# HUMAN MAST CELL RESPONSES TO RESPIRATORY SYNCYTIAL VIRUS

by

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DEDICATION PAGE

I dedicate my master's thesis to the memory of my favorite pal, Shamil Hamid. He lived a life filled with contagious laughter and joy and enriched the lives of everyone around him. Your sincerity, kindness and sense of humor will never be forgotten. I love and miss you!

Shamil Hamid (April 8th 1988 – December 1st 2010)

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# Abstract

Mast cells reside at tissue sites closely interfacing the environment and play a role in host defense against pathogens. Mast cell responses to respiratory syncytial virus (RSV), a major cause of severe respiratory tract infections and subsequent bronchiolitis, are not fully elucidated. Human cord blood-derived mast cells (CBMCs) and the HMC-1 mast cell line supported low levels of RSV antigen expression as compared with airway epithelial cells. RSV inoculated mast cells up-regulated the expression of several chemokines such as CCL4, CCL5 and CXCL10, as well as type I and III interferons. Type I interferon receptor blockade on RSV-inoculated HMC-1 cells had no effect on chemokine production or viral antigen expression. These data show that mast cells respond to RSV by expressing various cytokines and chemokines that 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 | alkaline phosphatase |
| ATCC | American Type Culture Collection |
| ATP | adenosine triphosphate |
| BAL | bronchoalveolar lavage |
| BCIP/NBT | nitro-blue tetrazolium chloride/5-bromo-4-chloro-indolyphosphate p-toluidine |
| 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 |
| 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 initiaion 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 |
| HUVEC | human umbilical vein endothelial cell |
| IFN | interferon |
| IFNAR | interferon alpha recentor |
| Ig | immunoglobulin |
| IL- | interleukin |
| 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 |
| NK cell | natural killer cell |
| NKT cell | natural killer T cell |
| NF-κB | nuclear factor-κB |
| NS1/2 | non-structural protein 1/2 |
| 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 |
| RD domain | receptor domain |
| 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 |
| SeV | Sendai virus |
| SH protein | small hydrophobic protein |
| shRNA | short hairpin RNA |
| siRNA | short interfering RNA |
| ssRNA | single-stranded RNA |
| STAT | signal transducer and activator of transcription |
| 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 |
| TRIF | TIR domain-containing adaptor-inducing interferon-β |
| TRP | tricopeptide |
| URTI | upper respiratory tract infection |

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# Chapter 1 – Introduction

## 1.1 Thesis Scope

Viral diseases remain a major cause of morbidity and mortality worldwide. Respiratory syncytial virus (RSV) is an important global pathogen and the main causative agent of severe lower respiratory tract infections (LRTIs) and hospitalizations in children and infants. Our understanding of certain aspects of the immune response to viruses such as RSV has greatly expanded over the last decades. Nevertheless, several questions regarding the early cellular responses to viral infection at the mucosal surfaces remain unanswered. Innate immune cells at mucosal surfaces, such as mast cells, play a sentinel role in host defense against viral infections. Mast cells are of special interest as potential effector cells in the development of severe airways disease due to their ability to contribute to the innate immune response and recruit immune cells. However, the role for, and responses of, mast cells in viral disease remains generally undefined. The content of this thesis examines the inflammatory innate responses of mast cells to RSV in an *in vitro* setting.

## 1.2 Respiratory Syncytial Virus

Blount *et al.* isolated a viral agent from nasal secretions of young chimpanzees with sneezing and rhinorrhea. The virus was named chimpanzee coryza agent (CAA), as it was able to induce the same symptoms upon inoculation in other chimpanzees (Blount *et al.*, 1956). Only a year later Chanock *et al.* isolated a CAA-like virus from infants presenting with pneumonia and bronchiolitis. Chanock *et al.* also observed that when cultured *in vitro*, the virus characteristically produced large, multinucleated cells commonly referred to as syncytia (Chanock *et al.*, 1957; Chanock, 1957). As a consequence of these observations, the human CAA-like virus was named respiratory syncytial virus (RSV). Since its discovery and isolation, RSV has been the focus of much research over the past decades and has been identified globally as the most common respiratory pathogen in infants and young children (Wright and Piedimonte, 2010).

### 1.3 RSV Epidemiology and Disease

RSV accounts for up to 60% of acute LRTIs in children and 80% of LRTIs in infants, making it the leading cause of bronchiolitis and pneumonia in children worldwide (Shay *et al.*, 1999). RSV is also the leading cause of hospitalization of infants in the first year of life. It is reported that 70% of children are infected with RSV by the age of one, and almost all children are affected by the virus by the age of two (Rogovik *et al.*, 2010). A recent analysis reported that 33.8 million cases of RSV were recorded in 2005, resulting in over 3.3 million hospitalizations and 120,000 deaths worldwide (Nair *et al.*, 2010).

There are two distinct subtypes of RSV, RSV A and RSV B. Both subtypes are known to cause disease but exhibit only approximately 25% antigenic relatedness owing to large variability in the G protein (Wright and Piedimonte, 2010). RSV infection in almost all children leads to clinical manifestations, the severity of which greatly varies depending on age, environmental exposure, history of infection and other co-morbidities (Anderson *et al.*, 2010). Infants typically present with mild fever and upper respiratory tract infection (URTI) symptoms, such as rhinorrhea and congestion, which can last for up to 14 days. In some cases, these symptoms can quickly progress to more severe LRTI symptoms which include coughing, wheezing, breathing difficulties, cyanosis and more life-threatening forms of pneumonia (Checchia, 2008).

RSV results in higher morbidity and mortality for premature-born infants, immunocompromised children and children with underlying congenital heart disease (high-risk groups for severe RSV disease). RSV is also emerging as a significant cause of severe LRTIs in the elderly populations and immunocompromised individuals due to lack of immunological memory following infection and the absence of a protective vaccine (Anderson *et al.*, 2010; Wright and Piedimonte, 2010).

### 1.4 RSV Morphology and Replication

RSV is a pneumovirus belonging to the *Paramyxovirus* family, with a non-segmented, negative-sense, single-stranded RNA genome (Cowton *et al.*, 2006; Wright and Piedimonte, 2010). The RSV genome is incorporated into helical nucleocapsids, which are surrounded by a matrix (M) protein and an envelope studded with viral

glycoproteins. During infection of a host cell, the viral genome is delivered to the cytoplasm by direct fusion of the virus membrane with the host cell membrane. The genome is transcribed in the cytoplasm by the viral polymerase to 11 sub-genomic messenger RNAs (mRNAs). The mRNAs are translated by the host ribosome to 10 different viral proteins (Cowton *et al.*, 2006). Genome replication involves the generation of an intermediate positive-sense antigenome template, which is replicated to the negative-sense genome by the viral polymerase and simultaneously incorporated into nucleocapsids for assembly into mature virions (Cowton *et al.*, 2006; Fields *et al.*, 2007).

## 1.5 RSV Proteins

### 1.5.1 The L, P and N Proteins

The RSV nucleocapsid is a complex of the large (L) protein, phosphoprotein (P), nucleoprotein (N) and viral RNA (Hacking and Hull, 2002). The 391 amino acid N protein remains associated with the viral RNA genome, forming the nucleoprotein complex. This interaction protects the viral RNA from degradation and prevents the formation of secondary structures. RNA transcription and genome replication is initiated by the polymerase complex, which consists of the large (L) protein and phosphoprotein (P). The L protein consists of 2165 amino acids and contains catalytic polymerase and nucleotide-binding motifs that are essential for the production and capping of viral mRNA, respectively (Fields *et al.*, 2007). The P protein (241 amino acids) is thought to be an essential cofactor for mRNA synthesis, and is capable of directly interacting with the N protein (Cowton *et al.*, 2006). Studies have suggested that the phosphorylated form of the P protein is required for stabilizing the viral polymerase complex during RSV infection, which is essential for the transcription of viral mRNA (Dupuy *et al.*, 1999).

### 1.5.2 The M2-1 and M2-2 Proteins

The M2-1 (194 amino acids) and M2-2 (90 amino acids) proteins are processivity factors that are essential in regulating the activity of the RSV polymerase complex between gene transcription and genome replication. The M2-1 protein is a transcription factor that is essential for viral viability (Fields *et al.*, 2007). It functions to promote the

transcription of viral mRNA. Studies have demonstrated that in its absence, the RSV polymerase terminates the transcription of viral genes prematurely and non-specifically (Collins *et al.*, 1996). Conversely, the M2-2 protein is thought to drive genome replication, as the absence of this protein favors the accumulation of greater amounts of viral mRNA rather than genome and antigenome (Bermingham and Collins, 1999). It is also noteworthy to mention that RSV lacking the M2-2 protein grows more slowly *in vitro* as compared to wild type strains of the virus (Bermingham and Collins, 1999; Cartee and Wertz, 2001; Fearns and Collins, 1999).

### 1.5.3 The M Protein

The matrix (M) protein of RSV is a non-glycosylated viral protein that consists of 256 amino acids. It has been shown to attach to the cytoplasmic leaflet of the host cell membrane (Marty *et al.*, 2004). The M protein is known to interact with other viral proteins during infection, such as the N protein, making it essential for coordinated virion assembly and budding from host cells (Ghildyal *et al.*, 2002). Intriguingly, it was demonstrated that the M protein localizes to the nucleus during RSV infection. This is thought to reduce the transcriptional activity within the nuclei of infected host cells which could help dampen the antiviral immune response (Ghildyal *et al.*, 2003).

### 1.5.4 The SH Protein

The 64 amino acid small hydrophobic (SH) protein of RSV is a type II transmembrane protein located primarily within the viral membrane (Gan *et al.*, 2008). Nevertheless, multiple forms of the SH protein can be detected intracellularly (Fields *et al.*, 2007). Notably, mutant RSV lacking the SH protein (ΔSH) grows as well as wild type virus and induces the formation of syncytia (Bukreyev *et al.*, 1997; Karron *et al.*, 1997). Very little is known about the function of the SH protein (Hacking and Hull, 2002). However, studies have suggested a potential role for the SH protein in viral fusion or in modifying membrane permeability (Heminway *et al.*, 1994; Perez *et al.*, 1997). It was recently shown that the SH protein of RSV is involved in preventing cell apoptosis by interfering with tumor necrosis factor (TNF) signaling (Fuentes *et al.*, 2007).

### 1.5.5 The F Protein

The fusion (F) glycoprotein is a highly conserved protein of 67 kDa. It is essential for virus replication and the generation of infectious progeny particles (Teng and Collins, 1998). The F protein mediates the fusion of viral and host membranes and the formation of syncytia later during infection (Hacking and Hull, 2002; Harris and Werling, 2003). The F protein is synthesized as a precursor $F_0$ protein. Proteolytic cleavage of the $F_0$ precursor generates two disulphide-linked polypeptides, $F_1$ and $F_2$. Fusion is initiated when the N terminus of $F_1$ polypeptide inserts into the target cell membrane. Located adjacent to the $F_1$ and $F_2$ proteins are two heptad repeat sequences that are essential for orienting opposing host and viral membranes for fusion (Collins and Mottet, 1991; Lambert *et al.*, 1996). It is noteworthy that the F protein is required, but not sufficient, for syncytia formation. It has been shown that the co-expression of F, G and SH glycoproteins is required for optimal syncytia formation (Heminway *et al.*, 1994). Within the host cell, the F protein also interacts with RhoA-GTPase, which results in actin mobilization, syncytia formation and signal transduction through the JNK and p38 mitogen-activated protein kinases (MAPKs) (Harris and Werling, 2003; Pastey *et al.*, 1999).

### 1.5.6 The G Protein

The G glycoprotein of RSV is a type II transmembrane protein, of 289 to 299 amino acids depending on the virus strain, with an N-terminal cytoplasmic domain and a C-terminal ectodomain (Hacking and Hull, 2002). The G protein is important in virus binding to host cells. During RSV infection, cells also secrete a soluble truncated form of the G protein (sG), which is hypothesized to divert the immune response away from infected cells (Cane, 2001). RSV mutant viruses lacking functional membrane-bound and soluble G protein forms have severely abrogated infectivity in respiratory epithelial cells *in vitro* and *in vivo* mouse models (Karron *et al.*, 1997; Teng *et al.*, 2001). The receptor for the G protein has not been identified, although cell membrane bound glycosaminoglycans (GAGs) such as heparan sulphate and chondroitin sulphate are

thought to play a role in binding (Harris and Werling, 2003). The G protein ectodomain is highly variable between different strains and is heavily glycosylated, which is thought to be important in masking protein residues from recognition by the immune system (Hacking and Hull, 2002).

### 1.5.7 The NS1 and NS2 Proteins

The NS1 and NS2 genes are the first to be transcribed during infection due to their proximity to the 3' end of the genome. Consequently, NS1 and NS2 are the most highly expressed viral proteins (Cowton *et al.*, 2006; Fields *et al.*, 2007). The NS1 protein consists of 139 amino acids and the NS2 protein consists of 124 amino acids (Klein Klouwenberg *et al.*, 2009). Recombinant RSV (rRSV) strains lacking NS1 (ΔNS1), NS2 (ΔNS2) or both (ΔNS1/NS2) exhibit severely attenuated replication *in vitro* and *in vivo* suggesting their necessity for replication efficiency (Jin *et al.*, 2000). NS1 and NS2 function to dampen the innate antiviral response, suppressing cell apoptosis, favoring virus growth and replication (Bitko *et al.*, 2007). Specific functions of the NS1 and NS2 proteins in inhibiting host antiviral responses are further discussed in section 1.11 (Cytokine and Chemokine Production during RSV Disease) of this chapter.

## 1.6 RSV Link to Allergy and Asthma

Numerous studies have highlighted a correlation between severe RSV disease and subsequent development of asthma and airways inflammation (Becker, 2006). Pioneering work by Sigurs *et al.* showed that 30% of a group of children hospitalized for RSV disease developed asthma by the age of 7.5 years, as compared with only 3% of a control group (Sigurs *et al.*, 2000). Although such studies show a strong correlation between severe RSV disease and the development of asthma, they do not implicate RSV as a causative agent for asthma. More recently, an epidemiological study showed that the greatest risk factor for the development of asthma following severe RSV or rhinovirus (RV) infection was a history of atopy in the first two years of life (Kusel *et al.*, 2007). This study indicates that a combination of immunological predisposition to allergy/asthma and viral pathogenesis is important for the development of asthma

following severe viral infection. To this extent, much work in this field has investigated genetic polymorphisms in genes including Toll-like receptor (TLR4), interleukin (IL)-4 and IL-13 that are associated with severe RSV disease and can contribute to the development of asthma and allergy later in life (Miyairi and DeVincenzo, 2008).

### 1.7 RSV Disease Treatment

Most children with RSV infections develop mild, self-limiting symptoms that do not require hospitalization. For children with more severe symptoms, supportive care remains the most preferred treatment option in a hospital setting and involves supplemental oxygen administration, hydration and monitoring nutritional requirements. Several pharmacological interventions have been assessed for the treatment of severe RSV disease, including corticosteroids and $\beta_2$-adrenergic agonists. However, such pharmacological agents have shown minimal efficacy in ameliorating disease symptoms and severity (Wright and Piedimonte, 2010). Ribavirin (1-β-$_D$-ribofuranosyl-1-2-4-triazole-3-carboxamide) is the only antiviral agent approved for the treatment of severe RSV disease. It is a non-specific inhibitor of replication of several DNA and RNA viruses such as RSV, influenza virus and Hepatits C virus. Ribavirin is thought to inhibit the 5' capping of viral RNA and also functions as a nucleoside analog, whereby its incorporation into viral genomic transcripts terminates virus transcription (Crotty *et al.*, 2002). *In vitro* studies have demonstrated the capability of Ribavirin to inhibit RSV replication in epithelial cells and the production of CXCL8 (Fiedler *et al.*, 1996). However, several clinical studies have shown that Ribavirin treatment does not ameliorate disease severity (Checchia, 2008).

### 1.8 RSV Vaccine Development

Several attempts to generate a protective vaccine against RSV have been unsuccessful despite some understanding of the virus and its pathogenesis. RSV is known to elicit poor immunological memory in the host, by blocking the generation of functional RSV-specific memory $CD8^+$ T-lymphocytes (Bueno *et al.*, 2008; Munir *et al.*, 2011). This creates a barrier to the development of long-lasting immunity which could

necessitate annual booster immunizations to maintain protection against RSV infection (Murata, 2009). Adding to this complexity, several studies using animal models of the disease have demonstrated that immunization with specific RSV antigens invokes an exaggerated, T helper 2 ($T_H2$)-biased immune response upon encountering the virus a second time (Anderson *et al.*, 2010). This phenomenon, known as vaccine-enhanced RSV disease, was behind the failure of 'Lot100' formalin-inactivated RSV vaccine in the 1960s. Administration of formalin-inactivated virus failed to generate high titers of virus-specific neutralizing antibodies and furthermore resulted in an exaggerated response to the virus in subsequent infections. Two vaccine recipients died, and further examination of lung biopsies revealed a severe immune response in the lungs, characterized by high neutrophil and eosinophil infiltration (Anderson *et al.*, 2010). Several approaches to creating a vaccine against RSV are being investigated, including subunit and vector-based vaccines, which incorporate full viral proteins or specific epitopes, DNA vaccines and live-attenuated virus vaccines. In light of previous attempts to generate a vaccine against RSV, safety is essential and long-term protection against A and B subtypes of RSV would be ideal (Murata, 2009).

### 1.9 RSV Passive Prophylaxis

Respiratory Syncytial Virus Intravenous Immunoglobulin (RSV-IVIG) was adapted for use as a prophylactic agent against RSV disease for patients with a high-risk of developing severe disease. It is collected from donors with high titers of RSV-specific immunoglobulins, and infused (over a 4-6 hour period) into patients at a high dose of 15 mL/kg. Although the administration of RSV-IVIG resulted in a great reduction in RSV-related hospitalizations it had adverse side effects in certain high-risk patients. Furthermore, its administration was rather cumbersome and risked the transmission of blood-borne pathogens between donors and recipients. Such disadvantages warranted further investigation into safer candidates for prophylaxis against RSV (Wright and Piedimonte, 2010). Palivizumab (Synagis®) is a humanized monoclonal IgG1 antibody that recognizes a neutralizing epitope on the RSV F protein. The antibody was developed by Johnson *et al.* (Johnson *et al.*, 1997). The same group was able to show that palivizumab prevented virus replication in HEp-2 cells. The abrogation of RSV

replication was achieved whether the antibody was administered alongside the virus or after infection. The same study also showed that lung titers of RSV in cotton rats were significantly diminished when palivizumab was used as a prophylactic agent. Through a series of elegant experiments, Huang *et al.* recently investigated the mechanism of action of palivizumab. Interestingly, palivizumab pretreatment of the virus inhibited virus transcription and replication in HEp-2 cells, but had no effect on virus binding to the host cell membrane. Palivizumab was also shown to inhibit syncytia formation and viral fusion to the host cell membrane which are mediated by the F protein (Huang *et al.*, 2010). Palivizumab showed great promise in pre-clinical trials resulting in great reductions in hospitalization and illness severity. Since its approval in 2002, palivizumab has offered a safer and more easily applied alternative to RSV-IVIG for high-risk patients (Wright and Piedimonte, 2010).

### 1.10 Innate Immune Responses to RSV

The innate immune system provides the host with a non-specific, primary line of defense against viruses and other pathogens. Activation of the innate immune system leads to the increased resistance of cells to infection, recruitment of effector cells to sites of inflammation to help clear the pathogen and development of a more effective and long-lasting adaptive response. Pathogens are recognized by the innate immune system through conserved pattern recognition receptors (PRRs), which are able to sense a variety of pathogen-associated molecular patterns (PAMPs). PAMPs are signature molecular structures that are intrinsic to, or produced during infection by, certain pathogens (Oshansky *et al.*, 2009). TLRs are an example of PRRs that can recognize viral protein motifs at the cell surface (such as TLR4 and TLR2) or viral nucleic acid within endosomal compartments (such as TLR3, TLR7 and TLR8), but not within the cytosol. Cytosolic PRRs include the RIG-I-like receptors (RLRs), which include RIG-I and MDA5, and PKR that are able to sense viral nucleic acid and its intermediates during viral replication (Kawai and Akira, 2010).

### 1.10.1 Toll-Like Receptors (TLRs)

TLRs are composed of extracellular leucine-rich domains used to recognize PAMPs, a transmembrane region and an intracellular domain termed a Toll/IL-1 receptor (TIR) domain. Activation of TLRs leads to the recruitment of several TIR domain-containing adaptor proteins that are essential for initiating downstream signaling and activation of nuclear factor-κB (NF-κB). NF-κB activation culminates in the transcription of various pro-inflammatory cytokines and chemokines (Haynes *et al.*, 2001). Signaling through TLR2, -4, -5, -7 and -9 is dependent on the recruitment of the adaptor molecule MyD88, while TLR3 recruits TIR-domain containing adaptor inducing interferon (IFN)-β (TRIF). TLR2, -3, -4 and -7 have been implicated in RSV disease (Klein Klouwenberg *et al.*, 2009). TLR2 and TLR4 are primarily expressed at the cell surface, while TLR3 and TLR7 are primarily expressed within intracellular compartments, such as endosomes and lysosomes, allowing for nucleic acid detection (Kawai and Akira, 2010). TLR3, -4 and -7 have been shown induce the production of IFN-α and IFN-β by activating interferon regulatory factor (IRF)3 and IRF7, which are essential for the transcription of type I IFN genes. TRIF signaling downstream of TLR3 can activate IRF3 and IRF7 as well as NF-κB, leading to the production of type I IFNs and other pro-inflammatory cytokines (Kawai and Akira, 2010).

