PRIMARY HUMAN MAST CELL ANTIVIRAL AND PRO-INFLAMMATORY RESPONSES TO RESPIRATORY SYNCYTIAL VIRUS (RSV)

by

Raidan Alyazidi

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
August 2014

DEDICATION PAGE

I dedicate my master thesis to my parents for they have always believed in me unconditionally, and never restricted my thinking and questioning of the world. For them I dedicate my passion for science!

Love and Care,

Your daughter

Table of Contents

LIST OF TABLES vi

LIST OF FIGURES vii

ABSTRACT ix

LIST OF ABBREVIATIONS USED x

ACKNOWLEDGEMENTS xiv

CHAPTER 1 – INTRODUCTION 1

1.1 THESIS SCOPE 1

1.2 RESPIRATORY SYNCYTIAL VIRUS 1

1.3 RSV EPIDEMIOLOGY, DISEASE, AND LINK TO AIRWAY HYPERREACTIVITY 2

1.4 RSV MORPHOLOGY, AND REPLICATION CYCLE 3

1.5 RSV RNA AND PROTEINS 6

1.5.1 ENVELOPE GLYCOPROTEINS: F, G, SH 6

1.5.2 NUCLEOCAPSID/POLYMEARSE PROTEINS: N, P, L, M2-1 7

1.5.3 OTHER VIRAL PROTEINS 8

1.6 RSV DISEASE PREVENTION: VACCINE AND PASSIVE PROPHYLAXIS 9

1.7 RSV DISEASE MANAGEMENT 11

1.8 RSV DISEASE PATHOGENESIS 11

1.8.1 IMMUNE CELLS 13

1.8.2 MEDIATORS PRODUCED DURING RSV INFECTION 17

1.8.3 INNATE IMMUNE RESPONSES TO RSV 20

1.9 PATTERN RECOGNITION RECEPTORS AND DOWNSTREAM SIGNALLING 20

1.9.1 TOLL-LIKE RECEPTORS (TLR) 21

1.9.2 RETINOIC ACID-INDUCIBLE GENE (RIG-I)-LIKE RNA HELICASES (RLH) OR RLR 22

1.9.3 NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN (NOD)-LIKE RECEPTORS (NLR) 23

1.9.4 TYPE I INTERFERONS (TYPE I IFN) 23

1.10 RSV DISEASE MODELS IN MICE: VIRAL AND MOUSE STRAINS 25

1.11 MAST CELLS 26

1.11.1 MAST CELL LOCALIZATION AND DEVELOPMENT 27

1.11.2 MAST CELL MODELS IN VITRO 29

1.11.3 MAST CELL DEFICIENT MICE 30

1.12 MAST CELL MEDIATORS 31

1.12.1 PRE-STORED, GRANULE-ASSOCIATED MEDIATORS 32

1.12.2 DE NOVO SYNTHESIZED MEDIATORS 33

1.13 PATTERN RECOGNITION RECEPTORS AND MAST CELLS 35

1.14 MAST CELL RESPONSES TO PATHOGENS 37

1.14.1 MAST CELL RESPONSES TO RSV 40

1.15 THESIS HYPOTHESIS, RATIONALE AND OBJECTIVES 41

CHAPTER 2 – MATERIALS AND METHODS 44

2.1 CELL LINES 44

2.2 PRIMARY MAST CELL CULTURES 44

2.3 CELL COUNT AND VIABILITY 44

2.4 RSV PROPAGATION 45

2.5 INOCULATION CONDITIONS 45

2.6 RSV PLAQUE ASSAY 46

2.7 IMMUNOFLUORESCENCE AND FLOW CYTOMETRIC STAINING 46

2.8 QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR) 47

2.9 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) 47

2.10 BIOPLEX ASSAY 48

2.11 STATISTICAL ANALYSIS 49

CHAPTER 3 – RESULTS: HUMAN MAST CELL RESPONSES TO RSV INFECTION 54

CHAPTER 4 – DISCUSSION 80

4.1 SUMMARY OF MAJOR FINDINGS 80

4.2 IMPLICATIONS FOR RSV INFLAMMATION 84

4.3 LIMITATIONS AND FUTURE DIRECTIONS 85
4.4 CLINICAL IMPLICATIONS 88
4.5 CONCLUDING REMARKS 89
APPENDIX I 91
I.1 INTRODUCTIN 91
I.2 METHODOLOGY 91
I.3 PRELIMINARY RESULTS 94
I.3.1 IMPACT OF DIFFERENT RSV STRAINS ON MOUSE INFECTION 94
I.3.2 SELECTED MEDIATOR ANALYSIS IN RSV INFECTED MICE 94
I.4 FUTURE DIRECTION 95
REFERENCES 101

List of Tables

Table 1. List of antibodies used for flow cytometry. 50

Table 2. List of commercially available primers used for qPCR. 51

Table 3. List of customized ordered primers for qPCR. 52

Table 4. List of capture and detection antibodies used for ELISAs**.** 53

Table 5. RSV infection of CBMCs at various time points. 64

List of Figures

Figure 1. Schematic representation of the RSV replication cycle. 42

Figure 2. TLR signaling in the mast cell. 43

Figure 3. RSV infection of CBMC, HMC-1 and HEp-2 cells at 24 hours. 62

Figure 4. Expression of intracellular RSV antigen in CBMC. 63

Figure 5. Expression of 3 RSV genes in CBMC, HMC-1 and HEp-2. 65

Figure 6. Expression of 7 RSV genes in CBMC and HMC-1. 67

Figure 7. An example of plaque formation. 69

Figure 8. Viability of CBMC after RSV exposure. 70

Figure 9. Reduction in CBMC granularity in RSV infection. 71

Figure 10. Type I Interferon response following CBMC infection with RSV. 73

Figure 11. Levels of chemokines in CBMC-RSV infection with and without plaivizumab neutralization. 74

Figure 12. Chemokine upregulation by CBMC post-RSV infection. 75

Figure 13. Effect of blocking type I IFN receptor on chemokine production by CBMC during RSV infection. 76

Figure 14. Type I IFN receptor blockade efficiency. 77

Figure 15. VEGF-A protein production in RSV infection of CBMC. 78

Figure 16. IL-1Ra protein production in RSV infection of CBMC. 79

Figure 17. A proposed model for a mast cell role in RSV disease. 90

Figure I. The experimental design for a mouse infection with RSV. 93

Figure II. Impact of infection with different RSV strains on mice weight and airway inflammation. 96

Figure III. Chemokines and growth factors involved in neutrophil chemotaxis and maintenance. 98

Figure IV. Other chemokines increased in RSV-infected mice. 99

Figure V. IL-12 family of cytokines related IFN-γ in RSV infection in mice. 100

Abstract

Respiratory syncytial virus (RSV) is a major cause of infant bronchiolitis and the leading cause of their hospitalization worldwide. It is also linked to airway hyper-responsiveness. Mast cells are essential in allergies and the immune response to pathogens, and can produce various mediators that influence vascularity, bronchoconstriction, and immune cell recruitment to sites of infection. Since mast cells are abundant in the airways at the site of RSV infection, we examined the human mast cell response to RSV *in vitro* and the role type I interferons, major antiviral cytokines, play in such a response.

Our data show that human mast cells responded to restricted RSV infection by producing pro-inflammatory chemokines and cytokines some of which were dependent on type I IFN response (CXCL10 and CCL4), while others were not (CCL5 and VEGF-A). Mast cell production of these mediators may enhance inflammation and effector cell recruitment during RSV disease.

List of Abbreviations Used

2-ME	2-mercaptoethanol
2’5-OAS	2’5-oligoadenylate synthetase
AHR	airway hyperresponsiveness
ANOVA	analysis of variance
AP-1	activation protein
ATF	activating transcription factor
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BMDC	bone marrow-derived dendritic cells
BMMC	bone marrow-derived mast cells
BSA	bovine serum albumin
CAA	chimpanzee coryza agent
CARD	caspase activation and recruitment domain
CBMC	cord blood-derived mast cell
CC	cysteine-cysteine chemokine domain
CPE	cytopathic effect
CRE	cAMP-responsive element
CT	threshold cycle
CTL	cytotoxic T-lymphocyte
CX3C	cysteine- 3 other amino acids -cysteine chemokine domain
CXC	cysteine-other amino acid-cysteine
DC	dendritic cell
dsRNA	double-stranded RNA
DV	dengue virus
eIF	eukaryotic initiation factor
ELISA	enzyme-linked immunosorbant assay
F protein	fusion protein
FACS	fluorescence-acquired cell sorting

FBS	fetal bovine serum
FCS	fetal calf serum
GAG	glycosaminoglycan
GAH-AP	alkaline-phosphatase-conjugated goat anti-human antibody
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GTP	guanine triphosphate
HCV	hepatitis C virus
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HIV	human immunodeficiency virus
HMC-1	human mast cell line -1
HPRT1	hypoxanthine phosphoribosyl transferase 1
IFN	interferon
IFNAR	interferon alpha receptor
Ig	immunoglobulin
IκB	inhibitor of κB
IKK	IκB kinase
IL-	interleukin
IL-1Ra	interleukin-1 receptor antagonist
IMDM	Iscove's modified Dulbecco's Medium
IP-10	interferon-γ-stimulated protein-10
IRF	interferon regulatory factor
ISG	interferon stimulated gene
ISRE	interferon-stimulated response element
JAK	Janus activated kinase
JNK	c-Jun N-terminus kinase
LAD	Laboratory of Allergic Diseases-2
LGP2	Laboratory of Genetics and Physiology 2
LPS	lipopolysaccharide
LRTI	lower respiratory tract infection

mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling protein
MC	mast cell
MCP	monocyte chemoattractant protein
MDA5	melanoma differentiation antigen 5
mDC	myeloid dendritic cell
MIP	macrophage inhibitory protein
MMP	matrix metalloproteinase
MOI	multiplicity of infection
NEMO	NF-κB modulator IKKγ
NK cell	natural killer cell
NKT cell	natural killer T cell
NF-κB	nuclear factor-κB
NS1/2	non-structural protein ½
ORF	open reading frame
pAb	polyclonal antibody
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with Tween-20
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PFU	plaque forming unit
PGE_2	prostaglandin E_2
PGN	peptidoglycan
PKR	protein kinase R
Poly I:C	polyinosinic:polycytidilic acid
PRR	pattern recognition receptor
PVM	pneumonia virus of mice
qPCR	quantitative polymerase chain reaction
RANTES	regulated on activation T cell expressed and secreted

RIG-I	retinoic acid-inducible gene-1
RPMI	Roswell Park Memorial Institute
rRSV	recombinant respiratory syncytial virus
RSV	respiratory syncytial virus
RSV-IVIG	respiratory syncytial virus intravenous immunoglobulin
RV	rhinovirus
SCC	side scatter
SCF	stem cell factor
SEM	standard error of the mean
SH protein	small hydrophobic protein
shRNA	short hairpin RNA
siRNA	short interfering RNA
ssRNA	single-stranded RNA
STAT	signal transducer and activator of transcription
TAK1	transforming growth factor beta activated kinase-1
TANK	TRAF-family member-associated NF-κB activator
TBK-1	TANK-binding kinase-1
TBS	TRIS-buffered saline
TBS-T	TRIS-buffered saline with Tween 20
T_H1	T helper 1
T_H17	T helper 17
T_H2	T helper 2
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
TRIF	TIR domain-containing adaptor-inducing interferon-β
URTI	upper respiratory tract infection
VCAM-1	Vascular cell adhesion protein 1
VEGF	vascular endothelial growth factor

Acknowledgements

I thank God for giving me this great opportunity; to use my mind in exploring the world and understanding it a little better!
I thank my supervisor Dr. Marshall for I have learnt from her so many valuable skills in the lab and life, and for being so nice all the time even when things go wrong. You are the spirit of this lab! I also thank our collaborator Dr. Anderson for his advice, time and effort as well as Yan Huang for her keen care and help which was instrumental in the *in vivo* experiments. I thank Ayham for introducing me to RSV & his valuable insight on a master student life.
As for the wonderful team of researchers I worked with for the past two years in the Marshall lab, you are more than colleagues to me! Your support, advice and friendship have been highly appreciated! I specifically would like to express my deepest gratitude to these people: Ian Haidl for his technical and scientific teaching, life advices, answering my endless questions, lending me a helping hand regardless of when it was needed, and helping me improving my presentations and course work. Nong Xu for being such a master in all sorts of lab stuff and teaching me the lab techniques I know! I actually owe a lot of what I know in lab to these two gentlemen. Also an endless thanks to Sharon Oldford for being there whenever needed and for listening and helping out when life gets complicated, as well as running all that bioplex for me, Liliana Portales-Cervantes for her kind friendship, continuous care and support and saving my day so many times, Ava Vila-Leahy for being the fun and supportive person who would not mind babysitting someone who's recovering from anesthetics & bringing them ice cream, Yisong for helping out with my ELISA and cell count, and Mary Ellen, you know you are the corner stone for our life in the lab… and to all the students who passed by the lab and enjoyed knowing them Ghaidah (my cheerleader ☺), Stephanie, and Marall… and the Johnston lab for their friendship, support and advice…
I also express my appreciation to my supervisory committee members Dr. Thomas Issekutz and Dr. Scott Halperin for their valuable instructions and support throughout the pre-master and master. As well, I extend my gratitude to the Clinician Investigator and Clinician Scientist Programs (Jess Kinney, Dr. Bob Bortulussi, and Dr. Jason Berman) for their crucial care and support. I send my greetings to my home university King Abdul Aziz University, and the Pediatric Department for entrusting me with this scholarship, and the Pediatrics in Dalhousie for supporting me in my time off clinical and back to it.…
To all my family and friends for their prayers, and good wishes, love you all!

Chapter 1 – Introduction

1.1 Thesis Scope

Respiratory illnesses have variable clinical presentations depending on the anatomical location inviolved and the host-pathogen interactions. A substantial number of respiratory infections is related to viruses. Of these viruses, respiratory syncytial virus (RSV) is one of the major respiratory pathogens that imposes significant risk on vulnerable populations like young children and the elderly (Tregoning and Schwarze, 2010). However, knowledge around RSV is ever growing and changing due to the complex pathology of RSV disease and the difficulties in obtaining accurate knowledge on the molecular mechanisms of human RSV disease.

Mast cells are increasingly recognized as sentinel cells in host defense against mucosal pathogens, including viruses. Their stratigic location around the vasculature and at the interface with the environment implies an important role in rapid response to pathogens. Nevertheless, the human mast cell's role in RSV disease is not defined. This thesis expands upon the M.Sc thesis work done previously (Al-Afif, 2012) and focuses around better understanding of human mast cell-RSV interactions.

1.2 Respiratory Syncytial Virus

In 1955, a virus that caused upper respiratory tract disease in chimpanzees was isolated and called chimpanzee coryza agent (CCA) (Blount et al., 1956). Soon after CCA was discovered, a related virus was isolated from infants with lower respiratory tract illness, and that was the first report of respiratory syncytial virus (RSV) in humans (Chanock et al., 1957). RSV was noticed to induce the formation of syncytia in infected cells *in vitro*, and hence the derivation of its name (Chanock et al., 1957).

Since its discovery, RSV continues to be associated with a significant global health and economic burden highlighted by the efforts directed towards developing a successful RSV vaccine. The advances in molecular and genetic analysis have enabled the scientifc

community to finally unravel important knowledge around the structure and genomic organization of RSV in the 1980s (Collins et al., 2013). Nevertheless, more understanding of RSV pathology in its primary host, humans, is needed to help in the development of a safe and effective vaccine and improved therapies.

1.3 RSV Epidemiology, Disease, and Link to Airway Hyperreactivity

By the age of two years, almost all people have been infected with RSV, and 20-30% of these had lower respiratory tract infection (LRTI) such as bronchiolitis or pneumonia. As well, one to three percent of infants under one year of age require hospitlization from RSV LRTI (Langley and Anderson, 2011). In fact, RSV is the leading cause of lower respiratory tract infection in young children, and the major cause for infant hospitalization. In 2005, there were around 33 million RSV LRTIs in children younger than five years old, 10% required hospitalization, and significant mortality was reported mainly in developing countries (Nair et al., 2010, Lozano et al., 2012). On the other hand, adults as well are affected with RSV disease. Almost 40% of adults in families with young children are infected with RSV, and of those adults, 40% missed work (Hall et al., 1976, O'Shea et al., 2005). The cost for infant and elderly hospitalization from RSV disease, in the United States alone, is around 4 billion dollars annually (Leader and Kohlhase, 2003, Falsey et al., 2005). Therefore, RSV disease is considered a huge health and economic burden globally.

RSV has two highly divergent antigenic subtypes: A and B (Mufson et al., 1985), and both are spread globally. In a temperate climate, RSV outbreaks peak generally in cold weather in the winter, spring and fall, while in a near tropical climate the disease occurs throughout the year associated with rain fall (Mullins et al., 2003, Omer et al., 2008).

The severity of RSV respiratory disease is largely related to the health status and living conditions of the host. The premature infants, those of very young or old age, with primary exposure to RSV, immunocomprimised status or cardiopulmonary disease, and male sex are at greater risk of severe disease (Langley and Anderson, 2011, Sommer et al., 2011). The mortality rate in immunocompromised patients infected with RSV

approaches 80% (Ison, 2009). Secondary RSV infection is common but usually milder. Nevertheless, elderly patients present for treatment usually with exacerbation of an underlying chronic condition (Falsey and Walsh, 2000, Falsey et al., 2005).

RSV disease in infants is characterized by the onset of URTI symptoms including low grade fever, nasal congestion, and cough that could develop into LRTI with assosicated persistent coughing, wheezing, tachypnea, laboured breathing, apnea as well as findings of pulmonary collapse. This might impact the patient's oxygenation and fluid intake and can lead to hypoxemia and dehydration. The acute disease lasts five to ten days with peak of symptoms after the third day. However, the cough may persist for several weeks. RSV disease can be complicated by secondary bacterial infection, and 30-50% of children hospitalized with RSV disease will have recurrent wheeze. Older children and adults present with a prolonged course of URTI, that could be complicated with a sinusitis or otitis media. However, one fourth of healthy adults with RSV URTI presents with wheeze (Hall et al., 2013).

In addition to the immediate clinical presentation of RSV infection, exposure to RSV at a young age and the severity of RSV at that age have been linked to the development of airway hyperreactivity (Stein et al., 1999, Sigurs et al., 2010). It is likely that patients with a higher baseline risk for asthma are more likely to have persistent wheeze into late adolescence after RSV disease (Chawes et al., 2012, Lotz et al., 2013).

1.4 RSV Morphology, and Replication Cycle

Human RSV is a single-stranded, negative-sense RNA virus from the genus pneumvirinae and the family paramyxoviridae. The virion helical structure is composed of a lipid envelope with spike-like glycoproteins. This envelope packages a matrix protein and a nucleocapsid core that carries the large polymerase protien along with a nucleoprotein and phosphoprotein (Collins et al., 2013).

The mechanism of fusion of the virus to the host cell membrane and its subsequent entry are not fully understood. However, it likely involves the binding of the fusion protein

with the receptor nucleolin and the interaction with a co-receptor or co-factor, like the adhesion molecule ICAM or the negatively charged matrix carbohydrate, by the viral attachment protein (Mastrangelo and Hegele, 2013, Tayyari et al., 2011). This fusion process is also dependent on the cell cytoskeleton proteins.

After the virus envelope fuses with the host cell membrane, it releases the nucleocapsid and the matrix protein into the cytosol to start primary transcription of the viral genes followed by secondary transcription and RNA replication. The 3' end of the genome has the conserved, single promoter sequence that is essential for both genomic replication and transcription. The viral polymerase enters the viral RNA from the 3' end to transcribe the genes into the corresponding messenger RNA (mRNA) in a polar gradient fashion, where the level of the gene transcription is reduced along the genes' order, or to replicate the genome into the complementary antigenome positive-sense strand. The genome and anti-genome are not 5' capped or 3' polyadenylated, while the viral mRNAs are polyadenylated by polymerase stuttering action. Throughout RNA replication, the genome and anti-genome are stabilized and protected from degradation and cellular recogniton by the nucelocapsid protein. Both trancription and replication for RSV can happen at the same time, early the infection. Viral mRNAs are detected as early as 4-6 hours after infection and peaks at 15-20 hours, after which the transcription is downregulated for RNA replication and packaging of viral progeny that could be released as early as 10-12 hours post infection (Collins et al., 2013) (Figure 1).

RSV infected cells contains large inclusion bodies that are believed to contain the viral RNA replication machinery but might function, as well, to entrap some cellular viral sensors. RSV assembly takes place at the apical surface of the plasma membrane in a polarized cell and preferably at a lipid raft (lipid microdomain). RSV might hijack the cellular endocytic pathway to bud out of the cell. During budding, RSV obtain its lipid envelope from the host cell membrane. Actually, selective lipid microdomain proteins as well as cellular cytoskeleton proteins are incorporated into the budding viral progeny. The resultant virus contains mainly the genomic RNA but could also contain the double-sranded RNA form. However, only the nucleocapsid without the polymerase along with

the matrix and fusion proteins are required for infectious viral particle formation (Shaikh and Crowe, 2013).

In vitro, RSV exists mainly in a long filamentous, or less commonly, a spherical form (Jeffree et al., 2003). The cellular cytoskeleton protein actin is found at the base of these filaments but not incorporated in them (Shaikh and Crowe, 2013). In cell lines, but less in primary cultures, the virus infected cells fuse to form syncytia. The majority of the virus in culture is associated with the cell surface. Therefore cell lysis is required to release the viral progenies. However, the viral particles become unstable which reduces the virus titre. For this reason, RSV extracellularly should be stored in excepients like magnesium sulphate or sucrose to prevent the virus from aggregating and losing infectivity (Ausar et al., 2007).

The phenotypic and kinetic features of RSV infection in epithelial cells were first described based on the infection of continuous epithelial cell lines like HEp-2 and A549. However, RSV infection of primary human airway epithelium (HAE) *in vitro* showed focal infection with minimal cytopathic effect and lower viral titres than observed in cell lines (Wright et al., 2005) where the whole monolayer becomes infected and the cytopathic effect e.g. cell elongation, giant cell formation and syncytia is much more prevalent. An *ex vivo/ in vitro* RSV model of well-differentiated primary bronchial epithelial cells (WD-PBECs), might better represent human RSV infection (Villenave et al., 2012). This model showed that RSV infection was non-contiguous, involved mainly apical and ciliated epithelial cells, resulting in apoptosis and sloughing of these cells with goblet cell hyperplasia and mucus hypersecretion. These new primary epithelial cell cultures are increasingly utilized in the study of RSV interactions with cells (Pickles, 2013).

1.5 RSV RNA and Proteins

RSV RNA is non-segmented genome carrying ten genes that encode eleven different monocistronic mRNA and proteins. It has nine highly divergent intergenic regions that generally have no impact on the viral replication. The 3’ end has a conserved extragenic

leader region and the 5' end has a moderately conserved trailer sequence, while each gene has gene-start (GS) and gene-end (GE) conserved peripheral signals. The M2 gene has two open reading frames (ORF) that encode two different proteins and overlap with the ORF for the following L polymerase gene. The viral RNA polymerase has a 10% "error" in reading the frame that leads to the transcription of a full length L mRNA. Otherwise, the polymerase reads through intergenic regions looking for GS sequences and never dissociates even at the GE signal (Collins et al., 2013).

1.5.1 Envelope Glycoproteins: F, G, SH

F and G are surface transmembrane proteins that are mainly responsible for viral entry into the cells but not required for viral replication. The fusion protein F is 574-amino acids and is found in the precursor form F0, which is cleaved by cellular endoproteases to give rise to the F form. This protein is heavily sialylated and guides virus-cell fusion and syncytium formation (McLellan et al., 2013). However, it also binds TLR-4 on host cells leading to possible immune activation (Haynes et al., 2001).

The large glycoprotein G variability gives rise to the antigenic subtypes of RSV. It is involved in viral attachment. It is composed of 298 amino acids and it has a small transmembrane anchor with large ectodomain composed of two highly divergent units and a central conserved region. This glycoprotein has been implicated in RSV host immune evasion. For example, the ectodomain contains variable lengths of O- and N-linked sugars that might render it similar to host matrix sugars and is rich in the amino acids proline, threonine and serine, which resembles the mucin structure (McLellan et al., 2013). Also, the central region contains a cysteine residue with two disulfide bonds which mimics the CX3C chemokine (fraktalkine), hence it could bind to immune cells carrying the fraktalkine receptor reducing their recruitment to infection sites and thereafter recognition of virus-infected cells as was shown in in a mouse lung (Tripp et al., 2001). G protein could also act as a decoy receptor for tumor necrosis factor (TNF) (Langedijk et al., 1998), as well as inhibit TLR (e.g. TLR-4) and alter human dendritic cell antigen presentation (Polack et al., 2005, Johnson et al., 2012). Almost 80% of G protein is secreted in a form that lacks the small anchor region, and this form is thought to

neutralize antibodies as well as FC receptors on immune cells, preventing viral recognition by the immune system (Bukreyev et al., 2008).
On the other hand the small or short hydrophobic protein **(**SH**)**, 64 amino acids, is not known to contribute to viral entry and its role is not clearly understood. It is believed to be a viroporin that alters cell permeability and affects viral budding. It has also a small inhibitory effect on apoptosis, and might inhibit TNF action on cells (Fuentes et al., 2007). RSV lacking SH is slightly attenuated *in vivo*, but replicates well *in vitro* (Collins et al., 2013, McLellan et al., 2013).

1.5.2 Nucleocapsid/Polymearse Proteins: N, P, L, M2-1

The N protein is a 391-amino acid and the genomic and anti-genomic packaging protein. It has a flexible structure to allow for the polymerase entry disturbing the nucleocapsid stability and RNA synthesis (Collins et al., 2013). It also inhibits the protein kinase PKR directly, preventing the phosphorylation of the cellular translation initiation factor eIF-2α. Therefore, cellular protein synthesis is maintained (Groskreutz et al., 2010).

The P protein is 241-amino acids long, and is phosphorylated. It is an essential co-factor for the polymerase activity, and it is an adaptor molecule that links the rest of the nucleocapsid components together. It seemingly promotes the dissociation of the M protein from the nucleocapsid early in the infection, and it binds the free N protein to prevent its aggregation and guides it, as well as M2-1 protein, to the RNA synthesis site (Collins et al., 2013).

The L is a large protein with 2,165 amino acids. It carries specific conserved sequences, and it is important for the all viral transcription and RNA synthesis (Collins et al., 2013). The M2-1 is 1 194-amino acid protein and a processivity factor (Fearns and Collins, 1999). In its absence viral gene transcription terminates prematurely. It is guided to site of viral RNA synthesis by the P protein and itself bind the M protein to transport it to inclusion bodies (Collins et al., 2013). It has an essential zinc finger motif similar in structure to that of the cellular zinc finger protein tristetraprolin (TTP) (Hardy and Wertz, 2000). This cellular protein is known to affect the stability of host response mRNA

including many cytokine mRNAs and it is involved in regulation of translation under stress conditions, but M2-1 is not yet known to specifically contribute to this regulation (Fricke et al., 2013).

1.5.3 Other Viral Proteins

Regulatory Protein M2-2, 88-90 amino acids, is another RNA synthesis factor required for the timely down-regulation of viral RNA transcription in favor of viral RNA synthesis, and it might be required for viral packaging. RSV lacking M2-2 has increased runaway transcription and delayed viral replication *in vitro* (Bermingham and Collins, 1999) and reduced viral titers *in vivo* (Teng et al., 2000). This protein is normally found in low levels in infected cells (Collins et al., 2013).

Matrix Protein M is a 256-amino acid, positively charged protein, located on the inner side of RSV envelope. It is found throughout the viral replication cycle with different function. Early in the infection, it inhibits the cellular RNA synthesis in the nucleus (Ghildyal et al., 2006). Later, it is found in the viral-induced inclusion bodies to inhibit viral RNA synthesis and signal RNA packaging into virions, then further transport to the plasma membrane. In its absence, infectious viral precursors or viral filaments are immature (Mitra et al., 2012).

Non –structural NS1 and NS2 proteins are less evident in the packaged virus, but they are found mainly in virus-infected (Evans et al., 1996). They are 139- and 124-amino acid proteins respectively, and they are important for inhibiting interferon responses and apoptosis of the infected cells, which eventually promote viral replication. NS1 and NS2 can work in synergy by a poorly understood mechanism (Swedan et al., 2011). They possibly play a negative feedback role on viral dsRNA formation by inhibiting viral RNA replication and transcription to prevent the activation of the cellular innate immune sensors. RSV lacking non-structural proteins is more susceptible to interferon and apoptosis, and replicates less efficiently *in vitro* and *in vivo* (Whitehead et al., 1999, Teng et al., 2000).

1.6 RSV Disease Prevention: Vaccine and Passive Prophylaxis

RSV is transmitted through respiratory secretions (large particle droplets) and fomites. The virus lasts for six hours on surfaces and for half an hour on hands. Therefore good hygiene practice is essential to reduce the viral transmission, at least in health care settings (Hall et al., 1980, Lindsley et al., 2010). However, control of the infection in the community is more complicated and reinforces the need for an effective RSV vaccine. Immunity against RSV is usually inadequate at the levels of both innate and adaptive immunity. RSV neutralizing antibodies are present in 50-75% of infants younger than 6 months of age and RSV antibodies decline rapidly over time (Brandenburg et al., 1997, Walsh and Falsey, 2004). Hence, reinfection is common (Varga and Braciale, 2013). Passively transferred maternal antibodies are associated with reduction in the severity and incidence of RSV disease in the first 3 months but are not fully protective (Kimman and Westenbrink, 1990). Additionally, the ability to mount a rapid secondary mucosal IgA response but not baseline level of IgA seems to be beneficial for protection in bovine RSV. There is some evidence for a correlation between protection from RSV pathology and the serum titer of neutralizing antibodies in adults but conflicting data are present for the elderly (Varga and Braciale, 2013, Falsey and Walsh, 1998, Falsey et al., 1990, Hall et al., 1991). Generally, there is compelling evidence that a high content of RSV-neutralizing antibodies in an immunoglobulin preparation given to high risk patients were significantly protective against severe RSV disease –as reported by the PREVENT Study Group- (1997). RSV affects mainly infants who already have immature immune system which further complicates development of effective immune protection.

Trials for an RSV vaccine started in the 1960s with the use of a formalin-inactivated RSV vaccine that led to morbidities and some deaths in the RSV-naive children. Infants who were not previously exposed to RSV developed enhanced RSV disease upon subsequent viral challenge. The disease was characterized by severe pulmonary Th2 cytokine response and eosinophilia. Currently, experimental RSV vaccines include live-attenuated RSV, replicative chimeric RSV, gene-based vaccine with a defective vector aiming to modulate T cell responses to RSV, and subunit proteins with adjuvants. In RSV naïve patients, protein-based vaccine (inactivated whole virus or purified viral protein)

are avoided because in experimental studies they were associated with enhanced disease (Karron et al., 2013, Morrison and Walsh, 2013, Anderson et al., 2013)

A monoclonal antibody against the RSV-F protein which was initially developed in 1989, Palivizumab reduced pulmonary RSV titre in cotton rats by >99% (Johnson et al., 1997). Palivizumab was approved for clinical use in the US in 1998. Patients with high risk for RSV severe disease are recommended to receive prophylactic palivizumab, starting before RSV season according to suggested protocols. These patients include preterm infants less than 32 weeks gestation or preterm 32-35 week gestation with specific risk factors, and patients with chronic lung disease, or congenital heart disease with hemodynamic instability. As well, patients with neuromuscular diseases, congenital diaphragmatic hernia, Downs Syndrome and cystic fibrosis might benefit from palivizumab prophylaxis. In randomized clinical trials, palivizumab reduced infant hospitalization by 43-55% depending on their disease category. However, the use of palivizumab in general population does not confer better clinical outcomes and therefore is not recommended (Lanari et al., 2013).

In addition to palivizumab, multiple monoclonal antibodies that target the F, the pre-F (the predominant form of neutralizing antibodies), or the shed G proteins are under development for clinical use (Chu and Englund, 2013). Blocking the soluble form of G protein in animals led to reduction of RSV infection and inflammation (Choi et al., 2012). As well, a hyperimmune immunoglobulin preparation is to be used in phase II clinical trial to assess prevention of LRTI development in immunocomprimised individuals (Empey et al., 2010)

1.7 RSV Disease Management

The management of RSV disease is generally supportive and depends on the severity of the clinical presentation. Patients with lower respiratory tract disease might need oxygen supplementation, and nutritional support to meet the increased respiratory effort (Chu and Englund, 2013). Hypertonic saline to relieve airway edema could help reduce the length of hospital stay and severity of bronchiolits from any viral cause (Zhang et al., 2008).

Also, bronchodilator might be benificial in a subset of patients with airway obstruction (Hammer et al., 1995).

The use of nonspecific anti-viral agents that interfere with viral transcription, such as ribavirin, is restricted to immunocomprimised patients, and not without significant side effects. In this population, ribavirin is used alone, or more recently in combination ,with palivizumab and or intravenous immunoglobulin (IVIg), and corticosteroids to treat or prevent disease progression. There is no known beneficial effect of using these antivirals in otherwise healthy patients admitted with RSV disease. Fusion inhibitors targeting RSV F protein, molecules neutralizing G and L protein or blocking the nucleolin receptor, small interfering RNA (siRNA) drugs that prevent the coding for specific genes by viral mRNA (Zhang et al., 2005), as well as the antibodies under development against F and G proteins are potential new therapies that could also be used for prevention in special cases and are under investigation (Chu and Englund, 2013).

1.8 RSV Disease Pathogenesis

Data on the mechanisms and biological impact of human RSV disease are limited due to the invasiveness of the procedures required to retrieve the necessary samples. In addition, RSV LRTI presents in two heterogeneous groups; the very young and the old, which complicates the description of RSV pathology, not to mention that experimental animal models might be difficult to interpret since animals are generally semi-permissive for human RSV (Bem et al., 2011).

