

A)
B)
C)
D)
E)
5 µg/mL
20 µg/mL
100 µg/mL
plaque forming units/mL
0
50
100
150
200
250
230 254 RSV 384 448 465 466 481 483
Group 1
230 254 RSV 394 408 416 418 419
****
****
Group 2
230 254 RSV 497 498 499 500 501 502
Group 3
230 254 RSV 494 495 496
Group 4
230 254 RSV 229 486 510 511A 511B 512D 512E
****
Group 5

Figure A.4. Heparan sulfate (HS) *in vitro* (Condition B) treatment of respiratory syncytial virus (RSV) infection

The assay was carried out in 24-well flat bottom plate. Group 1 (heparins and modified heparins (~12kDa)) (A), Group 2 (low molecular weight heparins (LMWH) (5kDa and 3kDa) and enoxaparin with/without glycol split) (B), Group 3 (fucoidans and modified fucoidan) (C), Group 4 (carrageenans) (D) and Group 5 (other) (E) HS mimetics were tested at 100, 20 or 5 µg/mL and 100 µl/well of HS mimetics was incubated with Vero E6 cell monolayer for 10 minutes at 37°C and 5% CO_2. Following the incubation, RSV (200 plaque forming units/well) was added to Vero E6 cell monolayer with HS mimetics and allowed to incubate for 1 hour at 37°C and 5% CO_2. Plate was processed by immunostaining RSV plaque assay (section 2.10.). Data are represented as a mean of RSV plaques recovered (n = 4) ± standard deviation. HS 230 (negative) and 254 (positive) were used as HS mimetic controls. For each graph, the controls HS 230, HS 254 and RSV results are presented in boxed area for comparison. Data were analysed using ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (***, $p < 0.001$ and ****, $p < 0.0001$).