TLR2 and TLR4 are expressed by many cell types including mast cells (Brzezinska-Blaszczyk and Wierzbicki, 2010) macrophages, dendritic cells (DCs) and epithelial cells. The implication of TLR2 and TLR4 in RSV inflammation is somewhat surprising. This is due to the fact that TLR2 and TLR4 are activated at the cell surface by primarily bacterial, fungal and parasitic PAMPs. TLR4 recognizes LPS (lipopolysaccharide), TLR2 recognizes peptidoglycan (PGN), lipoteichoic acid and several other bacterial and fungal structures (Kawai and Akira, 2010). The F protein has been shown to bind to, and signal through, TLR4 and CD14. These signaling events are important for triggering the host innate response to the virus (Haynes *et al.*, 2001). F protein interaction with TLR4 has been shown to elicit the release of various pro-inflammatory cytokines from primary human monocytes, such as CXCL8, IL-6 and tumor necrosis factor (TNF) (Kurt-Jones *et al.*, 2000). Furthermore, *in vivo* experiments have demonstrated that TLR4-deficient mice have severely diminished immune

responses to RSV infection, resulting in persistence of the virus in the lungs and impaired recruitment of natural killer (NK) cells (Haynes *et al.*, 2001). TLR2 was also shown to mediate the immune response to RSV infection. It was demonstrated that TLR2, but not TLR4, is responsible for the production of TNF by macrophages following RSV stimulation. TLR2-deficient mice also showed a severe reduction in CCL2 levels following RSV infection, as compared to wild type mice. TLR2 had no effect on IFN-α levels produced during infection. Mice deficient in TLR2 also showed greater viral burden in the lungs than did wild type mice (Murawski *et al.*, 2009).

TLR3 is expressed in macrophages, DCs, epithelial cells and B cells (Klein Klouwenberg *et al.*, 2009). TLR3 is activated by double-stranded RNA (dsRNA), and dsRNA intermediates that form during ssRNA virus infection. TLR3 is also activated by synthetic dsRNA analogues such as polyinosinic:polycytidilic acid (polyI:C) (Kawai and Akira, 2010). A role for TLR3 in RSV infection has been established *in vitro* and *in vivo*. In contrast to TLR4-deficient mice, experiments using TLR3-deficient mice of C57BL/6 background demonstrated that a deletion of TLR3 has no effect on the ability of mice to clear the virus. Furthermore, TLR3-deficient mice showed significantly higher levels of $T_H2$ cytokines in response to RSV infection than wild type mice, and greater eosinophil lung infiltration by day 14 post inoculation. These data demonstrate that although TLR3 may not play a significant role in virus clearance, it is crucial in influencing the lung cytokine microenvironment in mouse models of RSV infection (Rudd *et al.*, 2006). *In vitro* studies have shown that RSV infection of A549 lung epithelial cells up-regulated the expression of TLR3 and intracellular expression of the RNA sensor PKR (Groskreutz *et al.*, 2006). Subsequent studies have also demonstrated that knocking down TLR3 during RSV infection of A549 cells reduced the production of CXCL10 and CCL5, but not CXCL8, indicating the importance of TLR3 in chemokine production during RSV disease (Rudd *et al.*, 2005).

TLR7 and TLR8 are phylogenetically similar, are responsible for sensing single-stranded (ssRNA) virus infections and are expressed in various tissues as well as in plasmacytoid dendritic cells (pDCs) and monocytes (Kawai and Akira, 2010). A growing body of literature supports a role for TLR7 in the immune response to RSV. Studies have shown that TLR7-deficient mice exhibit more severe inflammation and elevated mucus

secretion during RSV infection, as well as an increase in cytokines IL-4 and IL-13 and IL-17 (Lukacs *et al.*, 2010). Intriguingly, it was shown using mouse models that eosinophils respond to RSV through a TLR7-MyD88 pathway, leading to the up-regulation of several antiviral genes such as IRF7 and IFN-β, enhancing virus clearance from the lungs (Phipps *et al.*, 2007). A very recent study investigated the role for TLR7 in the pathogenesis of pneumonia virus of mice (PVM), which mimics RSV infection in humans. In response to PVM infection, TLR7-deficient mice showed a delayed production of type I, II and III IFNs, higher virus burden in the lungs and diminished pDC recruitment. Reconstitution with TLR7-sufficient, but not TLR7-deficient, pDCs restored the immune response to wild type, highlighting an important role for TLR7 in the early innate immune response to viral infection (Davidson *et al.*, 2011).

### 1.10.2 RIG-I-like Receptors

The RIG-I-like Receptors (RLRs) are able to sense viral infection in the cytoplasm (Wilkins and Gale, 2010). The 3 members of the RLR family of intracellular RNA receptors are retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA5 have 2 different N-terminus caspase activation and recruitment (CARD) domains that are essential for downstream signaling activity. The DExD/H-box RNA helicase domain of RIG-I and MDA5 mediates the binding to RNA ligands and the intrinsic ATPase activity of this domain is essential for downstream signaling. Despite the absence of CARD domains in LGP2, studies have shown that LGP2 is essential for mediating MDA5 and RIG-I signaling and IFN-β transcription in response to RNA virus infection (Satoh *et al.*, 2010). LGP2 and RIG-I also contain a receptor domain (RD) at the C-terminus which is essential for maintaining the protein in an inactive state prior to RNA ligand binding (Onoguchi *et al.*, 2011). A significant body of research has enhanced our understanding of the ligands identified by RIG-I and MDA-5. The ligand for RIG-I was found to be uncapped 5' triphosphate RNA (Hornung *et al.*, 2006), which is absent from host RNA. RIG-I recognize a variety of viruses such as Hepatitis C virus (HCV), RSV and influenza virus. MDA5 was shown to recognize high molecular weight RNA containing single- and double-stranded structures such as that of picornaviruses and

polyI:C (Pichlmair *et al.*, 2009). Upon activation, RIG-I and MDA5 interact with the adaptor protein mitochondrial antiviral signaling protein (MAVS), which leads to the activation of a transcription complex that includes IRF3, IRF7 and NF-κB. Such downstream signaling events lead to the induction of pro-inflammatory cytokines as well type I and III IFNs (Onoguchi *et al.*, 2011). The work of Mikkelsen *et al.,* demonstrated that RIG-I induction of type I IFNs and IL-12 is dependent on activation of p38 MAPK in DCs (Mikkelsen *et al.*, 2009). Furthermore, studies have shown that RIG-I activation can induce CXCL8, CXCL9, CXCL10, IL-1β, IL-6 and interferon-stimulated gene (ISG)15 (Cui *et al.*, 2004; Kawaguchi *et al.*, 2009; Kubota *et al.*, 2006).

An important role for RIG-I in the recognition and response to RSV has been highlighted through several studies. A role for MDA-5 remains to be deciphered (Yoboua *et al.*, 2010). Most recently, it was shown that RIG-I, but not MDA5, was essential for NF-κB activation during RSV infection (Yoboua *et al.*, 2010). RIG-I-dependent IFN-β production was shown to be essential for the paracrine induction of TLR3. The small interfering RNA (siRNA) knockdown of RIG-I expression in RSV-infected airway epithelial cells inhibited the activation of NF-κB. RIG-I silencing also abrogated the translocation of IRF3 and NF-κB to the nucleus, and significantly reduced IFN-β, CCL5 and CXCL10 production at early time points (Liu *et al.*, 2007; Liu *et al.*, 2008). Interestingly, MAVS deficient mice exhibited a severely diminished innate response to RSV infection characterized by lower levels of several mediators including IFN-α/β, CCL2, TNF and IL-6, and a slowed clearance of the virus. However, a deficiency in MAVS did not hamper the generation of an RSV-specific $CD8^+$ response, indicating its importance in early innate responses (Bhoj *et al.*, 2008).

### 1.10.3 Protein Kinase R (PKR)

Protein kinase R (PKR), PKR belongs to a family of proteins capable of phosphorylating eukaryotic initiation factor 2α (eIF2α). PKR is encoded by the *eIF2αk2* gene, which is constitutively expressed at low levels in all tissues. Due to the presence of an interferon-signal response element (ISRE) upstream of *eIF2αk2*, its transcription is enhanced in response to IFN-α/β stimulation during the antiviral response (Pindel and Sadler, 2011). Several questions regarding the structure of the ligand recognized by PKR

remain unanswered. However, it has been shown that PKR recognizes viral dsRNA due to the presence of a 5' uncapped triphosphate domain, which is absent from mature host RNA. Ligand recognition happens through two dsRNA-binding domains, and a catalytic subunit able to bind ATP. Upon activation by binding dsRNA, PKR forms a homodimer which can phosphorylate several target proteins in the cell (Nallagatla *et al.*, 2007). The most studied target of PKR activation is eIF2α, which is responsible for initiating protein synthesis in eukaryotic cells by aiding in the delivery of methionine-transfer (t)RNA to the ribosome. Upon phosphorylation, eIF2α is rendered inactive and therefore viral and host protein synthesis in the cell is halted markedly (Dever, 2002). PKR is also able to activate several proteins such as p38 and c-Jun N-terminal Kinases (JNK) MAPKs as well as NF-κB, leading to cytokine and chemokine production (Goh *et al.*, 2000; Kumar *et al.*, 1994).

RSV infection of the A549 airway epithelial cell line was shown to up-regulate the expression of PKR (Groskreutz *et al.*, 2006). PKR-knockout mice infected with RSV showed diminished levels of several mediators such as IFN-α/β, CCL2, CCL5 and CXCL10 as compared to wild type mice and had less severe lung injury and immune cell infiltration (Minor *et al.*, 2010). Groskreutz *et al.* observed that RSV infection of the A549 airway epithelial cell line resulted in low levels of PKR-induced eIF2α phosphorylation. Very interestingly, it was shown that PKR was sequestered in the cell by RSV N protein, preventing PKR phosphorylation of eIF2α (Groskreutz *et al.*, 2010).

## 1.11 Cytokine and Chemokine Production During RSV Disease

### 1.11.1 Cytokines

The airway epithelium constitutes the first site of interaction between RSV and the host (Bueno *et al.*, 2011). The interaction of the virus with the epithelium through TLRs and other PRRs leads to the production several cytokines and chemokines that help in the recruitment of a variety of effector cells such as neutrophils, monocytes, eosinophils, T-lymphocytes, and NK cells to the airways. Recruited effector cells can then further enhance the inflammatory response by secreting more cytokines and chemokines (Bueno *et al.*, 2011). Elevated levels of a variety of cytokines have been reported in response to RSV using *in vitro* and *in vivo* models of infection as well as

human clinical samples; IFN-α, IFN-β, IFN-γ, IL-1β, TNF, IL-4, IL-6, IL-12 and IL-13 have been reported to be elevated (Bueno *et al.*, 2011; Welliver, 2008).

Type I IFNs, which include IFN-α and IFN-β, are the first cytokines made following the onset of RSV infection, making them an integral part of the host antiviral response. Although all nucleated cells are capable of producing type I IFNs, DCs are known to be the highest producers. There are 13 different subtypes of IFN-α and a single subtype of IFN-β. IFN-α and IFN-β signal through the IFN-α/β receptor which is made of two different subunits, IFNAR1 and IFNAR2. Following receptor binding, signal transduction occurs via the intracellular domain of IFNAR and commences by activating the janus kinases (JAK) JAK1 and TYK2. JAK1 and TYK2 then activate STAT1 and STAT2 transcription factors that dimerize and bind to IRF9, forming the ISGF3 transcription complex (Horvath *et al.*, 1996). The ISGF3 complex translocates to the nucleus where it can bind to ISREs and initiate the transcription of more than 100 ISGs with antiviral activity (Fensterl and Sen, 2011).

Type III IFNs are a recently described class of cytokines with similar functions to IFN-α and IFN-β. IL-28A (IFN-λ2), IL-28B (IFN-λ3) and IL-29 (IFN-λ1) are members of this family of cytokines that are produced by nearly all nucleated cells in the body, with DCs being the most prominent sources. Type III IFNs signal through a receptor complex consisting of the IL-28R1 and IL-10R2 receptor chains. IL-28R1 is ubiquitously expressed in various human organs and cell types. Similar to type I IFNs, type III IFN signaling leads the activation of IRF3, IRF7 and NF-κB, which can enhance the surface expression of MHC-I and lead to the induction of various ISGs including MxA and 2'5-OAS. RSV NS1 protein has been shown to suppress the production of IL-28A, IL-28B and IL-29 in A549 cells during RSV infection (Munir *et al.*, 2008).

Several studies have highlighted that NS1 and NS2 interfere with the induction and signaling of type I and type III IFNs (Munir *et al.*, 2011; Spann *et al.*, 2004). Specifically, NS2 has been shown to interact with RIG-I and inhibit its downstream signaling. This results in a decrease in IFN-β production during RSV infection (Ling *et al.*, 2009). Furthermore, NS1 has been shown to prevent the phosphorylation of the STAT1/STAT2 heterodimer downstream of the IFNR. This abrogates its localization to the nucleus during RSV infection of mouse bone marrow-derived DCs (BMDCs) which

is essential for the transcription of ISGs (Jie *et al.*, 2011). Other studies have shown that NS1 can promote the degradation of STAT2 downstream of the IFNAR1 (Elliott *et al.*, 2007). NS1 and NS2 also play an essential role in shaping the T-lymphocyte response in RSV infection. The maturation of primary human DCs infected with RSV is suppressed by NS1 and NS2 (Munir *et al.*, 2008). It was shown that the maturation of human autologous T-lymphocytes to $CD8^+$ cytotoxic T-lymphocytes (CTLs) and T helper 17 ($T_H17$) cells was enhanced in the presence of DCs infected with ΔNS1 RSV, but not wild type RSV. Furthermore, the same studies showed that the activation of IL-4-producing $CD4^+$ T- lymphocytes, which are linked to severe RSV disease, was diminished in response to ΔNS1 RSV indicating a pathogenic role for NS1 (Munir *et al.*, 2011).

ISGs, which are induced in response to type I and III IFN stimulation, generate proteins that have direct antiviral effects and are therefore essential in controlling viral infections. ISGs include the dsRNA sensor PKR and 2'5-oligoadenylate synthetase (2'5-OAS), an activator of RNase L which cleaves ssRNA halting the transcription of viral proteins. ISG56 (P65) was the first IFN-induced protein to be cloned and is one of the most well studied and characterized (Fensterl and Sen, 2011). The ISG56 family of genes encodes several proteins that function to inhibit virus replication. Proteins belonging to this family have no enzymatic activity but contain several tricopeptide repeats (TPRs), which enable their interaction with other proteins in the cell. One of the mechanisms by which ISG56/P65 inhibits virus replication is through binding to the eIF3 initiation factor, which shuts down protein translation and cell growth (Fensterl and Sen, 2011). It is also noteworthy that ISG56 can be induced in responses to dsRNA in an IFN-independent manner (Bandyopadhyay *et al.*, 1995). RSV infection has been shown to induce ISG15 (Moore *et al.*, 2008). Type I IFNs can also induce the Mx family of GTPases. Two Mx GTPases are expressed in human cells, MxA and MxB. While no function has been identified for MxB, MxA is known to confer antiviral properties against a wide variety of viruses such as orthomyxoviruses and paramyxoviruses. Interestingly, transgenic mice that constitutively express Mx1 were shown to be resistant to influenza virus infection (Arnheiter *et al.*, 1990). The mechanism of action of MxA remains elusive. However, MxA is thought to interact with viral components and proteins to prevent their association and assembly, which inhibits viral replication (Haller *et al.*,

2007). While type I IFNs are potent inducers of Mx genes, studies have shown that MxA can be induced in response to TLR4 stimulation with LPS in an IFN-independent manner (Malcolm and Worthen, 2003).

IFN-γ is characteristically produced by immune cells such as $T_H1$ $CD4^+$ T-lymphocytes, $CD8^+$ T-lymphocytes and NK cells. IFN-γ is important in controlling the host response to viral infections through a variety of mechanisms such as enhancing MHC I expression and activating NK cells. Although higher levels of IFN-γ in the lungs of children with bronchiolitis correlate with less severe disease, several studies recorded low IFN-γ levels in the lungs of children with RSV bronchiolitis (Zeng *et al.*, 2011).

### 1.11.2 Chemokines

Chemokines, or 'chemotactic cytokines', are soluble proteins capable of recruiting leukocytes, endothelial and epithelial cells to sites of infection and inflammation. The classification of chemokines is based on the arrangement of conserved cysteine (C) residues in their structure; the four main chemokine families are CC, CXC, CX3C and C, where X denotes intervening amino acids. In addition to their role in cell recruitment, chemokines have homeostatic roles and are involved in wound healing, angiogenesis and tumour development and metastisis. Chemokines mediate their effects by binding chemokine receptors on target cells. CXC chemokines interact with CXC-specific receptors (CXCRs), while CC chemokines interact with CC-specifc receptors (CCRs). Chemokine receptors are transmembrane G protein-coupled receptors (GPCRs). Upon activation of chemokine GPCRs, downstream signaling events can lead to cell activation, degranulation or extravasation through the endothelium to sites of inflammation. Gradients of chemokine are presented to infiltrating leukocytes on glycosaminoglycan networks by activated endothelial cells. Upon binding chemokine receptors, leukocytes up-regulate surface integrin expression, leading to firm adhesion to the endothelium which facilitates extravasation into sites of inflammation (Lortat-Jacob, 2009).

Interestingly, several chemokines are elevated in RSV disease. CXCL8 (also known as IL-8) is the most studied chemokine in the context of RSV inflammation (Bueno *et al.*, 2011). Several studies have showed that elevated levels of CXCL8 are

present in the lungs of children with RSV bronchiolitis. CXCL8 release in response to RSV has been documented in primary epithelial cells and epithelial cell lines (Yoon *et al.*, 2007), as well as in macrophages and in co-culture with neutrophils (Arnold *et al.*, 1994; Becker *et al.*, 1991). CXCL8 binds to the CXCR1 and CXCR2 chemokine receptors on target cells (Sharma, 2010) and is a potent activator and chemoattractant of neutrophils, which are the first cells to migrate to inflammation sites during RSV disease (Welliver, 2008). Elevated CXCL8 levels in patients have been linked to severe RSV disease and several polymorphisms in CXCL8 have been implicated in the development of severe bronchiolitis and asthma (Zeng *et al.*, 2011).

The levels of CXCL10, also known as interferon-γ-induced protein-10 (IP-10), were found elevated in the lungs of children hospitalized with RSV bronchioliotis as compared with controls (Roe *et al.*, 2011). Many *in vitro* studies have examined the production of this chemokine in response to RSV, and studies have shown that epithelial cells as well as DCs can make CXCL10 following RSV stimulation (Castro *et al.*, 2011). CXCL10 binds to the CXCR3 chemokine receptor on effector cells and is important for the chemotaxis of activated T helper 1 ($T_H1$) $CD4^+$ T-lymphocytes, NK cells as well as pDCs and myeloid dendritic cells (mDCs) to sites of inflammation (Muller *et al.*, 2010). In airway epithelial cells infected with RSV, the production of CXCL10 was dependent on RIG-I at early time points and TLR3 at later time points (Rudd *et al.*, 2006). Signaling through TLR7 has also been shown to enhance the production of CXCL10 by DCs incubated with RSV (Castro *et al.*, 2011). The importance of CXCL10 in RSV infection has been elucidated *in vivo* using murine models. Inhibiting CXCL10/CXCR3 using monoclonal antibodies in mice immunized with RSV lead to an increase in viral pathogenesis and airway hyperresponsiveness (AHR). It also lead to a reduction in DC recruitment and maturation in the lungs and a reduction in the recruitment of RSV-antigen specific $CD8^+$ T-lymphocytes. Furthermore, RSV-infected DCs treated with CXCL10 showed higher levels of IFN-α mRNA. This study was instrumental in highlighting a protective role for CXCL10 in RSV disease (Lindell *et al.*, 2008).

A great number of studies have focused on a role for CCL5 (regulated upon activation, normal T-cell expressed and secreted; RANTES) in RSV disease. This chemokine is produced in large amounts by primary epithelial cells and epithelial cell

lines in response to RSV stimulation in an IFN-independent, MyD88-dependent manner (Rudd *et al.*, 2005). Elevated levels of CCL5 are measured in nasopharyngeal secretions following RSV infection in children. Furthermore, elevated levels of CCL5 are associated with enhanced RSV disease the development of asthma (Chung and Kim, 2002; Zeng *et al.*, 2011). Through binding to CCR1, CCR3 and CCR5 chemokine receptors (Sharma, 2010), CCL5 facilitates the recruitment of several subsets of T-lymphocytes, as well as monocytes and eosinophils (Teran, 2000). Experiments utilizing *in vivo* mouse models showed that CCL5 was induced by IL-13 in the lungs, and that CCL5-blockade ameliorated lung inflammation during RSV infection (Tekkanat *et al.*, 2002).

CCL11 (eotaxin-1) levels were also elevated in nasopharyngeal secretions from infants with bronchiolitis compared to controls (Kim *et al.*, 2007). The importance of CCL11 lies in its ability to recruit eosinophils, which are associated with severe and allergic airway inflammation. BALB/c mice inoculated with RSV show elevated levels of CCL11 in bronchoalveolar lavage (BAL) samples. Antibody blockade of CCL11 resulted in less severe RSV disease (Matthews *et al.*, 2005). *In vitro* studies have not demonstrated epithelial cell production of CCL11 in response to RSV. The *in vivo* production of CCL11 is abolished with the depletion of $CD4^+$ T-lymphocytes, suggesting their potential role in the production of CCL11 (Johnson *et al.*, 2008).