RSV spreads down the respiratory tract either through cell to cell transmission or through direct inoculation by swallowing of respiratory secretions (Domachowske and Rosenberg, 1999). Johnson et al. (Johnson et al., 2007) described the histological features of non-fatal RSV bronchiolitis in a 15 months old patient who died in a motor vehicle accident on the third day of the disease and compared it to another three archived cases who died from severe RSV bronchiolitis in the pre-intensive care era. RSV antigen was detected throughout the superficial epithelium with patchier distribution on the larger airways and more circumferential staining as the airway becomes smaller, but sparing the

basal layers of the epithelium. RSV was detected as well in the alveolar pneumocytes and in the sloughed cells or intra-cytoplasmic in macrophages. Syncytia formation, which is hallmark of RSV infection in epithelial cell lines, was not observed in most patients similar to what was observed in primary cell culture infection; however, there were RSV-positive papillary projections of the superficial epithelium. Some of the infected cells were definitely ciliated and there was some *in vitro* evidence suggesting that ciliated cells might be better infected but not restrictively. Nevertheless, the circumferential staining of the airway for RSV antigen *in vivo* indicates that other non-ciliated cells might be involved depending on the anatomical location. As well, *in vitro* RSV can infect multiple different cell lines from different organs, which indicates that there are several factors that control RSV tropism *in vivo* other than the cell type and polarity or epithelial to mesenchymal transtion state, although budding is preferrable at polarized surfaces (Collins et al., 2013, Zhang et al., 2002)

Airway narrowing and blockage observed in human RSV infection is attributed mainly to mixed cellular debris from shed superficial epithelial cells and inflammatory cells, and fibrin and mucus plugging, as well as submucosal edema and follicular lymphoid agregates. Airway arteries, arterioles and capillaries were prominently congested and leaking inflammatory cells and fibrin down to the terminal airways/alveoli. Inflammatory infiltrate followed the vascular distribution, and was mainly localized to the submucosa, deeper to the muscularis layer of the airway, but still many inflammatory cells extended to the the epithelium and lumen. Pleural edema and lymphatic dilatation was also evident (Johnson et al., 2007). This autopsy report might be the most detailed and representative of the general cohort of RSV patients. Another post-mortem report by Welliver et al. (Welliver et al., 2007) decribed the pathological features in a fatal RSV bronchiolitis in 9 patients less than 1 year of age (median age 3 months, all but one were not ventilated, on median day 4 of their illness) and was compared to fatal influenza LRTI. In that report, more epithelial cell apoptosis as marked by caspase 3 positive cells, more sloughing, airway blockage giant cell formation, and more viral antigen were observed in RSV patients than in influenza patients. Consequently, severe RSV disease could result from exaggerated initial epithelial cell involvement and immediate immune response, or

alternatively, it could be due to lack of the ability to control the viral disease or regulate inflammation due to deficiency in some elements of the innate or adaptive immune system. Indeed, severe and prolonged RSV bronchilitis is associated with human T-cell deficiency.

We could speculate that early in the infection epithelial cells and other resident cells of the airway, including mast cells, initiate a sequence of inflammatory responses that are characterized by production of multiple mediators and inflammatory cellular infilitrate, and that this initial response is crucial for determining the subsequent long term response. In almost all the studies reviewed in the following section, the total cell and inflammatory mediator content of the RSV patient respiratory secretion correlate with RSV severity. At some point of the disease, one might expect that the recruitment of adaptive immune cells to the airways would help in establishing long term specfic immunity. However, as we mentioned, RSV evades immune system ability to efficiently present viral antigens in order to achieve protective long lasting immunity (Varga and Braciale, 2013).

1.8.1 Immune Cells

Immune responses to RSV include an early non-specific, innate immune response by epithelial cells and resident immune cells like macrophages, dendritic cells, and mast cells, as well as the early recruited leukocytes including neutrophils and monocytes, and possibly NK or NKT cells, and in some cases eosinophils. Following initial innate responses, specific, adaptive immune cells including T- and B-lymphocytes play a role in impacting long term immunity in most viral infections.

Johnson et al. (Johnson et al., 2007) reported in the case described above, a majority of mononuclear cellular infilitrate in the airway and interstitium consisting mainly of macrophages and few small or transformed lymphocytes, and in the lung parynchema consistent of CD68+ monocytes. Lymphoid aggregates were present intraluminally and peribronchial and composed mainly of CD20+ B lymphocytes. T lymphocytes were relatively abundant especially in the space between the vessels and the small airways, and were composed of uncharacterized CD3+ double-negative T cells (which could represent

NKT cells, intraepithelial lymphoid cells, or γδ-T cells), and CD8+ T cells. There was a minority of neutrophils (10-25% from total inflammatory cells) between the airway and the bronchial arteries, but were occasionally they seen in the luminal surface or traversing the muscularis layer of the airways. Eosinophils were infrequently observed in the peribronchilar areas, and dendritic cells (DCs) were not frequently detected in the patient lung but they might have migrated to the draining lymph nodes after maturation to present RSV antigen to naïve T-lymphocytes. In the previous Welliver et al. (Welliver et al., 2007) report of fatal RSV, T lymphcytes were almost not detected but $CD16^+$ cells (macrophages and neutrophils) were prevalent throughout the airways and interstitium.

In an older study (Everard et al., 1994) examining the broncheoalveolar lavage (BAL) from young infants intubated with RSV bronchiolitis and comparing it to nasal aspirates for non-intubated RSV-infected infants, neutrophils were prevalant in both groups (76% and 93%, respiectively), monocytes were (10% and 2%) and lymphocytes were (9% and 5%). However, eosinophils were occasionally present in few patients BAL (less than 1%). Again, the double-negative $CD3^+$ T cells were the majority of the lymphocytes observed, but was not found to be γδ-T cells. On the other hand, $CD8^+$ T cells were found in a lesser ration to $CD4^+$ T cells.

Another study comparing the BAL of RSV-bronchiolitis patients to asthmatic and control BAL, showed prominent macrophage (55%) and neutrophilic component (37.5%), and some lymphocytes (Kim et al., 2003, McNamara et al., 2003). There was a subset with very few eosinophils but most RSV-bronchiolitis patient BAL had none, compared to asthmatic patients who had 3% eosinophils.

To put the observed histopathology together with the BAL findings, it should be noted that neutrophils can rapidly migrate to the airway lumen, and therefore are better detected in the respiratory secretions, as well BAL. Nasal aspirates could be selective for the cell types retrieved and do not necessarily reflect the inflammation within the lung parenchyma. In addition, different BAL techniques could result in differences in the percentages of the population observed frequently in the studies, along with patient

factors. Interestingly, a lot of similarities exist between the cell population in patients requiring intubation and the ones who did not. Taken together with Welliver et al. (Welliver et al., 2007) observation of the absence of T-lymphocytes and granzyme-positive cells in patients who died from severe RSV and the observation of higher CD8+ T-cells in the blood of RSV-infants who had milder disease (Isaacs et al., 1987), we might conclude that some subtypes of T-lymphocytes are important for viral clearance and control in human disease, while neutrophils with their high content of protein degrading enzymes could induce lung tissue damage that results in patient symptoms. Monocytes, as well, seem to play both a role in viral clearance and could potentially participate in patient symptoms. As Everard et al. suggested older children and adults with RSV had more alveolar macrophages in their BAL than neutrophils, which could relate to their milder symptoms.

There is, however, still a considerable gap in our understanding of human RSV disease in the very early stage of the disease (first two days), and the resolution phase, since multiple different events could dominate in these phases that are even less well understood than in the acute symptomatic stage.

Other than the indirect immune function of leukocytes in RSV disease, there are *in vitro* reports of direct RSV infection of primary human DC and human alveolar macrophages. Human lungs contain multiple types of DCs including conventional DCs (cDC), plasmacytoid DCs (pDC), and some myeloid-origin DCs (mDCs) that could be additionally recruited to the lung during infection (Plantinga et al., 2010, McDermott et al., 2011). DCs are varied in their ability to be infected by RSV, depending on their subset. For example, monocytic derived DCs (moDCs) expressed RSV antigen in 30% of 7-days infected cultures and produced infectious viral particles at $5\text{-}9x10^4$ pfu/mL (Jones et al., 2006). Johnson et al. (Johnson et al., 2011) showed that primary human mDCs and pDCs were infected with RSV maximally at 24 hours post-infection (6.8% and 0.9%, respectively). McDermott et al. (McDermott et al., 2011) reviewed all the *in vitro* studies of RSV infection of human DCs, and concluded that RSV infects 4% of DCs which can be increased to 25% in a multiplicity of infection $\geq$20, and this infection leads to the

production of less infectious viral particles than in epithelial cells. RSV-infected DCs mature and induce the maturation of the neighboring DCs. They up-regulate their expression of major histocompatibility complex molecules (MHC class I and II) and the co-stimulatory molecules CD83 and CD86. Nevertheless, despite the observation that these DCs produced multiple antiviral mediators, they lack the capacity to stimulate and induce the proliferation of naïve but not memory T-cells. This could be due to soluble factors produced by infected DCs and impaired synapse formation between DC and T-cells.

Human alveolar macrophages *in vitro* were productively infected by RSV up to 25 days post-infection, and the maximum infection was at 24 hours (around $6x10^3$ pfu/mL at MOI of 3) (Panuska et al., 1990). However, Cirino et al. (Cirino et al., 1993) showed that this infection was restricted to about 38% of freshly isolated alveolar macrophages that express MHC class II and that differentiation of the cells for 24-48 hours reduced the infection significantly. It should be noted that infection of murine alveolar macrophages is abortive (Franke-Ullmann et al., 1995).

Eventually, these *in vitro* reports concluded that the RSV infection of either human DCs or macrophages could be slow and less productive than in epithelial cells, and although RSV antigen was detected inside human lung macrophages in RSV bronchiolitis (Johnson et al., 2007), it was thought to represent mainly the phagocytic activity of the cell rather than infection.

1.8.2 Mediators produced during RSV infection

Multiple mediators are found in the respiratory secretions of patients with RSV disease. They include cytokines that regulate inflammation, chemokines that recruit different cells down a concentration gradient, proteolytic and matrix remodeling enzymes that could be released by granulocytes and monocytes among other cells, and even lipid mediators that result from arachidonic acid degradation in immune cells.

Kim et al. (Kim et al., 2005) showed that CXCL8 (IL-8), a chemoattractant for neutrophils and NK cells that binds to CXCR1 and CXCR2, was increased in BAL of RSV patients compared to asthmatics or control, while eosinophil chemotactic protein (ECP) was not significantly different from controls, consistent with the finding of the predominantly neutrophilic and monocytic cellular infiltrates mentioned above. As well Kim et al. reported that increased BAL IL-5, an eosinophil growth and survival factor, was only observed in asthmatic subjects and the subset of bronchiolitis patients with eosinophils (Kim et al., 2003). There was no difference between the IFN-γ concentrations in the BAL all the groups. However, Semple et al. showed that higher IFN-γ in nasal lavage in infants admitted with RSV bronchiolitis was protective (Semple et al., 2007). As well, Bennett et al. (Bennett et al., 2007) found increased levels of IFN-γ, IL-6, TNF, and IL-1β, but conversely there was an inverse correlation between duration of oxygen supplementation and IFN-γ as well as IL-6, CXCL8 and IL-10. This controversy among the studies regarding IFN-γ, which is a signature cytokine for NK, NKT, T cytotoxic lymphocytes and T helper 1, might be related to the different methods for the lavage, or that patient who had higher IFN-γ had more severe RSV disease associated with a general increase in all mediators. On the other hand, patients who were intubated had even worse disease severity possibly because they were not able to recruit sufficient T-cells to clear the infection (Kim et al., 2003). As Welliver et al. reported that Th2 cytokines (IL-4, 5, and 13) were inconsistently present in RSV patient's nasal lavage samples (Welliver et al., 2007).

Bennett et al. in (Bennett et al., 2007) reported chemokines (and neutrophil and macrophage growth factors) GM-CSF, G-CSF were also increased in patients' nasal lavage as well as CCL4, the monocytes, T-cells and NK cells chemoattractant (Maurer and von Stebut, 2004).

Harrison et al. (Harrison et al., 1999) also observed increased CXCL8, CCL3 –a similar chemokine to CCL4-, and CCL5 in RSV infected HEp-2 as well as the BAL from 10 intubated RSV bronchiolitis infants. Interestingly, CXCL8 and CCL5 production from the epithelial cell line was independent from viral activity, while CCL3 production

required replicating virus, indicating different mechanisms for chemokine induction in this epithelial cell line. CCL5 is a T-cell, monocyte and eosinophil chemotactic factor (Teran, 2000) that binds to CCR5, 3 and 1 (Sharma, 2010) and was reported to inhibit RSV replication in vitro (Elliott et al., 2004) but might be associated with the development of airway hyperreactivity in RSV patients (Chung and Kim, 2002, Zeng et al., 2011). Harrison et al. in (Harrison et al., 1999) also reported increased eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin, both are eosinophil ribonucleases, in the BAL. However, ECP could be stored and utilized by neutrophils as well (Kim et al., 2005). Another human respiratory epithelial cell line, A549, upregulated CCL5 and IL-6 expression in response to two RSV strains of the subtype B infection, but interestingly the amount IL-6 production was different between the 2 strains, independent of viral titer (Levitz et al., 2012). This could indicate some differences in the level of mediator and therefore the severity of RSV disease caused by different RSV strains.

Infection of well-differentiated primary bronchial epithelial cells (WD-PBECs) with different strains of RSV induced significant production of IL-6, the chemokines CXCL8, CCL5 and CXCL10, as well as TRAIL, the death ligand. CXCL10, binds CXCR3, is also called interferon-induced protein 10 (IP10) because it reflects type I and/or II IFN activity. CXCL10 produced by moDCs in response to IFN-α2a has a role in enhancing dendritic cell recruitment of CD8+ T-cells and macrophages (Muller et al., 2010, Padovan et al., 2002). As well, CXCL10 induced NK cell recruitment to clear Coxsackie B-3 myocarditis in mice (Yuan et al., 2009). Also CXCL10 with VCAM-1 (Vascular cell adhesion protein 1) contact allows human macrophages to induce B-cell differentiation into plasma cells *in vitro* and in mice. This CXCL10 from macrophages was induced by IL-6 from B-cells and dependent on STAT-3 phosphorylation (Xu et al., 2012). More importantly, CXCL10/CXCR3 interaction in RSV disease in mice modulates DC activation and recruitment of $CD8^+$ T-cells, and blocking this access leads to more severe disease (Lindell et al., 2008).

One of the observations of RSV pathology is submucosal edema and vascular congestion (Johnson et al., 2007). As Lee et al. observed this edema could be induced by local

cellular injury and cytotoxicity, contraction of the endothelium at post-capillary venules, or the action of vasoactive substances. RSV-induced edema could be mediated by several mechanisms one of which that could be amenable to modulation is the production of soluble factors from epithelial and immune cells (Lee et al., 2000). Lee et al. suggested vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) from epithelial cells to contribute to the increased vascular permeability and edema in RSV disease. VEGF is a glycoprotein which alters the vascular permeability through increasing endothelial fenestration, and also induces monocytes chemotaxis, angiogenesis and tissue remodeling in wound healing and in pathological conditions such as tumors, chronic inflammatory diseases and fibrosis (Ferrara et al., 2003). Lee et al. found higher VEGF/VPF concentrations in the nasal aspirates of patients with RSV than control and influenza patients. VEGF was also increased as early as 2 hours post-infection in the supernatants of RSV-infected A549 and normal human bronchial epithelium (NHBE) than baseline, however, the mRNA was not increased. In HIV, VEGF-A found in excess in the serum of patients and from infected T-lymphocytes in vitro. It was suggested to be implicated in the AIDS-associated vasculopathy and increased vascular permeability leading vascular leakage (Ascherl et al., 1999, Sporer et al., 2004).

Different subsets of the *in vitro* differentiated human DCs respond with different mediator profiles to RSV infection. For example, pDCs produce more IFN-α and CXCL8 than mDCs. Infection of moDCs leads to the upregulation of many pro-inflammatory cytokines including IL-1β, TNF, IL-6, IL-12 and IFN-γ, as well as the chemokines CCL2,CCL3, CCL4, CCL5, CXCL8, CXCL10 (McDermott et al., 2011). In addition, matrix metalloprotease MMP-9 and TIMP-1 and 2 were produced by mDC and pDC infected with RSV *in vitro* (Johnson et al., 2011).

There are some studies looking at the mediator profile of infected human macrophages. For example, human alveolar macrophages released the pro-inflammatory cytokines TNF in response to live RSV and IL-6, and CXCL8 in response to live and inactivated RSV (Panuska et al., 1990, Becker et al., 1991), as well as the anti-inflammatory cytokine IL-10 after live RSV infection (Panuska et al., 1995). Purified human macrophage-

monocytes also produced IL-1 "inhibitors" – recognized later as IL-1R antagonist or IL-1Ra- in response to RSV infection (McCarthy et al., 1989). Human macrophage production of these two soluble anti-inflammatory cytokines was postulated to inhibit further inflammatory responses to RSV *in vivo*.

1.8.3 Innate Immune Responses to RSV

The innate immune system is the first line of defense against pathogens including viruses since it is both loosely specific and rapidly activated. It is not only composed of resident innate immune cells (dendritic cells, mast cells and macrophages) and various recruited granulocytes and monocytes but includes anatomical and physiological barriers (e.g. surfactant proteins in lung alveoli and the complement system) as well as epithelial and endothelial cell responses. These structural cells can produce signals upon their infection or death that activate neighbouring immune cells. In addition, a pathogen is likely to be recognized rapidly through detecting certain conserved pathogen associated molecular patterns (PAMPs) by the innate immune pattern recognition receptors (PRRs) in structural and immune cells. Once the immune system is activated, an inflammatory cascade is initated to help contain the threat (for example induction of apoptosis of infected cell) or recruit other effector cells (e.g. neutrophil or NK cells) to enhance immune response and to establish a long term, highly specific immune response (adaptive immunity) through recruitment of lymphocytes. It is noticeable that immune cells have more PRR than structural cells (Blander and Sander, 2012). As well, agonists of these receptors can be utilized in vaccine production to enhance the immunity to the vaccine (Higgins and Mills, 2010).

Here I briefly review selected aspects of RSV interaction with the innate immune system in human and in mice, specifcally the PRRs.

1.9 Pattern Recognition Receptors (PRRs) and Downstream Signalling

Multiple components of an RSV particle can be detected by PRRs including envelope glycoproteins, single-stranded RNA (ssRNA), or the double-stranded RNA (dsRNA) structure formed during RSV replication cycle. The binding of these structures to their

respective “sensors” signals the initiation of the downstream activation of various transcription factors and genes to upregulate multiple antiviral effectors (mediators and intracellular proteins) as well as regulatory molecules. On the other hand, viruses - including RSV- have evolved different mechanisms to avoid the innate immune system recognition and the rapid halting of the viral replication and spread (Zeng et al., 2012).

1.9.1 Toll-Like Receptors (TLRs)

TLRs are type I transmembrane glycoproteins that are found in the plasma membrane (e.g. TLR1, 2, 4-6, and 10) or in the endoplasmic membranes like (TLR3, 7,8, 9). Ten TLRs have been identified in humans and twelve in mice. They all signal through myeloid differentiation primary-response protein 88 (MYD88) except for TLR3. TLR3 signals via Toll/IL‑1R (TIR)-domain-containing adaptor protein inducing IFNβ (TRIF) , while TLR4 signals through MYD88 and TRIF. TLR2 binds to bacterial lipoproteins or lipopeptides, TLR3 binds dsRNA, TLR4 to lipopolysaccharide (LPS), and TLR7 to ssRNA (Blander and Sander, 2012).

TLR2 was implicated in RSV infection of mice as a co-receptor to RSV. In conjuction wih TLR6 it also induced chemokines and cytokines production, neutrophil chemotaxis, DC activation, and controlled viral replication (Murawski et al., 2009). TLR3 is important in the induction of IFN-α, CCL5 and CXCL10 from epithelial cells but had no direct effect on viral clearance *in vitro* or *in vivo* (Rudd et al., 2005), and leads a Th1 prevalent immune response in mice versus an eosinophilic inflammation with mucus overproduction and increased IL-5 and IL-13 in TLR3 absence (Rudd et al., 2006). RSV infection of epithelial cells also upregulates TLR3, which could increase epithelial cell responsiveness to subsquent dsRNA challenges and produce CXCL8 through NFkB-dependent mechanism (Groskreutz et al., 2006).

TLR4’s role in RSV disease is controversal with studies suggesting a significant role for TLR4 in human and mouse immunity and others showing no significance. Part of this controversy, in mouse studies at least, could be related to differences in the mouse model used. For example, TLR4 gene polymorphism has been linked with the development of

severe RSV disease requiring hospitalization in human (Tal et al., 2004), yet the role of human polymorphism in TLR4 gene was also rejected in another study (Douville et al., 2010). Upregulated TLR4 on circulating blood monocytes in infants was associated with increased disease severity (Gagro et al., 2004), which could be related to specific upregulation of TLR4 in immune cells and their ability to recognize and response to the virus in severe disease, since another study found that TLR4 is required for infant innate immunity against RSV (Awomoyi et al., 2007). TLR4 and CD-14 as a co-receptor interact with RSV F protein. Subsequently it was found that C57BL/6 mice lack TLR4, had reduced NK function and chemotaxis to the lung, lower IL-12 levels, and delayed viral clearance (Kurt-Jones et al., 2000, Haynes et al., 2001). However, these findings are not found in BALB/c mice deficient in TLR4 (Ehl et al., 2004).

TLR7 deficiency in mice led to the development of Th17 environment in the lung with airway hyperreactivity, and increased mucus production along with the cytokines IL-13, IL-4 and IL-17, while type I IFN was not affected. DCs from these animals produced IL-23 preferential to IL-12. Blocking IL-17 resulted in reduced mucus production (Lukacs et al., 2010).

1.9.2 Retinoic Acid-Inducible Gene (RIG-I)-Like RNA Helicases (RLH) or RLR

These are cytosolic RNA sensors which include: retinoic acid-inducible gene I (RIG‑I) which likely binds ssRNA, melanoma differentiation associated gene 5 (MDA5) which binds dsRNA, and LGP2, the later is a regulator for the former two. RIG-I and MDA-5 contains caspase-recruitment domain (CARD) that interact wih IFNB-promoter stimulator 1 (IPS1, MAVS, or VISA) at the mitochondria to induce type I IFN production (Blander and Sander, 2012).

RSV leads to the upregulation of RIG-I mRNA proportionate to viral load in RSV infected patient nasal washes (Scagnolari et al., 2009). In epithelial cells, RSV infection leads to the upregulation of NF-κB activity and interferon-regulatory factor-3 (IRF3) and the production of IFN-β, CXCL10 and CCL5, and knockdown of RIG-I causes the downregulation of these mediators (Liu et al., 2007). However, NS1 and NS2 proteins of

RSV interfere with RIG-I interaction with MAVS leading to the reduced type I IFN production observed in RSV disease compared to other viruses (Ling et al., 2009).

1.9.3 Nucleotide-Binding Oligomerization Domain (NOD)-Like Receptors (NLR)

These are cytosolic receptors that recognize bacterial peptidoglycan and activate NF‑κB signalling. Several NLRs form multimeric complexes called inflammasomes for the activation of caspase 1 and the release of IL‑1β and IL‑18. However, they were recently found to play a role in type I IFN production as well (Blander and Sander, 2012).NOD2 was upregulated within 2 hours post ssRNA (RSV) exposure and interacted with MAVS at the mitochondria where it activated IRF3 and NF-κB. NOD2 deficient mce had more weight loss and viral lung pathology as well as pro-inflammatory mediator content (Sabbah et al., 2009, Vissers et al., 2012).

1.9.4 Type I interferons (Type I IFN)

There are three classes of interferon distinguished by the receptors they engage with; type I IFN including 13 human IFNα subtypes, IFNβ, IFNκ, IFNε, IFNo, IFNτ and IFNδ, and they all bind IFNAR (IFNα receptor) complex formed of IFNAR1 and IFNAR2. Type II IFN or IFN-g, and type III IFN that include three IFNλ subtypes (Sadler and Williams, 2008). Type I IFN are known to be potent antiviral cytokines, and also has apoptotic, antiproliferative and immunmodulatory effects. Type I IFN for example are important in the survival of memory T-cells and differentiation as well as the activation of NK cells (Malmgaard, 2004). When type I IFN bind their receptor, they activate more than 300 interferon-stimulated genes (ISG)s some of which are antiviral in nature like ISG15 (IFN-stimulated protein of 15 kDa), the GTPase Mx1 (myxovirus resistance 1), ribonuclease L (RNaseL) and protein kinase R (PKR). These antiviral molecules modulate cell cytoskeleton, cause cell apoptosis and modify cellular post-transcriptional and translational events. Some ISGs are PRRs that detect intracellular viral molecules, and others are transcription factors that amplify the type I IFN signaling (Noppert et al., 2007).

The classical pathway leading to type I IFN induction includes the RIG-I/MAVS co-localization and the recruitment of the adapter family TRAF. TRAF3 activates the NEMO-TANK-TBK1/IKKε kinase complex that phosphorylates IRF3 and IRF7, and TRAF2/TRAF6 complex activates thre kinases; IKK, p38, and JNK, upregulating the transcription factors, NF-κB, ATF-2 and c-Jun, respectively. These activated elements, bind to the IFN-stimulated response element (ISRE) sequence at the type I IFN promoters and induce IFN-α and IFN-β (Fensterl and Sen, 2009, Belgnaoui et al., 2011). IFNs released from the infected cells induce an antiviral state in the neighboring uninfected cells (bystander effect) (Sen and Sarkar, 2007, Aaronson and Horvath, 2002).

Downstream of type I IFN receptors signaling is the activation of the Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. ISGF3 complex composed of IRF-9, STAT1, and STAT2 translocates to the nucleus, binds to the ISRE and induces transcription of ISGs (Fensterl and Sen, 2009). RSV infected mice and A549 cells induce the IFIT (IFN-induced protein with tetratricopeptide repeat/TPR) family of ISGs. These include IFIT1 (ISG56), IFIT2 (ISG54) and IFIT3 (ISG60) (Hastie et al., 2012, Janssen et al., 2007). These IFIT can be directly induced by viral molecules without IFN effect, and could inhibit some viruses (Fensterl and Sen, 2011). Their role in RSV disease warrants attention since RSV is known to target several steps of type I IFN induction. RSV targets type I IFN production through NS1 and NS2 actions. RSV non-structural proteins prevent the RIG-I association with MAVS (Ling et al., 2009), degrades TRAF3 (Swedan et al., 2009, Swedan et al., 2011), sequester IRF3 (Ren et al., 2011), as well as it prevents the nuclear translocation of IRF3 (Bossert et al., 2003, Spann et al., 2005). They can also degrade STAT-2 downstream from type I IFN signaling therefore blocking some type I IFN responses (Swedan et al., 2009, Swedan et al., 2011).

On the other hand, it is worth pointing out that the recently described type III IFN might have a role in limiting the spread from the nasal mucosa down to the lower airways, since they were produced from nasasl epithelium more than type I IFN indicating some different location for the type I and type III IFN function (Okabayashi et al., 2011).

1.10 RSV Disease models in Mice: Viral and Mouse Strains

There are multiple lab animal models for RSV disease including chimpanzees, cattle, sheep, cotton rats, and mice. In addition, pneumonia virus of mice and bovine RSV in their respective host have been used as an approximation to hRSV in human. Nevertheless, all of these models have limited generalizablity to human and are not significantly affected with hRSV or respond differently than in human disease (Bem et al., 2011). Mice in particular are important to utilize in the study of RSV disease despite their limitations, since our knowledge of their immune system, the relatively wide availability of knockout strains, the lower cost, and the ethical convenience facilitate mouse use in various investigations (Bem et al., 2011). For example, the research that enabled the use of palivizumab in RSV prophylaxis was established in mice and cotton rats, as well as studies of RSV vaccine-enhanced disease (Openshaw, 2013).

However, modelling RSV disease in mice is compounded by many factors including differences in the immune response in mice from humans, reduced permissiveness to hRSV in mice, and the different anatomy of the respiratory tract between the two species. The most permissive mouse strain to hRSV is the BALB/c mouse (Bem et al., 2011). Unfortunately, this means that certain immune deficiencies established in other strains like C57BL/6 need to be crossed to BALB/c specifically to ensure the closest representation of the human disease. Therefore, considerable heterogenity might present in the hRSV mouse disease literature based on the mouse strain used in the study.

Mouse strain is not the only variable in altering the severity of clinical disease and pathology in mice but also there are RSV-strain specific differences (Stokes et al., 2011) For example, RSV-line 19 is a strain that was adapted from RSV-A2 strain through repeated intracranial injection in mice (Cavallaro and Maassab, 1966). It was found that RSV-A2 and long strains induced Th1 like responses in BALB/c mice with less airway hyperreactivity, compared to RSV-line 19 which presnted with goblet hyperplasia, IL-13 mediated airway hyperreactivity (Lukacs et al., 2006, Moore and Peebles, 2006). A recent study has also compared six strains of RSV including clinical isolates, and found

that RSV-line 19 (adapted experimental isolate) and RSV 2-20 (clinical isolate) were higher in neutrophilic infilitrateof the BAL, weight loss, airway hyperreactivity and mucus upregulation (Stokes et al., 2011).

1.11 Mast Cells

Mast cells are highly granular innate immune cells that are prevalent in tissues at the interface between the host and the environment as in the skin, gut and the airways. Mast cells are well known in the development of allergic diseases. However, they play an important role in the host defense against certain pathogens like bacteria and parasites. Recently, the involvement of mast cells in viral diseases has drawn more attention. Classical mast cell activation in allergic disease includes the cross-linkage of specialized IgE FC receptors (FCεRI), after binding of two or more IgE molecules, leading to mast cell degranulation and release of various mediators causing symptoms of allergy. However, further study of mast cell biology and function revealed multiple mechanisms for mast cell activation. Mast cells can not only be activated by allergic stimuli but also by cytokines, complement components, antibody light chains, neuropeptides, hormones and bacterial and viral components (Moon et al., 2010). In fact, mast cells can even respond to danger signals coming from structural cells (like IL-33 or alarmin from necrotic epithelial cells) (Enoksson et al., 2011). Additionally, mast cells could respond differently or more vigirously to different combinations of these stimuli (Theoharides et al., 2007). Mast cells could respond rapidly by the release of a wide range of preformed mediators or synthesize new ones. As well, they secrete exoxomes that contain shuttle RNAs (mRNA and microRNA) which were found to differentiate DCs *in vivo* and might affect B and T lymphocyte function as well (Moon et al., 2010). Mast cells could participate in immunity against pathogens either directly through destruction via phagocytosis and free radical formation or antimicrobial peptides, or indirectly through action on structural, innate and adaptive immune cells (Urb and Sheppard, 2012). This eventually enables mast cells to participate in many physiological and pathological processes from wound healing to autoimmune disorders and infections.

1.11.1 Mast Cell Localization and Development

Mast cells are granulocytes that arise from mast cell committed progenitors which originate in the bone marrow from multipotent hematopoietic progenitor cells. In humans, these immature mast cell progenitors circulate in the blood expressing the markers CD13, CD33, CD38, CD34 and most importantly CD117 or c-Kit. In mice, mast cells can also differentiate from granulocyte/monocyte progenitor into a mast cell-basophil common progenitor that can be found in spleen or a monopotent progenitor in the bone marrow or intestine (Okayama and Kawakami, 2006, Moon et al., 2010).

Mast cells finally reside in various tissues to mature where they downregulate their CD34 expression but retain CD117 (c-Kit) expression (Kumar and Sharma, 2010). In mice, these cells differentiate from either the resident committed progenitors like in the intestine, or more likely from the circulating progenitors as in the lung (Okayama and Kawakami, 2006, Moon et al., 2010).

Mast cell progenitors home to their final destination under the effect of various constitutive, homeostatic or induced factors including several chemokines and their receptors, growth factors, and adhesion molecules. Stem cell factor (SCF or Kit ligand), the membrane-bound or the soluble isoform, has been shown to be one chemotactic signal. Integrins support the process of migration for developing mast cells. Mast cell progenitors also respond to the ligands for CCR3, CCR5, CXCR2 and CXCR4 *in vitro*. In patients with asthma, CXCR3 expression is selectively dominant in the airway smooth muscle resident mast cell, likely responding to the high expression of its ligand CXCL10 in the airway smooth muscles (Okayama and Kawakami, 2006). As well, mast cell localization into specific tissues relys in part on the selective interaction between mast cell receptors and various adhesion molecules in the tissues. For example, recruitment of mast cells to the mouse lung during antigen-induced inflammation is dependent on vascular cell adhesion molecule-1 (VCAM-1) interaction with $\alpha4\beta1$ and $\alpha4\beta7$ integrins (Moon et al., 2010). Mast cells bearing specific IgE can be also recruited by different antigens (Okayama and Kawakami, 2006). Also, complement components C3a and C5a are chemotactic for mast cells (Dawicki and Marshall, 2007).

Human mast cells are rich in granules that contain multiple serine proteases, including typtase (α, β, or γ) and chymase. According to their protease content, mature human mast cells are divided into M_{TC} (trypase and chymase), M_T (tryptase), and Mc (chymase). Mouse-mast cells on the other hand were originally described to have two different subtypes (mucosal versus connective tissue mast cells) with protease content homologous to that in humans but have more recently been recognised to have a more complex protease profile. Specifically, M_T shares some characteristics with mucosal murine mast cells, while M_{TC} are somewhat similar to mouse connective tissue mast cells. Human tissues are likely to have mixed populations of different mast cell subtypes (Moon et al., 2010), but for example, alveolar septa in the lung have more M_T while M_{TC} are more abundant in skin and submucosa of small intestine (Kumar and Sharma, 2010).
Mast cells are very heterogenous, for example, in the same culture individual mast cells can respond differently to the same stimulus, and in different microenvironments, the same mast cells could switch phenotypes (Moon et al., 2010).

The microenvironment is critical to the nature of mast cell development. Generally, SCF enhances anti-apoptotic protein expression and hence is crucial for survival of murine mast cells and in the growth, development and proliferation of human mast cells. SCF binding to Kit on the surface of mast cells activates the Kit tyorisne kinase cytoplasmic tail which then phosphorylates multiple proteins that further recruit multiple transcription factors to regulate the experession of microenvironment-specific mast cell characteristics. Mast cells activated via IgE also maintain their survival (Okayama and Kawakami, 2006). SCF combined with other cytokines can differentiate mast cells *in vitro* into distinct phenotypes more than with SCF alone. For example, human mast cells exposed to IL-4 reduce c-Kit expression and upregulates FCεRI and chymase. As well, cord blood progenitors treated with IL-6 have increased cell size, histamine level and proportion of chymase-containing cells, while those treated IL-33 have accelerated tryptase expression. IL-9 was also reported to enhance cell size and number with SCF in mast cell derived from human mast cell progenitors. IFN-γ induces the expression of FCγRI on human peripheral blood-derived mast cell (Okayama and Kawakami, 2006, Moon et al., 2010).