Condition C

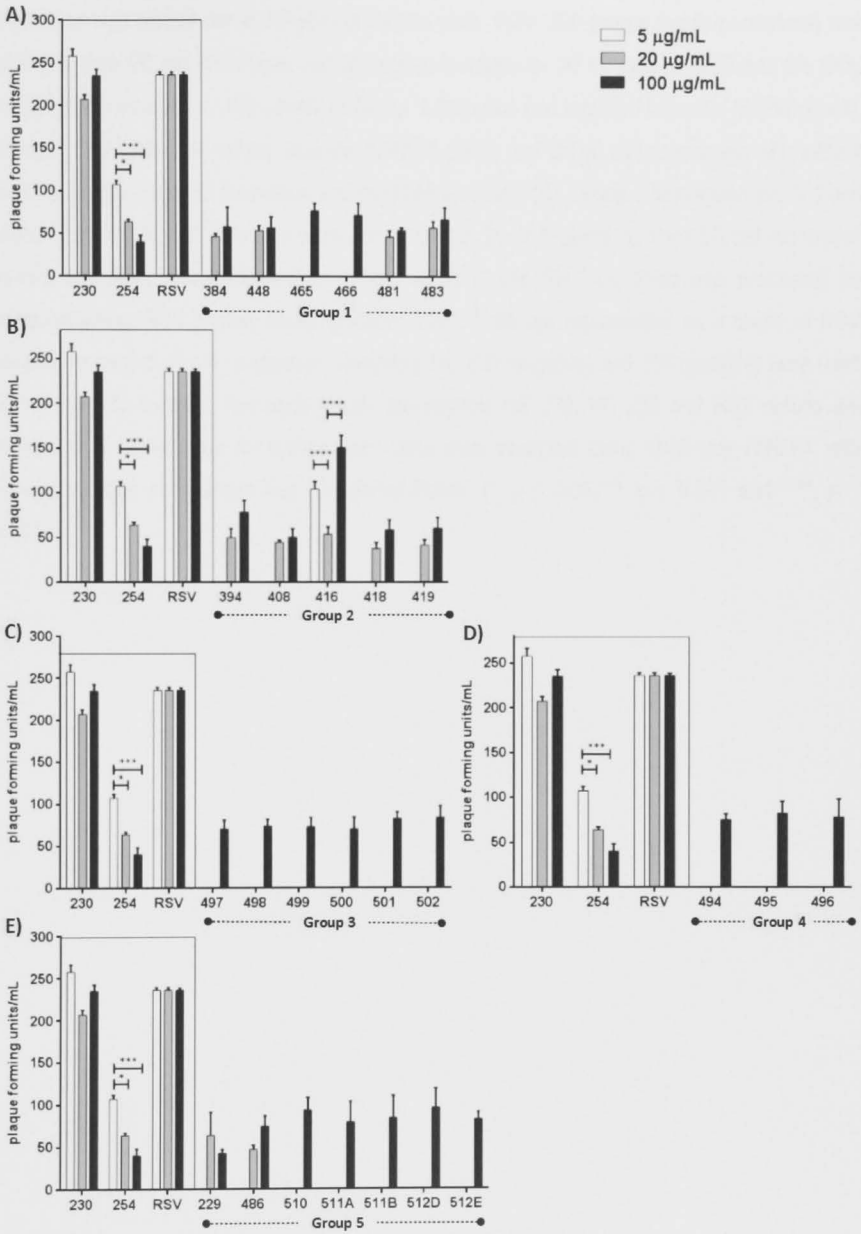

A)
B)
C)
D)
E)
5 µg/mL
20 µg/mL
100 µg/mL
plaque forming units/mL
Group 1
Group 2
Group 3
Group 4
Group 5
230
254
RSV
384
448
465
466
481
483
394
408
416
418
419
497
498
499
500
501
502
494
495
496
229
486
510
511A
511B
512D
512E

Figure A.5. Heparan sulfate (HS) *in vitro* (Condition C) treatment of respiratory syncytial virus (RSV) infection

The assay was carried out in 24-well flat bottom plate. RSV (200 plaque forming units/well) was added to Vero E6 cell monolayer and allowed to incubate for 10 minutes at 37°C and 5% CO_2. Following the incubation, 100 µl/well of Group 1 (heparins and modified heparins (~12kDa)) (A), Group 2 (low molecular weight heparins (LMWH) (5kDa and 3kDa) and enoxaparin with/without glycol split) (B), Group 3 (fucoidans and modified fucoidan) (C), Group 4 (carrageenans) (D) and Group 5 (other) (E) HS mimetics were added at 100, 20 or 5 µg/mL to Vero E6 cell monolayer treated with RSV and incubated for 1 hour at 37°C and 5% CO_2. Plate was processed by immunostaining RSV plaque assay (section 2.10.). Data are represented as a mean of RSV plaques recovered (n = 4) ± standard deviation. HS 230 (negative) and 254 (positive) were used as HS mimetic controls. For each graph, the controls HS 230, HS 254 and RSV results are presented in boxed area for comparison. Data were analysed using ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (*, $p < 0.05$,***, $p < 0.001$ and ****, $p < 0.0001$).