Respiratory epithelial cells make CCL2 (monocyte chemotactic protein-1; MCP-1), CCL3 (macrophage inflammatory protein-1α; MIP-1α) and CCL4 (macrophage inflammatory protein-1β; MIP-1β) when incubated with RSV (Zeng *et al.*, 2011). Interestingly, CCL4 levels in the lungs of children hospitalized with RSV disease inversely correlated with symptom severity and ventilator use (Garofalo *et al.*, 2001). However no studies to date have examined the specific role of CCL4 in RSV inflammation. CCL4 binds to the CCR5 chemokine receptor facilitating the recruitment of activated T-lymphocytes as well as monocytes (Cook, 1996; Robertson, 2002; Taub *et al.*, 1993).

Intriguingly, the G protein of RSV contains a CX3C chemokine motif, enabling it to bind to the fractalkine chemokine receptor (CX3CR1). This binding appears to influence the biology of the immune response against RSV by facilitating infection and altering the recruitment of immune cells to sites of inflammation (Tripp *et al.*, 2001).

## 1.12 Mast Cells

Since the discovery of mast cells in 1878 by Paul Ehrlich, great advancements have been made in understanding their development, function and morphology. Mast cells are now recognized as long-lived, tissue granulocytes with significant roles in allergic disease, wound healing and the defense against pathogens (Moon *et al.*, 2010).

### 1.12.1 Mast Cell Localization and Development

Mature human mast cells prominently reside in tissues that closely interface with the environment, including vascularized tissues of the skin, gastrointestinal tract and airways, which closely interface the environment. Within tissues, mast cells are close to blood vessels, nerves, smooth muscle cells and mucous producing cells, and are therefore amongst the first immune cells to interact with pathogens, allergens and other antigens at body surfaces (Galli *et al.*, 2005; Marshall, 2004).

Mast cells develop from $CD34^+$ hematopoietic progenitor stem cells in the bone marrow. Progenitor mast cells leave the bone marrow through circulation and home to various tissue sites throughout the body where the microenvironment provides the essential growth factors for their differentiation to mature mast cells. A wide variety of cytokines, chemokines and growth factors are required for efficient mast cell growth and development (Galli *et al.*, 2005). However, the most essential of factors for the differentiation of mast cells is stem cell factor (SCF), which is the ligand for CD117 (c-Kit). SCF is provided by several sources in tissues, including fibroblasts and stromal cells (Shea-Donohue *et al.*, 2010). Other important factors for mast cell development from $CD34^+$ progenitor cells include IL-6 (Saito *et al.*, 1996). IL-4, a $T_H2$ polarizing cytokine, was shown to be important for mast cell maturation, whereas the $T_H1$ cytokine IFN-γ inhibited mast cell growth (Kirshenbaum *et al.*, 1998; Nakahata and Toru, 2002). Upon maturation, the expression of CD34 is down-regulated and the expression of the high affinity FcεRI expression is enhanced. Uniquely, mast cells retain CD117 expression following maturation, making CD117 a suitable mast cell marker (Kumar and Sharma, 2010).

Human mast cells can be classified into 3 different subtypes based on the proteases contained in their granules. Mast cells of the subtype $MC_{TC}$ contain granules that are rich in both tryptase and chymase and are primarily located in the skin and the submucosa of the gastrointestinal tract. The $MC_T$ subtype of mast cells is predominantly found in the mucosa of the airways and gastrointestinal tract and contain only tryptase. The $MC_C$ subclass contains only chymase and is localized predominantly in the submucosa of the stomach, small intestines and colon. Despite differences between the subtypes, mast cell populations at tissue sites remain heterogeneous (Moon *et al.*, 2010).

### 1.12.2 Mast Cell Models

Several cell line models are available for the *in vitro* study of mast cells. The human mast cell (HMC)-1 cell line was generated in 1988 using cells from a patient with mast cell leukemia. HMC-1 cells share several phenotypic similarities with immature mast cells, including the lack of expression of the high affinity IgE receptor, FcεRI, and low histamine level expression (Butterfield *et al.*, 1988). Importantly, HMC-1 cells do not require SCF, as a mutation in CD117 constitutively activates downstream signaling pathways abrogating the need for extracellular SCF administration (Moon *et al.*, 2010). The Laboratory of Allergic Diseases-2 (LAD2) human mast cell line was produced using cells from a patient with mast cell sarcoma in 2003. Unlike HMC-1 cells, LAD2 cells are similar in phenotype to mature, differentiated mast cells and are responsive to SCF. LAD2 cells express FcεRI as well as granular chymase, trypsin and histamine (Kirshenbaum *et al.*, 2003). KU812 cells have mast cell- and basophil-like properties, and were developed from a patient with chronic myelogenous leukemia. KU182 cells express CD117 and histamine, but no Fc receptors (Kishi, 1985) .

Primary peripheral-tissue mast cells can be isolated from a variety of tissue sites around the body such as the lungs, peritoneum, skin and nasal tissue. These cells provide a useful model for studying mast cells, but are very difficult to isolate and culture. Furthermore, the effects of extraction on their phenotype have not been fully characterized (Moon *et al.*, 2010). Mast cells can also be generated from stem cells isolated from the bone marrow, umbilical cord and peripheral blood. The culture conditions for generating human mast cells generally require the presence of SCF and IL-

6. Human cord blood-derived mast cells (CBMCs) also require prostaglandin $E_2$ ($PGE_2$), which affects the granularity and the protease and histamine expression in the mast cells (Saito *et al.*, 1996).

The use of animal models is very useful for studying the function of mast cells *in vivo*. The WBB6F1-$Kit^{W}/Kit^{W\text{-}v}$ and C57BL/6-$Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ murine models are widely used for studying mast cells. $Kit^{W\text{-}v}$ and $Kit^{W\text{-}sh}$ mice are deficient in mast cells due to a general lack of CD117 signaling which is required for mast cell development. The usefulness of such models lies in the ability to reconstitute these animals with genetically compatible *in vitro* cultured mast cells (Galli *et al.*, 2005).

## 1.13 Mast Cell Mediators

### 1.13.1 Granule-associated Mediators

Mast cells contain several highly sulfated GAGs such as such as heparan sulphate and chondroitin sulphate, stored within granules. These granule-associated GAGs interact with several dyes, such as toluidine blue, giving mast cells a metachromatic appearance (Lundequist and Pejler, 2011). Critically, proteoglycans such as heparan sulphate have been shown to bind various chemokines in the microenvironment, such as CCL4, CCL5 and CXCL12. The binding of GAGs to chemokines generates a stable gradient that facilitates the activation of leukocytes and their chemotaxis to sites of inflammation. Furthermore, mast cells are a potent source of histamine and other biogenic amines. Histamine is the most highly studied mast cell mediator with a wide array of physiological functions such as vasodilation, bronchoconstriction and smooth muscle contraction, and can have direct effects on cell migration (Lortat-Jacob, 2009).

Mast cell granules contain a wide variety of proteases such as chymase, tryptase, cathepsin G and matrix metalloprotease-9 (MMP-9) (Lundequist and Pejler, 2011). Mast cell-derived proteases have been studied in various inflammatory responses including host defense again parasitic and bacterial infections, arthritis and airway and tissue remodeling (Caughey, 2007). An important function of mast cell proteases is the degradation or activation of several cytokines and chemokines. For example, the degradation of CCL11 and CCL5 by mast cell-derived β-tryptase was deemed essential for down-modulating eosinophil chemotaxis during airway inflammation (Pang *et al.*,

2006). Mast cell proteases are also capable of degrading IL-3, IL-5, IL-6 and TNF, which helps in controlling inflammatory responses (Zhao *et al.*, 2005). Alternatively, mast cell-derived tryptase was shown to invoke the release of CXCL8 and up-regulate cell adhesion molecules on epithelial cells (Cairns and Walls, 1996).

### 1.13.2 *De novo* Synthesized Mediators

Depending on the type and strength of activation, mast cells are capable of selectively secreting a plethora of *de novo* synthesized mediators, which include lipid mediators, cytokines and chemokines (Galli *et al.*, 2005). Lipid mediators (eicosanoids) include prostaglandins, leukotrienes, thromboxanes, and lipoxins, and are generated from arachidonic acid, a product of phospholipid membranes. Cyclooxygenases and lipooxygenases act on arachidonic acid leading to the generation of prostaglandins and leukotrienes respectively. Mast cells can make prostaglandins and leukotrienes which play an important role in asthma and airway inflammation by recruiting $T_H2$ cells and mediating smooth muscle contraction (Reuter *et al.*, 2010).

Mast cells can also secrete a myriad of cytokines that are known to affect inflammation, angiogenesis, endothelial activation and hematopoiesis. Examples of mast cell derived cytokines and growth factors include: TNF, IFN-γ, IFN-α, IFN-β, IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-12, IL-13, IL-15, VEGF and TGF-β (Galli *et al.*, 2005). The pro-inflammatory cytokines TNF, IL-1α and IL-1β are capable of up-regulating the expression of cell adhesion molecules on endothelial cells, affecting endothelial permeability and cell recruitment (Brown *et al.*, 2011; Chang *et al.*, 2003). IL-6 is an important cytokine in the development of plasma cells and the maturation of $T_H17$ cells. IL-4 and IL-5 are critical for the development of $T_H2$ cells. IFN-γ is a $T_H1$ polarizing cytokine and an important player in the host response to viral infections (Bueno *et al.*, 2011). The proliferation, survival, cytotoxicity and activation of NK cells are regulated by IL-15. IFN-α and IFN-β are important in antiviral responses (Zwirner and Domaica, 2010).

Mast cells can also synthesize and secrete a wide variety of chemokines. Such chemokines include CCL2, CCL3, CCL4, CCL5, CXCL8, CXCL10, and CXCL10 (Galli *et al.*, 2005). CCL2 is an essential chemokine for the chemotaxis of macrophages and

monocytes. Activated $CD4^+$ T-lymphocytes migrate to CCL3 and activated $CD8^+$ T-lymphocytes migrate to CCL4. CCL5 is capable of recruiting and activating $CD4^+$ and $CD8^+$ T-lymphocytes and eosinophils to sites of inflammation (Pang *et al.*, 2006; Taub *et al.*, 1993). CXCL2 and CXCL8 are potent neutrophil chemoattractants. CXCL10 is essential for the recruitment of activated $CD4^+$ and $CD8^+$ T-lymphocytes as well as NK cells (Lindell *et al.*, 2008).

**1.14 Mast Cell Responses to Viruses**

The strategic location of mast cells at sites interfacing the environment and their vast repertoire of PRRs and mediators make them ideal targets for viral infection or interactions with viral products. Surprisingly, however, few studies have examined mast cell interactions with viruses and/or viral products (Marshall *et al.*, 2003).

Human mast cells respond to polyI:C, a synthetic mimic of viral RNA, by secreting IFN-α and IFN-β. Furthermore, polyI:C stimulation of mast cells results in the production of several chemokines such as CCL2, CCL5, CCL4, CXCL8 and CXCL10 that are important in the recruitment of macrophages, activated T-lymphocytes and NK cells (Burke *et al.*, 2008; Kulka *et al.*, 2004). Recent work has shown that mast cells isolated from human skin can be infected with hantavirus, a ssRNA virus capable of causing hemorrhagic disease in humans. Hantavirus infection of mast cells resulted in the production of IFN-β and CCL5 (Guhl *et al.*, 2010).

Mast cell responses to dengue virus (DV) infection are well documented. Antibody-enhanced DV infection of KU812 cells results in the production of the IL-1β and IL-6 (King *et al.*, 2000). The antibody-blockade of TNF in supernatants of DV-infected mast cells abrogates the activation of human umbilical vein endothelial cells (HUVECs) (Brown *et al.*, 2011). These studies suggest an important role for mast cell-derived mediators in endothelial cell activation during antibody-enhanced DV infection. Furthermore, DV-infected KU812, HMC-1 and CBMCs generate significant amounts of CCL3, CCL4 and CCL5 (King *et al.*, 2002). It was very recently shown that the production of cytokines and chemokines, such as IFN-α, CCL5 and CXCL10 by DV-infected murine mast cells was dependent on RIG-I and MDA5. Furthermore, mast cell-deficient mice exhibited higher viral burden and impaired recruitment of NK and NKT

cells to sites of infection. (St John et al., 2011). Similarly, CBMCs generate substantial amounts of CXCL8 in response to reovirus infection *in vitro*, which was shown to promote the recruitment of $CD56^+$ NK cells. These studies are pivotal in stressing the importance of mast cells in effector cell recruitment during inflammation (Burke *et al.*, 2008).

Conversely, mast cells play a detrimental role for the host during human immunodeficiency virus (HIV) infection. $CD34^+$ mast cell progenitor cells express CXCR4, CCR5 and CD4 and can become infected with R5 tropic strains of HIV. Upon maturation, the expression of CD4, CCR5 and CXCR4 is down-regulated. Although this prevents re-infection, mature mast cells act as an inducible reservoir for HIV since reactivation of the virus is observed with TLR stimulation (Sundstrom *et al.*, 2007).

### 1.14.1 Mast Cell Responses to RSV

A small number of studies have examined a potential role for mast cells in RSV disease and inflammation, but the function of mast cells in RSV disease has not yet been investigated using mast cell-deficient animals. Early work using guinea pig and rat models of RSV infection found an increased number of mast cells in lung tissue samples of animals infected with the virus as compared with controls. It has also been proposed using a rat model of RSV disease that mast cell-secreted leukotrienes promote vascular permeability (Hegele *et al.*, 1994; Wedde-Beer *et al.*, 2002). BAL samples obtained from infants hospitalized with RSV had higher levels of mast cell tryptase (Everard *et al.*, 1995). Furthermore, infants with RSV bronchiolitis had higher levels of urinary 9α,11β-$PGF_2$, a marker of mast cell activation (Oymar *et al.*, 2006). These studies indicate that mast cells are activated during RSV bronchiolitis. It was recently observed that HMC-1 cell degranulation and TNF release occurs only in co-culture with RSV-infected A549 airway epithelial cells, and not in response to RSV alone. These findings highlight the important interaction between mast cells and the airway epithelium during RSV inflammation (Shirato and Taguchi, 2009).

The presence of RSV-specific IgE has been documented in humans with RSV-induced wheezing (Welliver *et al.*, 1981). To this extent, Dakhama *et al.* demonstrated that BALB/c mice develop virus-specific IgE during primary infection with RSV. Upon

reinfection, wild type but not FcεRI$^{-/-}$ mice exhibit asthmatic AHR. Bone marrow-derived mast cells (BMMCs) degranulate in response to RSV stimulation only in the presence of RSV-specific IgE (Dakhama *et al.*, 2009; Dakhama *et al.*, 2004). These observations highlight a potential role for mast cells as FcεRI-bearing cells in IgE-mediated AHR aggravated by RSV.

### 1.15 Thesis Hypothesis, Rationale and Objectives

A growing body of literature supports a sentinel role for mast cells in the innate immune response to bacterial, fungal and viral infections. Mast cells are localized to tissue sites in the body that directly interface the environment making them amongst the first cells to encounter invading pathogens. Studies have shown that viral infection of mast cells, or stimulation with viral products, triggers the release of various pro-inflammatory cytokines and chemokines that lead to the recruitment of monocytes, $CD4^+$ and $CD8^+$ T-lymphocytes and NK cells. Despite this knowledge, the responses of human mast cells to RSV and their role in RSV disease remain largely uncharacterized.

The aim of this project was to investigate the responses of human primary mast cells and mast cell lines to RSV. We hypothesized that mast cell stimulation with RSV will lead to the production of various pro-inflammatory cytokines and chemokines that are capable of effector cell recruitment and establishing an antiviral response. To this extent, specific objectives included:

1. Assessing whether human mast cells can support RSV antigen expression and viral infection.
2. Characterizing the pro-inflammatory cytokines and chemokines produced by human mast cells and airway epithelial cells in response to RSV.
3. Appraising the induction of antiviral IFNs and ISGs in response to RSV stimulation.
4. Evaluating the role of type I IFNs in mediator production by mast cells in RSV-stimulated mast cells.

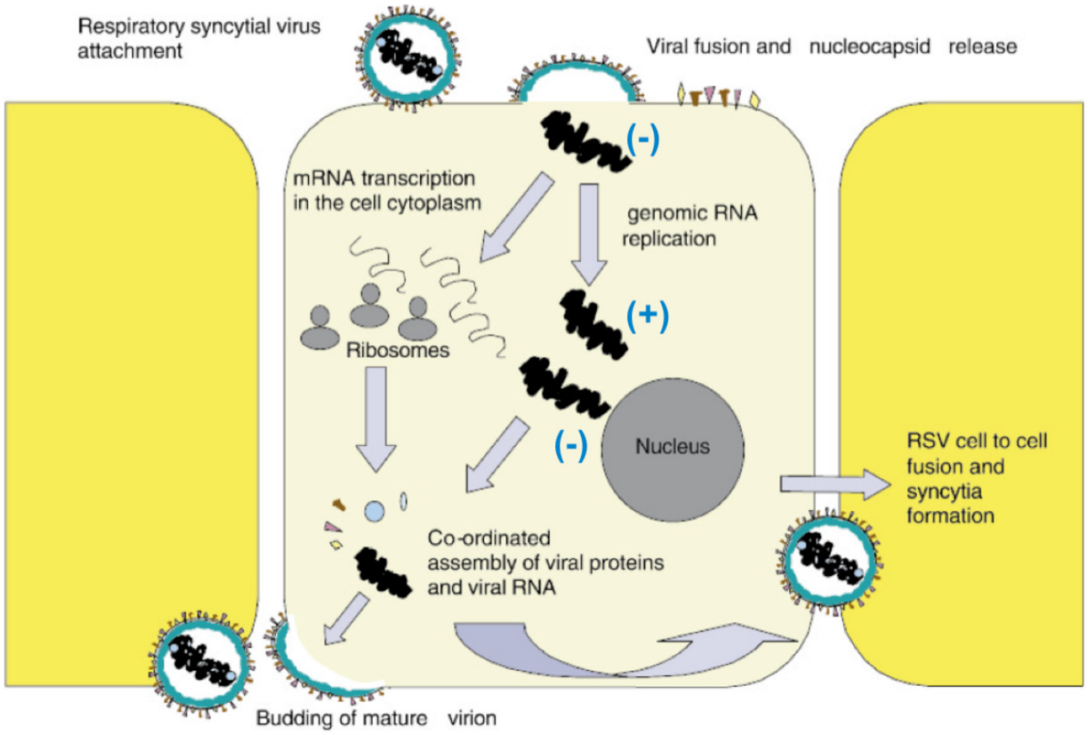



**Figure 1. Schematic representation of the RSV replication cycle**. The RSV virion attaches to the apical membrane of the host cell using the G protein. The F protein mediates the fusion of the viral membrane with that of the host delivering the negative-sense (-) ssRNA genome to the cytoplasm. Genome 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.

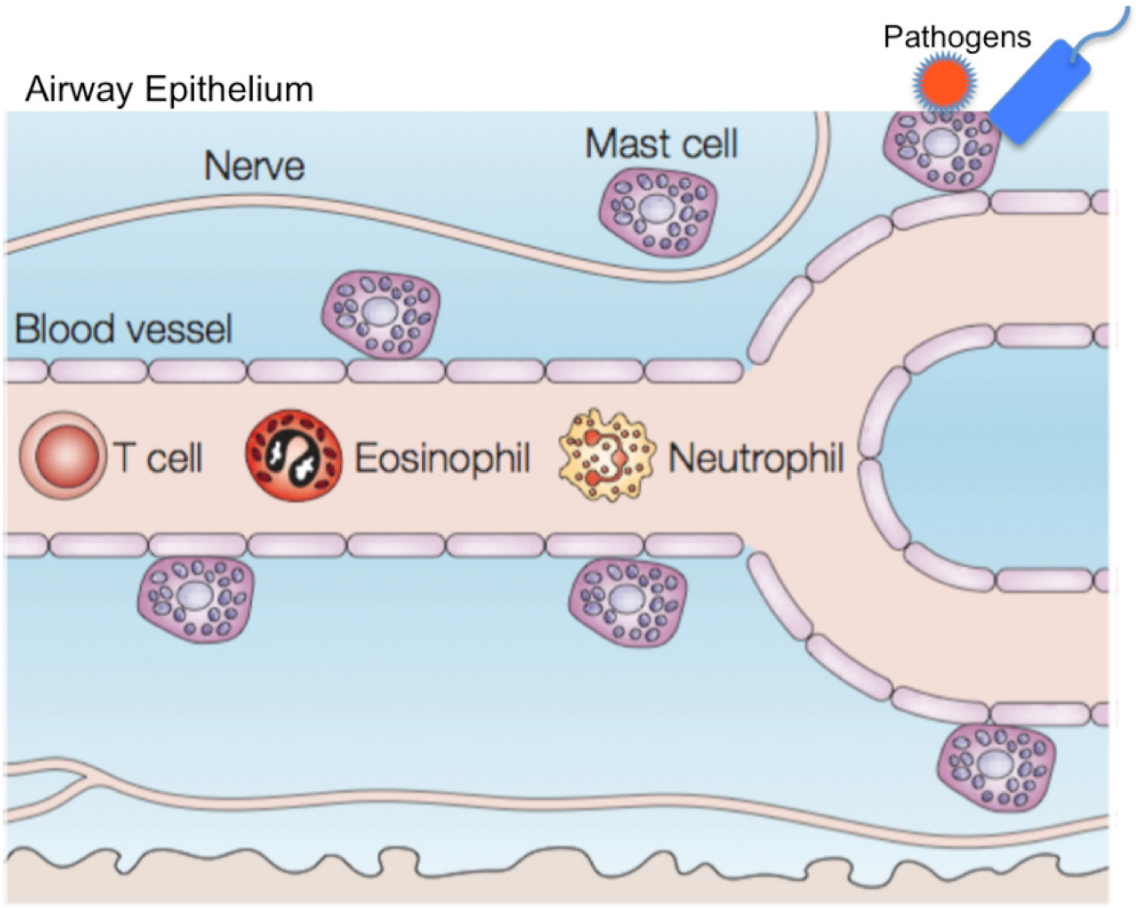



**Figure 2. Representation of mast cell distribution in tissue sites.** Mast cells reside at sites that closely interface the environment in the airways, skin and gastrointestinal tract. Within tissue sites, mast cells are located in close proximity to blood vessels, nerves and mucus producing cells. Adapted from *Nature Reviews Immunology*, Vol. 4, Marshall J.S., "Mast cell responses to pathogens", 787-799.