Therefore, we can infer that altering the microenvironment of mast cells could serve to change their phenotypes and their function.

1.11.2 Mast Cell Models *In Vitro*

HMC-1 cells were the first recognised human mast cell line, introduced in 1988 (Butterfield et al., 1988). They were isolated from a patient with mast cell leukemia. However, HMC-1 cells are immature with less-formed granules and lack some mast cell characteristics. Their growth is independent of SCF, they lack IgE receptors and other mast cell surface markers, yet they maintain a tryptase activity (Butterfield et al., 1988, Moon et al., 2010). Another cell line that resembles more the tissue mast cells is called LAD2 (Kirshenbaum et al., 2003). LAD2 was developed from the bone marrow of a patient with mast cell sarcoma/leukemia and they are dependent on SCF as well as respond to IgE activation with degranulation (Kirshenbaum et al., 2003, Moon et al., 2010). There are also several mouse and rat mast cell lines like mouse MC/9 and rat basophilic leukemia cells (Dawicki and Marshall, 2007).

Primary cultures of mast cells are more difficult to maintain but preferred to cell lines because they maintain most if not all mast cell characteristics. Yet they are still incompletely representative of *in vivo* mast cell phenotypes due to the different microenvironment from that of tissues (Moon et al., 2010). Human mast cells can be derived from stem cells in human cord blood (CBMC), fetal liver, peripheral blood CD34+ progenitors or bone marrow (Moon et al., 2010, Dawicki and Marshall, 2007). Human mast cell could also be retrieved from tissues like lung, skin, uterus and intestine. However, this process yields low numbers of mast cells and the isolation process leads to disruption of mast cell phenotype (Moon et al., 2010). Mast cells derived from mouse bone marrow (mBMMC) or from the mouse peritoneal cavity are not difficult to obtain, but results from these can be complex to interpret for human disease. Mouse BMMCs (mBMMC) can be cultured in different media to develop mucosal or connective tissue mast cell similarities (Moon et al., 2010).

Culturing of human mast cells in different cytokines could also improve the yield and phenotype of the derived cells. IL-6 in particular (enhanced by PGE_2) in the presence of SCF was found to enhance mast cell development from cord mononuclear cells or CD34+ cells. These cells were able to mature, secreted multiple mast cell mediators, and responded to IgE stimulation (Saito et al., 1996). IFN-γ and TNF or LPS can also induce expression of MHCII on HMC-1 and mBMMC (Moon et al., 2010).

1.11.3 Mast Cell Deficient Mice

Mast cell deficiency in mice was first observed in mice with a spontaneous mutation in the SCF receptor gene (c-Kit gene). Kit signaling is crucial for mast cell development form progenitors. $Kit^{W/Wv}$ mice have a deficiency in c-Kit function, however, they are anemic, sterile and have reduced numbers of neutrophils in blood and bone marrow (Moon et al., 2010, Katz and Austen, 2011). A similar phenotype is observed in mice deficient in production of SCF (the SLD mouse). $Kit^{W\text{-}sh/W\text{-}sh}$ C57Bl6 mice were described by Grimbaldeston et al. (Grimbaldeston et al., 2005) and since then have been used in the study of mast cell roles in various disease. These mice have an inversion mutation in the gene encoding Kit, but have relatively normal c-kit function during early development. They are fertile and not anemic, and can be crossed on BALB/c background with success. However, they were recently found to have increased myeloid-derived suppressor cells (MDSC) and neutrophilia of blood, bone marrow and spleen, specifically after 10 weeks of life (Michel et al., 2013). Therefore, selective reconstitution of these mice with BMMC from wild type are crucial to better understand the contribution of mast cell deficiency , specifically, rather than other defects to the experimental findings. Nevertheless, aberrant distribution of mast cells following reconstitution could develop (Katz and Austen, 2011).

Other than mutations in *Kit*, Dudeck et al. (Dudeck et al., 2011) has described mast cell protease (MCP)-5-*Cre*- inducible diphtheria toxin receptor transgenic mice, in which mast cells can be ablated without affecting other bone marrow lineages. However, due to restriction in MCP-5 distribution in mast cells, some mucosal tissues are not completely depleted of mast cells like in the stomach (Katz and Austen, 2011). Finally, mast cell

depletion was detected in carboxypeptidase A3-Cre+ mice ($Cpa^{Cre/+}$), with only some reduction on spleen basophils (Feyerabend et al. 2011). This last model seems to be the most reliable current representation of mast cell deficiency (Katz and Austen, 2011).

1.12 Mast Cell Mediators

Mast cells can respond to a wide range of stimuli with varying intensity, from the release of selected mediators (differential release, intagranular activation or piecemeal degranulation) to extensive degranulation, the latter is more seen in allergic conditions rather than in inflammatory or autoimmune disorders (Moon et al., 2010, Theoharides et al., 2007). Mast cell differential release of preformed mediators involves ultrastructural changes that leads to segregation of selected mediators into vesicular compartments and their further release independent from extracellular calcium. This process is different from the classical degranulation process of mast cells that involves the calcium dependent exocytosis of secretory granules. This is not surprising since the downstream signaling leading to degranulation is different from that leading to piecemeal degranulation. (Theoharides et al., 2007). However, even the classical triggers for degranulation could be modified to induce selective release of mediators in the presence of certain adaptor molecules that uncouple certain component of the down stream signaling pathway of degranulation, for example in the case of preformed IL-6 and TNF release in response to FCεRI stimulation without eicosanoid (e.g. leukotrienes and prostaglandins) production (Rivera, 2006).

In addition, mast cell newly generated mediators responses to a specific stimulus can be further modified under the effect of the microenvironment. For example, mast cells primed with IL-4 in the presence of SCF are more likely to produce Th2 cytokines like IL-13, while mast cells primed with IL-5 secrete higher pro-inflammatory cytokines and chemokines like TNF, GM-CSF and CCL3. (Theoharides et al., 2007). Generally, mast cell mediators can be divided into preformed, pre-stored mediators that can be released from the granules immediately after activation, and newly (de novo) synthesized mediators produced upon activation.

1.12.1 Pre-stored, Granule-Associated Mediators

Mast cell preformed mediators include: proteoglycans like heparin, multiple enzymes like chymase, tryptase, metalloproteinases and phospholipases, biogenic amines (e.g. histamine), polypeptides like corticotropin-releasing hormon (CRH), vascular endothelial growth factor (VEGF) and antibacterial peptides like cathelicidin, as well as the chemokines CXCL8 and CCL5 (Theoharides et al., 2007).

Proteoglycans are part of the extracellular matrix (ECM) and the glycocalyx of cells. The glycosaminoglycan (GAG) portion of the proteoglycan bind chemokines to stabilize them in the desired tissues. Therefore GAGs provide the required anchorage for a chemotactic gradient that prevents the chemokines (e.g. CCL3, CCL4, CCL5) from escaping into the blood stream, and subsequently enables leukocyte migration along that gradient. GAGs has been also participate in the transport of chemokines from the basolateral surface to the apical membrane of endothelial cells, hence approximating the chemokine to the passing leukocytes (Mortier et al., 2012).

Mast cells, on the other hand, have a wide range of proteases that function to degrade or process lipids, carbohydrates, proteoglycans in (ECM), and peptides like in antigen degradation, as part of remodeling (e.g. of ECM), to modify their activity (e.g. for cytokines and chemokines) or produce new mediators like the production of phospholipid metabolites (Theoharides et al., 2007). Additionally, mast cell tryptase potentiates bronchial hyper-responsiveness either through acting on special receptors in neuronal tissues and airway smooth muscles (proteinase-activated receptor-2 or PAR-2) or through lowering the threshold for other bronchoconstrictors also secreted by mast cells like histamine and LTC_4. It is notable that protease gene expression is the highest in asthmatic bronchial epithelial layer that includes mast cells. Mast cell tryptase cleaves CCL5 and CCL11 (Pang et al., 2006). Also, mast cell chymase can act in concert with antibacterial peptides in the skin to produce neutrophil chemotactic peptides. As well, it induces CXCL8 release from endothelial cells. Interestingly, mast cell proteases exhibit feedback regulation, for example, chymases could degrade metalloproteinases to inactivate them (Pang et al., 2006, Caughey, 2007, Dawicki and Marshall, 2007).

An extensively studied mast cell mediator is histamine which is a bronchoconstrictor, vasodilator, induces mucus production from epithelial cells, promotes angiogenesis and modulates T-cell responses as well as promoting DC migration (Theoharides et al., 2007, Shea-Donohue et al., 2010, Urb and Sheppard, 2012).

1.12.2 De novo Synthesized Mediators

Depending on the stimulus, mast cells can up-regulate the production of new mediators including the phospholipid metabolites (eicosanoids), multiple cytokines like IFN-α, IFN-γ, TNF, and interleukins, chemokines and growth factors such as GM-CSF, VEGF, and nitric oxide (NO).

Mast cells constitutively express multiple cytosolic phospholipases including phospholipase C and phospholipase A_2 ($cPLA_2$) which respond rapidly (5-10 minutes in allergic activation) to variable stimuli to generate arachidonic acid, the precursor of the phospholipid metabolites prostaglandins (PGs) and leukotrienes (LTs). $cPLA_2$ translocates to nuclear envelope and endoplasmic reticulum where PGs and LTs are produced under the effect of the cyclooxygenase and lipoxygenase enzymes respectively. Prostaglandin endoperoxide synthase enzyme (PGHS-1 and 2 or COX-1 and 2) generate unstable PGG_2 and PGH_2 which further breakdown to the active components PGD_2, PGE_2/PGF_2, prostacyclin (endothelium) and some thromboxane in mast cells. Lipoxygenases (like 5-LO) generate LTA_4, and LTB_4. However, under the action of LTC_4 synthase (or alternatively microsomal glutathione-*S*-transferase), LTA_4 is further metabolized into LTC_4 (in mast cells, basophils and eosinophils). LTC_4 is catalyzed extracellularly into the other cysteinyl leukotrienes (cysLTs) by transpeptidases LTD_4 and by dipetidase into LTE_4 which are the active metabolites (Bingham CO III, 1999). LTB_4 is important in chemotaxis of neutrophils for bacterial clearance and recruits subsets of CD8+ T-lymphocytes (Marshall et al. 2007, Kumar and Sharma et al. 2010). CysLT (LTD_4) is a 1000 times more potent brochoconstrictor and vasodilator than histamine and is crucial in bronchial asthma, while LTE_4 is very important in hypersenstitivity reaction. Initially, mast cell respond by the generation of both PGs and

LTs in the immediate phase but in the later phase PGs production is prevalent. However, the amount of production of each metabolite is affected by the phenotype of mast cells. For example human lung mast cells of M_T phenotype produce more LTC_4, while uterine M_{TC} cells generate more PGD_2. As well the maturity of mast cells, the presence of stem cell factor with other cytokines, and co-culturing with other cells like fibroblast affect the amount of metabolite produced as was demonstrated from studies on mBMMCs (Bingham CO III, 1999).

PGD_2 (receptor CRTh2) participates in vasodilatation and regulating smooth muscle contractility (e.g. in uterus or bronchi), as well it may selectively induce chemotaxis Th2 and eosinophils (Theoharides et al., 2007, Moon et al., 2010). Interestingly, PGE_2 (EP receptors) has a differential role where it can induce human mast cell release of VEGF and mouse mast cell protease (MCP) without degranulation, inhibit FCεRI mediated histamine release in human lung mast cells, yet induce degranulation in the skin mast cells of older but not younger mice (Theoharides et al., 2007, Moon et al., 2010).

Despite that mast cell-produced cytokines and chemokines overlap with that for structural cells and other innate immune cells, mast cells are distinguished with their ability to differentiate between various stimuli and respond selectively. For example, mast cells secrete IFN-γ, IL-12 and IL-18 and produce CXCL10 and CXCL8, all of which are associated with Th1 responses. On the other hand, mast cells can produce Th2 inducing cytokines like IL-4, 5 and 13, and CCL5 which is related to Th2 responses and eosinophil recruitment. Mast cells also produce CCL3 and CCL4 contributing to monocytes and NK cell recruitment (Dawicki and Marshall, 2007). Mast cells also secrete multiple pro-inflammatory cytokines such as IFN-α and β, IL-1β, IL-6 and TNF, and anti-inflammatory cytokines such as IL-10 and TGF-β. Mast cell chemokines and cytokines also recruit immature dendritic cells (like CCL20) and affect their function, and could affect B-lymphocyte antibody production which could further impact antigen presentation and adaptive immunity (Dawicki and Marshall, 2007, Kumar and Sharma, 2010, Moon et al., 2010). Collectively, mast cells could participate in the induction of inflammatory response characterized by granulocyte (neutrophil and eosinophil) or

mononuclear cell (monocytes, NK cells and lymphocytes) recruitment. It is expected that the nature of the damage resulting from inflammation is determined by the cells recruited and their subsequent activation, for example granulocytes are rich in digestive enzymes that could harm normal tissues leading to worsened inflammation outcome. Therefore, mast cells might have an important role in the inflammatory cell recruitment and activation process impacting the inflammation outcome.

1.13 Pattern Recognition Receptors (PRRs) and Mast Cells

As mentioned before, PRRs are important in sensing pattern-associated molecular patterns (PAMPs) related to different types of antigens and pathogens and are important components of the innate immune system. Mast cells are located near epithelial and endothelial cells and can secrete preformed mediators within seconds of stimulation or sensing the pathogen, which gives them an advantage in contributing to regulation of innate immune responses and the following adaptive immunity (St John and Abraham, 2013). Therefore, mast cell expression of various toll-like receptors (TLR)s, Nod-like receptors (NLR)s, RIG-I like helicases and others, is likely to have an important role in regulating innate immune responses. It is observed that TLR ligation *in vitro* usually leads to the release of chemokines and cytokines rather than the vigrous degranulation of mast cells (St John and Abraham, 2013). Non-immune cells often have lower PRR expression than immune cells to prevent their overstimulation (Sandig and Bulfone-Paus, 2012) emphasizing the important role of tissue resident sentinel cells such as mast cells in innate immune response. Human and murine mast cells have differential responses to different PAMPs. So, for example, a macrophage cytokine responses to lipopolysaccharide (LPS) and peptidoglycan (PGN) are similar, while mast cells have a different response to each of these stimuli (Sandig and Bulfone-Paus, 2012).

Human CBMCs express TLR1, 2, 4 and 6, while human peripheral blood derived mast cells express TLR1-7 and 9 mRNA. Murine mast cells express TLR1-13 with the exception of TLR-5 (Sandig and Bulfone-Paus, 2012, Kumar and Sharma, 2010). Surface TLRs include TLR1, 2, and 4-6, while intracellular TLRs include TLR3, 7, 8 and 9. Human CBMC and human mast cells derived from blood progenitors (PBDMC)s

stimulated with zymosan (TLR2/6 ligand) or PGN (TLR2 ligand) released GM-CSF, IL-1β, LTB_4, LTC_4, and TNF (Sandig and Bulfone-Paus, 2012). TLR2 ligation is likely to induce mast cell degranulation in CBMC, however, Pam3 CSK4 (synthetic ligand for TLR1/2) induced the same cytokines, but without leukotriene generation (McCurdy et al. 2003). While PBDMC produced TNF only with the TLR4-agonist (LPS) stimulation. This indicates that TLR2 is a more potent inducer for mast cell responses than TLR4. Indeed some mast cell cultures in humans do not express TLR4 without additional cytokine treatment (McCurdy et al., 2001). Mast cell pre-treatment with IFN-γ increases TNF produced in LPS stimulation of human lung mast cells. As well, IgE enhances LPS stimulation of TLR4. Flagillin (TLR5 ligand) causes human mast cells release of TNF and IL-1β (Sandig and Bulfone-Paus, 2012).

Intracellular TLRs are responsible for sensing viral and bacterial components, specifically RNA and DNA. Murine BMMC are less useful in delineating the effect of ligation of these TLRs on mast cell function than other mature cells like fetal skin derived mast cells (FSDMC)s, due to mBMMC immaturity and heterogenicity. TLR3 synthetic ligand poly I:C (resembles dsRNA) induced IFN-α and β from human PBDMCs . As well, ligand for TLR9 CpG-oligonucleotides released TNF, IL-1β, IFN-α and cysLTs from human PBDMCs (Kulka et al., 2004). On the other hand, TLR3 agonist, TLR7 agonist R848 (ssRNA like) and CpG-oligonucleotides induced TNF, IL-6, CCL2, CXCL2 and CCL5 from murine FSDMCs (Sandig and Bulfone-Paus, 2012).

In experimental models of viral infection TLRs and the downstream RNA/DNA sensors such as RIG-I, MDA5, MAVS, and PKR play a role in the function of mast cells. For example, mast cell recruitment of cytoxic T-cell in Newcastle virus model was dependent on TLR3 (Orinska et al., 2005). RIG-I and PKR were improtant for mast cell mediator production in dengue virus infection in vitro (Brown et al., 2011), as well as MDA5 (St John et al., 2011). Murine mast cell infection with influenza A showed that RIG-I/MAVS were important for cytokines and chemokines production but not for histamine release (Graham et al., 2013).

TLR signaling in mast cells is illustrated in Figure 2. It is noticable that mast cells appear to lack the MyD88-independent TLR4 signaling pathway maybe due to absence of CD14 expression by murine mast cells which might be required to internalize TLR4 in order to fet in contact with TRIF (Keck et al., 2011). As well, dectin-1 might act as a co-receptor for TLR in mast cells (Sandig and Bulfone-Paus, 2012).

1.14 Mast Cell Responses to Pathogens

A role for mast cells in host-pathogen interaction was first studied in models of helminth infection (Urb and Sheppard, 2012). Mast cell hyperplasia is observed in many *in vivo* models of helminth infection (Moon et al., 2010). Some of these models indicate mast cells have a crucial role in expelling the parasite such as in *Strongyloides* and *Trichinella spiralis* primary and chronic infection of the gut through mast cell proteases. Control of parasitemia has also been observed, via TNF, in the *Plasmodium berghii* model of malarial infection or in limiting cutaneous *Leshmania major* inflammation (Moon et al., 2010, Kumar and Sharma, 2010, Urb and Sheppard, 2012) . An IgE response leading to mast cell degranulation is important in preventing secondary helminth infection (Dawicki and Marshall, 2007).

Other than parasites, mast cell sense fungal wall components like zymosan via dectin-1 receptor (lectin receptor) (Olynych et al., 2006) and respond by either degranulation or the production of selective mediators. Mast cell degranulation in response to fungi is either IgE dependent, like in *Trichoderma viridae* infection of human bronchiolar cells, or IgE-independent like in *Aspergillus fumigatus* (Moon et al., 2010).

In addition, mast cells have an established a role in limiting gram-negative bacterial infection. Mast cell derived TNF and LTB_4 are crucial in neutrophil chemotaxis and clearance of bacteria in peritoneal sepsis mouse models (Echtenacher et al., 1996, Malaviya and Abraham, 2000). Mast cells limit infection in mouse models of *Klebsiella pneumoniae* and *Mycoplasma pneumoniae* lung infection, group A *streptococcus* skin infection, and *Haemophilus influenzae* otitis media (Urb and Sheppard, 2012). As well,

human mast cell IL-1 induces CXCL8-mediated neutrophil migration in *Pseudomonas aeruginosa* infections (Lin et al., 2002).

In recent years, mast cells have been identified as a potential players in viral infection. They could produce multiple mediators that could affect viral disease symptoms or impact immunity to viruses through induction of antiviral state in the tissues in which they reside or through the recruitment of effector immune cells. Sendai virus releases histamine from rat mast cells even with low extracellular calcium ion through lesions in the cell membrane (Gomperts et al., 1983). Dengue virus (DV) infection demonstrates increased endothelial permability leading to shock. In addition, DV infection is more deleterious in patients with pre-existing viral antibodies. Antibodies enhance dengue virus infection of human mast cells (HMC-1 and CBMC), which in turn secrete TNF and other factors that activate human endothelial cells as evidenced by their subsequent expression of adhesion molecules (Brown et al., 2011). Mast cell/basophil cell line KU812 expressed DV antigen which was virus-specific antibody dependent, and produced IL-1β and less IL-6 (King et al., 2000). In addition, KU812 and HMC-1 cells produced CCL3, CCL4, and CCL5 in response to DV in the presence of sub-neutralizing antibodies (King et al., 2002). Also, DV leads to caspase-dependent MC apoptosis specifically (Brown et al., 2009). Human cord blood mast cells (CBMC) infected with DV upregulate interferon stimulated genes and type I interferon which is protective from reinfection with DV, and CCL4, CCL5 and CXCL10 (Brown et al., 2012). There is a role of mast cells in limiting DV spread as was confirmed in mast cell deficient mice. These mice had increased viral burden in the draining lymph nodes. This effect was also dependent on mast-cell recruitment of NK and T cells into the infection site (St John et al., 2011). Most importantly, in patients with dengue virus hemorrhagic fever, serum levels of VEGF, chymase and tryptase (the later two are specific mast cell proteases indicating likely degranulation) were suggested to be used as predictive measures of disease severity (Furuta et al., 2012).

Human peripheral blood-derived primary cultured mast cells (HCMC) and LAD-2 cell line were abortively infected with influenza A (Marcet et al., 2013). Interestingly, *in vivo*,

mast cell deficient mice were resistant to the pathology induced in wild-type mice infected with a mouse-adapted strain of influenza A. This resistance was associated with reduced levels of some BAL mediators (TNF, CCL2, CCL3, CCL4, CXCL2, and CXCL10) and cellular infiltrate. Also, mBMMC were able to produce histamine, LTB_4 and IL-6 in response to *in vitro* activation with influenza virus for 6 hours (Graham et al., 2013). However, it is important to consider the doses of virus used in some of these studies.

Another virus infection that is characterized by vascular leakage is hantavirus infection. Differentiated human skin mast cells were susceptible to successful infection with hantavirus and upregulated interferon stimulated genes as well as CCL5 mRNA (Guhl et al., 2010). In a Newcastle virus model of peritonitis, mast cells recruited CD8+ T cells (Orinska et al., 2005). This could be an important process for the enhancement of local antiviral T cell effector responses.

Chemokines produced by mast cells could be particularly important for early effector cell recruitment during viral infection. CBMCs produce CXCL8 if infected with reovirus. This CXCL8 then induces the chemotaxis of NK cells (Burke et al., 2008). Also, supernatants of mast cells infected with reovirus induced NK cell activation through type I IFN and exogenous IL-18 *in vitro* (Portales-Cervantes et al. unpublished data).

Human skin mast cells inactivate vaccinia virus and protect against infection in mice. This protection occurs through the release of mast cell antimicrobial peptides (cathelicidin) secondary to mast cell degranulation following virus fusion to cell membrane and activation of sphingosine-1-phosphate G-coubled receptors (S1PR2) (Wang et al., 2012).

Mast cell progenitors can be latently infected with human immunodeficiency virus (HIV). When these cells reside in tissues and mature, they could form a reservoir of the virus that could help spread the infection if activated, even if the patient was on high activity antiretroviral therapy. That was evident from the existance of mast cell progenitors in the

blood and placental tissue mast cells infected with HIV in pregnant women (Sundstrom et al., 2007).

From the previous studies on mast cell role in viral disease, it seems that viruses induce mast cell production of chemokines that recruit monocytes and T cells rather than neutrophils, unlike what is observed in mast cell action in bacterial infections (Dawicki and Marshall, 2007).

1.14.1 Mast Cell Responses to RSV

The role of mast cells in RSV disease has not been investigated. There is some indirect evidence of mast cell activation in RSV disease, the significance of which is not fully understood. Everard et al. (Everard et al., 1995), found increased levels of tryptase specifically at the first two days in the BAL on infants with RSV bronchiolitis, yet IgE levels were not consistently detectable in the same group. As well, Urinary 9alpha,11beta-PGF(2), a marker of mast cell activation, and urinary LTE_4 were higher in infants admitted with RSV bronchiolitis and wheezing than in controls with no correlation between the two markers and the persistance of wheeze in RSV-positive infants (Oymar et al., 2006). *In vitro*, HMC-1 cells were not efficiently infected with RSV and produced TNF only when infected in co-culture with the airway epithelial cell line A549 (Shirato and Taguchi, 2009).

In relation to long term effects of RSV infection, nenonatal mice sensitized with RSV then re-infected five weeks later, had incresed RSV-specific IgE, airway hyper-responsiveness, airway eosinophilia, mucus hyperproduction and IL-4 and IL-13 production, which was attenuated in mast cell deficient mice (Dakhama et al., 2009). However, virus- specific IgE does not have a known role in human RSV infection.

1.15 Thesis Hypothesis, Rationale and Objectives

Respiratory syncytial virus (RSV) is the leading cause of bronchiolitis and infant hospitalization worldwide, and linked to airway hyperreactivity. Mast cells are granulocytes that induce allergic symptoms, but are important for the immune response to

bacteria and parasites. Mast cells are abundant in the lung, and can produce mediators that influence vasodilation, bronchoconstriction, and immune cell recruitment to sites of infection. There is some limited evidence of mast cell activity in RSV disease, and a growing literature on the role in other viral diseases.

Therefore, we hypothesized that human mast cells respond to RSV infection by the production of mediators that could contribute to viral clearance and/or disease symptoms.

For that purpose, we examined the human mast cell responses to RSV. We were first interested in further understanding the nature of RSV infection of mast cells that was reported by (Al-Afif, 2012) in his thesis, including the extent of viral replication and production of infectious viral particles. We were also interested in measuring the CCL4, CXCL10, CXCL8 and CCL5 responses by infected mast cells since these chemokines are implicated in RSV disease and recruit monocytes, neutrophils and T lymphocytes. We also investigated the production of vascular endothelial growth factor-A (VEGF-A) as it has a role in increasing vascular permeability, edema, and tissue remodeling, and leukotriene C_4 (LTC_4) as it causes bronchoconstriction and wheeze. In addition, since type I interferons (IFN) are important antiviral cytokines that can further induce the production of other mediators of inflammation, their role in RSV infection of mast cells was investigated.

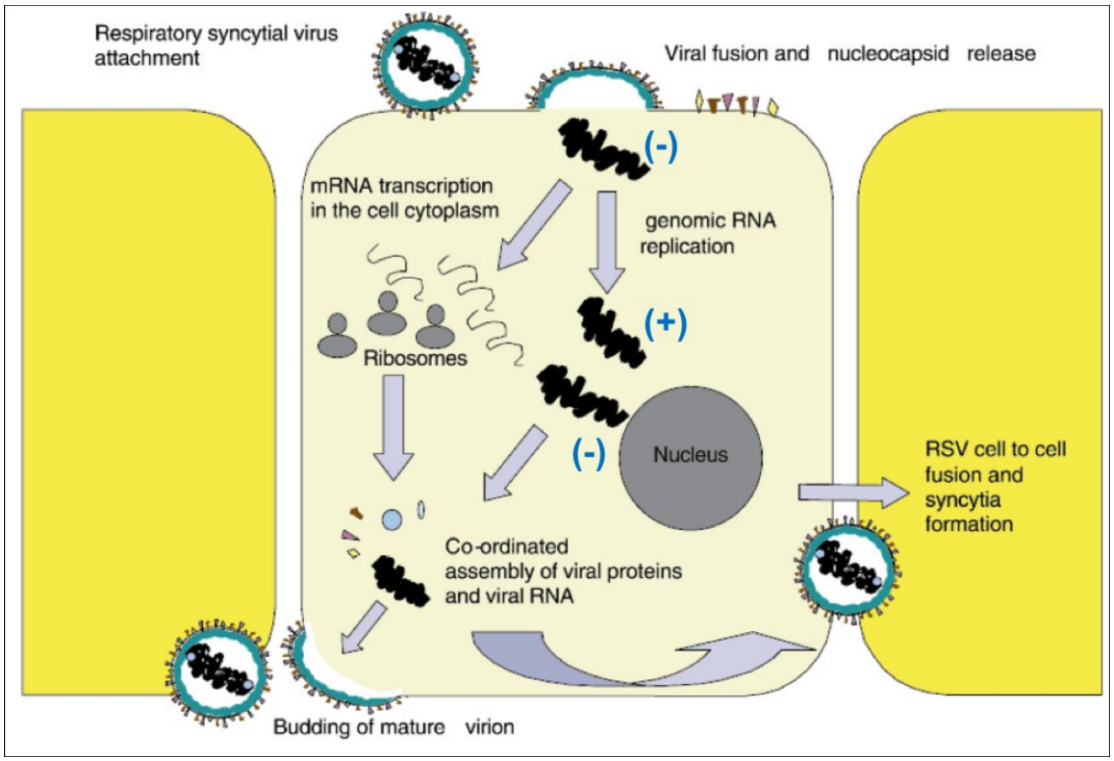


Figure 1. "Schematic representation of the RSV replication cycle. The RSV virion attaches to the apical membrane of the host cell using the G protein. The F protein mediates the fusion of the viral membrane with that of the host delivering the negative-sense (-) ssRNA genome to the cytoplasm. Genome transcription proceeds leading to the production of viral proteins. Genome replication gives rise to a positive-sense (+) ssRNA 'antigenome' intermediate which acts as a template for the (–) ssRNA genome synthesis. Upon synthesis, the genomes are integrated into nucleocapsids at the cell membrane where the budding of progeny virions occurs. Cell-cell fusion, mediated by the F protein, leads to the formation of syncytia. Adapted with permission from W.B./Saunders Co. Ltd. Publications: *The Journal of Infection*, Vol. 45, Hacking D. and Hull J., "Respiratory Syncytial Virus–Viral Biology and the Host Response", 18-24, copyright 2002." Adopted from (Al-Afif, 2012).

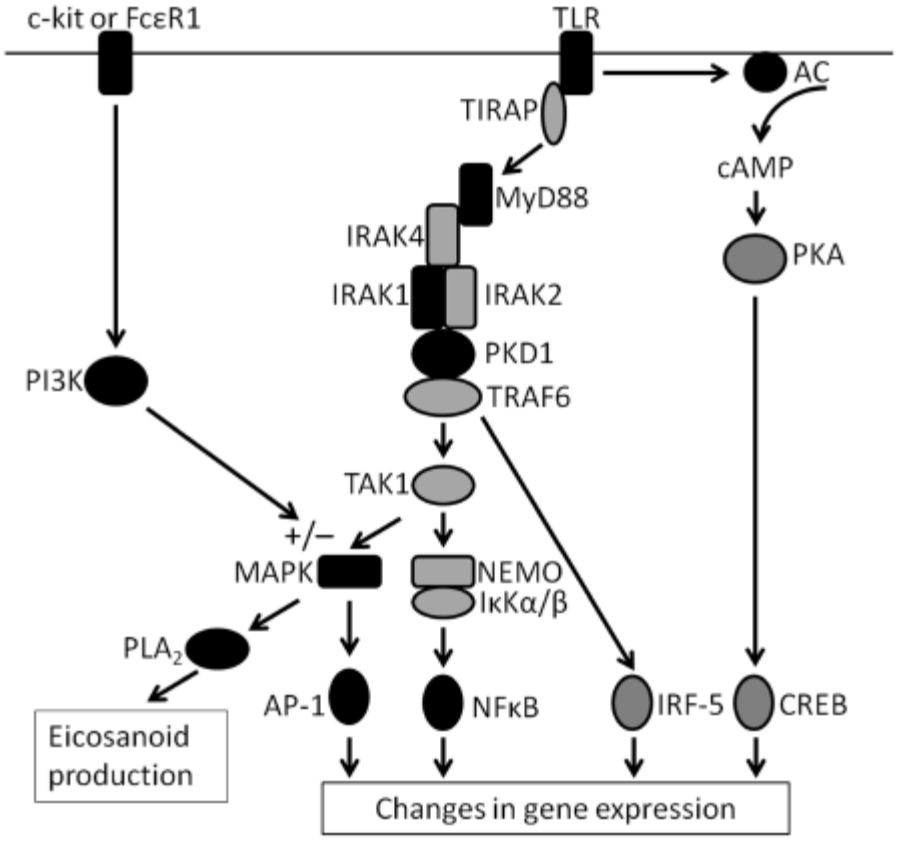


Figure 2. "**TLR signaling in the mast cell**. A scheme illustrating the signaling pathways triggered by TLR ligation in the mast cell. Where there is evidence in the literature for the involvement of a particular protein, the protein is filled in black. Proposed molecules are in grey (adapted from Akira et al., 2006; Lu et al., 2008; Park et al., 2009). The activation of PI3K via c-kit or FcεRI stimulation is shown, with its inhibitory and activating effects on MAPK activation." Adopted form (Sandig and Bulfone-Paus, 2012)

.

Chapter 2 – Materials and Methods

2.1 Cell Lines

HMC-1 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM; Thermo-Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich). HMC-1 cells were maintained at a concentration between $0.5x10^6$ and $1.5x10^6$ cells/mL. HEp-2 cells were maintained in Roswell Park Memorial Institute (RMPI)-1640 medium (Thermo-Fisher Scientific) with 15mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Thermo-Fisher Scientific) and 5% heat-inactivated FBS (Thermo-Fisher Scientific).

2.2 Primary Mast Cell Cultures

Human CBMCs were derived from umbilical cord blood obtained from subjects undergoing caesarian sections, as permitted by Izaak Walter Killam (IWK) Health Centre Research Ethic Board (Halifax, NS). Cells were cultured as described by (Saito et al., 1996) and (Radinger et al., 2010). In brief, umbilical cord blood was layered on Ficoll-Paque (GE Healthcare, Baie-d'Urfe, QC) to isolate mononuclear cells. Cells were passaged once a week for 6-8 weeks in RMPI-1640 supplemented with 10% FBS, 15mM HEPES, 1X MEM non-essential amino acids solution (HyClone), 1000 U/mL of penicillin-1000 μg/mL of streptomycin (HyClone), 10 ng/mL recombinant human IL-6 (eBioscience), and 100 ng/mL SCF (Peprotech, Rocky Hill, NJ). Cultures used for experiments were at least 90% viable and greater than 95% pure mast cells as was identified by expression of CD117 surface marker in flow cytometry (Table 1). Prior to use in experiments, CBMCs were cultured overnight in RMPI-1640 supplemented with 10% FBS, 15mM HEPES, 1X MEM non-essential amino acids solution, SCF and IL-6, both at 10 ng/mL.