Condition D

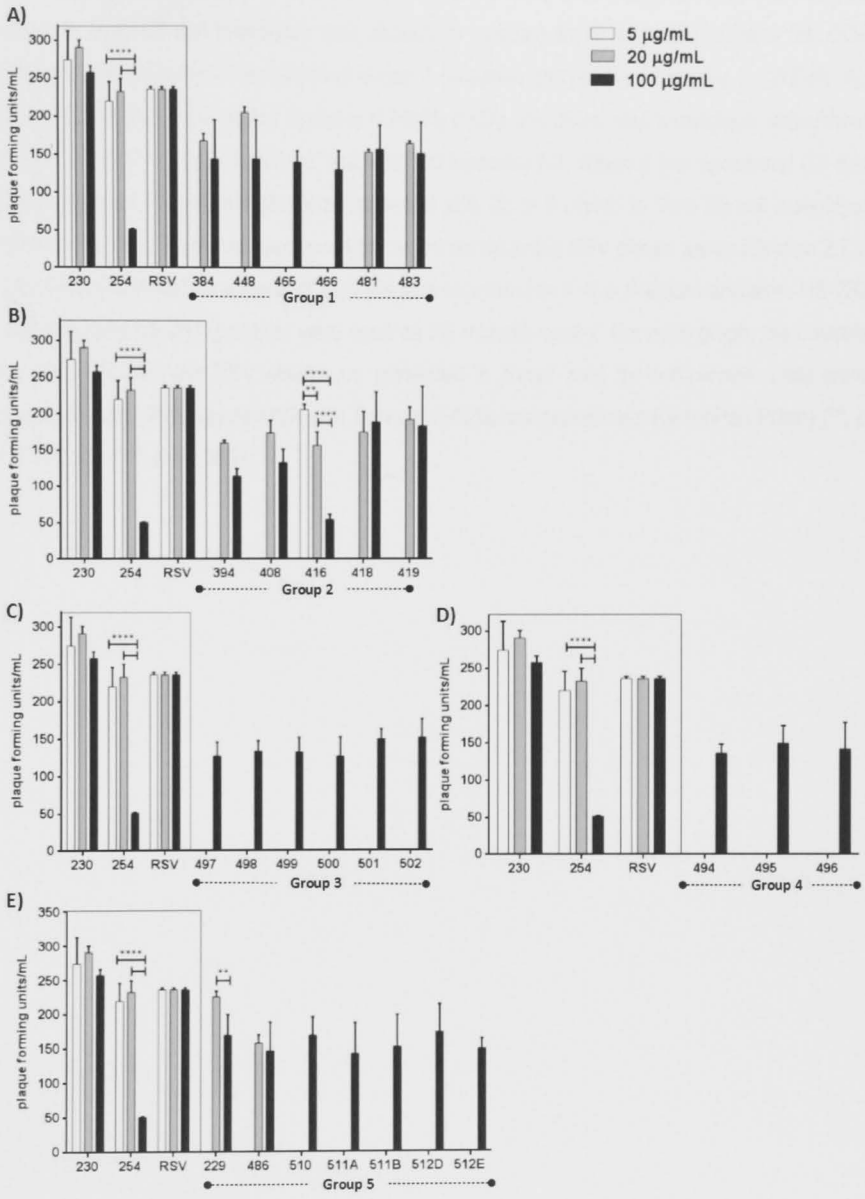

A)
B)
C)
D)
E)
5 µg/mL
20 µg/mL
100 µg/mL
plaque forming units/mL
230
254
RSV
384
448
465
466
481
483
Group 1
394
408
416
418
419
Group 2
497
498
499
500
501
502
Group 3
494
495
496
Group 4
229
486
510
511A
511B
512D
512E
Group 5

Figure A.6. Heparan sulfate (HS) *in vitro* (Condition D) treatment of respiratory syncytial virus (RSV) infection

The assay was carried out in 24-well flat bottom plate. RSV (200 plaque forming units/well) as added to Vero E6 cell monolayer and allowed to incubate for 1 hour at 37°C and 5% CO_2. Following the incubation, 100 µl/well of Group 1 (heparins and modified heparins (~12kDa)) (A), Group 2 (low molecular weight heparins (LMWH) (5kDa and 3kDa) and enoxaparin with/without glycol split) (B), Group 3 (fucoidans and modified fucoidan) (C), Group 4 (carrageenans) (D) and Group 5 (other) (E) HS mimetics were added at 100, 20 or 5 µg/mL to Vero E6 cell monolayer treated with RSV. Plate was processed as per immunostaining RSV plaque assay (Section 2.7.). Data are represented as a mean of RSV plaques recovered (n = 4) ± standard deviation. HS 230 (negative) and HS 254 (positive) were used as HS mimetic control. For each graph, the controls HS 230, HS 254 and RSV results are presented in boxed area for comparison. Data were analysed using ONE-way ANOVA with Tukey's multiple comparison test (GraphPad Prism) (**, $p < 0.005$ and ****, $p < 0.0001$).

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