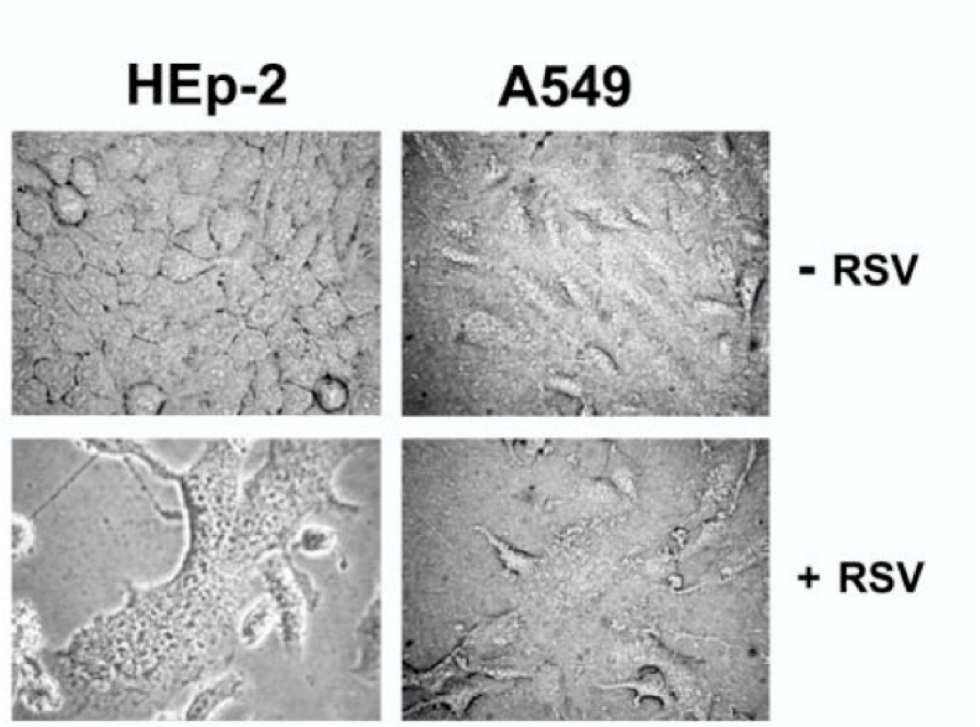



**Figure 3. Syncytia formation in RSV-infected HEp-2 and A549 epithelial cell lines.** A549 and HEp-2 cells were inoculated with RSV Long strain at MOI 3 and incubated at 37°C for 48 hours. Syncytia formation (cell-cell fusion) is a hallmark cytopathic effect (CPE) observed during RSV infection of epithelial cells, and is mediated by the viral F protein. Adapted with permission from BioMed Central Publications. *BMC Microbiology*, Vol. 3, V. Bitko *et al.,* "Profilin is required for viral morphogenesis, syncytium formation, and cell-specific stress fiber induction by respiratory syncytial virus", copyright 2002.

# Chapter 2 – Materials and Methods

## 2.1 Cell Lines

HMC-1 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS; Medicorp, Montreal, QC; Invitrogen; Sigma-Aldrich, Oakville, ON), 1% L-glutamine (Thermo-Fisher Scientific, Nepean, ON), 100U/mL penicillin G and 100 μg/mL streptomycin (Invitrogen). HMC-1 cells were maintained at a concentration between $0.2x10^6$ and $1.5x10^6$ cells/mL. HEp-2 cells were maintained in Roswell Park Memorial Institute (RMPI)-1640 medium (Sigma-Aldrich; Invitrogen) with 5% fetal calf serum (FCS; Thermo-Fisher Scientific). A549 cells were grown in RPMI-1640 supplemented with 10% FCS.

## 2.2 Primary Mast Cell Cultures

Human CBMCs were derived from umbilical cord blood obtained from subjects undergoing caesarian sections, as permitted by Izaac Walter Killam (IWK) Health Centre research ethics boards (Halifax, NS). Cells were cultured as described by Saito *et al* (Saito *et al.*, 1996). 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 20% FBS, 10mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Invitrogen), 100 U/mL of penicillin G, 1 μg/mL of streptomycin, 20% skin fibroblast culture supernatant as a source of IL-6 (CCL-204; Amerian Type Culture Collection [ATCC], Manassas, VA), $1x10^{-7}$ M prostaglandin $E_2$ ($PGE_2$; Sigma-Aldrich), and 75 ng/mL SCF (Peprotech, Rocky Hill, NJ). Cultures used for experiments were at least 90% viable and greater than 95% pure mast cells. Mast cell purity was determined using toluidine blue (pH 1.0) metachromatic staining of cytocentrifuged preparations. Prior to use in experiments, CBMCs were cultured overnight in normal culture medium devoid of $PGE_2$ with SCF at a concentration of 10 ng/mL.

## 2.3 Primary Mast Cell Sorting

CBMC cultures were sorted using fluorescence-acquired cell sorting (FACS) based on side scatter (SSC) to separate highly granulated mast cells (high SSC) from non mast cells (low SSC). For sorting procedures, cells were sorted by Sandy Edgar at the IWK Health Centre using FACSAria III cell sorter (BD Bioscience, Mississauga, ON). In brief, CBMCs were resuspended at $1x10^7$ cells/mL in sorting buffer (0.5% bovine serum albumin (BSA; Sigma-Aldrich), 15mM HEPES in PBS) and filtered through 40 μm nylon cell strainer to assure a single cell suspension. Cells were sorted in sterile conditions at 4°C and collected in 0.2 μm-filtered FBS. After sorting, cells were centrifuged at 200 x g for 10 minutes at 4°C. The cells were resuspended at $1x10^6$ cells/mL in RMPI-1640 supplemented with 20% FBS, 10mM HEPES, 100 U/mL of penicillin G, 1 μg/mL of streptomycin, 20% skin fibroblast culture supernatant as a source of IL-6, $1x10^{-7}$ M $PGE_2$, and 75 ng/mL SCF, and incubated at 37°C for 1 week. Cytocentrifuge preparations of cells prior to and after sorting were collected and stained with toluidine blue (pH 1.0) to determine mast cell purity. Prior to use in experiments, CBMCs were cultured overnight in culture medium devoid of $PGE_2$ with SCF at a concentration of 10 ng/mL.

## 2.4 RSV Propagation

RSV (Long strain) was used to infect 80% 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 FCS. Once the cell monolayer exhibited 70-80% fusion, the monolayer was disrupted by scraping. The supernatant was clarified from cellular debris by centrifugation at 4°C for 10 minutes at 2000 x g, and was used for each experiment. For UV-inactivation, an undiluted aliquot of clarified supernatant was exposed to a germicidal lamp at a distance of 10 cm for 15 minutes.

## 2.5 RSV Plaque Assay and Titration

HEp-2 cells were used as a reference cell line to determine viral titer, in plaque forming units (PFU)/mL, and multiplicity of infection (MOI) conditions. Serial dilutions

of virus were adsorbed to HEp-2 cells in a 24-well plate for 90 minutes at 4°C, washed and incubated at 37°C for 30 hours in RPMI-1640 with 2.5% FCS. The RSV infected monolayers were fixed with methanol for 2 minutes and incubated in phosphate-buffered saline (PBS; Invitrogen) at 4°C until the staining procedure. The cells were permeabilized with TNP (20 mM Tris, 0.1% Nonident P-40 (Roche; Mississauga ON), 0.8% NaCl in distilled water, pH 7.5) for 10 minutes and incubated in a 1:200 dilution of pooled human serum in blocking buffer (20 mM Tris-HCl (pH 7.5), 7% normal goat serum (R&D Systems, Minneapolis, MN), 2% BSA, 0.8% NaCl, 0.02% $NaN_3$ in distilled water) for 30 minutes at room temperature on a rocker. The HEp-2 cells were rinsed with TNP and incubated in a 1:1000 dilution of alkaline-phosphatase-conjugated goat anti-human antibody (GAH-AP) in blocking buffer for 30 minutes at room temperature on a rocker. The cells were washed with TNP and incubated in alkaline phosphatase (AP) buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris in distilled water, pH 9.5) for 5 minutes at room temperature on a rocker. Plaques were stained with 1-step nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP/NBT; Thermo-Fisher Scientific) and monitored through a microscope. Following a rinse with water, the immunostained monolayers were incubated with AP stop solution (0.5M EDTA in distilled water) for 5 minutes at room temperature, rinsed with water again and dried. The dilution of virus giving rise to 100-200 plaques following staining was used to determine viral titer (PFU/mL), which was calculated using the formula: number of plaques / (volume of virus inoculum added to well (mL) x dilution factor).

**2.6 Inoculation Conditions**

RSV alone, or mixed with Palivizumab (Synagis®; Abbot, QC) or human IgG at 10 μg/mL, was incubated at 4°C for 30 minutes. Cells were inoculated with RSV (with or without Palivizumab or IgG), UV-inactivated RSV or medium (RPMI with 2.5% FCS) for 90 minutes at 4°C to allow for virus adsorption to cells. One million CBMCs were inoculated at MOI 3-4, and $0.5x10^6$ HMC-1 cells at MOI 6-8 unless otherwise specified. A549 and HEp-2 cells (approximately $2x10^5$ cells in 24 well tissue culture plates purchased from Fisher Scientific) were inoculated at various MOIs as indicated. Fresh RPMI with 2.5% FCS was used as medium control. Following adsorption cells were

washed and incubated at 37°C for various time points in RPMI-1640 with 2.5% FCS (containing 10 ng/mL SCF for CBMCs). CBMCs were incubated at a concentration of $1x10^6$ cells/mL, HMC-1 cells at $0.5x10^6$ cells/mL and A549 and HEp-2 cells at a concentration of $0.2x10^6$ cells/mL. Where indicated, HMC-1 cells were incubated in the presence of B18R (eBioscience) at various concentrations to neutralize type I IFNs. Following incubation, supernatants were harvested and stored at -80°C and the cells prepared for flow cytometry or RNA extraction.

**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). Fc receptors were blocked with 50 μg/mL of total rabbit IgG (Dako; Burlington, ON) and cells were incubated for 25 minutes on ice with APC-conjugated anti-human CD117 monoclonal antibody (mAb) (eBioscience; San Diego, CA) at a final concentration of 1 μg/mL. APC-conjugated mouse IgG1 (eBioscience), at the same concentration, was used as an isotype control. Cells were washed, and fixed with 1% PFA in PBS. For intracellular RSV antigen staining, fixed cells were permeabilized in 0.1% saponin (Sigma-Aldrich) in FACS buffer for 30 minutes on ice. Following permeabilization, cells were incubated for 25 minutes with biotin-conjugated goat anti-RSV serum (Meridian Life Sciences, Saco, ME) or biotin-labeled goat IgG (Jackson Immunoresearch, West Grove, PA) at a final concentration of 5 μg/mL. The cells were washed and incubated with streptavidin-conjugated PE (SA-PE; eBioscience) at a concentration of 0.5 μg/mL for 25 minutes. Cells were rinsed, fixed in 1% PFA in PBS, and stored at 4°C, and acquired using a FACSCalibur (BD Bioscience). Analysis was performed using FCS Express 3 (*Denovo* Software, Los Angeles, CA). Markers used to quantify the percentage of RSV antigen expressing cells included less than 0.1% of istotype antibody-stained CBMC samples and less than 0.5% of isotype antibody-stained HMC-1, A549 and HEp-2 samples.

### 2.8 Enzyme-linked Immunosorbent Assay (ELISA)

Sandwich ELISAs were used to quantify protein levels in cell supernatants. Polystyrene-based ninety six-well microtiter plates, purchased from Fisher Scientific, were coated with capture antibody (50 µL per well) diluted to working concentration (Table 1) in bicarbonate buffer (0.05M NaCl, 0.01M $NaHCO_3$ in $H_2O$, pH 8.5). Plates containing coating solution were sealed with parafilm (Picheny Plastic Packaging Company, Chicago, IL) and incubated at 4°C overnight. Wells of coated plates were washed 3 times with 200 µL of PBS-T buffer (0.02% Tween-20 (Sigma-Aldrich) in PBS), and incubated for 1 hour 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 curves were added to duplicate wells and plates were incubated at 4°C overnight. Wells were then washed and incubated with 50 µL of detection antibody, diluted to working concentration (Table 1) in 0.02% BSA in PBS, for 1 hour at room temperature. ELISA plates were developed using the Invitrogen ELISA Amplification System according to the manufacturer's protocol. Data were analyzed using Softmax Pro (Molecular Devices, Sunnyvale, CA). All samples and standards were diluted in RPMI-1640 containing 2.5% FCS.

### 2.9 Chemokine Antibody Array

Chemokines in supernatants from CBMCs incubated with RSVor medium control were quantified using Chemokine Antibody Array I (RayBiotech, Norcross, GA) according to manufacturer's protocol. Five hundred µL of supernatant were diluted 1 in 2 with RPMI-1640 containing 2.5% FCS prior to addition to the membrane. Membranes were then processed according to the manufacturer's instructions. Membranes were exposed to Fujifilm Super RX Medical X-ray Film. Signal intensity on developed films was analyzed using Quantity One Software (Bio-Rad Laboratories, Mississauga, ON). Following background subtraction, intensities of internal positive controls on each membrane were normalized to allow for the comparison between individual spots. Signals were deemed detectable if spot densities following background subtraction exceeded 10% of negative control. The fold increases in spot intensity between

RSV/Medium membranes were analyzed and signals that were increased or decreased more than 2-fold in response to RSV were considered significant.

### 2.10 Quantitative Polymerase Chain Reaction (qPCR)

Following incubation for 12 hours, cells were lysed in RLT buffer with 3% 2-mercaptoethanol (2-ME) and RNA was extracted using the RNeasy Plus Mini Kit purchased from Qiagen (Mississauga, ON) 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 at an annealing/extension temperature of 60°C. Validated primers for CXCL10, CCL5, MxA, ISG56, IL-28b, IL-29, IFN-α1 and IFN-β were used with GAPDH as a control (SABiosciences, Qiagen). qPCR reactions were performed in duplicate using the MX-3000P QPCR System and relative gene expression was analyzed using MxPro Software (Stratagene, La Jolla, CA). Fold induction of gene expression in response to RSV treatment as compared with medium control was calculated using the formula $(\text{Efficiency}_{\text{Target Gene}})^{\Delta Ct\ (\text{Mean medium - treatment})} / (\text{Efficiency}_{\text{Reference Gene}})^{\Delta Ct\ (\text{Mean medium - treatment})}$. To compare the levels of target gene expression between mast cells and a known system, Sendai virus (SeV) infected A549 cells were utilized as a reference. SeV infection of A549 cells has been shown to induce the expression of IFN-α, IFN- β, IL-28B and IL-29 (Matikainen *et al.*, 2006). Briefly, A549 cells were inoculated with 100 hemaglutinating units/mL of SeV (Cantell Strain; Charles River, Wilmington, MA). The virus was adsorbed to A549 cells for 2 hours at 37°C, and following adsorption, cells were washed and incubated for 12 hours at 37°C. RNA was harvested and cDNA generated Dr. Christine King as described above.

### 2.11 Statistical Analysis

For experiments where 6 or more donors/repeats were assessed, the data were first tested for normality using a D'Agostino's-Pearson test. Normally distributed data were further analyzed using a repeated-measures one-way analysis of variance (ANOVA),

followed by Bonferroni's post test to compare differences between specific groups. Data not normally distributed were analyzed using a Friedman test, followed by a Dunn multiple comparison test to compare differences between specific groups. For experiments where less than 6 donors/cultures were examined, the data were analyzed using a repeated-measures one-way ANOVA. Specific groups were compared using a Bonferroni post test. Multiple groups were compared with medium control using a Dunnet post-test. P values greater than 0.05 ($p<0.05$) were considered significant. All figures represent the mean ± the standard error of the mean (SEM). All statistical analyses were performed using Prism 4 (GraphPad Software Inc).

**Table 1. List of capture and detection antibodies and kits used for ELISAs.**

| ELISA | Capture Antibody | | Detection Antibody | |
|---|---|---|---|---|
| | **Specifications** | **Concentration** | **Specifications** | **Concentration** |
| CXCL10 | Anti-CXCL10 mAb, clone 4D5 (BD Bioscience) | 2 μg/mL | Biotin-labeled anti-CXCL10 mAb, clone 6D4/D6/G2 (BD Bioscience) | 0.1 μg/mL |
| CCL4 | Anti-CCL4 mAb, clone 24006 (R&D) | 1 μg/mL | Biotin-labeled anti-CCL4 affinity purified pAb (R&D) | 50 ng/mL |
| CCL5 | Anti-CCL5 mAb, clone VL-1 (Endogen, Woburn, MA) | 1 μg/mL | Biotin-labeled goat anti-CCL5 pAb (R&D) | 0.1 μg/mL |
| CCL11 | Antigen-affinity purified rabbit anti-hEotaxin [1] | 0.5 μg/mL | Biotin-labeled antigen-affinity purified rabbit anti-hEotaxin [1] | 0.25 μg/mL |
| IFN-γ | Human IFN-γ ELISA MAX TM Capture Antibody [2] | 1X | Human IFN-γ ELISA MAX TM Detection Antibody [2] | 1X |
| TNF | Human TNF-α ELISA MAX TM Capture Antibody [3] | 1X | Human TNF-α ELISA MAX TM Detection Antibody [3] | 1X |
| IFN-α2 | Purified sheep anti-human IFN-α pAb (Endogen) | 0.5 μg/mL | Biotin-labeled mouse anti-human IFN-α2 mAb, clone M710B (Thermo Scientific) | 125 ng/mL |

[1] Part of human Eotaxin/CCL11 ELISA Development Kit (Peprotech)

[2] Part of IFN- γ ELISA Max Deluxe Set (BioLegned, San Diego, CA)

[3] Part of TNF ELISA Max Deluxe Set (BioLegned)

# Chapter 3 – Results

## CBMCs support RSV antigen expression in response to infectious virus as shown by flow cytometry

Mast cells are prominently located in vascularized tissues of the skin, gastrointestinal tract and airways which closely interface the environment. Mast cells are therefore amongst the first immune cells to interact with pathogens at body surfaces (Galli *et al.*, 2005; Marshall, 2004). To address whether primary human mast cells can support RSV infection, the intracellular expression of RSV antigen in CBMCs was quantified by flow cytometry. For this purpose, CBMCs were inoculated with infectious RSV (MOI 3-4), UV-inactivated RSV or treated with a medium control and incubated for 24 hours at 37°C. Following incubation, the cells were fixed, permeabilized and stained for viral antigen expression using goat anti-RSV serum (Figure 4). Gates that were used to quantify the percentage of RSV antigen expressing cells included less than 0.1% of normal goat serum stained cells. The percentage of cells expressing RSV antigen was higher in CBMCs inoculated with infectious virus as compared with cells treated with UV-inactivated RSV (Figure 4) or RSV pretreated with palivizumab, a neutralizing monoclonal anti-RSV antibody (data not shown). The proportion of cells with detected viral antigen expression after live virus inoculation varied between the CBMC cultures, but was consistently low (between 0.2% and 1.3% of cells). The use of gates offers a stringent and consistent method for assessing the percentage of RSV antigen expressing cells. However, it is notable that other analyses such as histogram cumulative subtraction could have also been used. The data presented in Figure 4 were obtained from a single CBMC donor. However, similar responses were observed using cells from 3 different donors. These data highlight that CBMCs are capable of supporting RSV antigen expression.

## RSV antigen expression is detected in mature CD117$^{hi}$ –expressing CBMCs

We next aimed to identify whether or not mature mast cells supported viral antigen expression. Cell sorting was performed on CBMC cultures to isolate mature mast

cells from contaminating cells using side scatter for highly granulated cells. Sorted CBMCs were inoculated with RSV, UV-inactivated RSV or medium control and incubated for 24 hours at 37°C. Following incubation, CBMCs were stained with APC-conjugated anti-CD117 antibody (FL4 channel) which was used as a mast cell marker. Goat anti-RSV serum (FL2 channel) was used to stain intracellular RSV antigen (Figure 5). Viral antigen expression was detected only in response to live virus and not UV-inactivated virus in approximately 2% of CBMCs from this particular donor. Furthermore, the intracellular expression of RSV proteins co-localized with CD117 expression by mature mast cells (upper right quadrant), thus indicating that mature mast cells can support RSV infection. Notably, the percentage of CD117 expressing mast cells was higher in CBMCs treated with live RSV (approximately 92%) than CBMCs treated with UV-inactivated or medium controls (approximately 80%). The data in Figure 5 represent a single CBMC culture, similar results were observed using cells from a second donor These data demonstrate that a subset of primary, mature CD117-expressing mast cells are capable of supporting RSV antigen expression.