2.3 Cell Count and Viability

In some experiments, after adsorbing the virus and before incubation (0 hour) as well as 24 hours after incubation, cells were harvested and resuspended in PBS then a sample

was diluted 1:1 in trypan blue stain (0.5% in PBS) then counted using hemocytometer (Bright-Line, Reichert, USA) under light microscopy. Dead cells allow for trypan blue to enter the cell while live cells exclude it (Strober, 2001). Dead cells were defined by dark staining, and viability was estimated as the percent of viable cells at 24 hours compared to 0 hour post infection.

2.4 RSV Propagation

RSV (Long) was used to infect 80-90% confluent HEp-2 cells. The virus was adsorbed to the monolayer for 90 minutes at 4°C while rocking, washed and cultured at 37°C in RPMI-1640 medium containing 2.5% heat-inactivated FBS. Once the cell monolayer exhibited 70-80% fusion under light microscopy, the monolayer was disrupted by scraping. The supernatant was clarified from cellular debris by centrifugation at 4°C for 10 minutes at 2095 x g. In experiments where we wanted to isolate the non-specific viral binding to the surface of the mast cells from the actual uptake of the virus, we further purified the virus with centrifugation at 9000 x g for 10 minutes at 4°C to remove some cell membrane debris then we infected the cells similar to what was described above. For UV-inactivation, an undiluted aliquot of clarified supernatant was exposed to a germicidal lamp (40W) at a distance of 10 cm for 15 minutes. UV inactivates the virus by cross linking the viral genome preventing viral replication (Lytle and Sagripanti, 2005).

2.5 Inoculation Conditions

Cells were inoculated with RSV (with or without palivizumab or IgG), UV-inactivated RSV or medium (RPMI with 2.5% FBS) for 90 minutes at 4°C to allow for virus adsorption to cells. For RSV neutralization, the virus was mixed with palivizumab (Abbott, Saint-Laurent, Canada) -or human IgG pooled from different donors as a control- at 10 μg/mL for 30 minutes at 4°C. One million CBMCs were inoculated at MOI 3-4, and $0.5x10^6$ HMC-1 cells and $1.5x10^5$ of HEp-2 cell were inoculated at MOI 6-8, all in tissue culture plates. The higher initial MOI for HMC-1 and HEp2 is due to the duplication of these cells during incubation, so that the MOI is eventually similar to the initial MOI of CBMCs. Following adsorption, cells were washed and incubated at 37°C for various time points in RPMI-1640 with 2.5% FBS, containing 10 ng/mL SCF for

CBMCs, soybean trypsin inhibitor (Sigma-Aldrich) at 100 μg/ml to minimize degradation of CXCL10 in some experiments, and peptidase inhibitor (Acivicin from Sigma-Aldrich) at 0.5mM in all the experiments where LTC_4 was measured.

The calcium ionophore A23187 activation of CBMC was used as a positive control for degranulation. The ionophore was incubated with CBMC at 37°C at a concentration of 0.1 μM for 4 hours or 0.5 μM for 30 minutes in the same infection/activation medium described above.

CBMCs were incubated at a concentration of $1x10^6$ cells/mL, HMC-1 cells at $0.5x10^6$ cells/mL and HEp-2 cells at a concentration of $0.15x10^6$ cells/mL. Where indicated, HMC-1 cells and CBMC cells were incubated in the presence of an anti-interferon-α/β receptor (IFNAR) antibody (MMHR-2 clone; Calbiotech, California) at a concentration of 5 μg/mL or purified mouse IgG2a-κ (BioLegend) at the same concentration as an isotype control. Following incubation, supernatants were harvested and stored at -80°C and the cells prepared for flow cytometry and RNA extraction.

2.6 RSV Plaque Assay

HEp-2 cells were used as a reference cell line to determine viral titer, in plaque forming units (pfu)/mL. For all viral titer determination on the cells harvested in the *in vitro* experiments, aliquots of clarified virus HEp-2 cell or CBMC cell lysates from control and RSV-treated cells, were serially diluted and inoculated onto 80-90% confluent HEp-2 cell monolayers. Plaque assays were performed as described (McKimm-Breschkin, 2004, Boukhvalova et al., 2010).

2.7 Immunofluorescence and Flow Cytometric Staining

For c-Kit/CD117 staining cells were resuspended in FACS buffer (0.5% BSA, 0.1% NaN_3 in PBS). Cells were incubated for 20 minutes on ice with the antibodies detailed in Table 1. Cells were washed, and fixed with 1% PFA in PBS. For intracellular RSV antigen staining, fixed cells were permeabilized in 0.2% saponin (Sigma-Aldrich) in

FACS buffer for 20 minutes on ice. Following permeabilization, cells were incubated for 20 minutes with biotinylated antibodies as per Table 1. Then cells were washed and incubated with secondary antibodies for 20 minutes. Cells were rinsed, fixed in 1% PFA in PBS, and stored at 4°C, and acquired using a FACS Calibur (BD Bioscience). Analysis was performed using FCS Express 3 (*Denovo* Software, Los Angeles, CA). The percentage of RSV antigen expressing cells was determined approximately relative to readings at 0 hour post adsorption.

2.8 Quantitative Polymerase Chain Reaction (qPCR)

Following incubation with RSV or controls for variable time points, cells were lysed in RLT buffer with 3% 2-mercaptoethanol (2-ME) and RNA was extracted using the RNeasy Plus Mini Kit (250) (Qiagen, Cat.#74136) as per the manufacturer's protocol. Genomic DNA was depleted and complementary DNA (cDNA) generated using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. cDNA was stored at -20°C. qPCR was performed on cDNA using Qiagen QuantiFast SYBR Green PCR Kit or SsoAdvanced Universal Syber Green Supermix (Bio-Rad) at an annealing/extension temperature of 60°C. qPCR reactions were performed in duplicate using the MX-3000P QPCR System or CFX-96 Real Time System (Bio-Rad) and relative gene expression was analyzed using MxPro Software (Stratagene, La Jolla, CA) or Bio-Rad CFX Manager 3.1. The fold induction of gene expression was calculated using the Pffafl Method (Pfaffl, 2001), or when appropriate, they were normalized to the reference gene and expressed as 2 to the power of the difference between the threshold cycles for amplification of reference gene and target gene. GAPDH and HPRT1 were the reference genes used as indicated –review table 2 and 3 for all the used primers-.

2.9 Enzyme-linked Immunosorbent Assay (ELISA)

Sandwich ELISAs were used to quantify protein levels in cell supernatants. Polystyrene-based ninety six-well microtiter plates (Thermo Fisher Scientific), were coated with capture antibody (50 μL per well) diluted to working concentration (Table 4) in borate buffer (pH 8.2-8.3). Plates containing coating solution were sealed with parafilm and

incubated at 4°C overnight. Wells of coated plates were washed 3 times with 300 µL of PBS-T buffer (0.02% Tween-20 (Sigma-Aldrich) in PBS), and incubated for 1-2 hours at room temperature with 100 µL of blocking buffer (2% BSA in PBS) to prevent non-specific antibody binding. Following further washing dilutions of samples and standard were added to duplicate wells and plates were incubated at 4°C overnight. All samples and standards were diluted in the same culture medium. Wells were then washed and incubated with 50 µL of biotin-labeled detection antibody, diluted to working concentration (Table 4) in 0.02% BSA in PBS, for 1 hour at room temperature.

LTC_4 was measured also by a sandwich ELISA according to a modified protocol from (Volland et al., 1994). In summary, after the cross- linking step (epitope immobilization) with glutaraldehyde, sodium borohydride and methanol were used for epitope release in the LTC4 ELISA. The ELISA amplification system from Invitrogen was used in all assays according to manufacturer protocol. Generally, after incubation with the detection antibody, the plate was washed then incubated for 30 minutes with alkaline phosphatase, then washed and incubated with the kit-provided substrate containing nicotinamide adenine dinucleotide phosphate (NADPH) followed by the kit amplifier solution. Bound alkaline phosphatase reduces NADPH to NADH and the resultant primary product goes into a secondary cycle of enzymatic reaction, therefore amplifying the signal of the colored end product. Finally, plates were read and analyzed using Gen5 software (Bio-Tek, USA).

2.10 Bioplex Assay

Bioplex assays were used to detect the human mediators: IFN-α2 and IFN-β (eBioscience), and selected CXCL10, VEGF-A and IL-1Ra measurements (BioRad) according to manufacturer protocols. Briefly, beads coupled to antibodies against the targeted protein were added to 96-well-plate, and then washed. After which, samples and standards were added and incubated for an hour, washed, then biotin-labeled detection antibodies specific to epitope on the target protein were added then washed, and a fluorescent streptavidin reporter was added to bind detection antibodies. Finally, after a final wash, assay buffer was added and wells were read at the Bio-Plex Reader. Each

bead was identified by the internal fluorescent signature of the bead region, and the level of the target bound was identified by the intensity of the reporter.

2.11 Statistical Analysis

For experiments where the HMC-1 cell line was used, normality was assumed and data were analyzed by a repeated-measures one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. In experiments with CBMC, data were either log transformed and analyzed by paired t-test or, for multiple comparisons, analyzed using a repeated-measures one-way ANOVA, followed by Sidak's comparison for selected pairs. In some cases (as stated), in view of non-normal data distribution, data were directly analyzed by Friedman's test, followed by Dunn's selected pairs comparison. P values of less than 0.05 ($p<0.05$) were considered significant. All statistical analyses were performed using Prism 6 (GraphPad Software Inc, La Jolla, CA).

Table 1. List of antibodies used for flow cytometry.

Primary Antibodies		
Target	**Specifications**	**Isotype**
CD117	APC mAb, clone104D2, working concentration 1μg/mL Biolegend Cat.#313206	APC mouse IgG1$_{\kappa}$, clone P3.6.2.8.1 eBioscience Ref.17-4714-82
RSV	Biotin-conjugated goat anti-RSV serum, working concentration 5μg/mL Meridian Life Sciences, Saco, ME	Biotin-labeled goat IgG Jackson Immunoresearch, West Grove, PA
Secondary Antibodies		
Strept-avidin-PE, eBioscience Cat.# 12-4317-87		

Table 2. List of commercially available primers used for qPCR.

Gene	Other names	Company	Catalogue #
CCL4		QIAGEN SAB	PPH00563B
CCL5		QIAGEN SAB	PPH00703A
CXCL10		QIAGEN SAB	PPH00765E
GAPDH		Invitrogen	7387982
HPRT1		BioRad	100-25636
IFIT1	ISG56	QIAGEN SAB	PPH01332E
IFNA1		QIAGEN SAB	PPH01321B
IFNA2		QIAGEN SAB	PPH00379A
IFNB1		QIAGEN SAB	PPH00384E
MX1		QIAGEN SAB	PPH01325A

Table 3. List of customized ordered primers for qPCR.

Gene	Sequence	Company
RSV Genes		Sigma-Oligo
NS1	F: CACAACAATGCCAGTGCTACAA R: TTAGACCATTAGGTTGAGAGCAATGT	
N	F: AAGGGATTTTTGCAGGATTGTTT R: CTCCCCACCGTAGCATTACTTG	
M	F: ATGTGCTAATGTGTCCTTGGATGA R: TGATTTCACAGGGTGTGGTTACA	
G	F: CGGCAAACCACAAAGTCACA R: TTCTTGATCTGGCTTGTTGCA	
F	F: TAAGCAGCTCCGTTATCACATCTC R: ATTGGATGCTGTACATTTAGTTTTGC	
M_2	F: CATGAGCAAACTCCTCACTGAACT R: TCTTGGGTGAATTTAGCTCTTCATT	
L	F: CACTCTACAAAACAAAAAGACACAATCA R: AGGATGCTGCATTGAACACATT	

F: Forward, R: Reverse

Table 4. List of capture and detection antibodies used for ELISAs.

Target	Capture Antibody		Detection Antibody	
	Specifications	**Concentration**	**Specifications**	**Concentration**
CCL4	mAb, clone 24006 (R&D)	1 μg/mL	Biotinylated affinity purified pAb (R&D)	50 ng/mL
CCL5	mAb, clone VL-1 (Endogen, Woburn, MA)	1 μg/mL	Biotinylated goat pAb (R&D)	0.1 μg/mL
CXCL10	mAb, clone 4D5 (BD Bioscience)	2 μg/mL	Biotin-labeled mAb, clone 6D4/D6/G2 (BD Bioscience)	0.1 μg/mL
LTC_4	mAb, Medicorp, Neo Makers, Fermont,CA clone 6E7	1 μg/mL	Biotin-labeled prepared in Marshall lab	0.2 μg/mL

Chapter 3 – Results: Human Mast Cell Responses to RSV Infection

3.1 Evidence for Infection

Whether or not human mast cells can become infected with RSV is an important question, since it has implications for disease pathology. Other than the study published by (Shirato and Taguchi, 2009) that found no significant infection for HMC-1 by RSV, there are no previous studies investigating RSV infection of human mast cells and none that examined primary human mast cells. Therefore, we first analysed primary cultures of human cord blood mast cells (CBMC) for permisiveness to RSV infection.

3.1.1 RSV Protein Expression

Al-Afif et al. (Al-Afif, 2012) had demonstrated that a small percentage (generally below 6%) of CBMC and HMC-1 expressed RSV antigen 24 hours after exposure to the virus (Figure 3A). To further confirm these findings, we infected CBMC with RSV (MOI 3-4) for variable time points and we used medium-treated as well as UV-inactivated RSV CBMC as negative controls while RSV-infected HEp-2 cells (Figure 3B) were used as a positive control when required. MOI were calculated and based on dose-response experiments described in (Al-Afif, 2012). We also pre-treated RSV with the monoclonal antibody palivizumab to reduce RSV entry into mast cells. Subsequently, we co-stained for the expression of CD117, a specific surface marker of mast cells (Kumar and Sharma et al. 2010), and intracellular RSV antigen at 24 hours (0, 2-4 and 48 hours in some experiments) and quantified the dual-positive cells by flow cytometry.

Maximum viral antigen expression was detected in response to live but not UV-inactivated virus in a small proportion of CD117hi cells (i.e. mature mast cells) at 24 hours post-infection (median= 0.7%, interquartile range (IQR)= 0.5-1.2 in RSV-infected cells vs 0 % in UV-RSV treated cells, $p<0.01$, n= 12 donors; Figure 5). Pre-treatment of RSV with palivizumab reduced the percentage of cells expressing RSV antigen by

49±14.9% (mean± SEM, 95% CI= [13.9-84.1]), compared to isotype-control treated-RSV infection (p=0.04, n=8 donors; Figure 4).

Mast cells are highly granular and rich in extracellular calyx and Fc receptors, which constitute two major difficulties with flow cytometric readings for RSV infection. The first difficulty is increased mast cell auto-fluorescence leading to high background readings, and the second is sticking of the virus and antibodies to the surface of the cell. Therefore, mast cells were infected with further purified RSV preparation for 0 hour post adsorption, and 4 and 24 hours after starting adsorption. Then the flow cytometry readings taken right after adsorption at time 0 were considered a baseline for what would be considered high positive RSV antigen expression in mast cells (Table 5). Also, time 0 readings were needed to confirm that the shift in the mast cell positivity for RSV antigen observed at 24 hours was due to internalized-virus and subsequent replication rather than simple virus persistence on the surface of mast cells after adsorption.

3.1.2 RSV Gene Expression

In order to confirm that RSV undergoes some degree of replication in CBMC, the expression of RSV genes was examined by qPCR at 24 hours in CBMC exposed to RSV UV-RSV or medium-treated cells. RSV-exposed CBMCs showed significant expression of RSV genes compared to medium-treated cells (p= 0.02 for the RSV NS1, N, and F genes, n=7 donors; Figure 5A) and this was confirmed for all of the RSV genes expression in CBMC (Figure 6, n=3 donors). PCR analysis of HMC-1 infection in one experiment, and HEp-2 in 3 experiments are presented for comparison (Figure 5B and C).

3.1.3 Infectious RSV Viral Particles

RSV *in vitro* infection of other immune cells like human dendritic cells and human macrophages has been reported. However, the infection rate of those cell types is lower than what is observed in epithelial cells, and infected cells may lose some of their immune functions such as those involved in activating T-lymphocytes (McDermott et al.,

2011, Panuska et al., 1990). Specifically, monocytic-derived DCs (moDCs) are the most permissive for RSV replication and release of infectious viral particles (productive infection) (Jones et al., 2006). Peripheral human eosinophils were also reported to be infected *in vitro* slowly but productively by RSV, the significance of which is not clear in the *in vivo* setting (Dyer et al., 2009).

Plaque assays were performed to determine whether human mast cells were productively infected with RSV, giving rise to infectious virus particles. CBMC cell lysate was harvested after 24 hours (n=5 donors) and 48 hours (n=2 donors) of RSV infection and was inoculated into confluent HEp-2 monolayer for plaque assay. CBMC plaque formation was compared in plaque assays to plaques from RSV-infected HEp-2 stock and to parallel HEp-2 cells infected for 24 and 48 hours with same MOI as CBMC. No plaques were detected from RSV-inoculated CBMCs, while plaques were observed in the positive control HEp-2 viral stock ($\geq 1x10^7$ pfu/mL) and from parallel 48 hours HEp-2 cell infection ($\geq$ 1300 pfu/mL); (Figure 7, an example of positive plaque assays). This indicates that treatment of CBMCs with RSV does not lead to substantial infectious virus formation.

From these and previous findings we could conclude that RSV replicates to a limited extent in primary human mast cells (CBMC). However, no significant infectious viral particles are produced. In comparison to the previously reported *in vitro* low but productive RSV infection of other immune cells, human mast cells might be resistant to productive RSV infection.

3.2 Mast cells Viability

RSV leads to apoptotic death and shedding of infected epithelial cells (Villenave et al., 2012). On the other hand, human moDCs and human alveolar macrophage viability was not significantly reduced by RSV infection (Jones et al., 2006, Panuska et al., 1990, Cirino et al., 1993). Mast cell viability could be reduced by apoptosis or necrosis in the context of RSV. Therefore we first assessed gross mast cell viability at 0, 4 and 24 hours

from infection, and compared to cell count before virus adsorption, under light microscopy. We quantified dead mast cells based on their increased uptake of trypan blue. However, CBMCs infected with RSV maintained similar viability to negative controls at multiple time points (Figure 8). These findings were compared to a positive control, calcium ionophore treatment which at 30 minute of cell activation leads to 15-50% reduction in cell viability, n=3 donors).

3.3 Flow Cytometric Analysis of Mast Cell Granularity

Mast cells could secrete mediator contents through degranulation or slower selective secretion of specific mediators, such as piece meal degranulation, or classical Golgi dependent protein secretion (Moon et al., 2010, Theoharides et al., 2007). It is likely that these contrasting mechanisms give rise to different and selective mast cell responses to a range of pathogen associated stimuli. As we have reviewed earlier, mast cells generally respond to viruses and intracellular TLR stimulation with selective mediator release rather than degranulation, unless other stimuli are present like immunoglobulins and antigen, to induce cross linking of Fc receptors (St John and Abraham, 2013).
On analyzing the density of mast cell granules as reflected by larger side scatter on flow cytometry reading, some degree of reduced mast cell granularity was evident in RSV-treated CBMCs at 24 hours post-infection compared to baseline granularity and to that of the parallel medium-treated cells (median reduction in RSV-exposed cell granularity= -10.9%, IQR= -20.1 to -6.9%, vs in medium-treated cells= -4.4%, IQR= -9.145 to 0.1%, n= 5 donors; Figure 9A). Figure 9B shows stained mast cells at 0 and 24 hours from RSV infection.

3.4 Cysteinyl Leukotriene Production

Typical mediators that are produced in asthma and responsible for significant bronchoconstriction and wheeze are cysteinyl leukotrienes, mainly LTC_4 and its metabolite LD_4. Mast cells typically produce LTC_4 in response to IgE/antigen cross-linkage of FCεRI leading to degranulation, and the onset of LTC_4 synthesis from

arachidonic acid (Bingham CO III, 1999). However, LTC_4 could be produced without mast cell degranulation (Enoksson et al., 2011). Mast cell derived LTE_4 (the other LTC_4 metabolite) was found in wheezing infants, but specifically in higher amounts in RSV infected infants (Oymar et al., 2006).

Mast cells produced substantial amounts of LTC_4 following activation with calcium ionophore at 30 minutes (median= 8677pg/mL, IQR= 4682-17265, n=4 donors). However, in our *in vitro* infection system, no significant LTC_4 response was observed by CBMC from the same donors treated with RSV, either at 30 minutes or at later time points (4-6 hours, 12 hours and 18-24 hours).

3.5 Mast Cell Mediator Production in response to RSV Infection

(Al-Afif, 2012) showed that CBMC could produce substantial amounts of mediators including CXCL10, CCL4, and CCL5, in response to RSV exposure. We therefore aimed to confirm these findings and explore a mechanism for the observed mediator production.

3.5.1 Type I Interferon

Type I IFNs mediate their antiviral effector actions through the activation of a group of IFN response genes such as *ISG56* (*IFIT1*) and *MX1 (MxA)* (Sadler and Williams, 2008). To elucidate a potential mechanism for regulation of chemokine production in mast cells exposed to RSV, we first investigated the type I interferon responses in CBMC 24 hours post-infection with RSV. There was higher expression of *IFNA1* ($p<0.05$), *IFNB*, *ISG56*, and *MX1* mRNA ($p<0.01$) in CBMCs inoculated with RSV than in UV-RSV treated cells (n=5 donors; Figure 10A). Supernatants from CBMCs treated with RSV had consistently higher protein levels of IFN-α2a ($p< 0.05$) and IFN-β compared to medium or UV-inactivated virus treated cells (n=6 donors; Figure 10B).

3.5.2 Chemokines (CXCL10, CCL4, CCL5)

Several pro-inflammatory chemokines are expressed in RSV disease. The levels of CCL4, CCL5, CCL11, CXCL10, and CXCL8 are elevated in nasal and bronchoalveolar lavage (BAL) samples of children hospitalized with severe RSV bronchiolitis (Zeng et al., 2011, Garofalo et al., 2001, Kim et al., 2007, Matthews et al., 2005, Chung and Kim, 2002, Roe et al., 2011, Yoon et al., 2007). The mast cell production of these cytokines in response to RSV was therefore examined. CBMCs were stimulated for 24h with medium, RSV, UV-inactivated RSV or RSV pretreated with either palivizumab or a human IgG control. Protein levels of chemokines were quantified in cell free supernatants, 24 hours post RSV treatment. CBMCs inoculated with RSV produced significantly higher amounts of CXCL10 and CCL4 at 24 hours as compared with medium control and UV-RSV treated cells ($p<0.05$ and <0.01, n=9 and 12 donors respectively; Figure 11). This response was highly selective, for example CXCL8 production was not upregulated in response to RSV (n=4 donors; Figure 12A). The amounts of CCL5 produced were much lower than CCL4 and CXCL10 in response to RSV. However, CCL5 production from CBMC was still elevated in response to RSV as compared with medium control or UV-inactivated virus treated cells ($p<0.01$, n=10 donors; Figure 11). In addition, induction of chemokine mRNA relative to control treated cells was measured by qPCR for CXCL10, CCL4 and CCL5. These mRNA's were significantly elevated for the RSV group compared to the UV-RSV group ($p<0.01$, n=5 donors; Figure 12B).

These findings confirm (Al-Afif, 2012) observations on CBMC production of CXCL10, CCL4, and CCL5. It is worth mentioning that CCL11 was not produced from mast cells in response to RSV (Al-Afif, 2012). The level of these chemokines decline significantly at 48 hours which could be due to their digestion by mast cell proteases (Al-Afif, 2012).

3.5.3 The Effect of Blocking Early Type I IFN Production on Chemokine Production

In order evaluate the role of the type I IFN response by mast cells in their chemokine profile, following RSV exposure, we blocked the type I IFN receptor, using specific antibodies, after virus adsorption, then collected supernatants for ELISA or Luminex

chemokine assays at 24h post infection. CBMCs activated with RSV produced significantly lower levels of CXCL10 and CCL4 protein when pretreated with type I IFN blocking antibody compared to when pretreated with an isotype control antibody ($p \leq 0.01$, n=7 donors; Figure 13). In contrast, CCL5 production by mast cells in response to RSV was not affected by type I IFN receptor blockade (n=7 donors; Figure 13). No significant inhibitory effect on viral antigen expression was noted with type I IFN receptor blockade (data not shown). The efficacy of type I IFN receptor blockade was confirmed in the KU812 cell line stimulated with IFN-α2 (Figure 14).

3.5.4 Vascular Endothelial Growth Factor-A (VEGF-A)

Patients with RSV disease were reported to have increased VEGF in their nasal secretions (Lee et al., 2000). However, VEGF production has not been extensively studied in RSV pathology. From the pathology reports on RSV patients, vascular oedema was an obvious feature of RSV disease (Johnson et al., 2007).There are multiple mechanisms for oedema one of which is through mediators like VEGF-A, which also acts on monocyte chemotaxis, and tissue remodeling (Ferrara et al., 2003), which could have impact on long term sequel of RSV disease.

CBMCs produced significant VEGF-A in response to RSV infection compared to medium-treated and UV-RSV treated CBMCs ($p < 0.01$ and <0.001, respectively, n=7 donors; Figure 15). VEGF-A production by RSV-infected mast cells was independent from early type I IFN response (Figure 15).

3.5.5 Interleukin-1 Receptor antagonist (IL-1Ra)

IL-1α and β are known pro-inflammatory cytokines and they are produced by epithelial cells in response to RSV infection (Chang et al., 2003). To explore the potential of an anti-inflammatory/regulatory role for mast cells in RSV disease, IL-1Ra was measured in infected CBMC supernatants. IL-1Ra is produced by human alveolar macrophages in

response to RSV infection *in vitro* (McCarthy et al., 1989). It was speculated to contribute to anti-inflammatory effect by macrophages in RSV disease.

Interestingly, mast cells produced significant IL-1Ra in response to RSV infection compared to medium-treated and UV-RSV treated CBMCs ($p < 0.05$, n=7 donors; Figure 16). IL-1Ra secretion by RSV-infected mast cells was dependent on early type I IFN response. IL-1Ra production was significantly reduced when type I IFN receptor was blocked compared to treatment of RSV-infected cells with isotype-control antibody instead ($p < 0.05$ Figure 16).

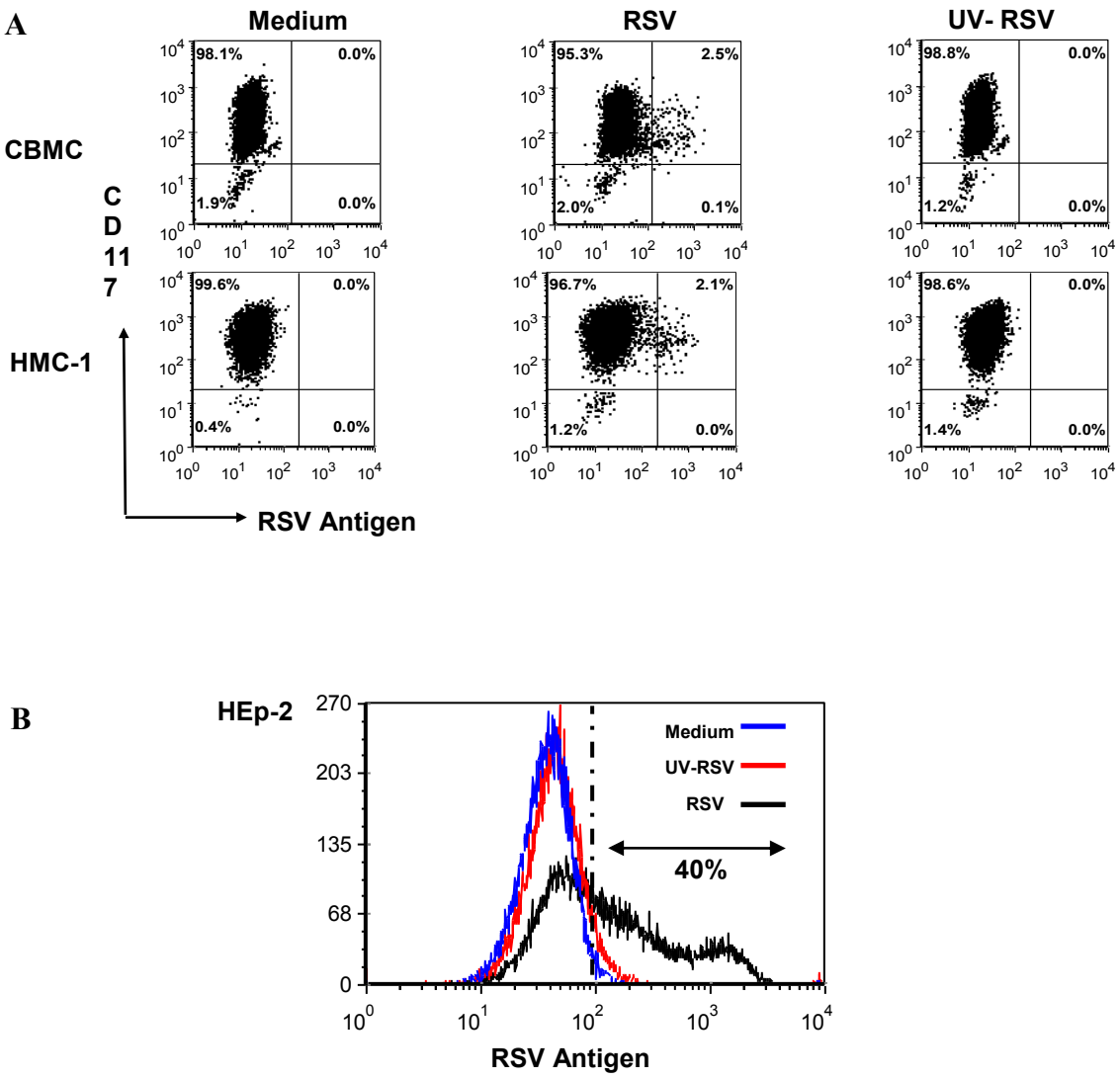


Figure 3. RSV infection of primary human mast cells (CBMCs), HMC-1 and HEp-2 cells at 24 hours post-adsorption. **A,** Intracellular RSV antigen expression in a donor's CBMC representative of n=12 donors, in HMC-1 (Al-Afif et al., 2011), and in **B,** in HEp-2 (representative of 6 experiments).
Cells were fixed and permeabilized to allow for intracellular staining of RSV antigen with polyclonal goat antibody against RSV or control goat IgG. CBMC and HMC-1 were also co-stained for expression of surface marker CD117, specific for mast cells.

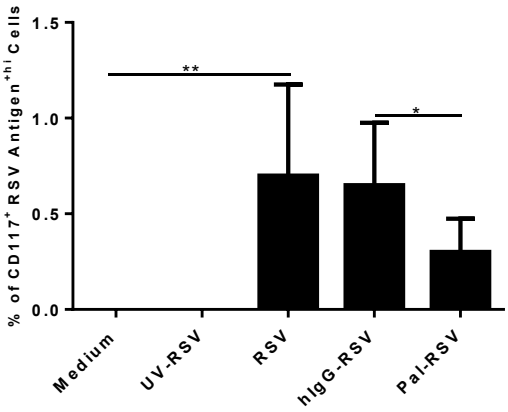


Figure 4. Expression of intracellular RSV antigen (stained with polyclonal goat anti-RSV) in CD117+ cells (CBMCs) in 12 donors as quantified by flow cytometry 24 hours after infection with RSV, UV-RSV or medium only. RSV was also neutralized using palivizumab (or control pooled human IgG) then was incubated with CBMC for 24 hours (n=8 donors). Data represents median ±IQR (interquartile range).

	0hr p.i.	4hr p.i.	24hr p.i.
% of CBMC RSV^{+hi}	0.2	0.1	0.6
	1.1	0.5	2.4
	0.1	0.1	0.5

Table 5. RSV infection of CBMCs at various time points as quantified by flow cytometry using the same staining method as in Figure 1, n=3 donors. Cells were infected with RSV purified with centrifugation at 9000 x g, then collected for co-staining of intracellular RSV antigen and CD117 at the end of 1 hour and 30 minutes of adsorption at 4°C (0hr) when no virus uptake should have occurred yet. Then the remaining cells were incubated at 37 °C for another 2 hours (4hr), and 24 hours from the start of adsorption (24hr), after which cells were collected as well for staining. $CD117^{+}$ RSV-$Antigen^{+hi}$ were quantified using flow cytometry.

Figure 5. Expression of 3 RSV genes (NS1, N, F), compared to medium-treated cells, 24 hours post RSV infection in **A,** CBMC (mean±SEM, n=7 donors), **B,** HMC-1 (n=1 experiment), and **C,** HEp-2 (mean±SEM, n=3 experiments). Housekeeping gene is HPRT-1.
The data presented are the CT values for each RSV gene normalized to the cell housekeeping gene, therefore the scale varies between cell types due to variable levels of housekeeping gene expression in each of them.