**CBMCs produce various chemokines in response to RSV**

Chemokines play an important role in recruiting effector cells during inflammation, and several chemokines are known to be up-regulated during RSV disease (Bueno *et al.*, 2011). Several studies have shown that mast cells are able to produce chemokines in response to viruses that can aid in the recruitment of activated T-lymphocytes and NK cells (Burke *et al.*, 2008; Kulka *et al.*, 2004). Chemokine antibody arrays were used as a screening tool to assess mast cell chemokine production following RSV stimulation. Forty-eight hour supernatants from RSV- and mock-inoculated CBMCs were applied to separate membranes. Following development, spot density analysis was used to quantify the relative expression of each chemokine in response to RSV as compared with medium. Signals were considered detectable if spot densities, following background subtraction, were greater than 10% of the negative control. The fold increases in spot intensity between RSV- and medium-treated membranes were analyzed and signals that were increased or decreased more than 2-fold in response to RSV were considered significant (Figure 6; Table 2). CBMCs incubated with RSV produced higher

levels of CCL2 (5.0 fold), CCL4 (2.7 fold), CXCL1 (3.9 fold), CXCL8 (2.1 fold) and CXCL10 (3.4 fold) over mock medium control.

More specifically, we examined the production of CCL4, CCL5 and CXCL10 in response to RSV in the absence and presence of palivizumab. These chemokines are implicated in RSV disease and are essential for the recruitment of activated $CD4^+$ and $CD8^+$ T-lymphocytes, NK cells as well as eosinophils (Lindell *et al.*, 2008; Taub *et al.*, 1993; Tekkanat *et al.*, 2002). CBMCs were stimulated with medium alone, infectious RSV, UV-inactivated RSV or RSV pretreated with either 10 μg/mL of palivizumab or human IgG control (Figure 7). Supernatants were harvested at 24 and 48 hours post-infection, and chemokine levels were quantified by ELISA. CBMCs inoculated with RSV produced significantly higher amounts of CXCL10 (over 1000 pg/mL) at 24 hours as compared to cells stimulated with medium alone. Also, CXCL10 production was significantly diminished in response to UV-inactivated RSV and RSV pretreated with palivizumab as compared with RSV treatment. The chemokine levels detected in CBMC supernatants at 48 hours were greatly diminished, but a similar trend in chemokine production was observed. Similarly, CCL4 production was significantly elevated in cells treated with RSV as compared with medium treated cells at 24 hours. RSV pretreatment with palivizumab or UV-inactivation elicited a diminished CCL4 response as compared with infectious virus treatment alone. CCL5 production was also elevated by CBMCs in response to RSV as compared with UV-killed RSV and palivizumab-pretreated RSV. However, the measured amounts of CCL5 were significantly lower than CCL4 and CXCL10 in response to RSV. Notably, pretreatment of RSV with human IgG resulted in an elevated CCL4 and CCL5 production as compared with RSV alone, and the levels of both chemokines were also diminished by 48 hours.

Time course analysis of mediator production by CBMCs in response to RSV was performed. Cells from a single CBMC culture were inoculated with RSV, medium and UV-inactivated RSV and the levels of CCL4, CCL5, CXCL10 and TNF were measured by ELISA at various time points over 24 hours (Figure 8). An increase in the production of CCL4, CCL5 and CXCL10 was observed in response to infectious RSV, but not UV-inactivated virus or medium controls. CXCL10 and CCL4 levels started to rise sharply after 6 hours, and continued to peak until 24 hours. CCL5 production was detectable at

later time points, after 18 hours, and reached much lower concentrations as compared with CXCL10 and CCL4 in response to RSV. Notably, no TNF was produced by CBMCs, as background levels were detected in CBMC supernatants treated with medium, live and UV-inactivated virus. It is possible that the chemokine levels measured in CBMC supernatants may have been reduced by degradation due to the lack of effective protease inhibition during cell incubation. These data demonstrate that primary human mast cells selectively produce a variety of pro-inflammatory chemokines in response to stimulation with infectious RSV.

**CBMCs do not produce CCL11 or IFN-γ in response to RSV**

Elevated levels of CCL11 (eotaxin -1) are detected in nasopharengeal secretions from children with severe RSV bronchiolitis and animal models have showed that CCL11 antibody blockade ameliorates disease severity (Matthews *et al.*, 2005). We therefore investigated whether CBMCs produce CCL11 after inoculation with RSV (Figure 9B). Background levels of CCL11 were detected by ELISA in 24 and 48 hour supernatants from CBMCs stimulated with medium, UV-killed RSV and RSV in the presence and absence of palivizumab or IgG. This indicates that CBMCs do not produce CCL11 when inoculated with RSV. The cytokine IFN-γ plays an essential role in the host antiviral response by promoting the activation and recruitment of NK cells and up-regulating MHC-I expression (Zeng *et al.*, 2011). IFN- γ can be produced by mast cells (Gupta *et al.*, 1996). Upon investigation, we found that mast cells do not produce IFN-γ following RSV stimulation (Figure 9A) at 24 or 48 hours.

**RSV stimulation of primary human mast cells induces the production of type I and III IFNs and ISGs**

Mast cells produce type I IFNs in response to viral infection or stimulation with viral products, which are important for combating viral infection (Kulka *et al.*, 2004). Therefore we examined whether CBMCs produce IFN-α in response to RSV (Figure 10). Supernatants from infectious-, UV-inactivated-RSV and medium-stimulated CBMCs were harvested at 48 hours and the levels of IFN-α2 were measured by ELISA. In comparison to medium control, RSV-inoculated CBMCs produced elevated levels of

IFN-α2 that did not reach statistical significance. Background levels of IFN-α2 were produced by CBMCs in response to UV-inactivated RSV. We were interested in measuring the relative gene expression of type I and III IFNs, as well as various ISGs by CBMCs in response to RSV treatment. CBMCs from 2 different donors were sorted to isolate mature mast cells and inoculated with infectious RSV or medium control. Following a 12 hour incubation period, the cells were harvested, lysed and RNA was extracted. The relative expression levels of ISG56, MxA, IFN-α1, IFN-β, IL-28B, IL-29, CXCL10 and CCL5 in response to RSV as compared with medium were quantified by qPCR. SeV infection of A549 cells is known to induce the expression of IFN-α, IFN-β, IL-28 and IL-29 (Matikainen *et al.*, 2006). Therefore, the relative induction of the target genes in RSV-inoculated CBMCs treatment was also compared in reference to SeV-inoculated A549 cells. GAPDH was used as control/reference gene. The data presented in Table 3 show that CBMCs from two different donors express higher levels of ISG56, MxA, IFN-α1, IFN-β, IL-28B, IL-29, CXCL10 and CCL5 in response to RSV treatment as compared with medium control. In comparison to SeV-infected A549 cells, the gene expression of IFN-α1, CXCL10, MxA and ISG56 was particularly elevated in CBMCs from both donors in response to RSV. Notably, IL-29, IL-28B and CCL5 expressed by RSV-inoculated CBMC was substantially lower in reference to SeV-infected A549 cells. These data highlight that mast cells express type I and III IFNs in response to RSV. These cytokines are capable of inducing various ISGs, such as MxA, ISG56 and CXCL10, which have direct and indirect antiviral effects.

**HMC-1 cells support RSV antigen expression**

To examine whether the HMC-1 cell line supports RSV antigen expression, intracellular staining was performed to measure viral antigen expression by flow cytometry at 24 hours post-inoculation. Low levels of viral antigen expressing cells (4.3%) were measured in response to infectious RSV (MOI 6-8), but not UV-inactivated RSV or medium (Figure 11). The data provided in Table 4 show dose-dependent expression of viral antigen by HMC-1 cells stimulated with RSV at various MOI levels. There were no significant differences in the percentage of RSV-antigen positive cells at

MOI 6-8 and MOI 3-4 (3.7 %). However, lower levels of virus antigen-expressing cells were detected at MOI 1.5-2 (2.8 %) and MOI 0.6-0.8 (2.3 %).

**RSV stimulation of HMC-1 cells induces the expression of several chemokines and IFN-stimulated genes**

The production of CCL4, CCL5 and CXCL10 in response to RSV by HMC-1 cells was also investigated. Cells inoculated with various doses of RSV (MOI of 6-8, 3-4, 1.5-2 and 0.6-0.8), UV-inactivated RSV and medium controls were incubated for 24 hours, and chemokine levels were quantified by ELISA. The data in Figure 12 show that HMC-1 cells produce CCL4, CCL5 and CXCL10 in a dose-dependent manner following RSV stimulation. HMC-1 cells produced higher levels of CCL4, CCL5 and CXCL10 in response to RSV as compared with medium control and UV-inactivated RSV controls at all MOIs tested, but these differences only reached significance at MOI 6-8 and 3-4. These data suggest that HMC-1 cells produce chemokines and express viral antigen in a dose-dependent manner following RSV inoculation.

We were also interested in measuring the relative gene expression of type I and III IFNs and various ISGs by RSV-stimulated HMC-1 cells. HMC-1 cells were inoculated with infectious RSV or medium control and incubated for 12 hours. Following incubation, the cells were harvested, lysed and RNA was extracted. The HMC-1 expression of ISG56, MxA, IFN-α1, IFN-β, IL-28B, IL-29, CXCL10 and CCL5 in response to RSV relative to medium and SeV-infected A549 cells was quantified by qPCR (Table 5). RSV treatment of HMC-1 cells induced higher levels of ISG56, MxA, IFN-α1, IFN-β, IL-28B, IL-29, CXCL10 and CCL5 expression as compared with medium control. However, in comparison to SeV-infected A549 cells, the expression of ISG56, MxA, IFN-α1, IFN-β, IL-28B, IL-29 and CCL5 in RSV-inoculated HMC-1 cells was at least ten-fold lower, whereas CXCL10 expression was approximately four-fold lower. These data indicate that HMC-1 cells express type I and III IFNs and various ISGs in response to RSV.

**B18R-mediated type I IFN blockade has no effect on chemokine production or antigen expression in RSV stimulated HMC-1 cells**

IFN-α/β is known to induce the expression of chemokines such as CXCL10 and CCL2 (Megjugorac *et al.*, 2004). To assess the effect of type I IFNs on chemokine production by HMC-1 cells in response to RSV, IFN-blockade experiments were performed. HMC-1 cells were stimulated with infectious RSV and incubated for 24 hours in the presence of various concentrations of B18R (1 ng/mL, 10 ng/mL and 100 ng/mL), an inhibitor of type I IFNs. B18R is a soluble protein encoded by vaccinia virus that shares a significant degree of homology with type I IFN receptors. By acting as a decoy receptor, B18R neutralizes type I IFNs preventing their receptor binding and biological activity (Colamonici *et al.*, 1995; Symons *et al.*, 1995). B18R treatment of RSV-inoculated HMC-1 cells had no significant effect on CCL4, CCL5 or CXCL10 production as compared with HMC-1 cells stimulated with RSV in the absence of B18R (Figure 13).

It is widely acknowledged that IFN-α/β stimulation induces an antiviral state, rendering cells resistant to viral infection. We therefore assessed whether blocking type I IFNs affected RSV antigen expression by HMC-1 cells (Figure 14). HMC-1 cells were stimulated with infectious RSV and incubated for 24 hours in the presence of various concentrations of B18R (1 ng/mL, 10 ng/mL and 100 ng/mL). Type I IFN blockade on RSV-inoculated HMC-1 cells had no significant effect on the percentage of viral antigen-expressing cells at any concentration of B18R.

The use of B18R to block the effects of type I IFN had no effect on viral antigen expression or chemokine expression by RSV-stimulated HMC-1 cells. These data indicate that RSV-induced mediator production by HMC-1 cells may occur in an type I IFN-independent manner.

**Higher levels of viral antigen-expressing A549 and HEp-2 epithelial cells are measured in response to RSV as compared with CBMC and HMC-1 cells**

The airway epithelium is at the forefront of the innate immune response to RSV, as it constitutes the first site of interaction between the virus and the host. Consequently, epithelial cell responses to RSV are well documented (Bueno *et al.*, 2011). In order to

compare epithelial and mast cell responses to RSV, viral antigen expression in RSV-infected epithelial cells was measured by flow cytometry (Figure 15). HEp-2 and A549 epithelial cells were inoculated with RSV (MOI 3.5-4.5), UV-RSV or medium and incubated for 24 hours. Following incubation cells were stained with goat anti-RSV serum to detect intracellular RSV antigen expression. The gates used to assess the percentage of RSV antigen expressing cells included less than 0.5% of isotype antibody-stained cells. High levels of RSV antigen expressing-cells were detected in HEp-2 (21.2 %) and A549 (17.6 %) cells in response to infectious RSV, but not UV-inactivated RSV or medium controls. These data indicate that A549 and HEp-2 epithelial cells are more capable of supporting RSV antigen expression as compared with CBMC or HMC-1 cells inoculated at a similar MOI.

**RSV-infected A549 and HEp-2 epithelial cells produce CCL4, CCL5 and CXCL10, but not CCL11, in response to RSV**

Airway epithelial cells produce a myriad of chemokines in response to RSV infection including CCL2, CCL3, CCL5, CXCL8 and CXCL10 (Bueno *et al.*, 2011). In mouse models of RSV infection, CCL11 production is abrogated with the depletion of $CD4^+$ T-lymphocytes, suggesting that the CCL11 is produced by T-lymphocytes and not epithelial cells (Johnson *et al.*, 2008). To compare epithelial and mast cell chemokine responses to RSV, the production of CCL4, CCL5, CCL11 and CXCL10 by A549 and HEp-2 cells in response to various doses of the virus was examined. Cells were treated with UV-inactivated RSV and medium controls and varying doses of RSV (MOIs of 15-18, 7.5-9 and 3.5-4.5). Chemokine levels in cell supernatants were quantified using ELISA at 6 and 24 hours. HEp-2 cells produced significantly higher levels of CCL5 in a dose dependent manner in response to RSV at 6 hours. Background levels of CCL4 and CXCL10 production were detected at this time point. At 24 hours post infection, HEp-2 cells produced significantly higher levels of CCL4, CCL5 and CXCL10 in response to RSV at all MOIs as compared with medium. The expression of CCL5 by HEp-2 cells was remarkably higher than CCL4 and CXCL10 (reaching approximately 12000 pg/mL) in response to RSV at 24 hours. The specific concentration of each chemokine did not vary greatly in response to different doses of RSV (Figure 16). Similarly, A549 cells produced

background levels of CXCL10 and CCL4 at 6 hours, but significantly elevated levels of CCL5 in response to RSV at all MOIs tested (Figure 17). CCL5 levels in response to RSV at this time point increased in a dose-dependent manner. Significantly elevated levels of CCL4, CCL5 and CXCL10 were measured at 24 hours following stimulation with infectious RSV as compared with medium control. The measured levels of CCL4 and CXCL10 were increased in response to infectious RSV in a dose-dependent manner. Interestingly, CCL5 production in response to RSV was highest at MOI 7.5-9. In comparison, CBMCs (Figure 7) and HMC-1 cells (Figure 12) produced much lower levels of CCL5 than CCL4 or CXCL10 in response to RSV. Conversely, HEp-2 and A549 cells produced substantially higher levels of CCL5 than CCL4 or CXCL10 in response to RSV.

However, similarly to CBMCs (Figure 9), background levels of CCL11 were detected in HEp-2 and A549 cell supernatants at 6 and 24 hours in response to all doses of RSV, reaffirming the finding that epithelial cells do not produce CCL11 following RSV stimulation.

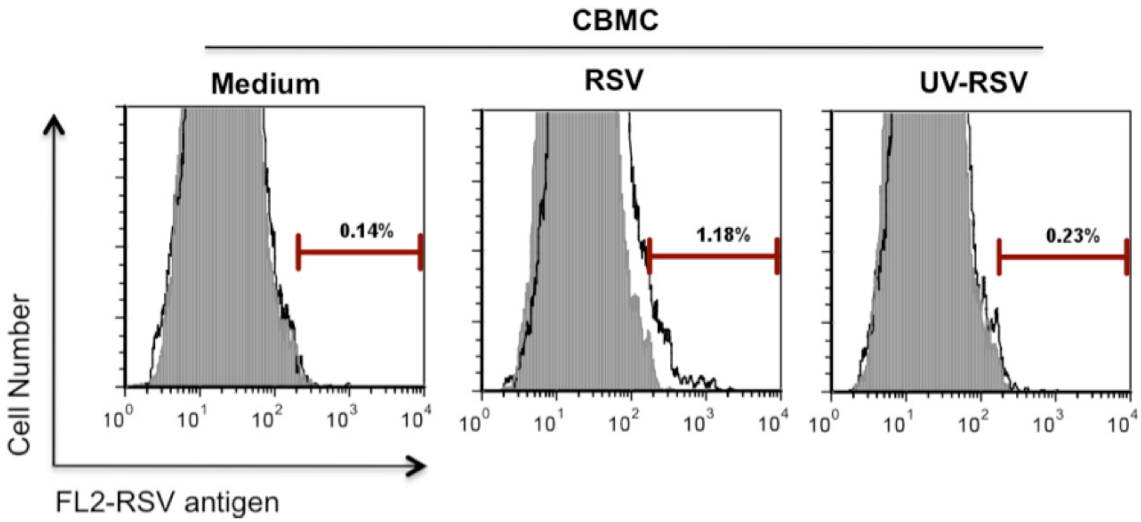
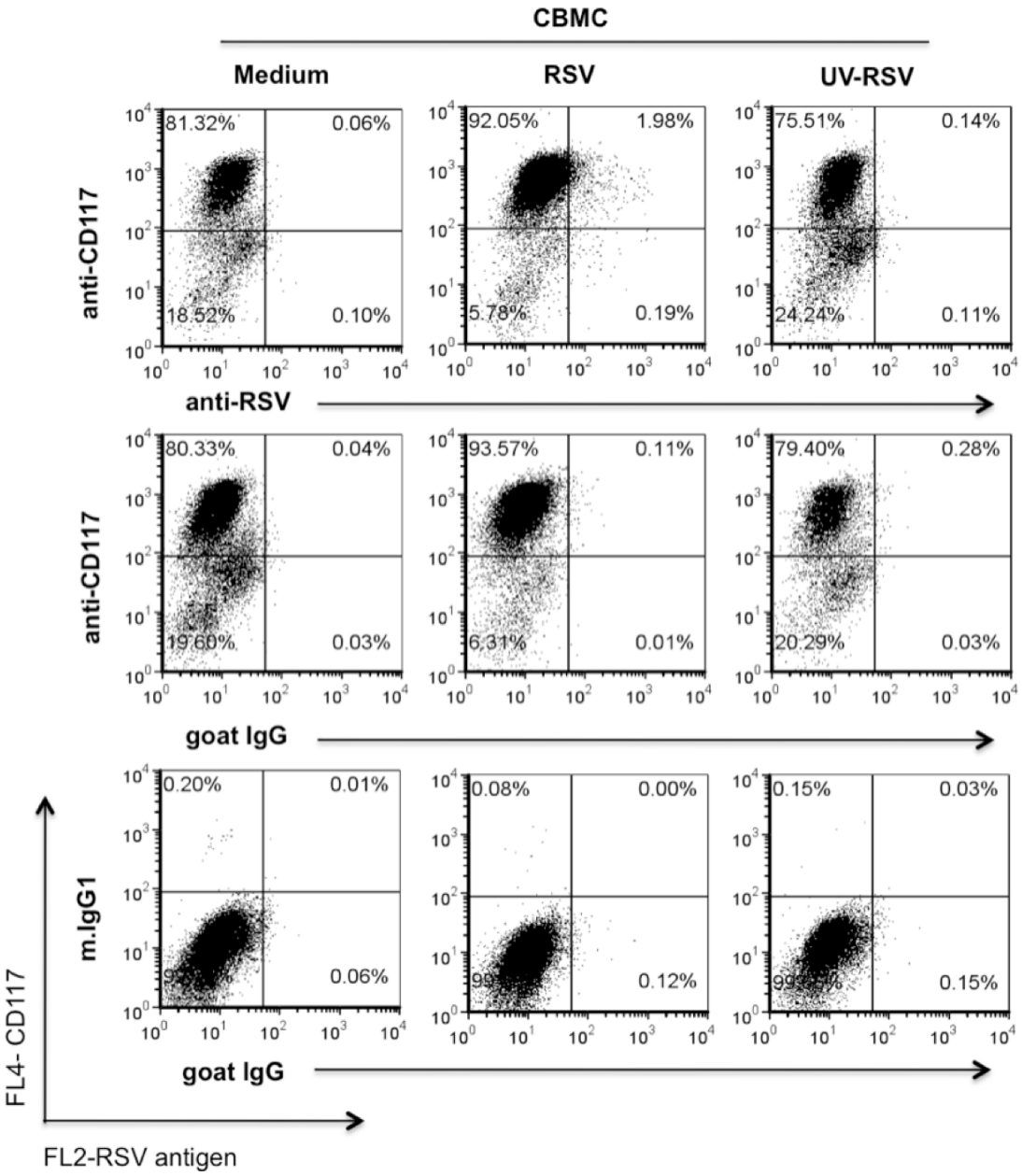



**Figure 4. CBMCs express intracellular RSV antigen only in response to infectious RSV.** CBMC ($1.0x10^6$ cells/mL) were inoculated with RSV, UV-inactivated RSV or treated with medium as a control. Following incubation for 24 hours at 37°C, cells were fixed, permeabilized and intracellular RSV antigen expression was measured by flow cytometry. Shaded histograms represent cells stained with goat IgG conrol; non-shaded histograms represent cells stained with goat anti-RSV serum (FL2 channel). Markers encompass less that 0.1% of isotype stained cells; percentage values depict RSV antigen positive cells stained with goat anti-RSV serum. Plots represent a single CBMC donor. Similar responses were observed with CBMCs from 3 other donors.

**Figure 5. RSV antigen is expressed by CD117$^{hi}$ mast cells in response to infectious, but not UV-inactivated virus.** Cells from a single CBMC culture were sorted to isolate mature mast cells, and incubated in the presence of SCF at 37°C for one week. Sorted CBMCs were then stimulated with infectious RSV, UV-RSV or medium control and were incubated for 24 hours (at $1.0x10^6$ cells/mL). Cells were stained for CD117 and intracellular RSV antigen expression using an APC-conjugated anti-CD117 mAb (FL4 channel) and goat anti-RSV serum (FL2 channel). Mouse IgG1 (m.IgG1) and goat IgG antibodies were used as isotype controls respectively. Flow cytometric plots are representative of 2 separate experiments using two different CBMC cultures.