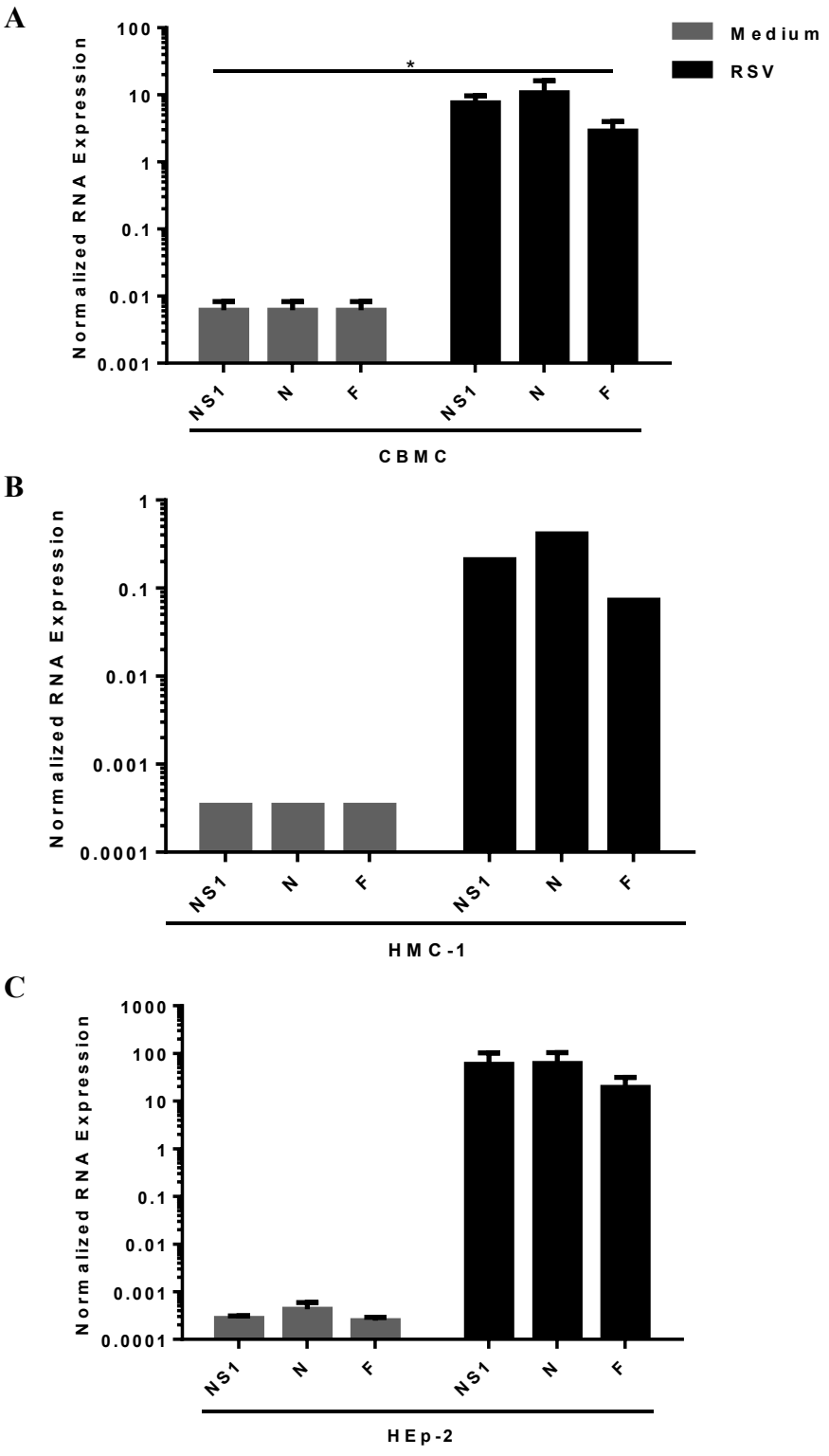
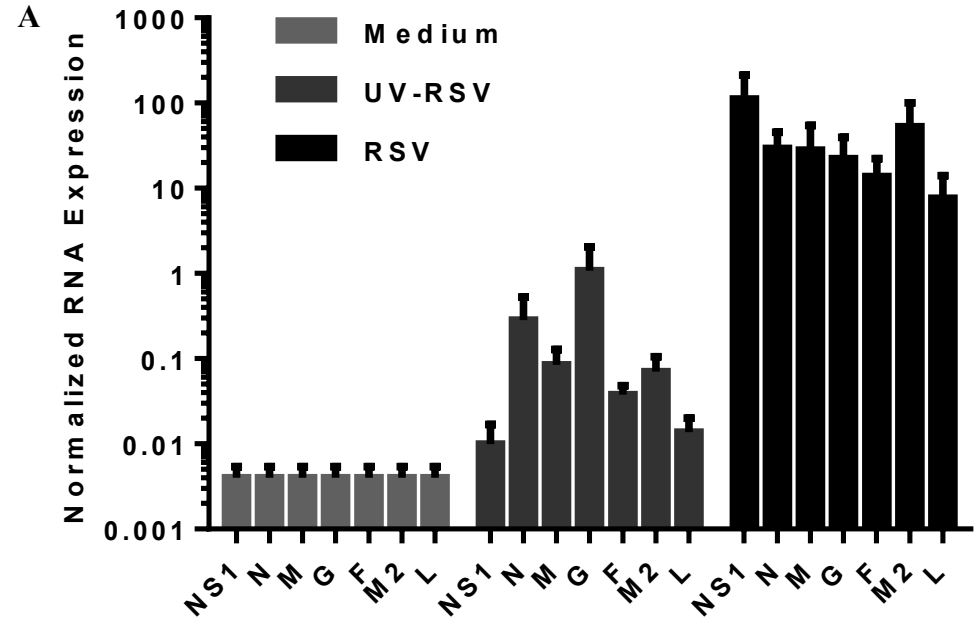
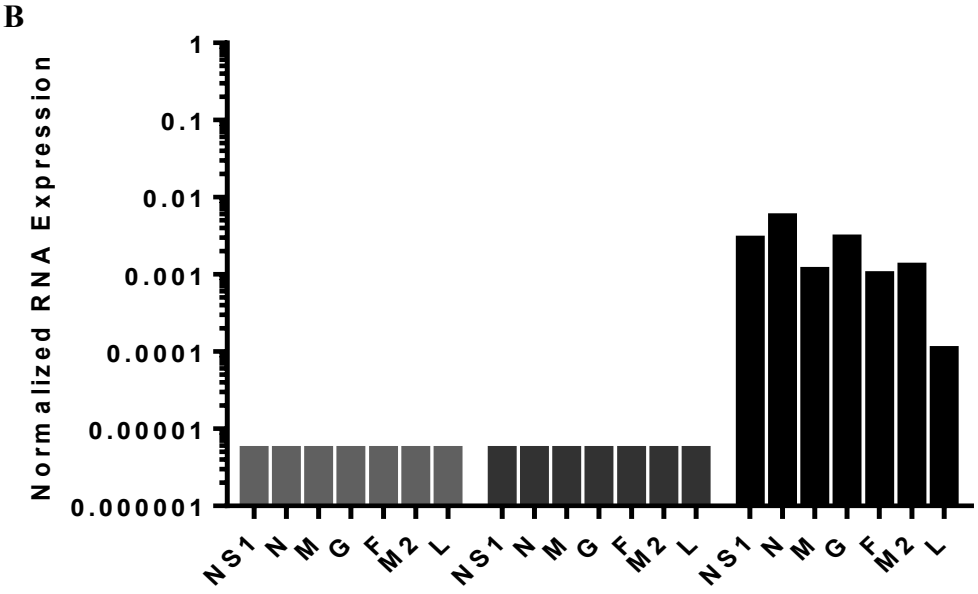

A
Medium
RSV
Normalized RNA Expression
100
10
1
0.1
0.01
0.001
*
NS1
N
F
NS1
N
F
CBMC
B
Normalized RNA Expression
1
0.1
0.01
0.001
0.0001
NS1
N
F
NS1
N
F
HMC-1
C
Normalized RNA Expression
1000
100
10
1
0.1
0.01
0.001
0.0001
NS1
N
F
NS1
N
F
HEp-2

Figure 6. Expression of 7 RSV genes 24 hours post-infection, compared to medium-treated cells, in **A,** CBMCs (mean±SEM, n=3 donors)**,** and **B,** HMC-1 (n=1 experiment). Housekeeping gene was GAPDH.


A
Normalized RNA Expression
1000
100
10
1
0.1
0.01
0.001
Medium
UV-RSV
RSV
NS1 N M G F M2 L
NS1 N M G F M2 L
NS1 N M G F M2 L


B
Normalized RNA Expression
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
NS1 N M G F M2 L
NS1 N M G F M2 L
NS1 N M G F M2 L

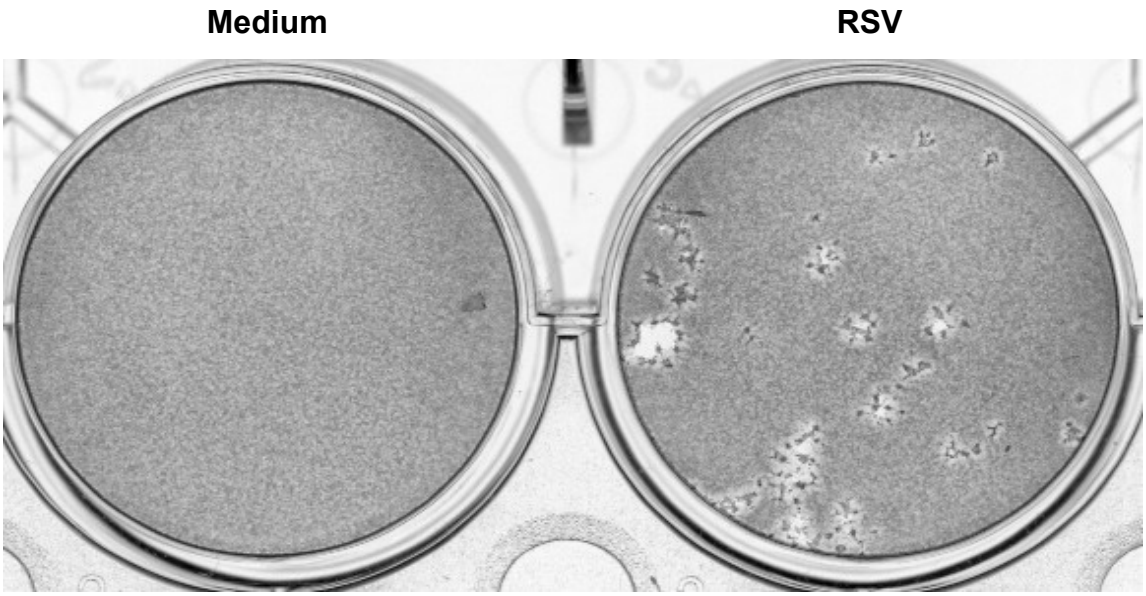


Figure 7. An example of plaque formation is seen on the right well compared to medium-treated cells on the left. HEp-2 monolayer was infected with RSV stock (dilution of $5x10^{-4}$), then cells were fixed and stained. The viral titer shown here $=2.2x10^{7}$ pfu/mL.

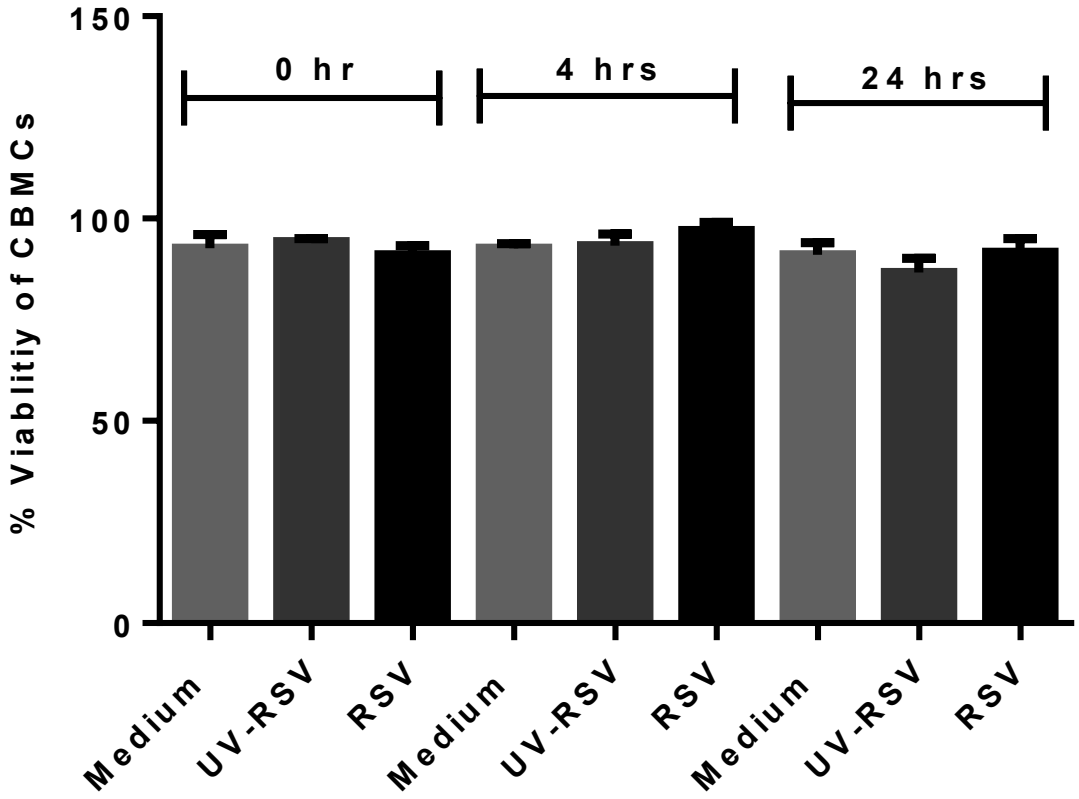
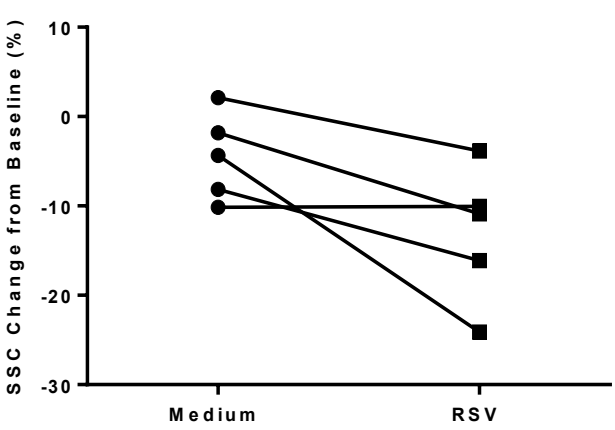
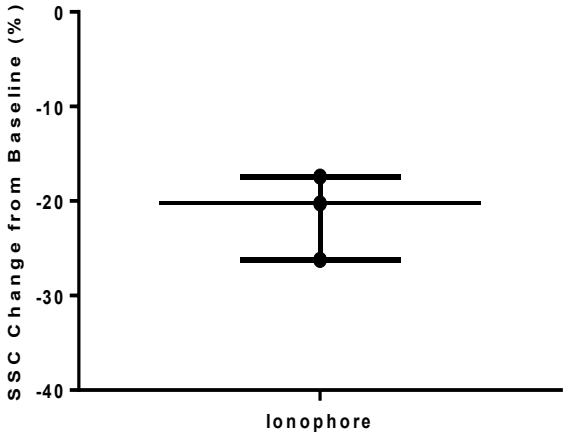


Figure 8. Viability of CBMCs after RSV exposure. Mast cells were ≥ 90% viable at 0, 4 and 24 hours post RSV-infection, which was similar to mast cells treated with medium and UV-RSV (mean±SEM, n=3 donors).

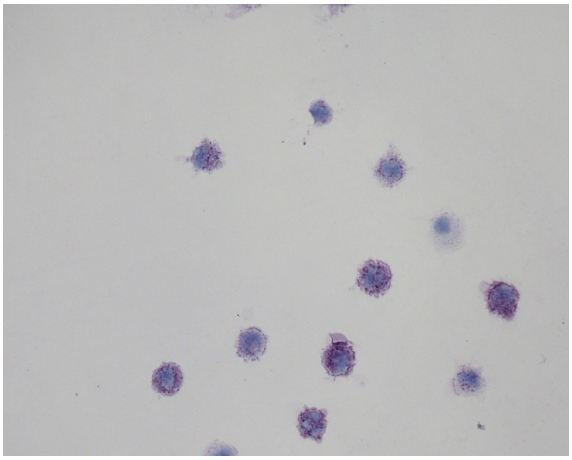
Figure 9. A, Reduction in CBMC granularity from baseline at 24hrs in RSV- vs medium-treated cells (left graph, n=5 donors), calcium ionophore activation of CBMC for 4 hours was used as a positive control for degranulation (right graph, n=3 donors, median±IQR) The change of CBMC granularity was calculated as percent of the difference between 0 and 24 hour granularity to the 0 hour (baseline) granularity for infected CBMCs. For ionophore activation, CBMC at 4 hours of activation was compared to matched medium-treated cells at 4 hours as well, and the reduction in granularity in the ionophore group is presented. Granularity was measured as the mean of the side scatter (SSC) value on flow cytometric readings.
B, An example of RSV-exposed CBMC appearance at 0 hour (left panel) and at 24 hours post-infection (right panel). CBMC were stained with toluidine blue, fixed, and then visualized under light microscopy (1000X).

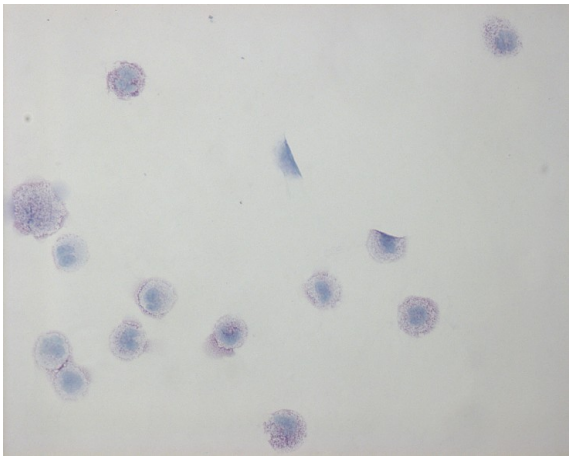
A


SSC Change from Baseline (%)
10
0
-10
-20
-30
Medium
RSV


SSC Change from Baseline (%)
0
-10
-20
-30
-40
Ionophore

B





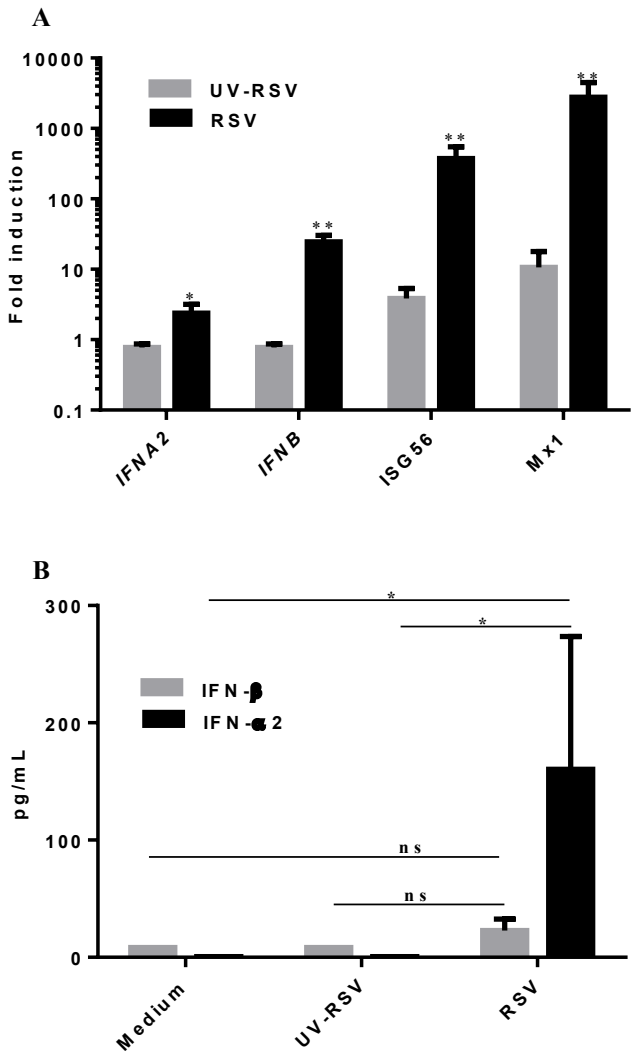


Figure 10. Type I IFN response following CBMC infection with RSV. **A,** IFN-a2 and IFN-β, as well as IFN-related genes fold induction in UV-RSV and RSV-treated CBMCs, relative to medium-treated CBMCs (n=5 donors, mean ± SEM), housekeeping gene is GAPDH. **B,** IFN-α2 and IFN-β secreted from CBMC 24 hours post-infection (n=6 donors, mean ± SEM).

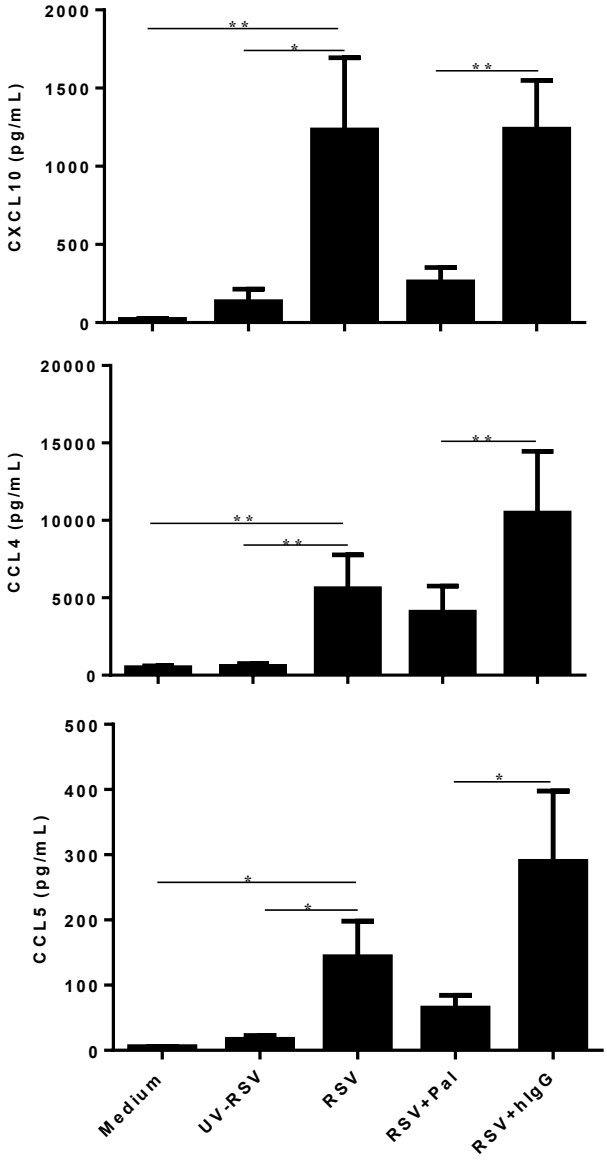


Figure 11. Levels of chemokines 24 hours post RSV infection with and without neutralization of infection with palivizumab (Pal), (for CXCL10 n=9; CCL4 n=12; CCL5 n=10; mean ±SEM). New data compiled with data from Al-Alfif et al. (2011).

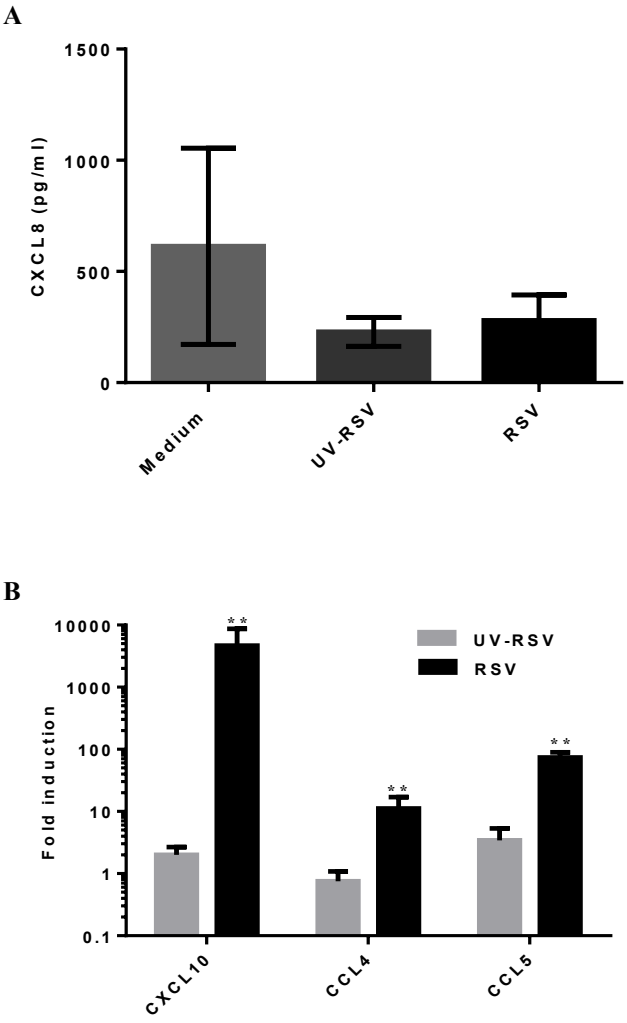


Figure 12. Chemokine upregulation by CBMCs 24 hours post-RSV infection. **A,** RSV does not induce significant CXCL8 production by CBMCs compared to controls (mean+/-SEM, n=4).
B, CXCL10, CCL4 and CCL5 genes upregulation in RSV treated CBMC and UV-inactivated virus treated CBMC, compared to medium- treated CBMC, (n=5 donors, mean ±SEM).

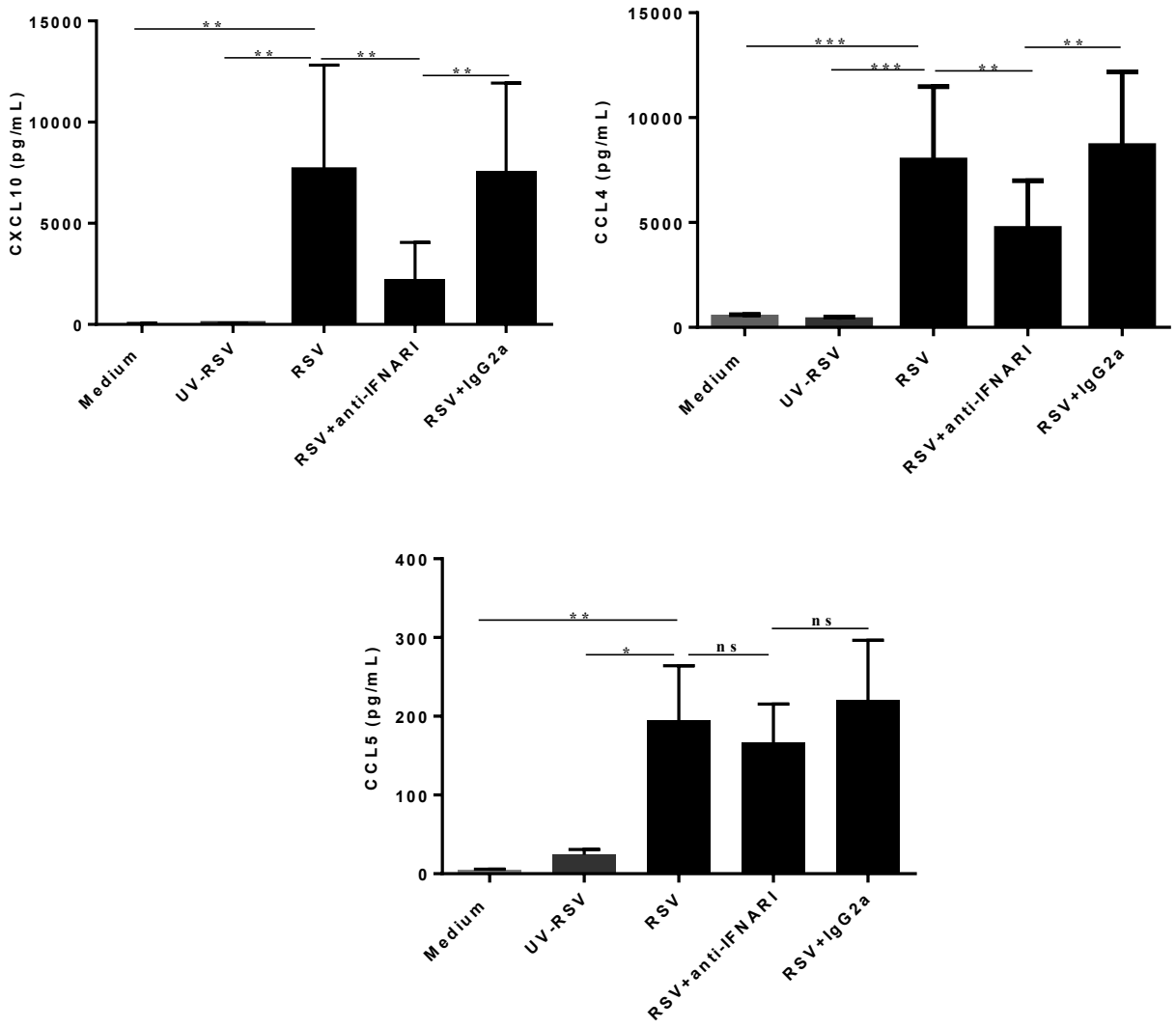


Figure 13. Effect of blocking type I IFN receptor on chemokine production by CBMC during RSV infection at 24 hours.
To block type I IFN early response, anti-IFN-α/β receptor (IFNAR) antibody was used to block the autocrine and paracrine action of type I IFN on cells. After adsorbing the virus, CBMCs were incubated for 24 hours in medium containing IFNAR antibody or isotype control antibody, (n=7 donors, mean±SEM).

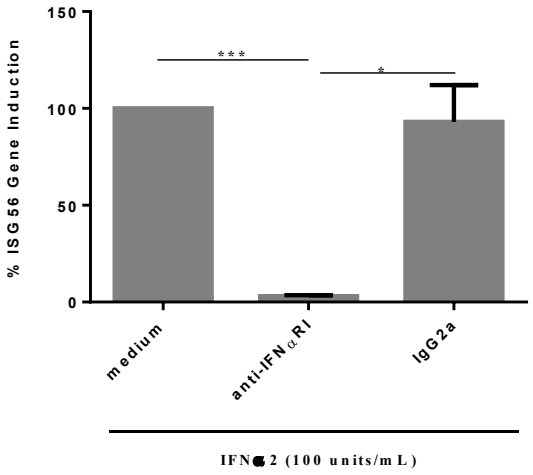


Figure 14. Type I IFN receptor blockade efficiently inhibits IFNα2-mediated induction of IFN stimulated gene, ISG56. KU812 cells were either unstimulated or incubated with IFNα2 for 24 hours alone, with antibody against IFNaRI, or with an isotype control antibody IgG2α.

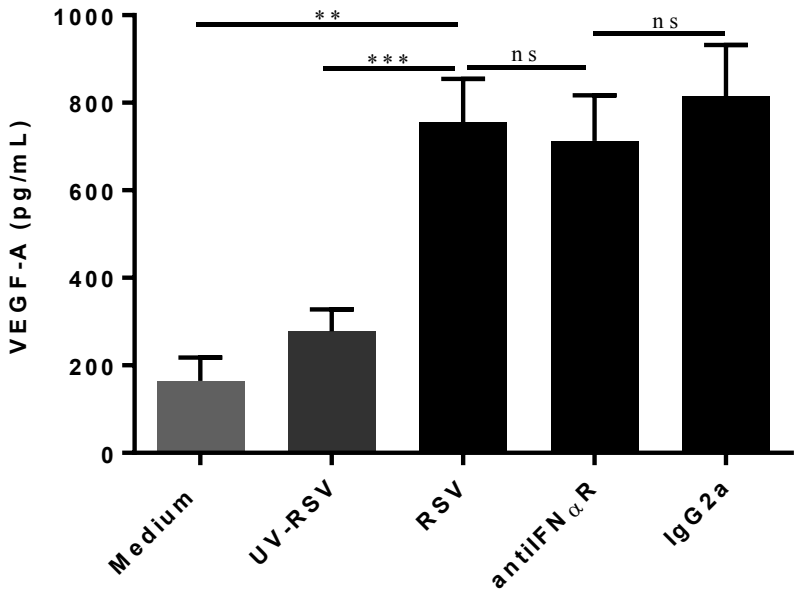


Figure 15. VEGF-A protein production (as detected in bioplex) is significantly upregulated in RSV-exposed CBMCs compared to control-treated cells. RSV-exposed cells were also incubated with anti-IFNAR mAb for 24 hours or with isotype control antibody (IgG2a). However, VEGF-A production at 24 hours was independent from early type I IFN response (n=7 donors, mean±SEM).

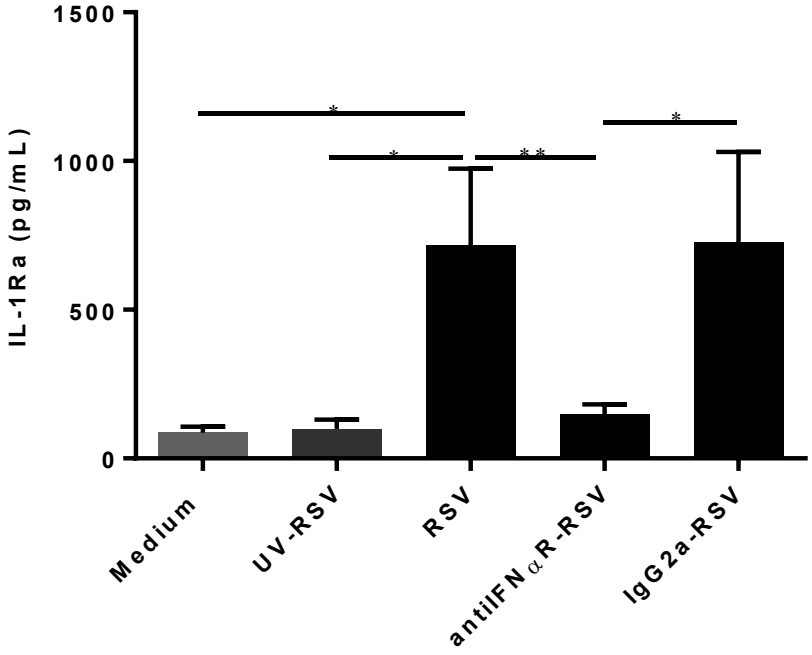


Figure 16. IL-1Ra is significantly upregulated in RSV-exposed CBMCs compared to control-treated cells (as detected in bioplex). RSV-exposed cells were also incubated with anti-IFNAR mAb for 24 hours or with isotype control antibody (IgG2a). IL-1Ra production at 24 hours was significantly reduced when blocking early type I IFN response (n=7 donors, mean±SEM).

Chapter 4 – Discussion

4.1 Summary and Discussion of Major Findings

We have demonstrated above that primary human mast cells show limited infection with RSV at 24 hours, as indicated by a very small percentage of RSV antigen-positive cells with evidence of RSV replication intracellularly without significant viral particle formation. NS1 protein is produced mainly during infection and is not present in the mature virus, and the mRNA for NS1 is highly expressed in infected cells (Evans et al., 1996), therefore the expression of NS1 RNA in mast cells may be taken as an indication of RSV mRNA transcription. The expression of RSV genes in CBMCs suggests that some degree of RSV replication takes place in mast cells leading to RSV mRNA production and low RSV protein expression as shown by flow cytometry. In addition, reduced side scatter on flow cytometry indicates some degree of loss of mast cell granularity potentially due to degranulation. Kulka et al (Kulka et al., 2004) found that human mast cells derived from peripheral blood did not degranulate in response to poly (I:C) stimulation (analogue of viral dsRNA). However, some types of TLR2 stimulation can lead to stimulation of low levels of degranulation by CBMCs (McCurdy et al., 2003) and Murawski et al. (Murawski et al., 2009) suggested that TLR2 and 6 could be co-receptors for RSV as they showed in knockout mice. Alternatively, reduced granularity following RSV treatment could reflect the viral inhibition of host mRNA translation and protein synthesis leading to loss of granule integrity.

Notably, RSV has been demonstrated to inhibit the PKR-mediated phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α), hence allowing for cellular but as well viral translation to occur (Groskreutz et al., 2010). On the other hand, it is known that RSV induces stress granule formation in epithelial cells 18 hours post-infection around the time when RSV switches from transcription to replication of its genomic RNA (Lindquist et al., 2010). Stress granule formation represents a cellular anti-viral response. Although the mechanism of inhibition is controversial, stress granules might induce shut down of mRNA translation, through sequestering mRNA and subjecting it to degradation in nearby structures (Mollet et al., 2008). Conversely, RSV

requires stress granule formation for its replication despite the observation that viral replication is likely to occur in inclusion bodies rather than stress granules (Lindquist et al., 2010). In our case, inhibition of translation at least before 18- 24 hours of infection seems unlikely due to the large amount of mediator production by cells as was shown by (Al-Afif, 2012). Notably, *in vivo* infection with RSV could provide other stimuli that induce gross mast cell degranulation, specifically since mast cell degranulation with tryptase release (sensitive mast cell degranulation markers) was reported in human RSV disease (Everard et al., 1995).

In our infection system mast cells did not produce LTC_4. However, it is possible that mast cells could be primed *in vivo* by other mediators to produce cysLTs. For example, IL-33 released from necrotic cells, such as infected epithelial cells, could be sensed by mouse BMMCs leading to mast cell production of TNF and LTs (Enoksson et al., 2011).

Despite low levels of infection, RSV induced a significant type I IFN response as well as production of the chemokines CXCL10, CCL4 and CCL5, and VEGF-A through both type I IFN dependent and independent mechanisms. (Kulka et al., 2004) showed that poly I:C (dsRNA analogue), RSV and other viruses were able to induce IFN-α and β mRNA and IFN-α2 protein secretion by human mast cell lines, mouse BMMCs, and human peripheral blood-derived cultured mast cells. Type I IFN production could potentially be induced through detection of dsRNA formed during the virus replication cycle by TLR3 or the protein kinase PKR (Alexopoulou et al., 2001), ssRNA by TLR7 or less likely in the case of mature mast cells TLR8 (Kulka et al., 2004, Heil et al., 2004). It has been observed that surface TLR stimulation of mast cells by bacterial components is generally less likely to produce type I IFN than the intracellular impact of viral analogues (Kulka et al., 2004) . Type I IFN could also be produced in TLR-independent pathways like through RLH (e.g. RIG-I), and through intracellular host or bacterial DNA (Noppert et al., 2007).

Type I IFNs signal through their IFNAR on various cells including the cell of origin (autocrine and paracrine action) that can further activate a multitude of effectors. These effectors enhance antiviral molecules (interferon-response genes e.g. PKR and RNaseL), alter cell cycle like promoting memory T-cell survival, and apoptosis, activate immune cells like NK cell, as well as induce chemokine production including CXCL10, CCL3, CCL4 and maybe CCL5 (Noppert et al., 2007, Salazar-Mather et al., 2002). Compared to well-differentiated primary pediatric bronchial epithelial cells (WD-PBECs), the primary target of RSV infection, mast cells induced more IFN-α at 24 hours when infected with RSV (Villenave et al., 2012). In fact, WD-PBECs showed a poor type I IFN response consistent with that seen in infants with RSV disease (Villenave et al., 2012, Hall et al., 1978).

Johnson et al. (Johnson et al., 2011) looked at the production of IFN-α by different subsets of human DCs, and reported variable production of the cytokine between donors and subsets. For example when mDCs and pDCs produced higher level of IFN-α, it was detected less frequently and more inconsistently between donors, compared to our observations in mast cells.

It is worth mentioning that (Al-Afif, 2012) could not detect significant IFN-γ protein production from RSV-infected CBMCs at 24 or 48 hours from infection, and found upregulation of type III signal in infected mast cells. However, the production of a number of other IFN subtypes has not yet been examined.

CXCL10 and CCL4 are synthesized de novo in mast cells, while CCL5 and CXCL8 can both be found stored in mast cell granules (Theoharides et al., 2007). Epithelial cells and DCs were reported to produce significant CXCL8 in response to RSV *in vitro*, and comparable levels of CCL5, CXCL10 and CCL4 at 24 hours of infection to CBMC (Johnson et al., 2011, Villenave et al., 2012). However, it seems that mast cell and potentially human alveolar macrophage production of CXCL8 is insignificant or even reduced as we have reported here and Panuska et al. (1995) (in contrast to Becker et al., (1991) reporting of early increased CXCL8 production by alveolar macrophages). As

well, as (Al-Afif, 2012) found that CCL5 peaked at 18 hours post RSV infection of mast cells and yielded lower levels, compared to CXCL10 and CCL4 which peaked at 6 hours of infection and reached higher levels. This could reflect the lack of consistent feedback on mast cells to produce CCL5 during the infection.

The production of CXCL10 and CCL4 and not CXCL8 in response to RSV might indicate the nature of mast cell responses to RSV infection which involve predominantly the recruitment of mononuclear cells like monocytes, $CD3^+$ T-lymphocytes, and maybe NK cells as well. This is in contrast to the role of mast cells in bacterial infection where they contribute in the recruitment of granulocytes (e.g. neutrophils). This could impact the picture of the inflammation in RSV disease since neutrophils are known to be rich in digestive enzymes that can further worsen inflammation and cause tissue damage (reviewed in details in section 1.15).

Chemokines could be produced directly in response to TLR and other RNA sensing molecules detection of the virus prior to and independently of type I IFN production. For example, CCL5 could be produced in mice secondary to direct TLR 2/6 or TLR4 stimulation by RSV (Murawski et al. 2009, Kurt-Jones et al. 2000), or it could still be under direct intracellular TLR control (e.g. TLR3 as reported by Rudd et al., 2005 in A549, and RIG-I by Liu et al. 2007 and 2008). In addition, it was noted that CCL5 was only produced from the cells that were infected with RSV and not the RSV-negative epithelial cells (Becker et al., 1997) indicating that CCL5 might need a more direct interaction with the active virus to be produced which is somewhat proved by the reduction of CCL5 observed after palivizumab treatment of RSV. This could explain the lower level of CCL5 production in our system, because CCL5 could be less sensitive to type I IFN augmentation and paracrine feedback upstream of TLRs, RIG-I and IRF-3/7, unlike CXCL10 and CCL4.

The amount of VEGF-A produced by mast cells was similar to or slightly below the amounts produced by A549 cell line and normal human bronchial epithelial cells infected with RSV at MOIs similar to those used on CBMC and at 24 hours post-infection (Lee et

al., 2000). However, it seems that epithelial cells do not store preformed VEGF or upregulate VEGF mRNA synthesis after RSV infection. Epithelial cells might enhance translational and/or post-translational modification of VEGF to increase its secretion (Lee et al., 2000). On the other hand, mast cells maintain preformed stores of VEGF protein in their granules (Theoharides et al., 2007), and therefore can play a role in the early production of VEGF in RSV disease.

Furthermore, we have shown that RSV infection of human mast cells in *vitro* induced IL-1 Ra in a type I IFN dependent manner. IL-1Ra was reported in the nasopharyngeal aspirates of infants with RSV disease (Bermejo-Martin et al., 2007). IL-1Ra was also produced by alveolar macrophages *in vitro* in response to RSV (McCarthy et al., 1989). CXCL8 secretion, mediated by IL-1 produced by monocytes, from primary epithelial cultures and cell lines infected with RSV was inhibited by exogenous IL-1Ra (Thomas et al., 2000). Indeed human mast cells and isolated human lung mast cells contained preformed IL-1Ra and were able to upregulate its secretion in response to IgE mediated or specific antigen activation (Hagaman et al., 2001). Whether mast cells play a role in controlling RSV inflammation is also not known but might warrant investigation. As well, we have not investigated the role of IL-1 or IL-1 receptor expression in our model.

4.2 Implications for RSV Inflammation

Our results could support an *in vivo* model of RSV disease in which mast cells augment type I IFN response early on in the infection, enhancing inflammation and antiviral status in the lung. As well, mast cells could contribute to the induction of certain chemokines to help recruit mononuclear cells like monocytes and lymphocytes. Direct interaction with RSV or even priming of mast cells by factors produced by neighboring structural cells could lead to production of multiple other mediators rapidly from mast cell granules like CCL5 and VEGF-A. While epithelial cells die in response to RSV infection, mast cells could maintain their viability and response to continuing RSV infection. It must be noted however, that compared to epithelial cells, mast cells are much less permissive to RSV infection and presumably any RSV-mediated cell-killing.

Mast cells might be effectively resistant to RSV infection. This could be due to reduced uptake of the virus but as well it seems that even when viral uptake occurs, no significant infectious viral particles are produced. Therefore analyzing the antiviral mechanisms that could limit intracellular viral replication, packaging or release in mast cells could potentially be interesting for comparison with epithelial and immune cells fully or somewhat permissive to RSV. It is not unlikely that cells could limit their permissiveness to RSV replication due to antiviral molecular signals, for example, Bhoj et al. (Bhoj et al., 2008) reported that although RSV infection in mice was restricted to type I alveolar pneumocytes, knockout of MAVS and MyD88 led to the abrogation of type I IFN and the permissiveness of bronchial epithelial cells for RSV infection. The mechanisms by which RSV triggers recognition by cellular PRRs is not fully understood and therefore comparison of epithelial cell infection with RSV to infection in a resistant cell type that produces multiple antiviral effectors could help understanding some of these mechanisms. Mast cells infected with RSV upregulated *ISG56*, which was found at least to be inhibitory for related viruses such as Sendai virus (Fensterl and Sen, 2011). A potential role for this gene in mast cell interaction with viruses would be interesting to explore.

RSV-infected mast cell cultures produced CXCL10, CCL4 and modest CCL5 in a somewhat similar fashion to other successfully infected cells. Therefore, mast cells could play a role in the rapid innate immune response to RSV *in vivo*. This innate immune response would further impact the adaptive immune responses via selective recruitment and activation of various subsets of antigen presenting cells and lymphocytes. For example CXCL10 could play a role in activating DCs and ensuring efficient $CD8^+$ T-cell recruitment (Lindell et al., 2008). RSV-activated mast cells could contribute to the outcome of RSV disease and immunity.

4.3 Limitations and Future directions

Our results should be interpreted with caution since mast cell phenotypes and their ability to produce specific mediators depend on their microenvironment as we reviewed previously. Therefore, an RSV infection in a co-culture system resembles the lung microenvironment in which mast cells are in close proximity to primary epithelial cells like the WD-PBECs would be more ideal and will allow for the evaluation of mast cell production of more mediators like cysLTs that were not produced from RSV infection only, or prostaglandins which we have not measured.

In addition, we need to interpret evidence of low levels of infection with caution since CBMCs are not 100% pure. However limited RSV gene expression was also detected in the HMC-1 mast cell line with no contaminating cells but additional repeat experiments are required to further confirm this finding.

Also, as we discussed in the introduction, RSV-induced oedema could be caused by multiple factors, one of which could be soluble factors such as VEGF, but could also include lipid mediators and cytokines produced by mast cells. (Theoharides et al., 2007, Moon et al., 2010)

Furthermore, a better assessment of degranulation of mast cells in response to RSV infection via tryptase and/ or chymase release assay, and assessment of early apoptosis with the detection of the caspase activity assays would be needed to detail our findings regarding mast viability and degranulation.

The production of other type I IFNs, including a more detailed look at type III interferons is needed to understand interferon response by mast cells for RSV disease and for other ssRNA and dsRNA viruses.

We also do not know yet the mechanism by which RSV initiates the antiviral response in mast cells. Therefore, we have started screening for the possible antiviral effector contributing to the reduced infectivity of mast cell with RSV. Then following this,

siRNA systems might be utilized to knock down the desired target specific antiviral genes in mast cells to confirm our observations.

In addition, the observed low rate of mast cell infection with RSV might indicate that mast cells lack some of the requirement for RSV entry, such as reduced receptor/co-receptor expression or localization. This limited infection might also indicate impaired replication or progression of RSV infection in mast cells due to enhanced antiviral immunity and effector function or deficiency in the requirement for RSV replication or budding. These aspects of RSV interactions with mast cells require further investigation.

In addition, discrepancies in the findings around RSV infection from those by others in this field of research could result from virus strain differences. For example, pDCs isolated from PBMCs produced IFN-α in a fusion- and replication-dependent manner, but independent of PKR and TLR7/8 when infected with RSV-Long strain (Hornung et al., 2004). Therefore, it could be reasonable to investigate interactions of other RSV strains with human mast cells.

Therefore, ideally different viral strains infection in an *in vivo* model of mast cell deficiency like the one described by (Feyerabend et al. 2011) might give a better insight into the significance of mast cell interaction with RSV *in vivo*. The first step we have taken to achieve this, is to replicate the findings of Stokes et al. (2011) in BALB/c infection with different RSV strains, and preliminary data suggest that RSV-Line 19 has provided the most clinically relevant picture for RSV disease in BALB/c mice (see Appendix I).

Mast cells indeed are characterized by their ability to secrete multiple preformed and newly formed mediators in a timely manner, which justify study of their role in early and long term RSV disease pathology (Figure 17 shows a proposed model for a mast cell role in RSV disease).

4.4 Clinical Implications

Mast cells are abundant in the airway as well as at many mucosal surfaces and their interactions with different antigens in allergies are known to induce multiple inflammatory responses (Moon et al., 2010).

The profile of mediators produced by human mast cell infected with RSV include not only mediators that enhance antiviral immunity but also mediators that could induce airway remodeling like VEGF-A (Ferrara et al.; 2003). This could impact patient symptoms in the short run through airway obstruction and wheeze, or through chronic changes in the lung tissues that might link to the development of airway hyperreacivity in the future. Notably, RSV could sensitize the epithelial cells for further viral challenge (Groskreutz et al., 2006). As well, mast cells maintain their viability after RSV infection which could lead to their contribution to persistent patient symptoms. In addition, investigating the production of cysLTs and PGs in animal models in response to RSV infection could help clarify mast cell contribution to mutltiple inflammatory events leading to patients symptoms such as vascular congestion and wheeze.

Innate immune cells are known to have more PRRs than structural cells and they are more selective in their responses than non-immune cells. Therefore, understanding the specificity of mast cell innate immune response to RSV is useful for the utilization of specific vaccine adjuvants that could be selective or exclusive for mast cell responses.

(Al-Afif, 2012) showed that human mast cells could upregulate type III interferon in response to RSV infection. Since interferons are used as antiviral treatment (Buster et al., 2007), the mast cell production of these mediators and the selectivity by which mast cell produce these cytokines could aid in better control of severe RSV disease.

4.5 Concluding Remarks

Despite the overlap between the symptoms of RSV and allergic symptoms, as well as possible relationships between airway hyperreactivity and the close proximity of RSV infection to mast cells in the airways and around the vasculature, there has not been significant study of the role of mast cells in RSV disease. The work we present here sheds some light on potential aspects of mast cell contribution to human RSV disease. Given our findings, additional investigation into the pro- and anti-inflammatory mast cell responses to RSV and the consequence of these interactions is indicated.

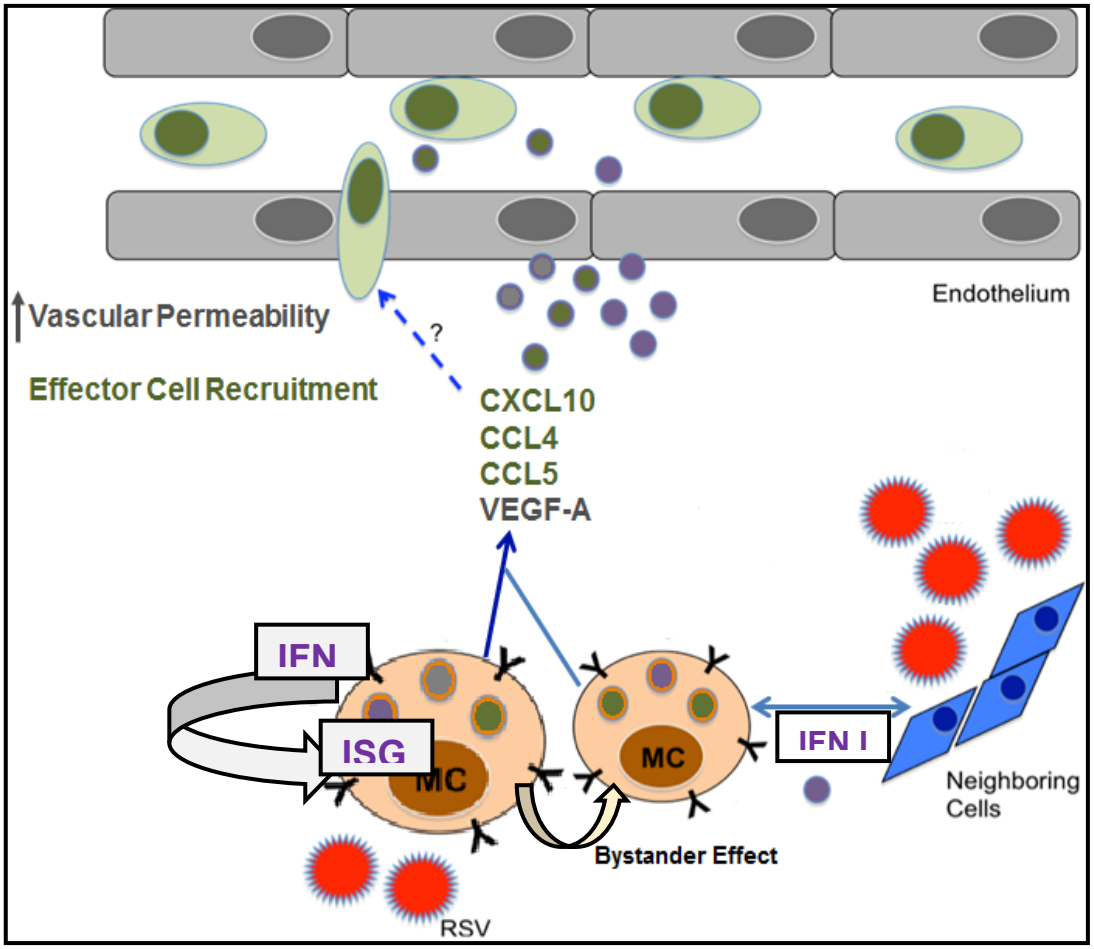


Figure 17. A proposed model for mast cell role in RSV disease. Lung mast cells respond to RSV infection by producing type I interferon (IFN I), which could further feedback on the same mast cells and neighboring cells (bystander effect) to produce type I IFN as well as other mediators like CXCL10 and CCL4. Also, in an interferon independent mechanisms, mast cells produce CCL5 and VEGF-A. These mediators will recruit effector immune cells or induce vascular permeability and tissue remodeling, and enhance inflammation. While epithelial cells are destroyed by RSV infection, they produce mediators like type I IFN that also activate mast cells. Mast cells continue to enhance inflammation and resist destruction and immune evasion by RSV.

Appendix I: Evaluating Infection of Different RSV Strains in Mice

I.1 Introduction

Many difficulties exist with RSV modeling in mice, one of which is to establish a clinical RSV infection in mice that simulate the immune response in human, especially since human RSV is not fully permissive in most mice strains. Stokes et al. (Stokes et al., 2011) published a comparison between six different RSV strains' infection in BALB/c mice, which are considered the most permissive mouse strain for RSV. In that comparison two RSV strains stood out in their induction of clinical disease, mucus production, and neutrophilic infiltrate in the respiratory secretions. These strains were a clinical isolate RSV 2-20, and an adapted experimental isolate RSV line 19. Therefore we aimed at comparing RSV line 19, RSV 2-20, and one of the most commonly used experimental strains RSV long infection in mice. After establishing the most appropriate RSV strain in approximating human RSV disease, we plan to use this model in the context of mast cell deficiency to further explore mast cell role in RSV pathology.

Here, we are presenting preliminary data compiled from a pilot study comparing the three RSV strains and two dose response studies for RSV line 19.

I.2 Methodology

RSV (long, line 19, or 2-20 strain) were grown as previously described in section 2.5 of this thesis and harvested in the same day of the infection. RSV line 19 and 2-20 were kind gifts from (Martin Moore, PhD, Division of Infectious Disease, Department of Pediatrics, Emory University School of Medicine, USA). All the experiments were performed by Dr. R. Anderson, Dr. Yan Yan Huang and R. Alyazidi.

Female BALB/c mice (purchased from Charles River Laboratories (St Constance, QC, Canada) were 6-8 week old for the start of infection, and they were and housed in the Animal Care Facility in the IWK Hospital, Halifax, according to ethically approved institutional guidelines.

In the following Figure I, the infection process and time course is described. UV-inactivated RSV and/or RPMI+2.5% FBS medium-treated mice were used as controls. Generally, we collected data and samples aiming at comparing the infection clinically (weight, morbidity signs, respiratory rate, and temperature), histologically (lungs and BAL cellular infiltrate), and for mediator induction (ELISA/luminex on sera, BAL & lung homogenate for selected chemokines & cytokines; and qPCR on the lung and spleen tissues for chemokines and cytokines). Tissue and BAL collection was similar to what was described in (Huang et al., 2009).

Determination of the mouse inoculation dose of RSV was performed in the day of the infection by Dr. R. Anderson based using the plaque assay and immunostaining described in details in (Al-Afif, 2012) thesis.

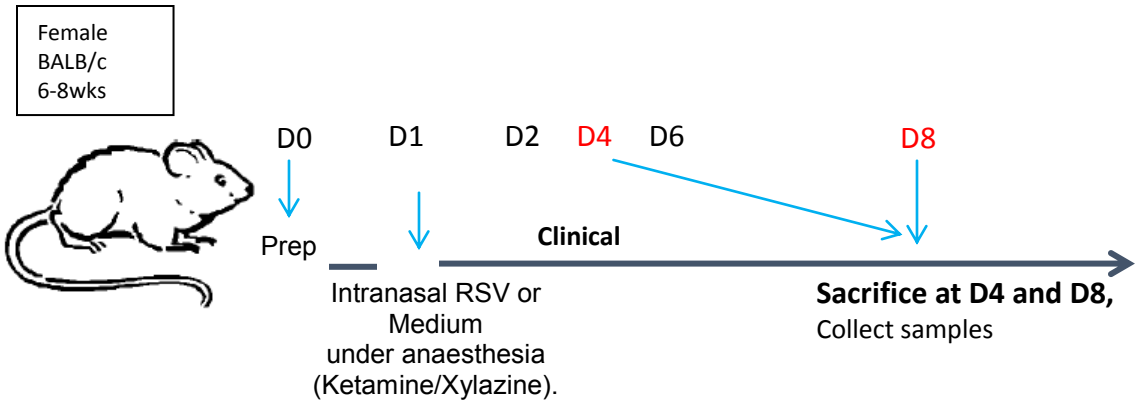


Figure I. The experimental design for mouse infection with RSV.

I.3 Preliminary Results

I.3.1 Impact of Different RSV Strains on Mouse Infection

Clinical data from our experiments as well as analysis of the BAL cellular infiltrate showed that RSV line 19 infections of mice were more symptomatic and inflammatory even at doses lower than the one used with RSV 2-20 or long strain infection. For example, a lower dose of RSV line 19 caused more weight loss and induced more inflammatory infiltrate in mice compared to higher dose from RSV 2-20 and long on day 8 (Figure IIA and B). In addition, RSV line 19 had the most impact on morbidity signs and respiratory effort than the other two strains. The pathological symptoms had two peaks, one after day 2 and the other after day 4.

I.3.2 Selected Mediator Analysis in RSV Infected Mice

We screened the BAL from control mice and mice infected with RSV 2-20 and long on day 8 post-infection with 23-mouse mediator-plex assay for pro-inflammatory mediators (BioRad). The assay included: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, CCL3, CCL4, CCL5, and TNF.

There was an expected trend toward increased chemokines and growth factors that contributes in neutrophil chemotaxis and maintenance, more pronounced in RSV 2-20 infected mice than in the RSV long infection (G-CSF, GM-CSF, and KC), as well as CCL3, CCL4, and CCL5. CXCL10 was analyzed in a separate ELISA and was not found to be increased in response to RSV 2-20 infection (Figure III and IV).

In addition, the highest level of cytokine detected was for IL-12p40 subunit of the IL-12 cytokine family. This subunit is shared between IL-12 and IL-23, but can also be found as a homdimer (Vignali and Kuchroo, 2012). This increase in IL-12p40 was not associated with increase in IL-12p70 subunit that refers to the IL-12 cytokine, and the level of IL-23 alternatively was not determined and the significance of this high level is not established yet. However, IFN-γ, which is a cytokine that frequently increases in the

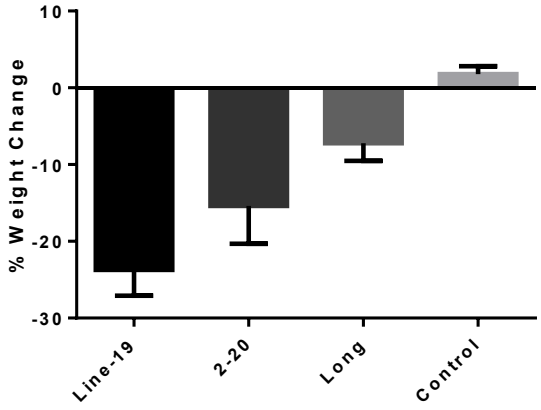
context of increased IL-12 and Th1 immune response, was also elevated in infected mice, more so in the group infected with RSV long strain (Figure V). As well, we have not analyzed the BAL from the RSV line 19 infected mice collected in the subsequent dose-response studies, for the IL-12p40 level.

I.4 Future Direction

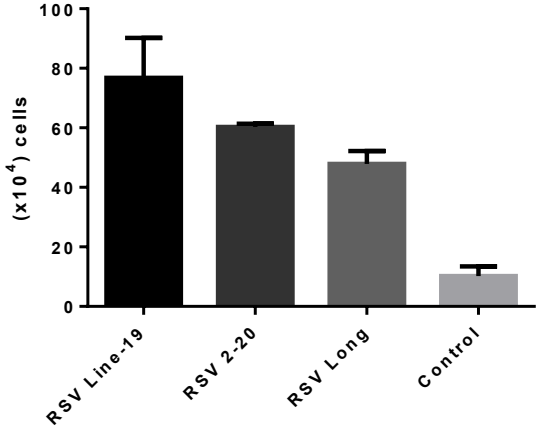
Further investigation of the IL-12p40 subunit significance in RSV infection of mice and the relation to IL-12 family members of related cytokines is needed. IL-12p40 homodimer is thought to function in regulation of cytokine production, while IL-12p40 subunit could be part of the IL-23 cytokine. IL-23 is related to Th17 cell mediated inflammation as oppose to IL-12 which id related to Th1 cell mediated inflammation (Vignali and Kuchroo, 2012). This investigation might unravel a new aspect of regulation of RSV inflammation in mice.

Figure II. Impact of different RSV strains infection on mouse weight and airway inflammation. Mice were infected with [$7x10^6$ pfu/ml of RSV line 19] in one experiment, and in another experiment mice were infected with [$1.7x10^7$ pfu/ml of RSV 2-20] and [0.9×10^7 pfu/mL RSV long].
A, Percentage of weight change in infected mice compared to medium-treated control mice (mean+/-SEM, n= 4 in RSV infected groups, n=6 in control).
B, Total cell count in BAL of infected and control mice. Cells from around 3 mL of BAL were spun down, re-suspended in PBS, and then counted (mean+/-SEM, n= 4 in RSV infected groups, n=6 in control).

A


10
0
-10
-20
-30
% Weight Change
Line-19
2-20
Long
Control

B


100
80
60
40
20
0
(x10^4) cells
RSV Line-19
RSV 2-20
RSV Long
Control

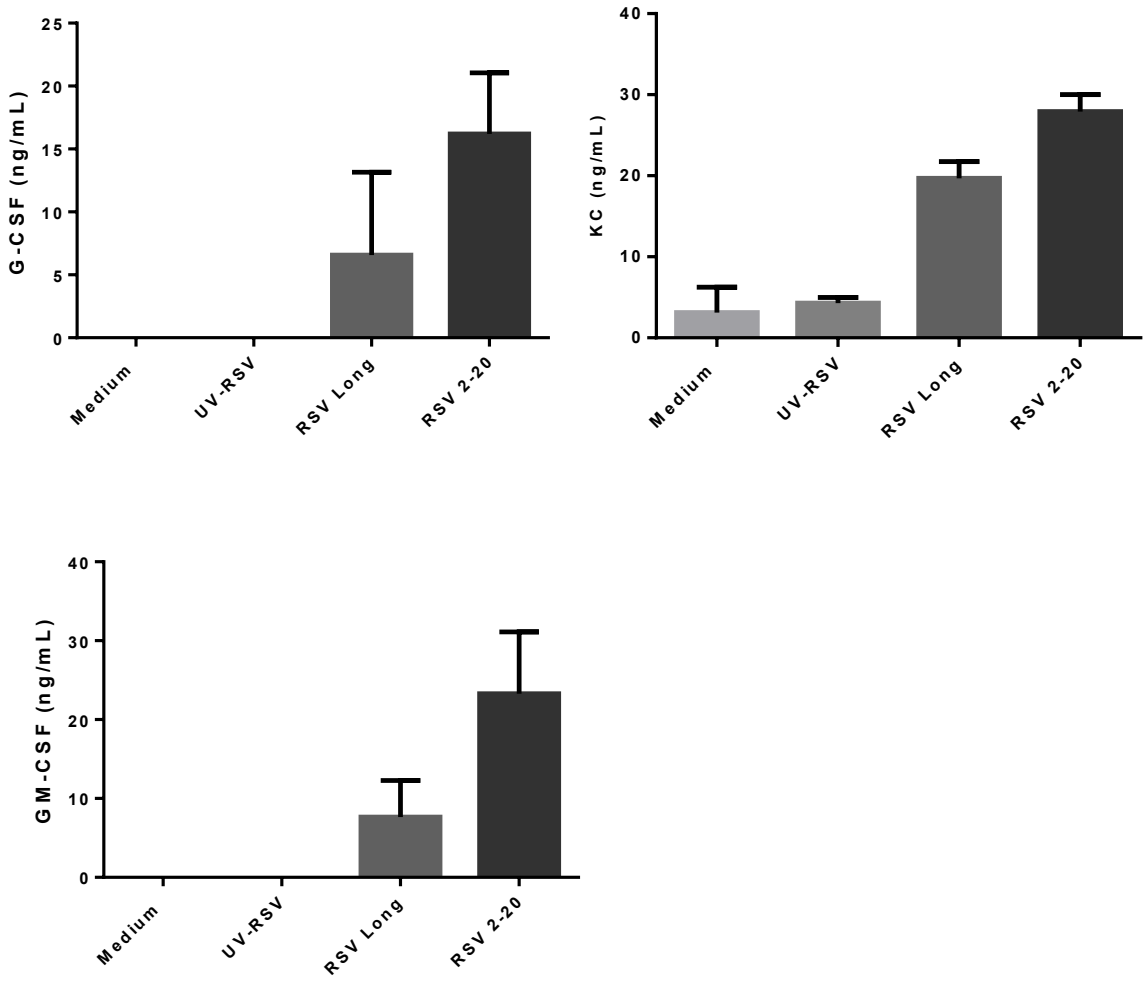


Figure III. Chemokines and growth factors involved in neutrophil chemotaxis and maintenance were increased in RSV-infected mice (mean+/-SEM, n= 4 in RSV infected groups, n=2 in UV-RSV and medium groups).