CBMC
Medium
RSV
UV-RSV
anti-CD117
anti-RSV
81.32%
0.06%
18.52%
0.10%
92.05%
1.98%
5.78%
0.19%
75.51%
0.14%
24.24%
0.11%
anti-CD117
goat IgG
80.33%
0.04%
19.60%
0.03%
93.57%
0.11%
6.31%
0.01%
79.40%
0.28%
20.29%
0.03%
m.IgG1
goat IgG
0.20%
0.01%
0.06%
0.08%
0.00%
0.12%
0.15%
0.03%
0.15%
FL4- CD117
FL2-RSV antigen

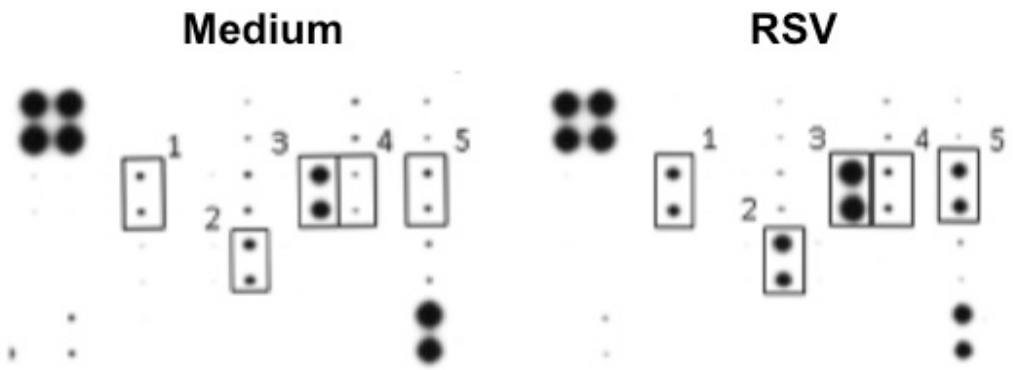


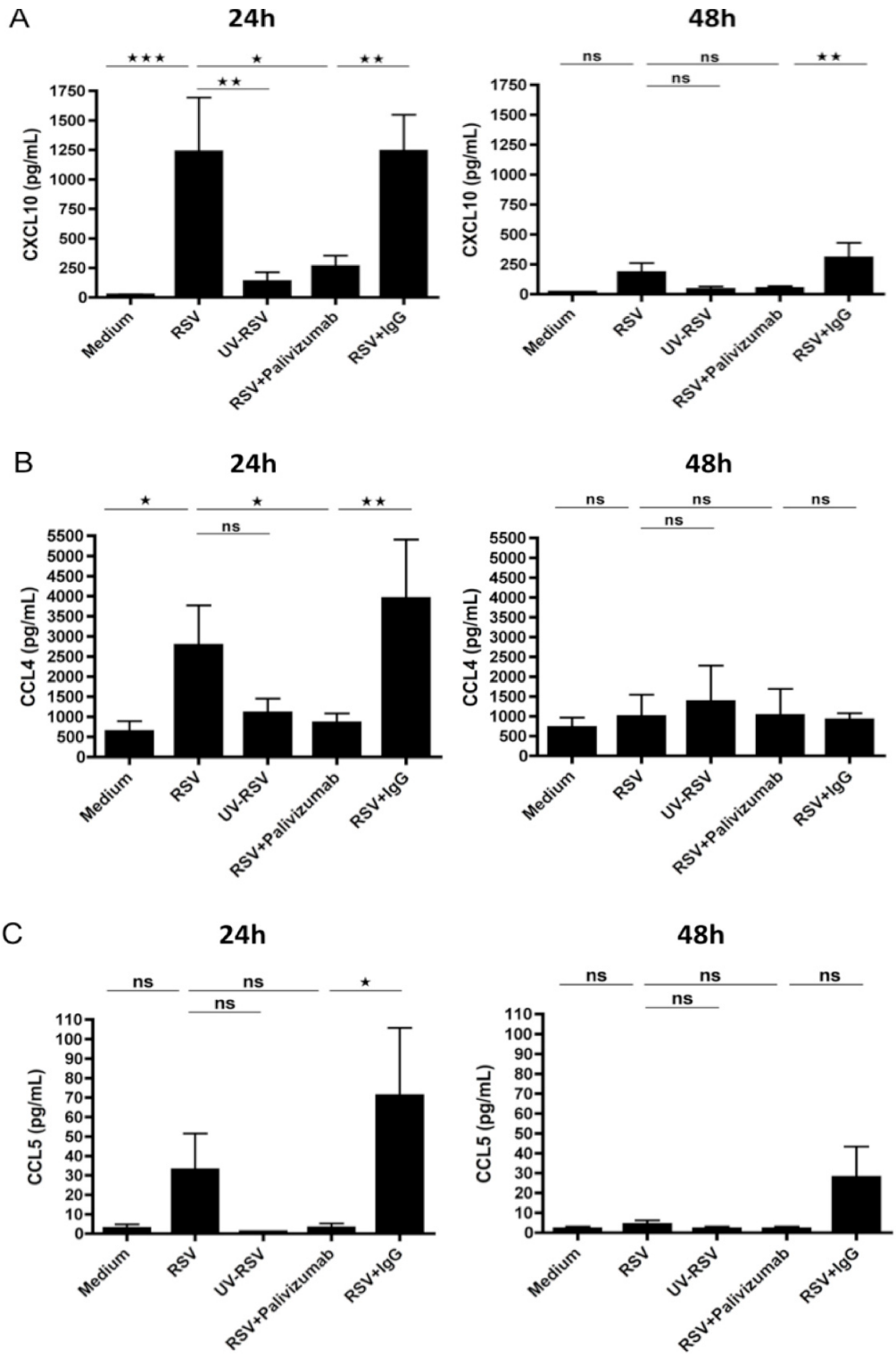

**Figure 6. CBMCs up-regulate the expression of several chemokines in response to RSV.** CBMCs ($1.0x10^6$ cells/mL) from a single donor stimulated with infectious RSV or fresh medium as a control. The cells were washed and incubated at 37°C for 48 hours. CBMC supernatants were harvested and applied to separate chemokine antibody array membranes, which were developed and scanned. Spot densities were analyzed using Quantity One Software and normalized between membranes. Boxes depict duplicate spots for chemokines at least 2 fold higher in supernatants from RSV-stimulated CBMCs compared to medium control. [1]CXCL1; [2]CCL4; [3]CXCL8; [4]CXCL10; and [5]CCL2.

**Table 2. Normalized spot densities for chemokine antibody array on supernatants from mock- and RSV-stimulated CBMCs**

| Chemokine | Sensitivity (pg/mL) | Normalized Spot Density | |
|---|---|---|---|
| | | Medium | RSV |
| XCL1 (Lymphotactin) | 1000000 | 6429 | 10837 |
| CX3CL1 (Fractalkine) | 1600 | 67536 | 54531 |
| CCL1 (I-309) | 1000 | 505204 | 329257 |
| **CCL2 (MCP-1)** | **1** | **608247** | **3032697** |
| CCL3 (MIP-1α) | 20 | 107929 | 90346 |
| **CCL4 (MIP-1β)** | **10** | **1155119** | **3158656** |
| CCL5 (RANTES) | 2000 | 388428 | 210457 |
| CCL7 (MCP-3) | 1000 | -5619 | 2015 |
| CCL8 (MCP-2) | 100 | -2974 | 52978 |
| CCL9/10 (MIP-1δ) | 100 | 45663 | 115477 |
| CCL11 (Eotaxin) | 1 | 25798 | 14024 |
| CCL13 (MCP-4) | 100 | 13214 | 6949 |
| CCL16 (HCC-4) | 1000 | 98010 | 53290 |
| CCL17 (TARC) | 100 | 7133 | 5026 |
| CCL18 (PARC) | 1000 | 2366 | 30738 |
| CCL19 (MIP-3β) | 1000 | 26171 | 13758 |
| CCL20 (MIP-3α) | 100 | 20220 | 16701 |
| CCL22 (MDC) | 1000 | 101326 | 56020 |
| CCL23 (Ckb8-1) | 1000 | 214998 | 188866 |
| CCL23 (MPIF-1) | 1000 | 28995 | 13990 |
| CCL24 (Eotaxin-2) | 1 | 214592 | 194756 |
| CCL25 (TECK) | 10000 | 21979 | 8910 |
| CCL26 (Eotaxin-3) | 320 | 112878 | 98976 |
| CCL27 (CTACK ) | 100 | 32870 | 31279 |
| CCL28 | 100000 | 38263 | 10175 |
| CXCL1 (GROα) | 1000 | 62144 | 73100 |
| **CXCL1/2/3 (GRO)** | **α-1000, β-1000, γ-1** | **480146** | **1854109** |
| CXCL5 (ENA-78) | 1 | 380810 | 326143 |
| CXCL6 (GCP-2) | 100 | 34353 | 37348 |
| CXCL7 (NAP 2) | 100 | 352022 | 238367 |
| **CXCL8 (IL-8)** | **1** | **3211331** | **6796924** |
| CXCL9 (MIG) | 1 | 37708 | 24561 |
| **CXCL10 (IP-10)** | **10** | **229673** | **791735** |
| CXCL11 (I-TAC) | 10000 | 54006 | 74772 |
| CXCL12α (SDF-1α) | 2000 | 19335 | 48335 |
| CXCL12β (SDF-1β) | 2000 | 25356 | 25030 |
| CXCL13 (BLC) | 10 | 37854 | 25341 |
| CXCL16 | 1000 | -20331 | -32451 |

1. **Bolded text**: Signals >10% of negative (>152332) and ≥ 2-fold change.

**Figure 7. CBMCs produce elevated levels of CXCL10, CCL4 and CCL5 in response to RSV.** CBMCs ($1.0x10^6$ cells/mL) were inoculated with RSV with or without Palivizumab or human IgG, UV-inactivated RSV or medium for 24 and 48 hours. Supernatants were harvested and the levels of CXCL10 (A), CCL4 (B) and CCL5 (C) were measured by ELISA. CXCL10 data represent 9 different CBMC cultures (inoculated at MOI 3-8). Data for CCL4 and CCL5 represent 4 different CBMC cultures (inoculated at MOI 3-4). Graphs represent the mean ± SEM; $^{***}p<0.001$; $^{**}p<0.01$; $^{*}p<0.05$; ns=not statistically significant.


A
24h
48h
CXCL10 (pg/mL)
B
CCL4 (pg/mL)
C
CCL5 (pg/mL)
Medium
RSV
UV-RSV
RSV+Palivizumab
RSV+IgG
ns

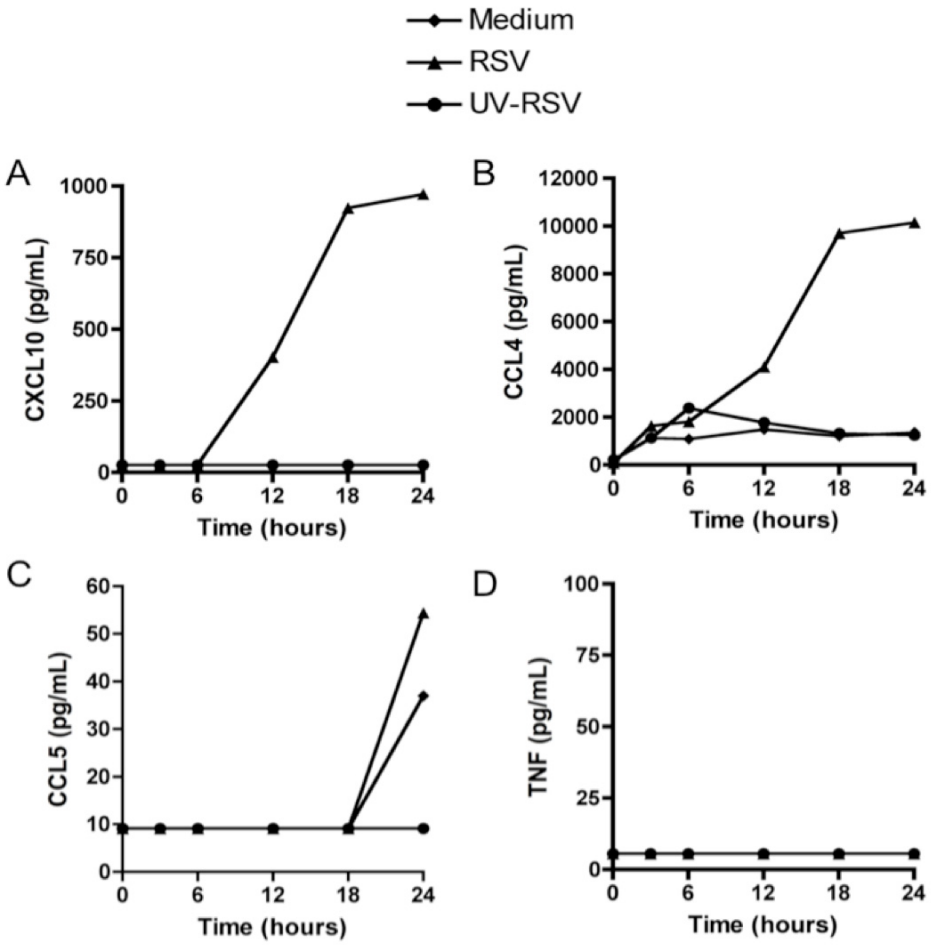



**Figure 8. Time course mediator production profile by CBMCs in response to RSV.** CBMCs ($1.0x10^6$ cells/mL) from a single donor were cultured with medium, UV-RSV and infectious RSV. Supernatants were harvested at various time points and the levels of CXCL10, CCL4, CCL5 and TNF were measured by ELISA. Graphs represent the levels of various chemokines produced.

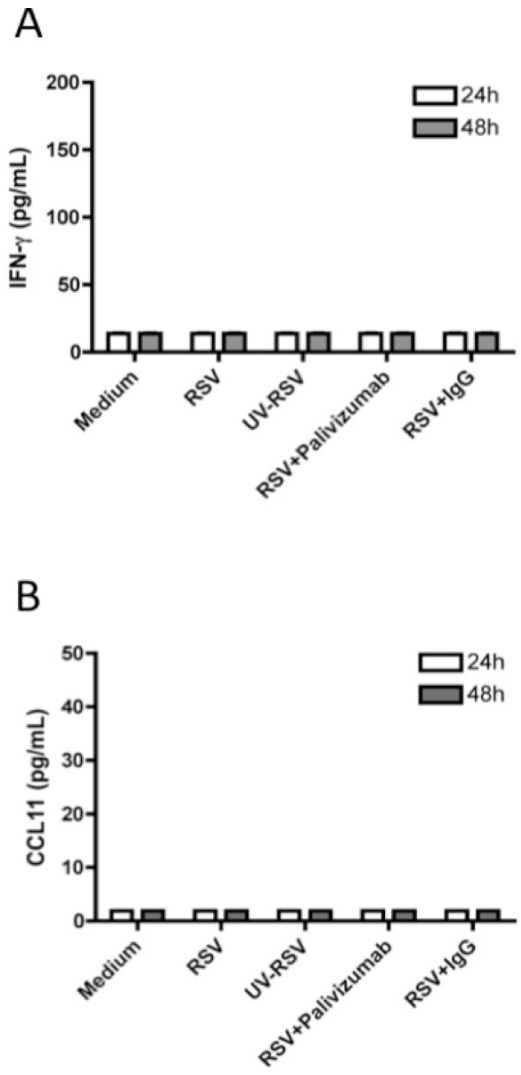



**Figure 9. CBMCs stimulated with RSV do not produce IFN-γ or CCL11**. CBMCs were treated with medium, UV-RSV and RSV with or without Palivizumab or human IgG and incubated for 24 and 48 hours ($1.0x10^6$ cells/mL). Graphs represent the mean ± SEM. The levels of IFN-γ and CCL11 in cell supernatants at 24 and 48 hours were quantified by ELISA. Data for IFN- γ represent 3 different CBMC cultures; data for CCL11 represent 2 different CBMC cultures. All values detected were at limit of detection (LD); LD IFN-γ=13.7 ± 1.0 pg/mL; LD CCL11=1.91 ± 0.0 pg/mL.

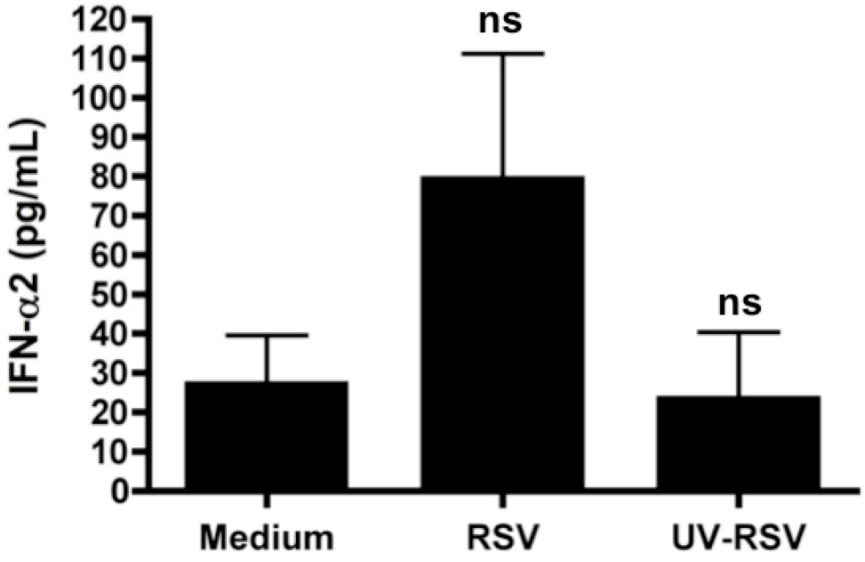



**Figure 10. CBMCs produce elevated levels of IFN-α2 in response to RSV**. CBMCs ($1.0x10^6$ cells/mL) were treated with medium, UV-RSV and incubated for 48 hours at 37°C. Graphs represent the mean ± SEM of 4 different CBMC donors. The levels of IFN-α2 in CBMC supernatants at 48 hours were quantified by ELISA. ns represents no statistical significance as compared with medium control.

**Table 3. CBMCs up-regulate expression of type I and III IFNs and various ISGs in response to RSV**

| Target Gene | Fold Induction CBMC-RSV / CBMC-Medium | | Fold Induction CBMC-RSV / SeV-A549 | |
|---|---|---|---|---|
| | Donor #1 | Donor #2 | Donor #1 | Donor #2 |
| IFN-α1 | 476.234 | 2466.300 | 7.766 | 78.663 |
| IFN-β1 | 150.907 | 3152.786 | 0.997 | 8.306 |
| IL-28B | 16.417 | 40.348 | 0.011 | 0.072 |
| IL-29 | 24.327 | 2500.221 | 0.037 | 0.706 |
| ISG56 | 759.922 | 796.179 | 2.932 | 10.489 |
| MxA | 3376.507 | 1455.662 | 13.907 | 67.048 |
| CXCL10 | 5056.784 | 88904.037 | 28.815 | 317.948 |
| CCL5 | 10.459 | 136.520 | 0.006 | 0.064 |

1. Sorted CBMCs (from 2 different CBMC cultures) stimulated with RSV or medium control were incubated for 12 hours at 37°C at a concentration of $1.0x10^6$ cells/mL. qPCR was used to measured the change in expression of various target genes in response to RSV as compared with medium-treated CBMCs, and expression relative to SeV-infected A549 cells.

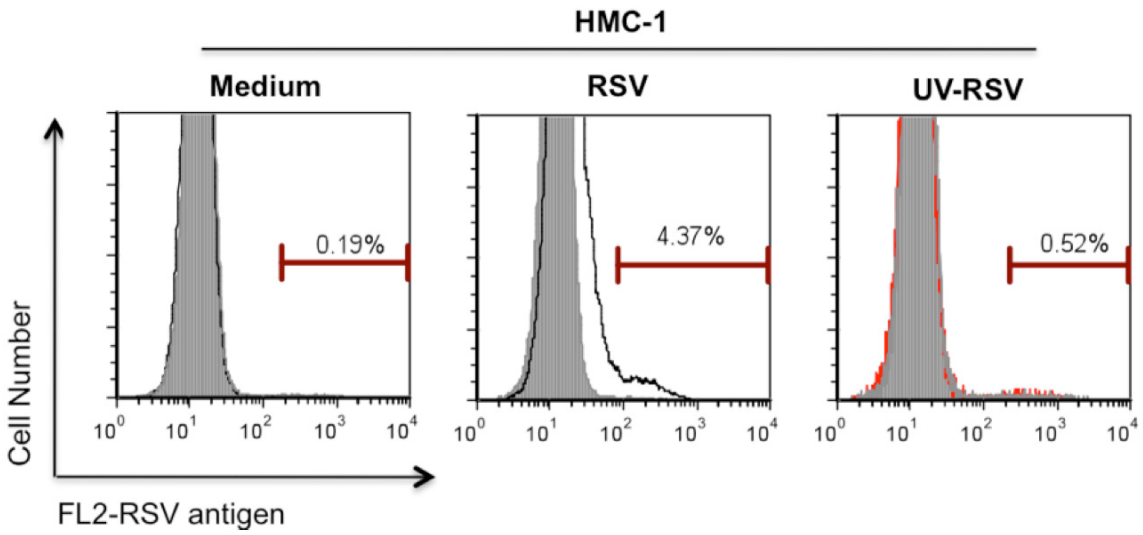



**Figure 11. HMC-1 cells express intracellular RSV antigen in response to infectious, but not UV-inactivated, RSV.** HMC-1 cells ($0.5x10^6$ cells/mL) stimulated with RSV, UV-inactivated RSV or medium control were incubated for 24 hours at 37°C. Following incubation cells were fixed and flow cytometry was used to quantify intracellular RSV antigen expression. Shaded histograms represent cells stained with normal goat IgG conrol; non-shaded histograms represent cells stained with goat anti-RSV serum (FL2 channel). Markers encompass less that 0.5% of isotype-stained cells; percentage values depict RSV antigen positive cells stained with goat anti-RSV serum. Data are representative of 4 independent experiments.