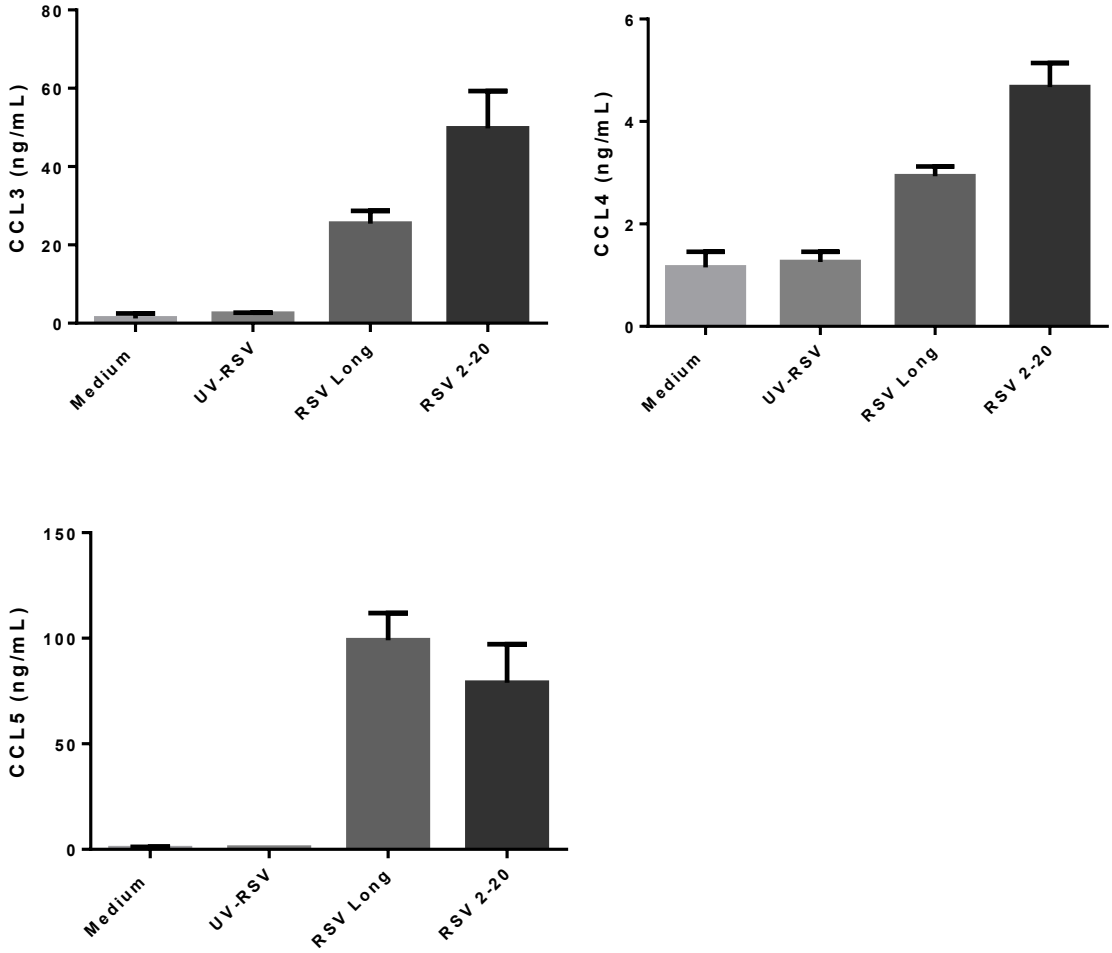


Figure IV. Other chemokines increased in RSV-infected mice (mean+/-SEM, n= 4 in RSV infected groups, n=2 in UV-RSV and medium groups).

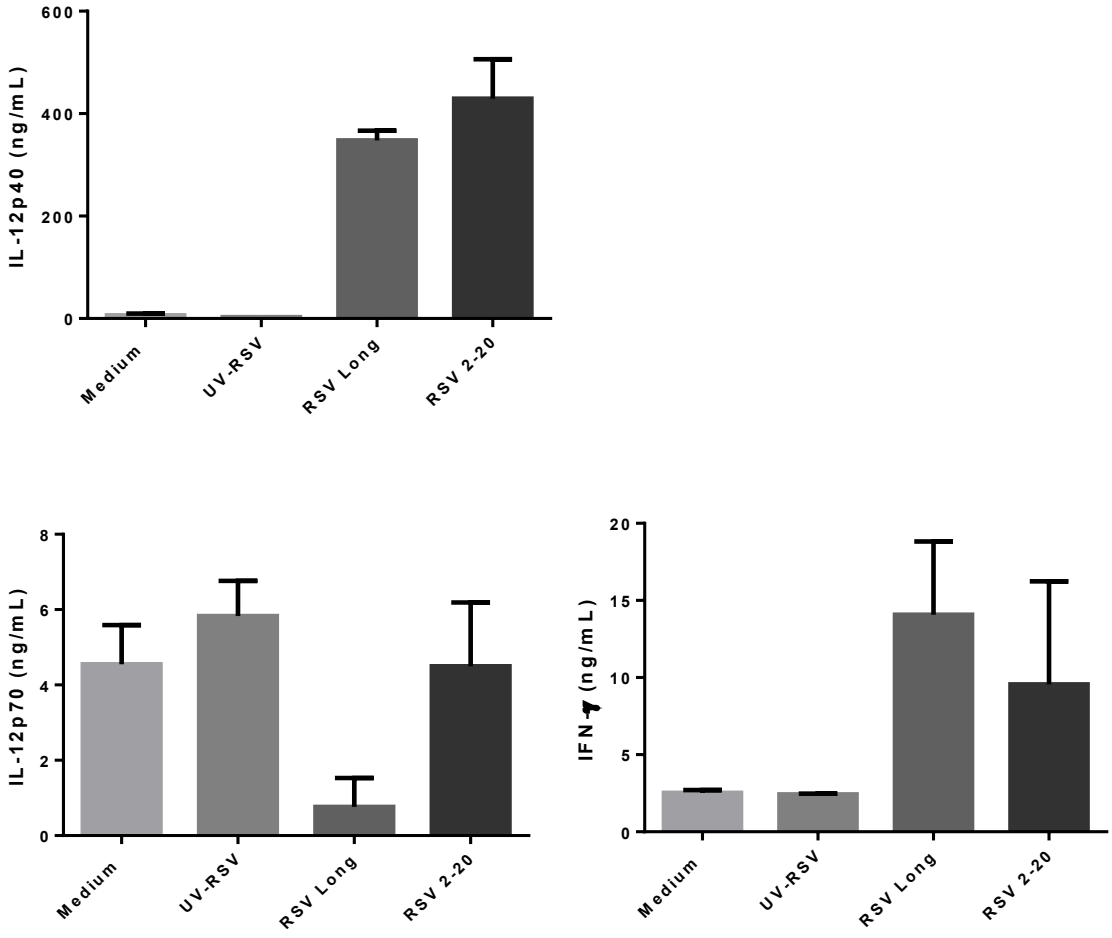


Figure V. IL-12 family of cytokines in RSV infection in mice and the related IFN-γ. IL-12p40 subunit was increased in the BAL RSV-infected mice, without a concomitant increased in IL-12p70, indicating that the increase subunit is either IL-12p40 homodimer or part of IL-23 cytokine (mean+/-SEM, n= 4 in RSV infected groups, n=2 in UV-RSV and medium groups).

References

1997. Reduction of respiratory syncytial virus hospitalization among premature infants and infants with bronchopulmonary dysplasia using respiratory syncytial virus immune globulin prophylaxis. The PREVENT Study Group. *Pediatrics,* 99**,** 93-9.

AARONSON, D. S. & HORVATH, C. M. 2002. A road map for those who don't know JAK-STAT. *Science,* 296**,** 1653-5.

AL-AFIF, A. 2012. *Human Mast Cell Responses to Respiratory Syncytial Virus.* M.Sc, Dalhousie University.

ALEXOPOULOU, L., HOLT, A. C., MEDZHITOV, R. & FLAVELL, R. A. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature,* 413**,** 732-8.

ANDERSON, L. J., DORMITZER, P. R., NOKES, D. J., RAPPUOLI, R., ROCA, A. & GRAHAM, B. S. 2013. Strategic priorities for respiratory syncytial virus (RSV) vaccine development. *Vaccine,* 31 Suppl 2**,** B209-15.

ASCHERL, G., HOHENADL, C., SCHATZ, O., SHUMAY, E., BOGNER, J., ECKHART, L., TSCHACHLER, E., MONINI, P., ENSOLI, B. & STURZL, M. 1999. Infection with human immunodeficiency virus-1 increases expression of vascular endothelial cell growth factor in T cells: implications for acquired immunodeficiency syndrome-associated vasculopathy. *Blood,* 93**,** 4232-41.

AUSAR, S. F., ESPINA, M., BROCK, J., THYAGARAYAPURAN, N., REPETTO, R., KHANDKE, L. & MIDDAUGH, C. R. 2007. High-throughput screening of stabilizers for respiratory syncytial virus: identification of stabilizers and their effects on the conformational thermostability of viral particles. *Hum Vaccin,* 3**,** 94-103.

AWOMOYI, A. A., RALLABHANDI, P., POLLIN, T. I., LORENZ, E., SZTEIN, M. B., BOUKHVALOVA, M. S., HEMMING, V. G., BLANCO, J. C. & VOGEL, S. N. 2007. Association of TLR4 polymorphisms with symptomatic respiratory syncytial virus infection in high-risk infants and young children. *J Immunol,* 179**,** 3171-7.

BECKER, S., QUAY, J. & SOUKUP, J. 1991. Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. *J Immunol,* 147**,** 4307-12.

BECKER, S., REED, W., HENDERSON, F. W. & NOAH, T. L. 1997. RSV infection of human airway epithelial cells causes production of the beta-chemokine RANTES. *Am J Physiol,* 272**,** L512-20.

BELGNAOUI, S. M., PAZ, S. & HISCOTT, J. 2011. Orchestrating the interferon antiviral response through the mitochondrial antiviral signaling (MAVS) adapter. *Curr Opin Immunol,* 23**,** 564-72.

BEM, R. A., DOMACHOWSKE, J. B. & ROSENBERG, H. F. 2011. Animal models of human respiratory syncytial virus disease. *Am J Physiol Lung Cell Mol Physiol,* 301**,** L148-56.

BENNETT, B. L., GAROFALO, R. P., CRON, S. G., HOSAKOTE, Y. M., ATMAR, R. L., MACIAS, C. G. & PIEDRA, P. A. 2007. Immunopathogenesis of respiratory syncytial virus bronchiolitis. *J Infect Dis,* 195**,** 1532-40.

BERMEJO-MARTIN, J. F., GARCIA-AREVALO, M. C., DE LEJARAZU, R. O., ARDURA, J., EIROS, J. M., ALONSO, A., MATIAS, V., PINO, M., BERNARDO, D., ARRANZ, E. & BLANCO-QUIROS, A. 2007. Predominance of Th2 cytokines, CXC chemokines and innate immunity mediators at the mucosal level during severe respiratory syncytial virus infection in children. *Eur Cytokine Netw,* 18**,** 162-7.

BERMINGHAM, A. & COLLINS, P. L. 1999. The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. *Proc Natl Acad Sci U S A,* 96**,** 11259-64.

BHOJ, V. G., SUN, Q., BHOJ, E. J., SOMERS, C., CHEN, X., TORRES, J. P., MEJIAS, A., GOMEZ, A. M., JAFRI, H., RAMILO, O. & CHEN, Z. J. 2008. MAVS and MyD88 are essential for innate immunity but not cytotoxic T lymphocyte response against respiratory syncytial virus. *Proc Natl Acad Sci U S A,* 105**,** 14046-51.

BINGHAM CO III, M. M., FUJISHIMA H, ET AL 1999. Signal Transduction in Mast Cells and Basophils. *In:* RAZIN E, R. J. (ed.). New York: Springer.

BLANDER, J. M. & SANDER, L. E. 2012. Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. *Nat Rev Immunol,* 12**,** 215-25.

BLOUNT, R. E., JR., MORRIS, J. A. & SAVAGE, R. E. 1956. Recovery of cytopathogenic agent from chimpanzees with coryza. *Proc Soc Exp Biol Med,* 92**,** 544-9.

BOSSERT, B., MAROZIN, S. & CONZELMANN, K. K. 2003. Nonstructural proteins NS1 and NS2 of bovine respiratory syncytial virus block activation of interferon regulatory factor 3. *J Virol,* 77**,** 8661-8.

BOUKHVALOVA, M. S., YIM, K. C., PRINCE, G. A. & BLANCO, J. C. 2010. Methods for monitoring dynamics of pulmonary RSV replication by viral culture and by real-time reverse transcription-PCR in vivo: Detection of abortive viral replication. *Curr Protoc Cell Biol,* Chapter 26**,** Unit26 6.

BRANDENBURG, A. H., GROEN, J., VAN STEENSEL-MOLL, H. A., CLAAS, E. C., ROTHBARTH, P. H., NEIJENS, H. J. & OSTERHAUS, A. D. 1997. Respiratory syncytial virus specific serum antibodies in infants under six months of age: limited serological response upon infection. *J Med Virol,* 52**,** 97-104.

BROWN, M. G., HERMANN, L. L., ISSEKUTZ, A. C., MARSHALL, J. S., ROWTER, D., AL-AFIF, A. & ANDERSON, R. 2011. Dengue virus infection of mast cells triggers endothelial cell activation. *J Virol,* 85**,** 1145-50.

BROWN, M. G., HUANG, Y. Y., MARSHALL, J. S., KING, C. A., HOSKIN, D. W. & ANDERSON, R. 2009. Dramatic caspase-dependent apoptosis in antibody-enhanced dengue virus infection of human mast cells. *J Leukoc Biol,* 85**,** 71-80.

BROWN, M. G., MCALPINE, S. M., HUANG, Y. Y., HAIDL, I. D., AL-AFIF, A., MARSHALL, J. S. & ANDERSON, R. 2012. RNA sensors enable human mast cell anti-viral chemokine production and IFN-mediated protection in response to antibody-enhanced dengue virus infection. *PLoS One,* 7**,** e34055.

BUKREYEV, A., YANG, L., FRICKE, J., CHENG, L., WARD, J. M., MURPHY, B. R. & COLLINS, P. L. 2008. The secreted form of respiratory syncytial virus G glycoprotein helps the virus evade antibody-mediated restriction of replication by acting as an antigen decoy and through effects on Fc receptor-bearing leukocytes. *J Virol,* 82**,** 12191-204.

BURKE, S. M., ISSEKUTZ, T. B., MOHAN, K., LEE, P. W., SHMULEVITZ, M. & MARSHALL, J. S. 2008. Human mast cell activation with virus-associated stimuli leads to the selective chemotaxis of natural killer cells by a CXCL8-dependent mechanism. *Blood,* 111**,** 5467-76.

BUSTER, E. H., HANSEN, B. E., BUTI, M., DELWAIDE, J., NIEDERAU, C., MICHIELSEN, P. P., FLISIAK, R., ZONDERVAN, P. E., SCHALM, S. W., JANSSEN, H. L. & GROUP, H. B. V. S. 2007. Peginterferon alpha-2b is safe and effective in HBeAg-positive chronic hepatitis B patients with advanced fibrosis. *Hepatology,* 46**,** 388-94.

BUTTERFIELD, J. H., WEILER, D., DEWALD, G. & GLEICH, G. J. 1988. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk Res,* 12**,** 345-55.

CAUGHEY, G. H. 2007. Mast cell tryptases and chymases in inflammation and host defense. *Immunol Rev,* 217**,** 141-54.

CAVALLARO, J. J. & MAASSAB, H. F. 1966. Adaptation of respiratory syncytial (RS) virus to brain of suckling mice. *Proc Soc Exp Biol Med,* 121**,** 37-41.

CHANG, C. H., HUANG, Y. & ANDERSON, R. 2003. Activation of vascular endothelial cells by IL-1alpha released by epithelial cells infected with respiratory syncytial virus. *Cell Immunol,* 221**,** 37-41.

CHANOCK, R., ROIZMAN, B. & MYERS, R. 1957. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). I. Isolation, properties and characterization. *Am J Hyg,* 66**,** 281-90.

CHAWES, B. L., POORISRISAK, P., JOHNSTON, S. L. & BISGAARD, H. 2012. Neonatal bronchial hyperresponsiveness precedes acute severe viral bronchiolitis in infants. *J Allergy Clin Immunol,* 130**,** 354-61 e3.

CHOI, Y., MASON, C. S., JONES, L. P., CRABTREE, J., JORQUERA, P. A. & TRIPP, R. A. 2012. Antibodies to the central conserved region of respiratory syncytial virus (RSV) G protein block RSV G protein CX3C-CX3CR1 binding and cross-neutralize RSV A and B strains. *Viral Immunol,* 25**,** 193-203.

CHU, H. Y. & ENGLUND, J. A. 2013. Respiratory syncytial virus disease: prevention and treatment. *Curr Top Microbiol Immunol,* 372**,** 235-58.

CHUNG, H. L. & KIM, S. G. 2002. RANTES may be predictive of later recurrent wheezing after respiratory syncytial virus bronchiolitis in infants. *Ann Allergy Asthma Immunol,* 88**,** 463-7.

CIRINO, N. M., PANUSKA, J. R., VILLANI, A., TARAF, H., REBERT, N. A., MEROLLA, R., TSIVITSE, P. & GILBERT, I. A. 1993. Restricted replication of respiratory syncytial virus in human alveolar macrophages. *J Gen Virol,* 74 (Pt 8)**,** 1527-37.

COLLINS, P. L., FEARNS, R. & GRAHAM, B. S. 2013. Respiratory syncytial virus: virology, reverse genetics, and pathogenesis of disease. *Curr Top Microbiol Immunol,* 372**,** 3-38.

DAKHAMA, A., LEE, Y. M., OHNISHI, H., JING, X., BALHORN, A., TAKEDA, K. & GELFAND, E. W. 2009. Virus-specific IgE enhances airway responsiveness on reinfection with respiratory syncytial virus in newborn mice. *J Allergy Clin Immunol,* 123**,** 138-145 e5.

DAWICKI, W. & MARSHALL, J. S. 2007. New and emerging roles for mast cells in host defence. *Curr Opin Immunol,* 19**,** 31-8.

DOMACHOWSKE, J. B. & ROSENBERG, H. F. 1999. Respiratory syncytial virus infection: immune response, immunopathogenesis, and treatment. *Clin Microbiol Rev,* 12**,** 298-309.

DOUVILLE, R. N., LISSITSYN, Y., HIRSCHFELD, A. F., BECKER, A. B., KOZYRSKYJ, A. L., LIEM, J., BASTIEN, N., LI, Y., VICTOR, R. E., SEKHON, M., TURVEY, S. E. & HAYGLASS, K. T. 2010. TLR4 Asp299Gly and Thr399Ile polymorphisms: no impact on human immune responsiveness to LPS or respiratory syncytial virus. *PLoS One,* 5**,** e12087.

DUDECK, A., DUDECK, J., SCHOLTEN, J., PETZOLD, A., SURIANARAYANAN, S., KOHLER, A., PESCHKE, K., VOHRINGER, D., WASKOW, C., KRIEG, T., MULLER, W., WAISMAN, A., HARTMANN, K., GUNZER, M. & ROERS, A. 2011. Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. *Immunity,* 34**,** 973-84.

DYER, K. D., PERCOPO, C. M., FISCHER, E. R., GABRYSZEWSKI, S. J. & ROSENBERG, H. F. 2009. Pneumoviruses infect eosinophils and elicit MyD88-dependent release of chemoattractant cytokines and interleukin-6. *Blood,* 114**,** 2649-56.

ECHTENACHER, B., MANNEL, D. N. & HULTNER, L. 1996. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature,* 381**,** 75-7.

EHL, S., BISCHOFF, R., OSTLER, T., VALLBRACHT, S., SCHULTE-MONTING, J., POLTORAK, A. & FREUDENBERG, M. 2004. The role of Toll-like receptor 4 versus interleukin-12 in immunity to respiratory syncytial virus. *Eur J Immunol,* 34**,** 1146-53.

ELLIOTT, M. B., TEBBEY, P. W., PRYHARSKI, K. S., SCHEUER, C. A., LAUGHLIN, T. S. & HANCOCK, G. E. 2004. Inhibition of respiratory syncytial virus infection with the CC chemokine RANTES (CCL5). *J Med Virol,* 73**,** 300-8.

EMPEY, K. M., PEEBLES, R. S., JR. & KOLLS, J. K. 2010. Pharmacologic advances in the treatment and prevention of respiratory syncytial virus. *Clin Infect Dis,* 50**,** 1258-67.

ENOKSSON, M., LYBERG, K., MOLLER-WESTERBERG, C., FALLON, P. G., NILSSON, G. & LUNDERIUS-ANDERSSON, C. 2011. Mast cells as sensors of cell injury through IL-33 recognition. *J Immunol,* 186**,** 2523-8.

EVANS, J. E., CANE, P. A. & PRINGLE, C. R. 1996. Expression and characterisation of the NS1 and NS2 proteins of respiratory syncytial virus. *Virus Res,* 43**,** 155-61.

EVERARD, M. L., FOX, G., WALLS, A. F., QUINT, D., FIFIELD, R., WALTERS, C., SWARBRICK, A. & MILNER, A. D. 1995. Tryptase and IgE concentrations in the respiratory tract of infants with acute bronchiolitis. *Arch Dis Child,* 72**,** 64-9.

EVERARD, M. L., SWARBRICK, A., WRIGHTHAM, M., MCINTYRE, J., DUNKLEY, C., JAMES, P. D., SEWELL, H. F. & MILNER, A. D. 1994. Analysis of cells obtained by bronchial lavage of infants with respiratory syncytial virus infection. *Archives of Disease in Childhood,* 71**,** 428-432.

FALSEY, A. R., HENNESSEY, P. A., FORMICA, M. A., COX, C. & WALSH, E. E. 2005. Respiratory syncytial virus infection in elderly and high-risk adults. *N Engl J Med,* 352**,** 1749-59.

FALSEY, A. R. & WALSH, E. E. 1998. Relationship of serum antibody to risk of respiratory syncytial virus infection in elderly adults. *J Infect Dis,* 177**,** 463-6.

FALSEY, A. R. & WALSH, E. E. 2000. Respiratory syncytial virus infection in adults. *Clin Microbiol Rev,* 13**,** 371-84.

FALSEY, A. R., WALSH, E. E. & BETTS, R. F. 1990. Serologic evidence of respiratory syncytial virus infection in nursing home patients. *J Infect Dis,* 162**,** 568-9.

FEARNS, R. & COLLINS, P. L. 1999. Role of the M2-1 transcription antitermination protein of respiratory syncytial virus in sequential transcription. *J Virol,* 73**,** 5852-64.

FENSTERL, V. & SEN, G. C. 2009. Interferons and viral infections. *Biofactors,* 35**,** 14-20.

FENSTERL, V. & SEN, G. C. 2011. The ISG56/IFIT1 gene family. *J Interferon Cytokine Res,* 31**,** 71-8.

FERRARA, N., GERBER, H. P. & LECOUTER, J. 2003. The biology of VEGF and its receptors. *Nat Med,* 9**,** 669-76.

FRANKE-ULLMANN, G., PFORTNER, C., WALTER, P., STEINMULLER, C., LOHMANN-MATTHES, M. L., KOBZIK, L. & FREIHORST, J. 1995. Alteration of pulmonary macrophage function by respiratory syncytial virus infection in vitro. *J Immunol,* 154**,** 268-80.

FRICKE, J., KOO, L. Y., BROWN, C. R. & COLLINS, P. L. 2013. p38 and OGT sequestration into viral inclusion bodies in cells infected with human respiratory syncytial virus suppresses MK2 activities and stress granule assembly. *J Virol,* 87**,** 1333-47.

FUENTES, S., TRAN, K. C., LUTHRA, P., TENG, M. N. & HE, B. 2007. Function of the respiratory syncytial virus small hydrophobic protein. *J Virol,* 81**,** 8361-6.

FURUTA, T., MURAO, L. A., LAN, N. T., HUY, N. T., HUONG, V. T., THUY, T. T., THAM, V. D., NGA, C. T., HA, T. T., OHMOTO, Y., KIKUCHI, M., MORITA, K., YASUNAMI, M., HIRAYAMA, K. & WATANABE, N. 2012. Association of mast cell-derived VEGF and proteases in Dengue shock syndrome. *PLoS Negl Trop Dis,* 6**,** e1505.

GAGRO, A., TOMINAC, M., KRSULOVIC-HRESIC, V., BACE, A., MATIC, M., DRAZENOVIC, V., MLINARIC-GALINOVIC, G., KOSOR, E., GOTOVAC, K., BOLANCA, I., BATINICA, S. & RABATIC, S. 2004. Increased Toll-like receptor 4 expression in infants with respiratory syncytial virus bronchiolitis. *Clin Exp Immunol,* 135**,** 267-72.

GAROFALO, R. P., PATTI, J., HINTZ, K. A., HILL, V., OGRA, P. L. & WELLIVER, R. C. 2001. Macrophage inflammatory protein-1alpha (not T helper type 2 cytokines) is associated with severe forms of respiratory syncytial virus bronchiolitis. *J Infect Dis,* 184**,** 393-9.

GHILDYAL, R., HO, A. & JANS, D. A. 2006. Central role of the respiratory syncytial virus matrix protein in infection. *FEMS Microbiol Rev,* 30**,** 692-705.

GOMPERTS, B. D., BALDWIN, J. M. & MICKLEM, K. J. 1983. Rat mast cells permeabilized with Sendai virus secrete histamine in response to Ca2+ buffered in the micromolar range. *Biochem J,* 210**,** 737-45.

GRAHAM, A. C., HILMER, K. M., ZICKOVICH, J. M. & OBAR, J. J. 2013. Inflammatory response of mast cells during influenza A virus infection is mediated by active infection and RIG-I signaling. *J Immunol,* 190**,** 4676-84.

GRIMBALDESTON, M. A., CHEN, C. C., PILIPONSKY, A. M., TSAI, M., TAM, S. Y. & GALLI, S. J. 2005. Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. *Am J Pathol,* 167**,** 835-48.

GROSKREUTZ, D. J., BABOR, E. C., MONICK, M. M., VARGA, S. M. & HUNNINGHAKE, G. W. 2010. Respiratory syncytial virus limits alpha subunit of eukaryotic translation initiation factor 2 (eIF2alpha) phosphorylation to maintain translation and viral replication. *J Biol Chem,* 285**,** 24023-31.

GROSKREUTZ, D. J., MONICK, M. M., POWERS, L. S., YAROVINSKY, T. O., LOOK, D. C. & HUNNINGHAKE, G. W. 2006. Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells. *J Immunol,* 176**,** 1733-40.

GUHL, S., FRANKE, R., SCHIELKE, A., JOHNE, R., KRUGER, D. H., BABINA, M. & RANG, A. 2010. Infection of in vivo differentiated human mast cells with hantaviruses. *J Gen Virol,* 91**,** 1256-61.

HAGAMAN, D. D., OKAYAMA, Y., D'AMBROSIO, C., PRUSSIN, C., GILFILLAN, A. M. & METCALFE, D. D. 2001. Secretion of interleukin-1 receptor antagonist from human mast cells after immunoglobulin E-mediated activation and after segmental antigen challenge. *Am J Respir Cell Mol Biol,* 25**,** 685-91.

HALL, C. B., DOUGLAS, R. G., JR. & GEIMAN, J. M. 1976. Respiratory syncytial virus infections in infants: quantitation and duration of shedding. *J Pediatr,* 89**,** 11-5.

HALL, C. B., DOUGLAS, R. G., JR. & GEIMAN, J. M. 1980. Possible transmission by fomites of respiratory syncytial virus. *J Infect Dis,* 141**,** 98-102.

HALL, C. B., DOUGLAS, R. G., JR., SIMONS, R. L. & GEIMAN, J. M. 1978. Interferon production in children with respiratory syncytial, influenza, and parainfluenza virus infections. *J Pediatr,* 93**,** 28-32.

HALL, C. B., SIMOES, E. A. & ANDERSON, L. J. 2013. Clinical and epidemiologic features of respiratory syncytial virus. *Curr Top Microbiol Immunol,* 372**,** 39-57.

HALL, C. B., WALSH, E. E., LONG, C. E. & SCHNABEL, K. C. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. *J Infect Dis,* 163**,** 693-8.

HAMMER, J., NUMA, A. & NEWTH, C. J. 1995. Albuterol responsiveness in infants with respiratory failure caused by respiratory syncytial virus infection. *J Pediatr,* 127**,** 485-90.

HARDY, R. W. & WERTZ, G. W. 2000. The Cys(3)-His(1) motif of the respiratory syncytial virus M2-1 protein is essential for protein function. *J Virol,* 74**,** 5880-5.

HARRISON, A. M., BONVILLE, C. A., ROSENBERG, H. F. & DOMACHOWSKE, J. B. 1999. Respiratory syncytical virus-induced chemokine expression in the lower airways: eosinophil recruitment and degranulation. *Am J Respir Crit Care Med,* 159**,** 1918-24.

HASTIE, M. L., HEADLAM, M. J., PATEL, N. B., BUKREYEV, A. A., BUCHHOLZ, U. J., DAVE, K. A., NORRIS, E. L., WRIGHT, C. L., SPANN, K. M., COLLINS, P. L. & GORMAN, J. J. 2012. The human respiratory syncytial virus nonstructural protein 1 regulates type I and type II interferon pathways. *Mol Cell Proteomics,* 11**,** 108-27.

HAYNES, L. M., MOORE, D. D., KURT-JONES, E. A., FINBERG, R. W., ANDERSON, L. J. & TRIPP, R. A. 2001. Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. *J Virol,* 75**,** 10730-7.

HEIL, F., HEMMI, H., HOCHREIN, H., AMPENBERGER, F., KIRSCHNING, C., AKIRA, S., LIPFORD, G., WAGNER, H. & BAUER, S. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science,* 303**,** 1526-9.

HIGGINS, S. C. & MILLS, K. H. 2010. TLR, NLR Agonists, and Other Immune Modulators as Infectious Disease Vaccine Adjuvants. *Curr Infect Dis Rep,* 12**,** 4-12.

HORNUNG, V., SCHLENDER, J., GUENTHNER-BILLER, M., ROTHENFUSSER, S., ENDRES, S., CONZELMANN, K. K. & HARTMANN, G. 2004. Replication-dependent potent IFN-alpha induction in human plasmacytoid dendritic cells by a single-stranded RNA virus. *J Immunol,* 173**,** 5935-43.

HUANG, Y., CYR, S. L., BURT, D. S. & ANDERSON, R. 2009. Murine host responses to respiratory syncytial virus (RSV) following intranasal administration of a Protollin-adjuvanted, epitope-enhanced recombinant G protein vaccine. *J Clin Virol,* 44**,** 287-91.

ISAACS, D., BANGHAM, C. R. & MCMICHAEL, A. J. 1987. Cell-mediated cytotoxic response to respiratory syncytial virus in infants with bronchiolitis. *Lancet,* 2**,** 769-71.

ISON, M. G. 2009. Respiratory syncytial virus and other respiratory viruses in the setting of bone marrow transplantation. *Curr Opin Oncol,* 21**,** 171-6.

JANSSEN, R., PENNINGS, J., HODEMAEKERS, H., BUISMAN, A., VAN OOSTEN, M., DE ROND, L., OZTURK, K., DORMANS, J., KIMMAN, T. & HOEBEE, B. 2007. Host transcription profiles upon primary respiratory syncytial virus infection. *J Virol,* 81**,** 5958-67.

JEFFREE, C. E., RIXON, H. W., BROWN, G., AITKEN, J. & SUGRUE, R. J. 2003. Distribution of the attachment (G) glycoprotein and GM1 within the envelope of mature respiratory syncytial virus filaments revealed using field emission scanning electron microscopy. *Virology,* 306**,** 254-67.

JOHNSON, J. E., GONZALES, R. A., OLSON, S. J., WRIGHT, P. F. & GRAHAM, B. S. 2007. The histopathology of fatal untreated human respiratory syncytial virus infection. *Mod Pathol,* 20**,** 108-19.

JOHNSON, S., OLIVER, C., PRINCE, G. A., HEMMING, V. G., PFARR, D. S., WANG, S. C., DORMITZER, M., O'GRADY, J., KOENIG, S., TAMURA, J. K., WOODS, R., BANSAL, G., COUCHENOUR, D., TSAO, E., HALL, W. C. & YOUNG, J. F. 1997. Development of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. *J Infect Dis,* 176**,** 1215-24.

JOHNSON, T. R., JOHNSON, C. N., CORBETT, K. S., EDWARDS, G. C. & GRAHAM, B. S. 2011. Primary human mDC1, mDC2, and pDC dendritic cells are differentially infected and activated by respiratory syncytial virus. *PLoS One,* 6**,** e16458.

JOHNSON, T. R., MCLELLAN, J. S. & GRAHAM, B. S. 2012. Respiratory syncytial virus glycoprotein G interacts with DC-SIGN and L-SIGN to activate ERK1 and ERK2. *J Virol,* 86**,** 1339-47.

JONES, A., MORTON, I., HOBSON, L., EVANS, G. S. & EVERARD, M. L. 2006. Differentiation and immune function of human dendritic cells following infection by respiratory syncytial virus. *Clin Exp Immunol,* 143**,** 513-22.

KARRON, R. A., BUCHHOLZ, U. J. & COLLINS, P. L. 2013. Live-attenuated respiratory syncytial virus vaccines. *Curr Top Microbiol Immunol,* 372**,** 259-84.

KATZ, H. R. & AUSTEN, K. F. 2011. Mast cell deficiency, a game of kit and mouse. *Immunity,* 35**,** 668-70.

KECK, S., MULLER, I., FEJER, G., SAVIC, I., TCHAPTCHET, S., NIELSEN, P. J., GALANOS, C., HUBER, M. & FREUDENBERG, M. A. 2011. Absence of TRIF signaling in lipopolysaccharide-stimulated murine mast cells. *J Immunol,* 186**,** 5478-88.

KIM, C. K., KIM, S. W., KIM, Y. K., KANG, H., YU, J., YOO, Y. & KOH, Y. Y. 2005. Bronchoalveolar lavage eosinophil cationic protein and interleukin-8 levels in acute asthma and acute bronchiolitis. *Clin Exp Allergy,* 35**,** 591-7.

KIM, C. K., KIM, S. W., PARK, C. S., KIM, B. I., KANG, H. & KOH, Y. Y. 2003. Bronchoalveolar lavage cytokine profiles in acute asthma and acute bronchiolitis. *J Allergy Clin Immunol,* 112**,** 64-71.

KIM, H. H., LEE, M. H. & LEE, J. S. 2007. Eosinophil cationic protein and chemokines in nasopharyngeal secretions of infants with respiratory syncytial virus (RSV) bronchiolitis and non-RSV bronchiolitis. *J Korean Med Sci,* 22**,** 37-42.

KIMMAN, T. G. & WESTENBRINK, F. 1990. Immunity to human and bovine respiratory syncytial virus. *Arch Virol,* 112**,** 1-25.

KING, C. A., ANDERSON, R. & MARSHALL, J. S. 2002. Dengue virus selectively induces human mast cell chemokine production. *J Virol,* 76**,** 8408-19.

KING, C. A., MARSHALL, J. S., ALSHURAFA, H. & ANDERSON, R. 2000. Release of vasoactive cytokines by antibody-enhanced dengue virus infection of a human mast cell/basophil line. *J Virol,* 74**,** 7146-50.

KIRSHENBAUM, A. S., AKIN, C., WU, Y., ROTTEM, M., GOFF, J. P., BEAVEN, M. A., RAO, V. K. & METCALFE, D. D. 2003. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. *Leuk Res,* 27**,** 677-82.

KULKA, M., ALEXOPOULOU, L., FLAVELL, R. A. & METCALFE, D. D. 2004. Activation of mast cells by double-stranded RNA: evidence for activation through Toll-like receptor 3. *J Allergy Clin Immunol,* 114**,** 174-82.

KUMAR, V. & SHARMA, A. 2010. Mast cells: emerging sentinel innate immune cells with diverse role in immunity. *Mol Immunol,* 48**,** 14-25.

KURT-JONES, E. A., POPOVA, L., KWINN, L., HAYNES, L. M., JONES, L. P., TRIPP, R. A., WALSH, E. E., FREEMAN, M. W., GOLENBOCK, D. T., ANDERSON, L. J. & FINBERG, R. W. 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol,* 1**,** 398-401.

LANARI, M., VANDINI, S., ARCURI, S., GALLETTI, S. & FALDELLA, G. 2013. The use of humanized monoclonal antibodies for the prevention of respiratory syncytial virus infection. *Clin Dev Immunol,* 2013**,** 359683.

LANGEDIJK, J. P., DE GROOT, B. L., BERENDSEN, H. J. & VAN OIRSCHOT, J. T. 1998. Structural homology of the central conserved region of the attachment protein G of respiratory syncytial virus with the fourth subdomain of 55-kDa tumor necrosis factor receptor. *Virology,* 243**,** 293-302.

LANGLEY, G. F. & ANDERSON, L. J. 2011. Epidemiology and prevention of respiratory syncytial virus infections among infants and young children. *Pediatr Infect Dis J,* 30**,** 510-7.

LEADER, S. & KOHLHASE, K. 2003. Recent trends in severe respiratory syncytial virus (RSV) among US infants, 1997 to 2000. *J Pediatr,* 143**,** S127-32.

LEE, C. G., YOON, H. J., ZHU, Z., LINK, H., WANG, Z., GWALTNEY, J. M., LANDRY, M. & ELIAS, J. A. 2000. Respiratory syncytial virus stimulation of vascular endothelial cell growth Factor/Vascular permeability factor. *Am J Respir Cell Mol Biol,* 23**,** 662-9.

LEVITZ, R., WATTIER, R., PHILLIPS, P., SOLOMON, A., LAWLER, J., LAZAR, I., WEIBEL, C. & KAHN, J. S. 2012. Induction of IL-6 and CCL5 (RANTES) in human respiratory epithelial (A549) cells by clinical isolates of respiratory syncytial virus is strain specific. *Virol J,* 9**,** 190.

LIN, T. J., GARDUNO, R., BOUDREAU, R. T. & ISSEKUTZ, A. C. 2002. Pseudomonas aeruginosa activates human mast cells to induce neutrophil transendothelial migration via mast cell-derived IL-1 alpha and beta. *J Immunol,* 169**,** 4522-30.

LINDELL, D. M., LANE, T. E. & LUKACS, N. W. 2008. CXCL10/CXCR3-mediated responses promote immunity to respiratory syncytial virus infection by augmenting dendritic cell and CD8(+) T cell efficacy. *Eur J Immunol,* 38**,** 2168-79.

LINDQUIST, M. E., LIFLAND, A. W., UTLEY, T. J., SANTANGELO, P. J. & CROWE, J. E., JR. 2010. Respiratory syncytial virus induces host RNA stress granules to facilitate viral replication. *J Virol,* 84**,** 12274-84.

LINDSLEY, W. G., BLACHERE, F. M., DAVIS, K. A., PEARCE, T. A., FISHER, M. A., KHAKOO, R., DAVIS, S. M., ROGERS, M. E., THEWLIS, R. E., POSADA, J. A., REDROW, J. B., CELIK, I. B., CHEN, B. T. & BEEZHOLD, D. H. 2010. Distribution of airborne influenza virus and respiratory syncytial virus in an urgent care medical clinic. *Clin Infect Dis,* 50**,** 693-8.

LING, Z., TRAN, K. C. & TENG, M. N. 2009. Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting with RIG-I. *J Virol,* 83**,** 3734-42.

LIU, P., JAMALUDDIN, M., LI, K., GAROFALO, R. P., CASOLA, A. & BRASIER, A. R. 2007. Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J Virol,* 81**,** 1401-11.

LOTZ, M. T., MOORE, M. L. & PEEBLES, R. S., JR. 2013. Respiratory syncytial virus and reactive airway disease. *Curr Top Microbiol Immunol,* 372**,** 105-18.

LOZANO, R., NAGHAVI, M., FOREMAN, K., LIM, S., SHIBUYA, K., ABOYANS, V., ABRAHAM, J., ADAIR, T., AGGARWAL, R., AHN, S. Y., ALVARADO, M., ANDERSON, H. R., ANDERSON, L. M., ANDREWS, K. G., ATKINSON, C., BADDOUR, L. M., BARKER-COLLO, S., BARTELS, D. H., BELL, M. L., BENJAMIN, E. J., BENNETT, D., BHALLA, K., BIKBOV, B., BIN ABDULHAK, A., BIRBECK, G., BLYTH, F., BOLLIGER, I., BOUFOUS, S., BUCELLO, C., BURCH, M., BURNEY, P., CARAPETIS, J., CHEN, H., CHOU, D., CHUGH, S. S., COFFENG, L. E., COLAN, S. D., COLQUHOUN, S., COLSON, K. E., CONDON, J., CONNOR, M. D., COOPER, L. T., CORRIERE, M., CORTINOVIS, M., DE VACCARO, K. C., COUSER, W., COWIE, B. C., CRIQUI, M. H., CROSS, M., DABHADKAR, K. C., DAHODWALA, N., DE LEO, D., DEGENHARDT, L., DELOSSANTOS, A., DENENBERG, J., DES JARLAIS, D. C., DHARMARATNE, S. D., DORSEY, E. R., DRISCOLL, T., DUBER, H., EBEL, B., ERWIN, P. J., ESPINDOLA, P., EZZATI, M., FEIGIN, V., FLAXMAN, A. D., FOROUZANFAR, M. H., FOWKES, F. G., FRANKLIN, R., FRANSEN, M., FREEMAN, M. K., GABRIEL, S. E., GAKIDOU, E., GASPARI, F., GILLUM, R. F., GONZALEZ-MEDINA, D., HALASA, Y. A., HARING, D., HARRISON, J. E., HAVMOELLER, R., HAY, R. J., HOEN, B., HOTEZ, P. J., HOY, D., JACOBSEN, K. H., JAMES, S. L., JASRASARIA, R., JAYARAMAN, S., JOHNS, N., KARTHIKEYAN, G., KASSEBAUM, N., KEREN, A., KHOO, J. P., KNOWLTON, L. M., KOBUSINGYE, O., KORANTENG, A., KRISHNAMURTHI, R., LIPNICK, M., LIPSHULTZ, S. E., OHNO, S. L., et al. 2012. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet,* 380**,** 2095-128.

LUKACS, N. W., MOORE, M. L., RUDD, B. D., BERLIN, A. A., COLLINS, R. D., OLSON, S. J., HO, S. B. & PEEBLES, R. S., JR. 2006. Differential immune responses and pulmonary pathophysiology are induced by two different strains of respiratory syncytial virus. *Am J Pathol,* 169**,** 977-86.

LUKACS, N. W., SMIT, J. J., MUKHERJEE, S., MORRIS, S. B., NUNEZ, G. & LINDELL, D. M. 2010. Respiratory virus-induced TLR7 activation controls IL-17-associated increased mucus via IL-23 regulation. *J Immunol,* 185**,** 2231-9.

LYTLE, C. D. & SAGRIPANTI, J. L. 2005. Predicted inactivation of viruses of relevance to biodefense by solar radiation. *J Virol,* 79**,** 14244-52.

MALAVIYA, R. & ABRAHAM, S. N. 2000. Role of mast cell leukotrienes in neutrophil recruitment and bacterial clearance in infectious peritonitis. *J Leukoc Biol,* 67**,** 841-6.

MALMGAARD, L. 2004. Induction and regulation of IFNs during viral infections. *J Interferon Cytokine Res,* 24**,** 439-54.

MARCET, C. W., ST LAURENT, C. D., MOON, T. C., SINGH, N. & BEFUS, A. D. 2013. Limited replication of influenza A virus in human mast cells. *Immunol Res,* 56**,** 32-43.

MASTRANGELO, P. & HEGELE, R. G. 2013. RSV fusion: time for a new model. *Viruses,* 5**,** 873-85.

MATTHEWS, S. P., TREGONING, J. S., COYLE, A. J., HUSSELL, T. & OPENSHAW, P. J. 2005. Role of CCL11 in eosinophilic lung disease during respiratory syncytial virus infection. *J Virol,* 79**,** 2050-7.

MAURER, M. & VON STEBUT, E. 2004. Macrophage inflammatory protein-1. *Int J Biochem Cell Biol,* 36**,** 1882-6.

MCCARTHY, D. O., DOMURAT, F. M., NICHOLS, J. E. & ROBERTS, N. J., JR. 1989. Interleukin-1 inhibitor production by human mononuclear leukocytes and leukocyte subpopulations exposed to respiratory syncytial virus: analysis and comparison with the response to influenza virus. *J Leukoc Biol,* 46**,** 189-98.

MCCURDY, J. D., LIN, T. J. & MARSHALL, J. S. 2001. Toll-like receptor 4-mediated activation of murine mast cells. *J Leukoc Biol,* 70**,** 977-84.

MCCURDY, J. D., OLYNYCH, T. J., MAHER, L. H. & MARSHALL, J. S. 2003. Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. *J Immunol,* 170**,** 1625-9.

MCDERMOTT, D. S., WEISS, K. A., KNUDSON, C. J. & VARGA, S. M. 2011. Central role of dendritic cells in shaping the adaptive immune response during respiratory syncytial virus infection. *Future Virol,* 6**,** 963-973.

MCKIMM-BRESCHKIN, J. L. 2004. A simplified plaque assay for respiratory syncytial virus--direct visualization of plaques without immunostaining. *J Virol Methods,* 120**,** 113-7.

MCLELLAN, J. S., RAY, W. C. & PEEPLES, M. E. 2013. Structure and function of respiratory syncytial virus surface glycoproteins. *Curr Top Microbiol Immunol,* 372**,** 83-104.

MCNAMARA, P. S., RITSON, P., SELBY, A., HART, C. A. & SMYTH, R. L. 2003. Bronchoalveolar lavage cellularity in infants with severe respiratory syncytial virus bronchiolitis. *Arch Dis Child,* 88**,** 922-6.

MICHEL, A., SCHULER, A., FRIEDRICH, P., DONER, F., BOPP, T., RADSAK, M., HOFFMANN, M., RELLE, M., DISTLER, U., KUHAREV, J., TENZER, S., FEYERABEND, T. B., RODEWALD, H. R., SCHILD, H., SCHMITT, E., BECKER, M. & STASSEN, M. 2013. Mast cell-deficient Kit(W-sh) "Sash" mutant mice display aberrant myelopoiesis leading to the accumulation of splenocytes that act as myeloid-derived suppressor cells. *J Immunol,* 190**,** 5534-44.

MITRA, R., BAVISKAR, P., DUNCAN-DECOCQ, R. R., PATEL, D. & OOMENS, A. G. 2012. The human respiratory syncytial virus matrix protein is required for maturation of viral filaments. *J Virol,* 86**,** 4432-43.

MOLLET, S., COUGOT, N., WILCZYNSKA, A., DAUTRY, F., KRESS, M., BERTRAND, E. & WEIL, D. 2008. Translationally repressed mRNA transiently cycles through stress granules during stress. *Mol Biol Cell,* 19**,** 4469-79.

MOON, T. C., ST LAURENT, C. D., MORRIS, K. E., MARCET, C., YOSHIMURA, T., SEKAR, Y. & BEFUS, A. D. 2010. Advances in mast cell biology: new understanding of heterogeneity and function. *Mucosal Immunol,* 3**,** 111-28.

MOORE, M. L. & PEEBLES, R. S., JR. 2006. Respiratory syncytial virus disease mechanisms implicated by human, animal model, and in vitro data facilitate vaccine strategies and new therapeutics. *Pharmacol Ther,* 112**,** 405-24.

MORRISON, T. G. & WALSH, E. E. 2013. Subunit and virus-like particle vaccine approaches for respiratory syncytial virus. *Curr Top Microbiol Immunol,* 372**,** 285-306.

MORTIER, A., VAN DAMME, J. & PROOST, P. 2012. Overview of the mechanisms regulating chemokine activity and availability. *Immunol Lett,* 145**,** 2-9.

MUFSON, M. A., ORVELL, C., RAFNAR, B. & NORRBY, E. 1985. Two distinct subtypes of human respiratory syncytial virus. *J Gen Virol,* 66 (Pt 10)**,** 2111-24.

MULLER, M., CARTER, S., HOFER, M. J. & CAMPBELL, I. L. 2010. Review: The chemokine receptor CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 in neuroimmunity--a tale of conflict and conundrum. *Neuropathol Appl Neurobiol,* 36**,** 368-87.

MULLINS, J. A., LAMONTE, A. C., BRESEE, J. S. & ANDERSON, L. J. 2003. Substantial variability in community respiratory syncytial virus season timing. *Pediatr Infect Dis J,* 22**,** 857-62.

MURAWSKI, M. R., BOWEN, G. N., CERNY, A. M., ANDERSON, L. J., HAYNES, L. M., TRIPP, R. A., KURT-JONES, E. A. & FINBERG, R. W. 2009. Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. *J Virol,* 83**,** 1492-500.

NAIR, H., NOKES, D. J., GESSNER, B. D., DHERANI, M., MADHI, S. A., SINGLETON, R. J., O'BRIEN, K. L., ROCA, A., WRIGHT, P. F., BRUCE, N., CHANDRAN, A., THEODORATOU, E., SUTANTO, A., SEDYANINGSIH, E. R., NGAMA, M., MUNYWOKI, P. K., KARTASASMITA, C., SIMOES, E. A., RUDAN, I., WEBER, M. W. & CAMPBELL, H. 2010. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. *Lancet,* 375**,** 1545-55.

NOPPERT, S. J., FITZGERALD, K. A. & HERTZOG, P. J. 2007. The role of type I interferons in TLR responses. *Immunol Cell Biol,* 85**,** 446-57.

O'SHEA, M. K., RYAN, M. A., HAWKSWORTH, A. W., ALSIP, B. J. & GRAY, G. C. 2005. Symptomatic respiratory syncytial virus infection in previously healthy young adults living in a crowded military environment. *Clin Infect Dis,* 41**,** 311-7.

OKABAYASHI, T., KOJIMA, T., MASAKI, T., YOKOTA, S., IMAIZUMI, T., TSUTSUMI, H., HIMI, T., FUJII, N. & SAWADA, N. 2011. Type-III interferon, not type-I, is the predominant interferon induced by respiratory viruses in nasal epithelial cells. *Virus Res,* 160**,** 360-6.

OKAYAMA, Y. & KAWAKAMI, T. 2006. Development, migration, and survival of mast cells. *Immunol Res,* 34**,** 97-115.

OLYNYCH, T. J., JAKEMAN, D. L. & MARSHALL, J. S. 2006. Fungal zymosan induces leukotriene production by human mast cells through a dectin-1-dependent mechanism. *J Allergy Clin Immunol,* 118**,** 837-43.

OMER, S. B., SUTANTO, A., SARWO, H., LINEHAN, M., DJELANTIK, I. G., MERCER, D., MONIAGA, V., MOULTON, L. H., WIDJAYA, A., MULJATI, P., GESSNER, B. D. & STEINHOFF, M. C. 2008. Climatic, temporal, and geographic characteristics of respiratory syncytial virus disease in a tropical island population. *Epidemiol Infect,* 136**,** 1319-27.

OPENSHAW, P. J. 2013. The mouse model of respiratory syncytial virus disease. *Curr Top Microbiol Immunol,* 372**,** 359-69.

ORINSKA, Z., BULANOVA, E., BUDAGIAN, V., METZ, M., MAURER, M. & BULFONE-PAUS, S. 2005. TLR3-induced activation of mast cells modulates CD8+ T-cell recruitment. *Blood,* 106**,** 978-87.

OYMAR, K., HALVORSEN, T. & AKSNES, L. 2006. Mast cell activation and leukotriene secretion in wheezing infants. Relation to respiratory syncytial virus and outcome. *Pediatr Allergy Immunol,* 17**,** 37-42.

PADOVAN, E., SPAGNOLI, G. C., FERRANTINI, M. & HEBERER, M. 2002. IFN-alpha2a induces IP-10/CXCL10 and MIG/CXCL9 production in monocyte-derived dendritic cells and enhances their capacity to attract and stimulate CD8+ effector T cells. *J Leukoc Biol,* 71**,** 669-76.

PANG, L., NIE, M., CORBETT, L., SUTCLIFFE, A. & KNOX, A. J. 2006. Mast cell beta-tryptase selectively cleaves eotaxin and RANTES and abrogates their eosinophil chemotactic activities. *J Immunol,* 176**,** 3788-95.

PANUSKA, J. R., CIRINO, N. M., MIDULLA, F., DESPOT, J. E., MCFADDEN, E. R., JR. & HUANG, Y. T. 1990. Productive infection of isolated human alveolar macrophages by respiratory syncytial virus. *J Clin Invest,* 86**,** 113-9.

PANUSKA, J. R., MEROLLA, R., REBERT, N. A., HOFFMANN, S. P., TSIVITSE, P., CIRINO, N. M., SILVERMAN, R. H. & RANKIN, J. A. 1995. Respiratory syncytial virus induces interleukin-10 by human alveolar macrophages. Suppression of early cytokine production and implications for incomplete immunity. *J Clin Invest,* 96**,** 2445-53.

PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res,* 29**,** e45.

PICKLES, R. J. 2013. Human airway epithelial cell cultures for modeling respiratory syncytial virus infection. *Curr Top Microbiol Immunol,* 372**,** 371-87.

PLANTINGA, M., HAMMAD, H. & LAMBRECHT, B. N. 2010. Origin and functional specializations of DC subsets in the lung. *Eur J Immunol,* 40**,** 2112-8.

POLACK, F. P., IRUSTA, P. M., HOFFMAN, S. J., SCHIATTI, M. P., MELENDI, G. A., DELGADO, M. F., LAHAM, F. R., THUMAR, B., HENDRY, R. M., MELERO, J. A., KARRON, R. A., COLLINS, P. L. & KLEEBERGER, S. R. 2005. The cysteine-rich region of respiratory syncytial virus attachment protein inhibits innate immunity elicited by the virus and endotoxin. *Proc Natl Acad Sci U S A,* 102**,** 8996-9001.

RADINGER, M., JENSEN, B. M., KUEHN, H. S., KIRSHENBAUM, A. & GILFILLAN, A. M. 2010. Generation, isolation, and maintenance of human mast cells and mast cell lines derived from peripheral blood or cord blood. *Curr Protoc Immunol,* Chapter 7**,** Unit 7 37.

REN, J., LIU, T., PANG, L., LI, K., GAROFALO, R. P., CASOLA, A. & BAO, X. 2011. A novel mechanism for the inhibition of interferon regulatory factor-3-dependent gene expression by human respiratory syncytial virus NS1 protein. *J Gen Virol,* 92**,** 2153-9.

RIVERA, J. 2006. Adaptors discriminate mast-cell cytokine production from eicosanoid production and degranulation. *Trends Immunol,* 27**,** 251-3.

ROE, M. F., BLOXHAM, D. M., COWBURN, A. S. & O'DONNELL, D. R. 2011. Changes in helper lymphocyte chemokine receptor expression and elevation of IP-10 during acute respiratory syncytial virus infection in infants. *Pediatr Allergy Immunol,* 22**,** 229-34.

RUDD, B. D., BURSTEIN, E., DUCKETT, C. S., LI, X. & LUKACS, N. W. 2005. Differential role for TLR3 in respiratory syncytial virus-induced chemokine expression. *J Virol,* 79**,** 3350-7.

RUDD, B. D., SMIT, J. J., FLAVELL, R. A., ALEXOPOULOU, L., SCHALLER, M. A., GRUBER, A., BERLIN, A. A. & LUKACS, N. W. 2006. Deletion of TLR3 alters the pulmonary immune environment and mucus production during respiratory syncytial virus infection. *J Immunol,* 176**,** 1937-42.

SABBAH, A., CHANG, T. H., HARNACK, R., FROHLICH, V., TOMINAGA, K., DUBE, P. H., XIANG, Y. & BOSE, S. 2009. Activation of innate immune antiviral responses by Nod2. *Nat Immunol,* 10**,** 1073-80.

SADLER, A. J. & WILLIAMS, B. R. 2008. Interferon-inducible antiviral effectors. *Nat Rev Immunol,* 8**,** 559-68.

SAITO, H., EBISAWA, M., TACHIMOTO, H., SHICHIJO, M., FUKAGAWA, K., MATSUMOTO, K., IIKURA, Y., AWAJI, T., TSUJIMOTO, G., YANAGIDA, M., UZUMAKI, H., TAKAHASHI, G., TSUJI, K. & NAKAHATA, T. 1996. Selective growth of human mast cells induced by Steel factor, IL-6, and prostaglandin E2 from cord blood mononuclear cells. *J Immunol,* 157**,** 343-50.

SALAZAR-MATHER, T. P., LEWIS, C. A. & BIRON, C. A. 2002. Type I interferons regulate inflammatory cell trafficking and macrophage inflammatory protein 1alpha delivery to the liver. *J Clin Invest,* 110**,** 321-30.

SANDIG, H. & BULFONE-PAUS, S. 2012. TLR signaling in mast cells: common and unique features. *Front Immunol,* 3**,** 185.

SCAGNOLARI, C., MIDULLA, F., PIERANGELI, A., MORETTI, C., BONCI, E., BERARDI, R., DE ANGELIS, D., SELVAGGI, C., DI MARCO, P., GIRARDI, E. & ANTONELLI, G. 2009. Gene expression of nucleic acid-sensing pattern recognition receptors in children hospitalized for respiratory syncytial virus-associated acute bronchiolitis. *Clin Vaccine Immunol,* 16**,** 816-23.

SEMPLE, M. G., DANKERT, H. M., EBRAHIMI, B., CORREIA, J. B., BOOTH, J. A., STEWART, J. P., SMYTH, R. L. & HART, C. A. 2007. Severe respiratory syncytial virus bronchiolitis in infants is associated with reduced airway interferon gamma and substance P. *PLoS One,* 2**,** e1038.

SEN, G. C. & SARKAR, S. N. 2007. The interferon-stimulated genes: targets of direct signaling by interferons, double-stranded RNA, and viruses. *Curr Top Microbiol Immunol,* 316**,** 233-50.

SHAIKH, F. Y. & CROWE, J. E., JR. 2013. Molecular mechanisms driving respiratory syncytial virus assembly. *Future Microbiol,* 8**,** 123-31.

SHARMA, M. 2010. Chemokines and their receptors: orchestrating a fine balance between health and disease. *Crit Rev Biotechnol,* 30**,** 1-22.

SHEA-DONOHUE, T., STILTZ, J., ZHAO, A. & NOTARI, L. 2010. Mast cells. *Curr Gastroenterol Rep,* 12**,** 349-57.

SHIRATO, K. & TAGUCHI, F. 2009. Mast cell degranulation is induced by A549 airway epithelial cell infected with respiratory syncytial virus. *Virology,* 386**,** 88-93.

SIGURS, N., ALJASSIM, F., KJELLMAN, B., ROBINSON, P. D., SIGURBERGSSON, F., BJARNASON, R. & GUSTAFSSON, P. M. 2010. Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. *Thorax,* 65**,** 1045-52.

SOMMER, C., RESCH, B. & SIMOES, E. A. 2011. Risk factors for severe respiratory syncytial virus lower respiratory tract infection. *Open Microbiol J,* 5**,** 144-54.

SPANN, K. M., TRAN, K. C. & COLLINS, P. L. 2005. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. *J Virol,* 79**,** 5353-62.

SPORER, B., KOEDEL, U., PAUL, R., EBERLE, J., ARENDT, G. & PFISTER, H. W. 2004. Vascular endothelial growth factor (VEGF) is increased in serum, but not in cerebrospinal fluid in HIV associated CNS diseases. *J Neurol Neurosurg Psychiatry,* 75**,** 298-300.

ST JOHN, A. L. & ABRAHAM, S. N. 2013. Innate immunity and its regulation by mast cells. *J Immunol,* 190**,** 4458-63.

ST JOHN, A. L., RATHORE, A. P., YAP, H., NG, M. L., METCALFE, D. D., VASUDEVAN, S. G. & ABRAHAM, S. N. 2011. Immune surveillance by mast cells during dengue infection promotes natural killer (NK) and NKT-cell recruitment and viral clearance. *Proc Natl Acad Sci U S A*.

STEIN, R. T., SHERRILL, D., MORGAN, W. J., HOLBERG, C. J., HALONEN, M., TAUSSIG, L. M., WRIGHT, A. L. & MARTINEZ, F. D. 1999. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet,* 354**,** 541-5.

STOKES, K. L., CHI, M. H., SAKAMOTO, K., NEWCOMB, D. C., CURRIER, M. G., HUCKABEE, M. M., LEE, S., GOLENIEWSKA, K., PRETTO, C., WILLIAMS, J. V., HOTARD, A., SHERRILL, T. P., PEEBLES, R. S., JR. & MOORE, M. L. 2011. Differential pathogenesis of respiratory syncytial virus clinical isolates in BALB/c mice. *J Virol,* 85**,** 5782-93.

STROBER, W. 2001. Trypan blue exclusion test of cell viability. *Curr Protoc Immunol,* Appendix 3**,** Appendix 3B.

SUNDSTROM, J. B., ELLIS, J. E., HAIR, G. A., KIRSHENBAUM, A. S., METCALFE, D. D., YI, H., CARDONA, A. C., LINDSAY, M. K. & ANSARI, A. A. 2007. Human tissue mast cells are an inducible reservoir of persistent HIV infection. *Blood,* 109**,** 5293-300.

SWEDAN, S., ANDREWS, J., MAJUMDAR, T., MUSIYENKO, A. & BARIK, S. 2011. Multiple functional domains and complexes of the two nonstructural proteins of human respiratory syncytial virus contribute to interferon suppression and cellular location. *J Virol,* 85**,** 10090-100.

SWEDAN, S., MUSIYENKO, A. & BARIK, S. 2009. Respiratory syncytial virus nonstructural proteins decrease levels of multiple members of the cellular interferon pathways. *J Virol,* 83**,** 9682-93.

TAL, G., MANDELBERG, A., DALAL, I., CESAR, K., SOMEKH, E., TAL, A., ORON, A., ITSKOVICH, S., BALLIN, A., HOURI, S., BEIGELMAN, A., LIDER, O., RECHAVI, G. & AMARIGLIO, N. 2004. Association between common Toll-like receptor 4 mutations and severe respiratory syncytial virus disease. *J Infect Dis,* 189**,** 2057-63.

TAYYARI, F., MARCHANT, D., MORAES, T. J., DUAN, W., MASTRANGELO, P. & HEGELE, R. G. 2011. Identification of nucleolin as a cellular receptor for human respiratory syncytial virus. *Nat Med,* 17**,** 1132-5.

TENG, M. N., WHITEHEAD, S. S., BERMINGHAM, A., ST CLAIRE, M., ELKINS, W. R., MURPHY, B. R. & COLLINS, P. L. 2000. Recombinant respiratory syncytial virus that does not express the NS1 or M2-2 protein is highly attenuated and immunogenic in chimpanzees. *J Virol,* 74**,** 9317-21.

TERAN, L. M. 2000. CCL chemokines and asthma. *Immunol Today,* 21**,** 235-42.

THEOHARIDES, T. C., KEMPURAJ, D., TAGEN, M., CONTI, P. & KALOGEROMITROS, D. 2007. Differential release of mast cell mediators and the pathogenesis of inflammation. *Immunol Rev,* 217**,** 65-78.

THOMAS, L. H., WICKREMASINGHE, M. I., SHARLAND, M. & FRIEDLAND, J. S. 2000. Synergistic upregulation of interleukin-8 secretion from pulmonary epithelial cells by direct and monocyte-dependent effects of respiratory syncytial virus infection. *J Virol,* 74**,** 8425-33.

TREGONING, J. S. & SCHWARZE, J. 2010. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. *Clin Microbiol Rev,* 23**,** 74-98.

TRIPP, R. A., JONES, L. P., HAYNES, L. M., ZHENG, H., MURPHY, P. M. & ANDERSON, L. J. 2001. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. *Nat Immunol,* 2**,** 732-8.

URB, M. & SHEPPARD, D. C. 2012. The role of mast cells in the defence against pathogens. *PLoS Pathog,* 8**,** e1002619.

VARGA, S. M. & BRACIALE, T. J. 2013. The adaptive immune response to respiratory syncytial virus. *Curr Top Microbiol Immunol,* 372**,** 155-71.

VIGNALI, D. A. & KUCHROO, V. K. 2012. IL-12 family cytokines: immunological playmakers. *Nat Immunol,* 13**,** 722-8.

VILLENAVE, R., THAVAGNANAM, S., SARLANG, S., PARKER, J., DOUGLAS, I., SKIBINSKI, G., HEANEY, L. G., MCKAIGUE, J. P., COYLE, P. V., SHIELDS, M. D. & POWER, U. F. 2012. In vitro modeling of respiratory syncytial virus infection of pediatric bronchial epithelium, the primary target of infection in vivo. *Proc Natl Acad Sci U S A,* 109**,** 5040-5.

VISSERS, M., REMIJN, T., OOSTING, M., DE JONG, D. J., DIAVATOPOULOS, D. A., HERMANS, P. W. & FERWERDA, G. 2012. Respiratory syncytial virus infection augments NOD2 signaling in an IFN-beta-dependent manner in human primary cells. *Eur J Immunol,* 42**,** 2727-35.

VOLLAND, H., VULLIEZ LE NORMAND, B., MAMAS, S., GRASSI, J., CREMINON, C., EZAN, E. & PRADELLES, P. 1994. Enzyme immunometric assay for leukotriene C4. *J Immunol Methods,* 175**,** 97-105.

WALSH, E. E. & FALSEY, A. R. 2004. Humoral and mucosal immunity in protection from natural respiratory syncytial virus infection in adults. *J Infect Dis,* 190**,** 373-8.

WANG, Z., LAI, Y., BERNARD, J. J., MACLEOD, D. T., COGEN, A. L., MOSS, B. & DI NARDO, A. 2012. Skin mast cells protect mice against vaccinia virus by triggering mast cell receptor S1PR2 and releasing antimicrobial peptides. *J Immunol,* 188**,** 345-57.

WELLIVER, T. P., GAROFALO, R. P., HOSAKOTE, Y., HINTZ, K. H., AVENDANO, L., SANCHEZ, K., VELOZO, L., JAFRI, H., CHAVEZ-BUENO, S., OGRA, P. L., MCKINNEY, L., REED, J. L. & WELLIVER, R. C. S. 2007. Severe Human Lower Respiratory Tract Illness Caused by Respiratory Syncytial Virus and Influenza Virus Is Characterized by the Absence of Pulmonary Cytotoxic Lymphocyte Responses. *The Journal of Infectious Diseases* 195**,** 1126-1136.

WHITEHEAD, S. S., BUKREYEV, A., TENG, M. N., FIRESTONE, C. Y., ST CLAIRE, M., ELKINS, W. R., COLLINS, P. L. & MURPHY, B. R. 1999. Recombinant respiratory syncytial virus bearing a deletion of either the NS2 or SH gene is attenuated in chimpanzees. *J Virol,* 73**,** 3438-42.

WRIGHT, P. F., IKIZLER, M. R., GONZALES, R. A., CARROLL, K. N., JOHNSON, J. E. & WERKHAVEN, J. A. 2005. Growth of respiratory syncytial virus in primary epithelial cells from the human respiratory tract. *J Virol,* 79**,** 8651-4.

XU, W., JOO, H., CLAYTON, S., DULLAERS, M., HERVE, M. C., BLANKENSHIP, D., DE LA MORENA, M. T., BALDERAS, R., PICARD, C., CASANOVA, J. L., PASCUAL, V., OH, S. & BANCHEREAU, J. 2012. Macrophages induce differentiation of plasma cells through CXCL10/IP-10. *J Exp Med,* 209**,** 1813-23, S1-2.

YOON, J. S., KIM, H. H., LEE, Y. & LEE, J. S. 2007. Cytokine induction by respiratory syncytial virus and adenovirus in bronchial epithelial cells. *Pediatr Pulmonol,* 42**,** 277-82.

YUAN, J., LIU, Z., LIM, T., ZHANG, H., HE, J., WALKER, E., SHIER, C., WANG, Y., SU, Y., SALL, A., MCMANUS, B. & YANG, D. 2009. CXCL10 inhibits viral replication through recruitment of natural killer cells in coxsackievirus B3-induced myocarditis. *Circ Res,* 104**,** 628-38.

ZENG, R., CUI, Y., HAI, Y. & LIU, Y. 2012. Pattern recognition receptors for respiratory syncytial virus infection and design of vaccines. *Virus Res,* 167**,** 138-45.

ZENG, R., LI, C., LI, N., WEI, L. & CUI, Y. 2011. The role of cytokines and chemokines in severe respiratory syncytial virus infection and subsequent asthma. *Cytokine,* 53**,** 1-7.

ZHANG, L., MENDOZA-SASSI, R. A., WAINWRIGHT, C. & KLASSEN, T. P. 2008. Nebulized hypertonic saline solution for acute bronchiolitis in infants. *Cochrane Database Syst Rev***,** CD006458.

ZHANG, L., PEEPLES, M. E., BOUCHER, R. C., COLLINS, P. L. & PICKLES, R. J. 2002. Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. *J Virol,* 76**,** 5654-66.

ZHANG, W., YANG, H., KONG, X., MOHAPATRA, S., SAN JUAN-VERGARA, H., HELLERMANN, G., BEHERA, S., SINGAM, R., LOCKEY, R. F. & MOHAPATRA, S. S. 2005. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. *Nat Med,* 11**,** 56-62.