**Table 4. Intracellular RSV antigen expression by HMC-1 cells in response to various doses of RSV.**

| Treatment | HMC-1 (24 hours) |
|---|---|
| | RSV antigen +ve cells (%) |
| Medium | 0.3 ± 0.1 |
| RSV (MOI 6-8) | 3.7 ± 0.7 ** |
| RSV (MOI 3-4) | 3.7 ± 0.8 ** |
| RSV (MOI 1.5-2) | 2.8 ± 0.6 ** |
| RSV (MOI 0.6-0.8) | 2.3 ± 0.4 ** |
| UV-RSV | 0.4 ± 0.2 $^{ns}$ |

1. HMC-1 cells were stimulated with various doses of RSV, UV-inactivated RSV and medium and were incubated for 24 hours at 37°C (at $0.5x10^6$ cells/mL). Intracellular RSV antigen expression was measured by flow cytometry using an goat anti-RSV serum and goat IgG as a control.
2. Data are presented as mean ± SEM and are representative of 4 different experiments; ns=not statistically significant, **$p<0.01$ as compared with medium.

**Figure 12. HMC-1 cells produce various chemokines in a dose-dependent manner in response to RSV.** HMC-1 cells (approximately $0.5x10^6$ cells/mL) were inoculated with various doses of RSV, UV-inactivated RSV or medium control for 90 minutes at 4°C, and were cultured for 24 hours at 37°C. Following incubation cell supernatants were harvested and the concentration of CXCL10 (A), CCL4 (B) and CCL5 (C) were measured by ELISA. Data are pooled from 4 independent experiments and presented as the mean ± SEM. $^{\star\star}p<0.01$; $^{\star}p<0.05$ and ns=no statistical significance as compared with medium control.

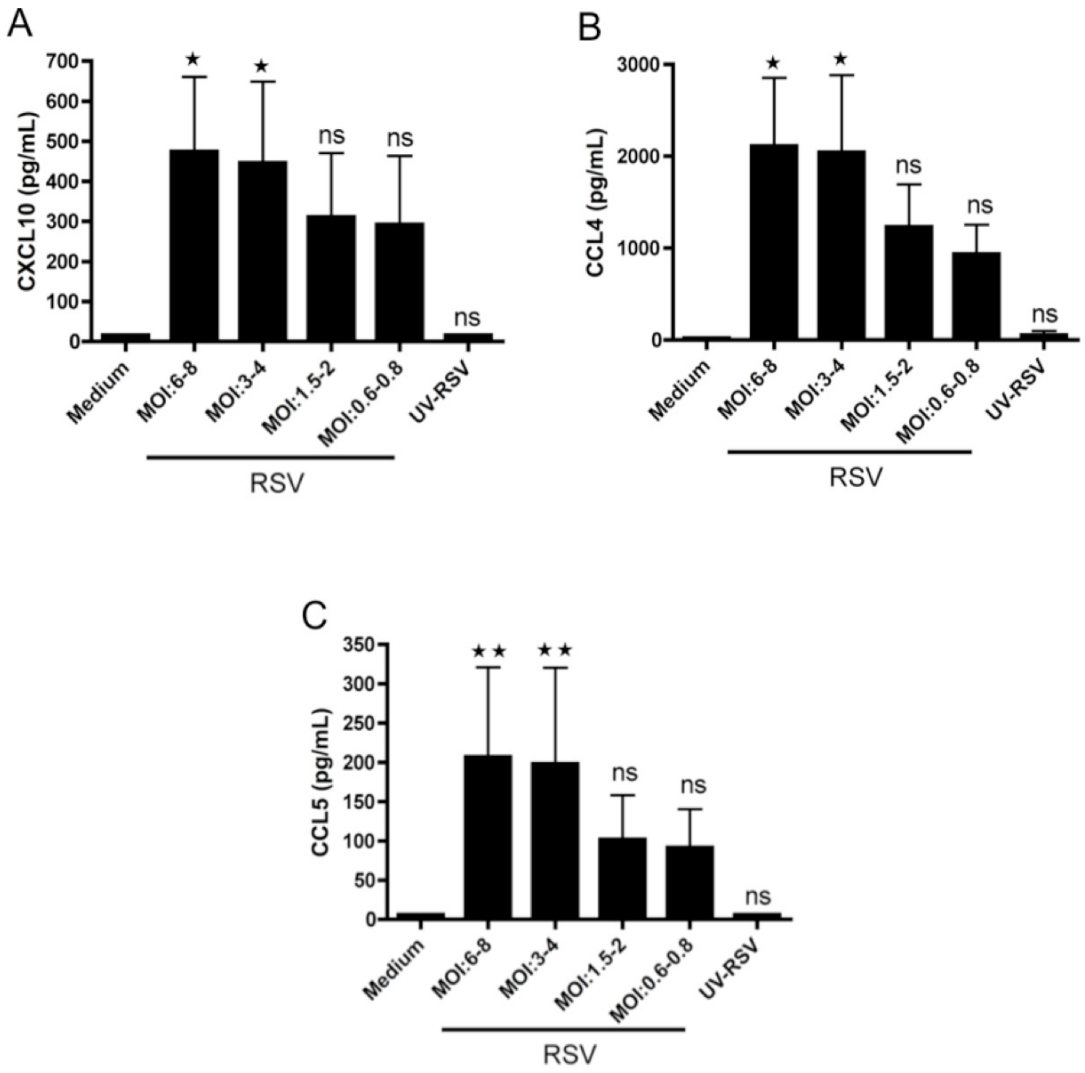
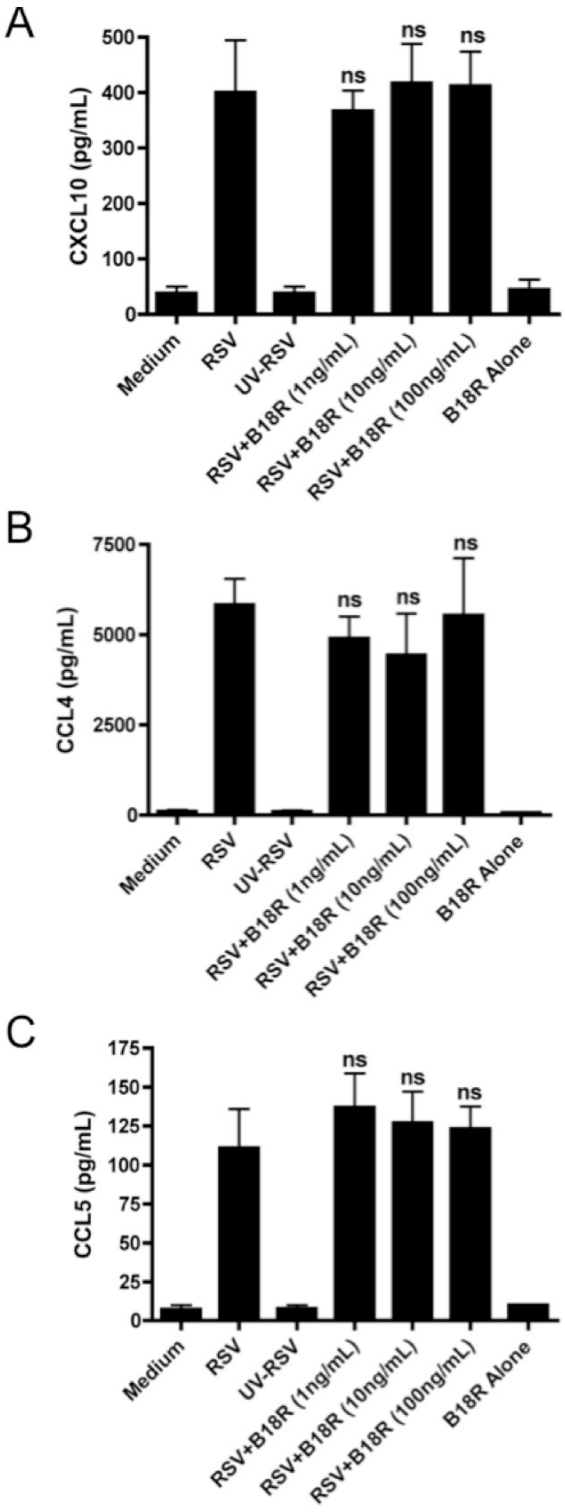

A
CXCL10 (pg/mL)
700
600
500
400
300
200
100
0
★
★
ns
ns
ns
Medium
MOI:6-8
MOI:3-4
MOI:1.5-2
MOI:0.6-0.8
UV-RSV
RSV
B
CCL4 (pg/mL)
3000
2000
1000
0
★
★
ns
ns
ns
Medium
MOI:6-8
MOI:3-4
MOI:1.5-2
MOI:0.6-0.8
UV-RSV
RSV
C
CCL5 (pg/mL)
350
300
250
200
150
100
50
0
★★
★★
ns
ns
ns
Medium
MOI:6-8
MOI:3-4
MOI:1.5-2
MOI:0.6-0.8
UV-RSV
RSV

**Table 5. HMC-1 cells up-regulate the expression of type I and III IFNs and various ISGs in response to RSV**

| Target Gene | Fold Induction HMC-1-RSV / HMC-1-Medium | Fold Induction HMC-1-RSV / SeV-A549 |
|---|---|---|
| IFN-α1 | 2.821 ± 1.735 | 0.006 ± 0.002 |
| IFN-β1 | 92.040 ± 42.640 | 0.065 ± 0.022 |
| IL-28B | 73.980 ± 63.620 | 0.001 ± 0.000 |
| IL-29 | 78.580 ± 20.730 | 0.005 ± 0.003 |
| ISG56 | 14.280 ± 1.901 | 0.098 ± 0.039 |
| MxA | 1.619 ± 0.331 | 0.001 ± 0.000 |
| CXCL10 | 89.840 ± 51.710 | 0.234 ± 0.079 |
| CCL5 | 102.700 ± 95.030 | 0.001 ± 0.000 |

1. HMC-1 cells stimulated with RSV or medium control were incubated for 12 hours at 37°C at a concentration of $0.5x10^6$ cells/mL. qPCR was used to measured the change in expression of various target genes in response to RSV as compared with medium-treated HMC-1 cells, and expression relative to SeV-infected A549 cells.
2. Data are presented as mean ± SEM and are representative of 3 different experiments.

**Figure 13. B18R treatment has no significant effect on chemokine production by RSV-treated HMC-1 cells.** HMC-1 cells ($0.5x10^6$ cells/mL) were stimulated with medium, UV-inactivated RSV or infectious RSV in the presence and absence of various concentrations of B18R. Supernatants were harvested following 24 hours of incubation and the levels of various chemokines were quantified by ELISA. Graphs represent the mean ± SEM from 3 different experiments, except for B18R alone condition (n=2); ns=no statistical significance when compared with RSV treatment.


A
CXCL10 (pg/mL)
500
400
300
200
100
0
ns
ns
ns
Medium
RSV
UV-RSV
RSV+B18R (1ng/mL)
RSV+B18R (10ng/mL)
RSV+B18R (100ng/mL)
B18R Alone
B
CCL4 (pg/mL)
7500
5000
2500
0
ns
ns
ns
Medium
RSV
UV-RSV
RSV+B18R (1ng/mL)
RSV+B18R (10ng/mL)
RSV+B18R (100ng/mL)
B18R Alone
C
CCL5 (pg/mL)
175
150
125
100
75
50
25
0
ns
ns
ns
Medium
RSV
UV-RSV
RSV+B18R (1ng/mL)
RSV+B18R (10ng/mL)
RSV+B18R (100ng/mL)
B18R Alone

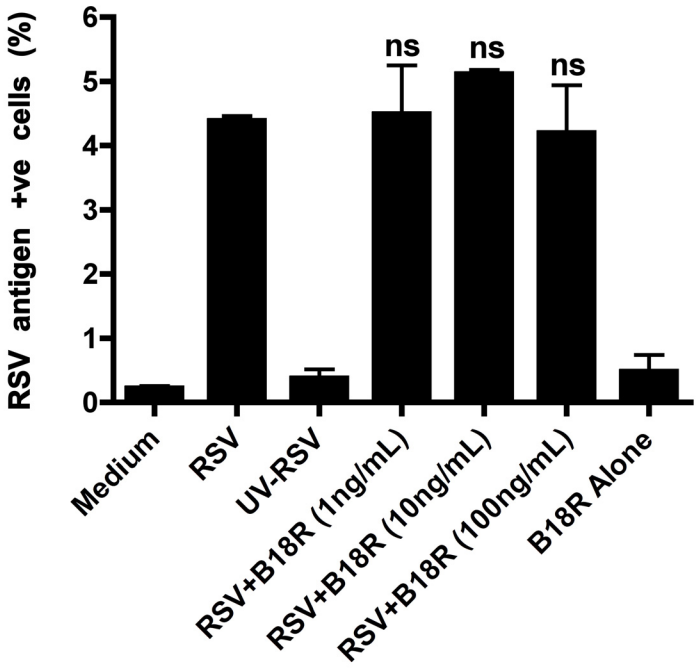



**Figure 14. Type I IFN blockade does not affect the percentage RSV antigen-expressing HMC-1 cells.** HMC-1 cells ($0.5x10^6$ cells/mL) were treated with medium, UV-inactivated RSV or RSV in the presence or absence of various concentrations of B18R. Following 24 hours of incubation cells were harvested and fixed. Intracellular RSV antigen expression was quantified by flow cytometry using biotin-conjugated anti-RSV serum and normal goat IgG control as a control. Data are pooled from 3 independent experiments and presented as the mean ± SEM; ns=no statistical significance as compared with RSV treatment.

**Figure 15. A549 and HEp-2 cells show high levels of RSV antigen expressing cells at 24 hours.** HEp-2 and A549 cells (approximately $0.2x10^6$ cells/mL) were stimulated with RSV (MOI: 3.5-4.5), UV-inactivated RSV and medium control for 90 minutes at 4°C, and were cultured for 24 hours at 37°C. Following incubation cells were fixed and permeabilized and the intracellular level of RSV antigen was quantified by flow cytometry. Shaded histograms represent cells stained with normal goat IgG control; non-shaded histograms represent cells stained with goat anti-RSV serum (FL2 channel). Markers encompass less that 0.5% of isotype stained cells; percentage values depict RSV antigen positive cells stained with goat anti-RSV serum.

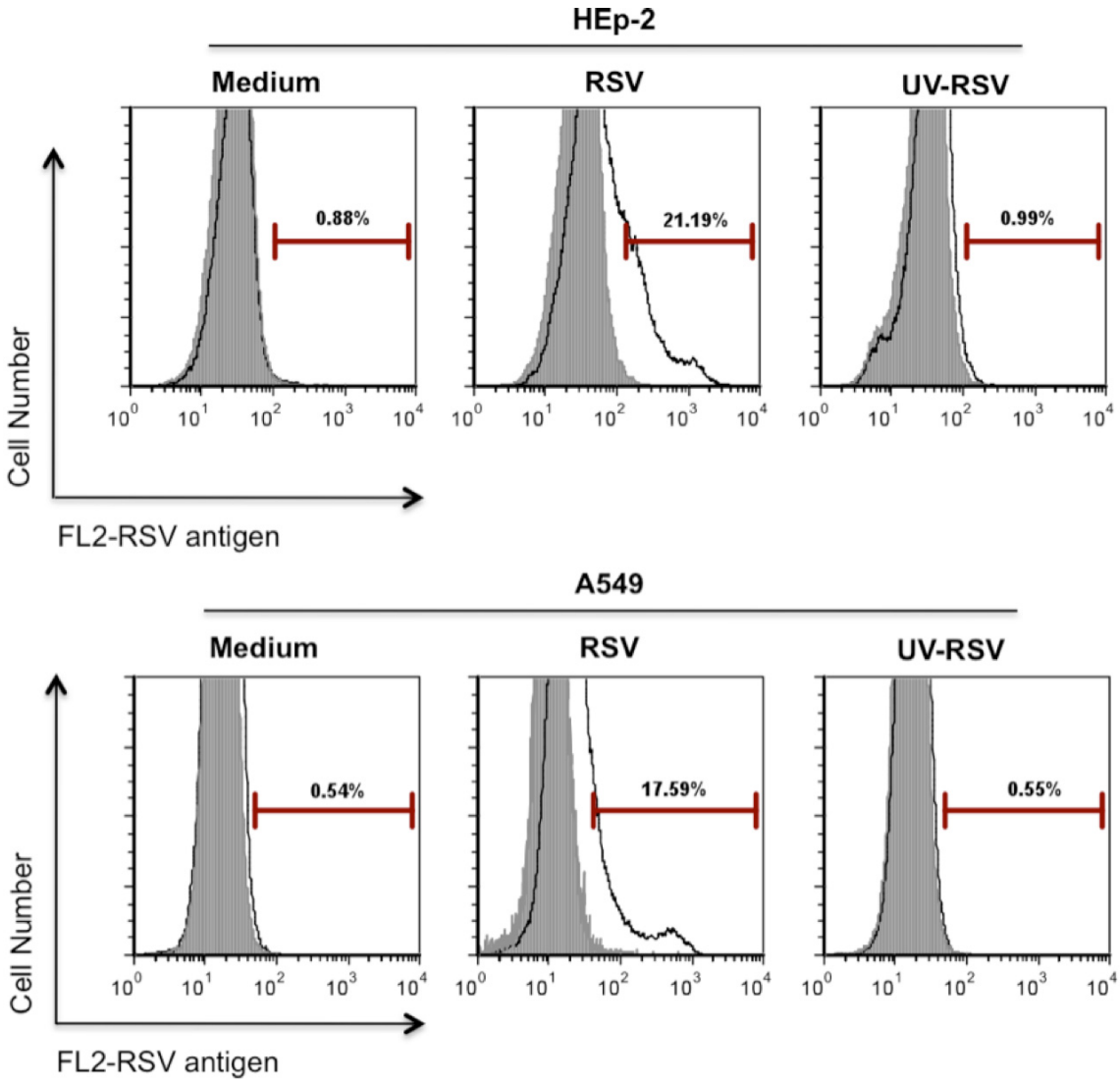
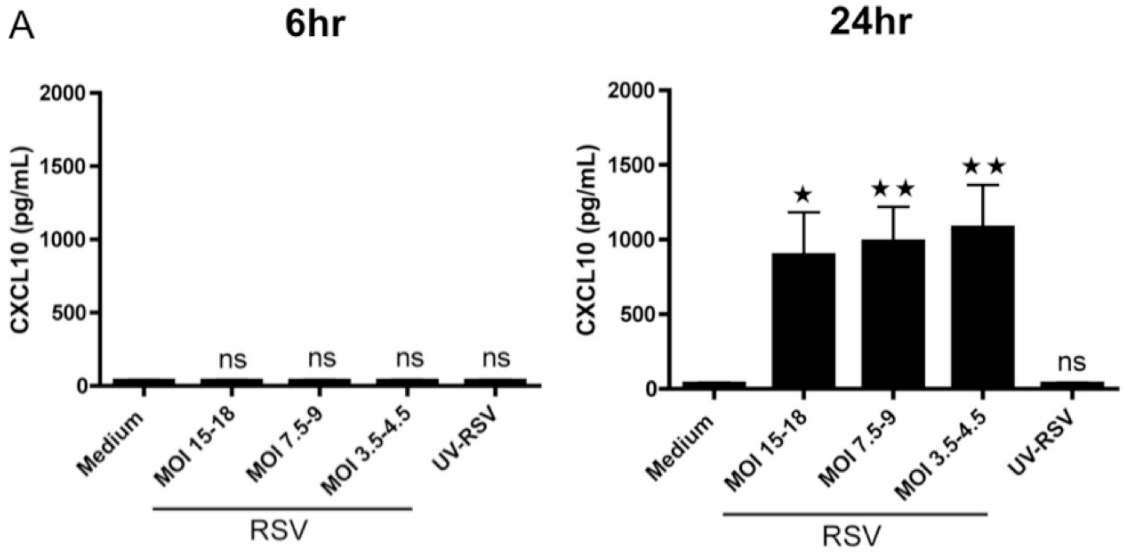
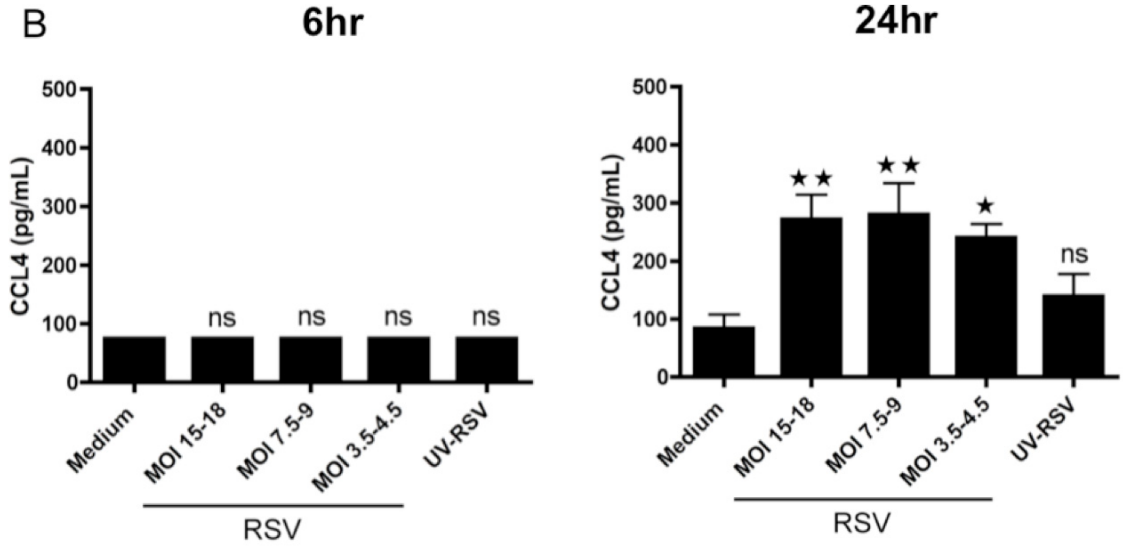

HEp-2
Medium
RSV
UV-RSV
0.88%
21.19%
0.99%
Cell Number
FL2-RSV antigen
A549
Medium
RSV
UV-RSV
0.54%
17.59%
0.55%
Cell Number
FL2-RSV antigen

**Figure 16. HEp-2 cells produce CXCL10, CCL4 and CCL5 in response to various doses of RSV.** HEp-2 cells (approximately $0.2x10^6$ cells/mL) were inoculated with RSV at various MOIs, UV-inactivated RSV or treated with medium control for 90 minutes at 4°C. Cells were cultured for 6 or 24 hours at 37°C. Following incubation, cell supernatants were harvested and the levels of CXCL10 (A), CCL4 (B) and CCL5 (C) were measured by ELISA. Graphs represent the mean ± SEM of 3 different experiments. $^{\star\star}p<0.01$; $^{\star}p<0.05$; ns=not statistically significant as compared with medium control.


A
6hr
24hr
CXCL10 (pg/mL)
2000
1500
1000
500
0
ns
ns
ns
ns
★
★★
★★
ns
Medium
MOI 15-18
MOI 7.5-9
MOI 3.5-4.5
UV-RSV
RSV


B
6hr
24hr
CCL4 (pg/mL)
500
400
300
200
100
0
ns
ns
ns
ns
★★
★★
★
ns
Medium
MOI 15-18
MOI 7.5-9
MOI 3.5-4.5
UV-RSV
RSV

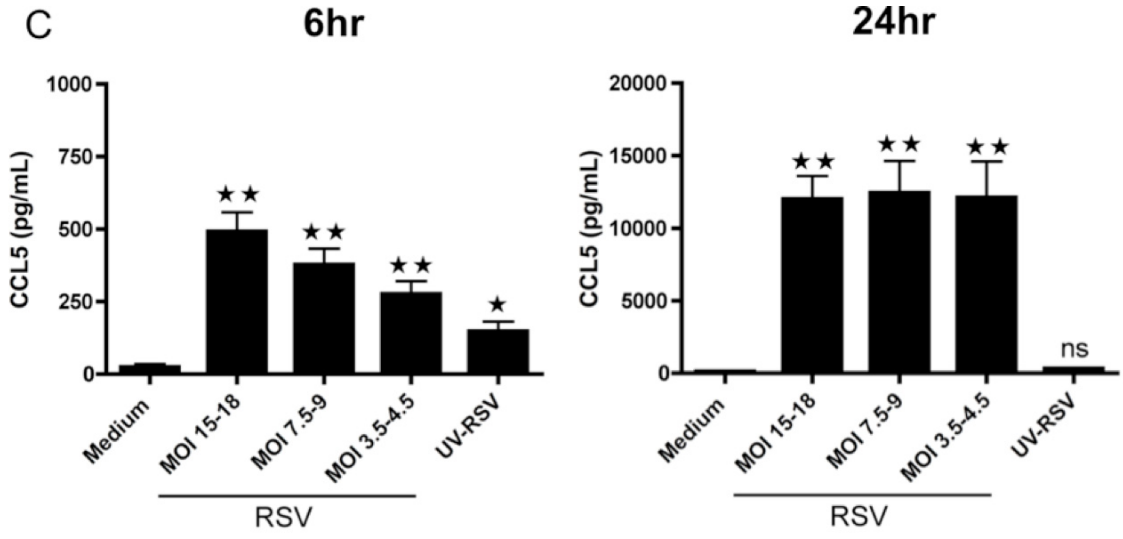
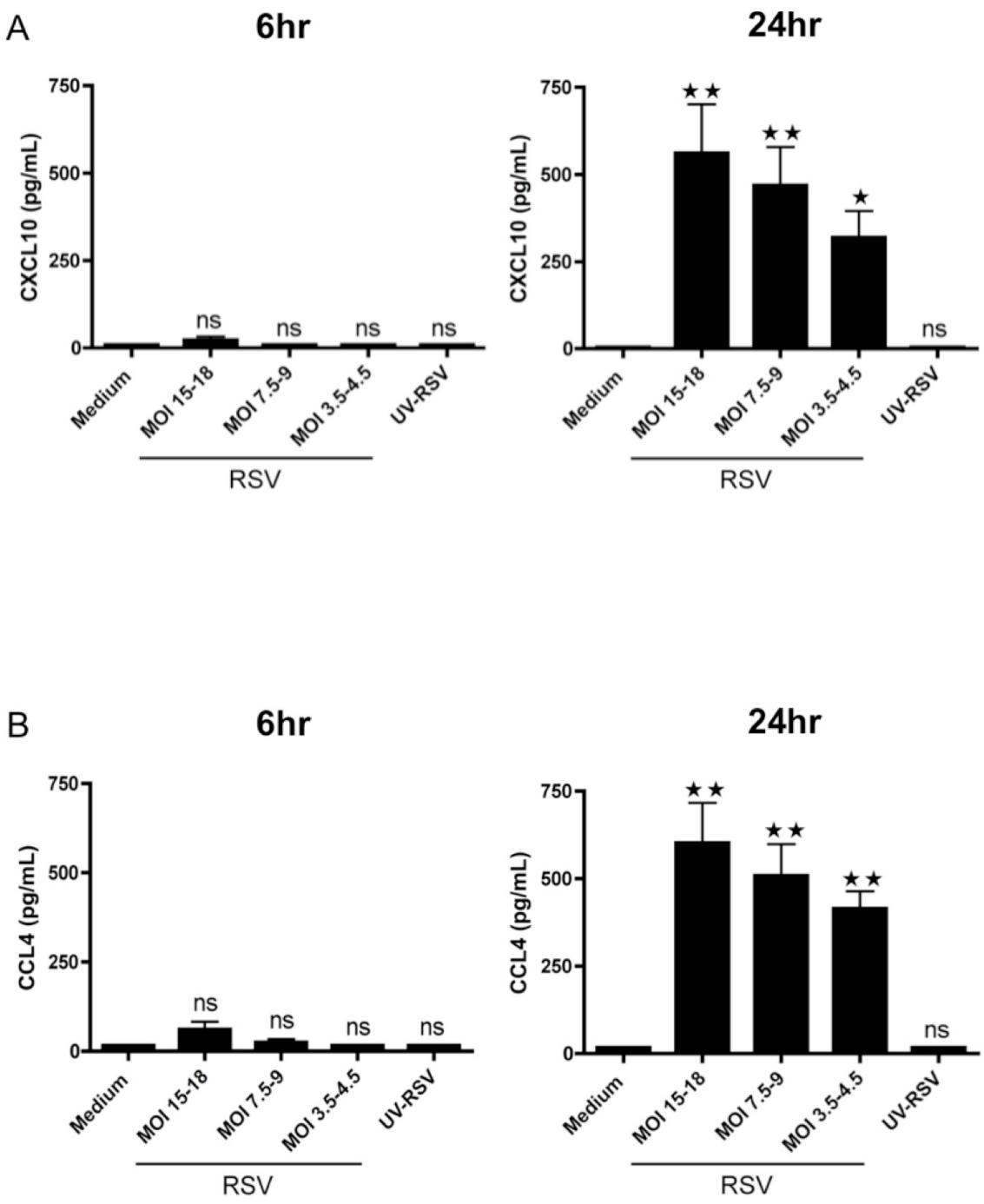
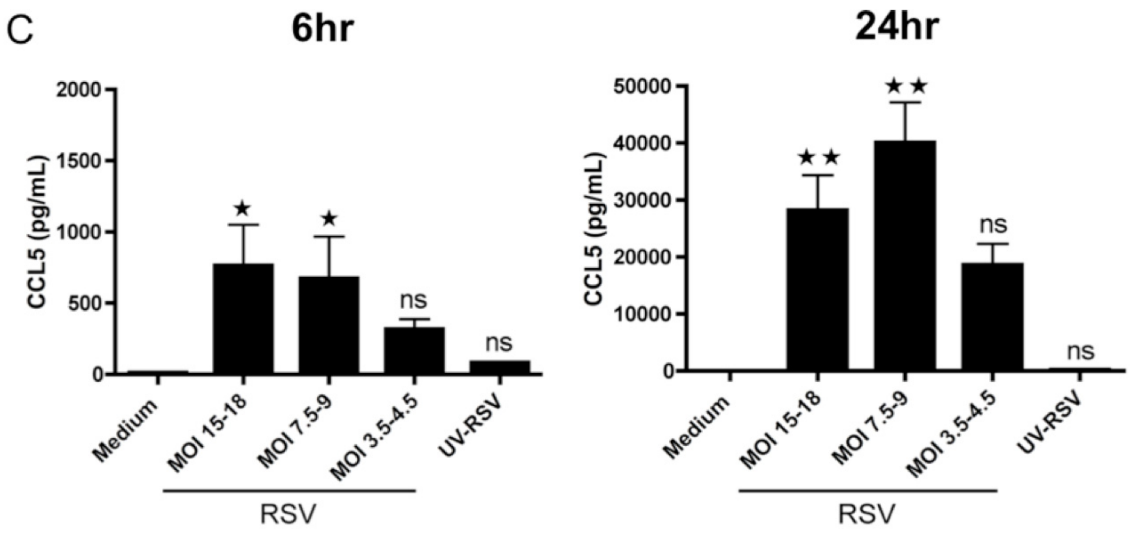

C
6hr
24hr
CCL5 (pg/mL)
1000
750
500
250
0
20000
15000
10000
5000
ns
Medium
MOI 15-18
MOI 7.5-9
MOI 3.5-4.5
UV-RSV
RSV

**Figure 17. A549 cells produce CXCL10, CCL4 and CCL5 in response to various doses of RSV.** A549 cells (approximately $0.2x10^6$ cells/mL) were inoculated with RSV at various MOIs, UV-inactivated RSV or treated with medium control for 90 minutes at 4°C. Cells were cultured for 6 or 24 hours at 37°C. Following incubation, cell supernatants were harvested and the levels of CXCL10 (A), CCL4 (B) and CCL5 (C) were measured by ELISA. Graphs represent the mean ± SEM of 3 different experiments. $^{\star\star}p<0.01$; $^{\star}p<0.05$; ns=not statistically significant as compared with medium control.


A
6hr
24hr
CXCL10 (pg/mL)
750
500
250
0
ns
★★
★
Medium
MOI 15-18
MOI 7.5-9
MOI 3.5-4.5
UV-RSV
RSV
B
CCL4 (pg/mL)


C
6hr
24hr
CCL5 (pg/mL)
2000
1500
1000
500
0
50000
40000
30000
20000
10000
★
★★
ns
Medium
MOI 15-18
MOI 7.5-9
MOI 3.5-4.5
UV-RSV
RSV

# Chapter 4 – Discussion

Mast cells are classically studied in the context of allergic disease and inflammation. However, recent advances in the understanding of mast cell biology and function support their role as sentinel immune cells with important functions in host defense against fungal, bacterial and viral pathogens. The location of mast cells at sites that interface with the environment and their vast repertoire of PRRs allows them to be at the forefront of pathogen recognition. Studies have shown that mast cells are capable of selectively producing a myriad of pro-inflammatory mediators that are essential in the recruitment of various cell subsets including neutrophils, NK cells and T-lymphocytes (Burke et al., 2008; Echtenacher et al., 1996; St John et al., 2011).

The contribution of mast cells to viral infection and inflammation, particularly during RSV disease, remains poorly characterized. This body of work aimed to investigate how human mast cells respond to RSV, with a specific focus on cytokine and chemokine production, as well as ISG expression. Based on the current understanding of mast cell biology and function, we hypothesized that mast cells produce pro-inflammatory mediators in response to RSV that are capable of recruiting effector cells and inducing an antiviral state.

## 4.1 Summary of major findings

Our work has demonstrated that mature $CD117^{hi}$-expressing human CBMCs (Figure 5) and the HMC-1 mast cell line (Figure 11; Table 4) are capable of supporting low levels of intracellular RSV antigen expression, indicative of viral infection, as measured by flow cytometry. We then aimed to characterize the chemokine responses of human mast cells to RSV. Using protein antibody arrays and ELISA techniques, we demonstrated that RSV-inoculated CBMCs selectively produce elevated levels of several chemokines including CCL2, CCL4, CXCL1, CXCL8 and CXCL10 (Figure 6; Table 2). We chose to focus on CCL4, CCL5 and CXCL10 as they have been shown to be chemoattractants for $CD4^+$ and $CD8^+$ T-lymphocytes as well as NK cells (Lindell *et al.*, 2008; Taub *et al.*, 1993). Upon further analysis, CBMCs were found to produce

substantial amounts of CXCL10 and CCL4 following inoculation with infectious, but not UV-inactivated, RSV (Figure 7). The production of these two chemokines started to increase after 12 hours. In comparison to CCL4 and CXCL10 generation, CBMCs produced much lower levels of CCL5 in response to infectious RSV, which peaked later during infection (18 hours) (Figure 8). HMC-1 cells produced significant amounts of CXCL10 and CCL4 and CCL5 following inoculation with RSV (Figure 12). However, RSV-inoculated CBMCs produced only background levels of CCL11 (eotaxin-1), IFN-γ and TNF, indicating that mast cell mediator responses to RSV are selective (Figure 8; Figure 9). These data highlight that a major mast cell response to RSV is the secretion of a variety of chemokines that are capable of recruiting effector cells.

Epithelial cell responses to RSV infection are well characterized and the airway epithelium constitutes the first line of defense against RSV infection (Bueno *et al.*, 2011). Therefore, we compared epithelial and mast cell responses to RSV. In comparison to mast cells, higher levels of RSV antigen-expressing A549 and HEp-2 epithelial cells were observed (Figure 15). Unlike CBMCs or HMC-1 cells, epithelial cells produced substantial amounts of CCL5, as early as 6 hours, as well as lower amounts of CXCL10 and CCL4 (Figure 7; Figure 16; Figure 17).

We then investigated mast cell antiviral responses to RSV in particular the role of type I IFNs in chemokine expression. RSV-inoculated CBMCs produced higher levels of IFN-α2 protein as compared with medium and UV-inactivated RSV controls (Figure 10). In addition, we demonstrated that the mRNA expression of IFN-α1, IFN-β, CCL5, CXCL10, ISG56, MxA and IL-28B and IL-29 was enhanced in RSV-inoculated CBMCs and HMC-1 cells (Table 3; Table 5). These data indicate that mast cells up-regulate the expression of type I and III IFNs and ISGs in response to RSV, which should help limit viral replication. Interestingly, type I IFN blockade did not affect chemokine production or viral antigen expression by RSV-inoculated HMC-1 cells (Figure 13; Figure 14). These findings suggest that chemokine production by RSV-inoculated mast cells may occur in a type I IFN-independent manner.

### 4.2 Implications for RSV inflammation

This study is amongst the first to address mast cell cytokine and chemokine responses to RSV. Our data demonstrates a remarkable capability for mast cells as sentinel immune cells with a significant capacity to produce chemokines leading to effector cell recruitment. In comparison to epithelial cells, which support much higher levels of infection, mast cells produce substantial levels of CXCL10 and CCL4. On a per cell basis, the production of CCL4 by mast cells in response to RSV is greater than epithelial cells. However, epithelial cells make higher levels of CXCL10 and CCL5 following RSV stimulation. Coupled with the close proximity of mast cells to blood vessels at tissue sites, these observations could translate to a superior ability for mast cells, as compared with epithelial cells, to recruit leukocytes during RSV infection. These findings highlight a beneficial role for mast cells through their potential recruitment of a wide variety of effector cell populations during RSV disease and inflammation. We have demonstrated that mast cells produce substantial amounts of CCL4 in response to infectious RSV. The role for CCL4 in RSV disease and inflammation has not yet been elucidated. However, CCL4 production by mast cells during RSV inflammation could be advantageous by aiding the recruitment of activated $CD8^+$ T-lymphocytes and the activation of NK cells (Robertson, 2002; Taub *et al.*, 1993). CXCL10 levels are elevated in nasopharyngeal secretion of infants with RSV brochiolitis (Roe *et al.*, 2011). Our data show that CBMCs release substantial amounts of CXCL10 following RSV inoculation. CXCL10 is essential in the recruitment of $CD8^+$ T-lymphocytes and NK cells to the lungs during RSV inflammation, which are essential in viral clearance and the establishment of immunological memory. Furthermore, CXCL10 is an ISG which has been shown to induce the production of IFN-α by RSV-infected murine DCs (Lindell *et al.*, 2008). The production of CXCL10 by mast cells may therefore play a beneficial role in human RSV disease. Our data also show that epithelial cells are major contributors of CCL5 in response to RSV, which agrees with published studies (Rudd *et al.*, 2005). High CCL5 production during RSV bronchiolitis is correlated with disease severity and later development of asthma (Chung and Kim, 2002; Zeng *et al.*, 2011). The low level of CCL5 production by mast cells in response to RSV suggests a potentially beneficial role for mast cells in RSV inflammation. Furthermore, RSV-inoculated CBMCs produced

only background levels of CCL11 and TNF, which are associated with severe RSV disease and eosinophil-mediated inflammation of the lungs (Choi *et al.*, 2010; Matthews *et al.*, 2005). These data also support the finding that CCL11 production during RSV disease is primarily mediated by $CD4^+$ T-lymphocytes, and not epithelial or mast cells (Johnson *et al.*, 2008).

We also observed that RSV induces CXCL8 production by CBMCs. CXCL8 is important for the chemotaxis of NK cells. Previous work from our laboratory has shown that CXCL8 production by CBMCs is essential for the chemotaxis of NK cells following poly I:C stimulation or reovirus infection (Burke *et al.*, 2008). CXCL8 is also a potent chemoattractant of neutrophils, which are the first cells to be recruited to the lungs during RSV disease. Although the role for neutrophils in RSV inflammation is not fully characterized, their influx is thought to enhance the pathophysiology of the disease (Welliver, 2008). IFN-γ controls the host response to viral infection by enhancing MHC I expression and activating NK cells (Welliver, 2008). The lack of IFN-γ production by CBMCs in response to RSV suggests that mast cells likely do not contribute to the antiviral response by the secretion of this cytokine. It is noteworthy to mention that the levels of cytokines and chemokines in RSV-stimulated CBMC supernatants might be higher than we have detected. This is due to the lack of protease inhibition throughout the experiments. Mast cell derived proteases have been described to degrade a number of cytokines and chemokines.

Type I IFNs, which include IFN-α and IFN-β, are essential in host defense against viruses and are amongst the first cytokines to be made during viral infection. We have demonstrated that RSV-inoculated CBMCs and HMC-1 cells express type I IFNs. Our work adds to published studies showing that primary human mast cell stimulated with poly I:C, or infected with hantavirus, produce IFN-α and IFN-β (Guhl *et al.*, 2010; Kulka *et al.*, 2004). Furthermore, we have demonstrated that RSV stimulates mast cells to produce type III IFNs, which include IL-28A, IL-28B and IL-29. This is the first report to our knowledge showing that mast cells are capable of producing type III IFNs. Types I and III IFNs signal in an autocrine and paracrine manner to induce the expression of various ISGs, which have direct and indirect antiviral effects. These include the ISG56 family of proteins, the MxA and MxB proteins and chemokines such as CXCL10 and

CCL2 (Bandyopadhyay *et al.*, 1995; Haller *et al.*, 2007; Megjugorac *et al.*, 2004). These data highlight a potential role for mast cells in establishing an antiviral state during RSV infection. IFN produced by mast cells could stimulate other mast cells as well as epithelial cells and dendritic cells to help curb viral infection.

In trying to explore the mechanism of chemokine production by RSV-stimulated HMC-1 cells, we examined the role of type I IFNs. B18R-mediated blockade of type I IFN signaling in RSV-inoculated HMC-1 cells had no effect on chemokine production. This finding is somewhat surprising considering that IFNs are potent inducers of chemokines such as CXCL10 and CCL2 (Megjugorac *et al.*, 2004). A substantial number of studies highlight a role for B18R in neutralizing various isoforms of human IFN-α, but not IFN-β (Colamonici *et al.*, 1995; Symons *et al.*, 1995; Waibler *et al.*, 2009). Since we have observed that HMC-1 cells express primarily IFN-β in response to RSV, the role of type I IFNs in chemokine production by HMC-1 cells warrants further investigation.

### 4.3 Limitations and future directions

Our findings have shown that mast cells are capable of expressing viral antigen following inoculation with live RSV. Viral antigen expression is indicative of infection and several studies exploit this feature of the virus replication cycle as a read-out for quantifying viral infection. For example, Johnson *et al.* used rRSV encoding green fluorescent protein (GFP) to assess RSV infection of different subsets of primary human DCs. Using flow cytometry to measure the percentage of GFP expressing cells, they were able to show low levels of RSV infection in mDCs and pDCs (Johnson *et al.*, 2011). While using flow cytometry to quantify the percentage of antigen expressing cells is a rapid way to assess viral infection, it does not confirm whether the infection is productive. To address this issue, plaque assays on RSV-infected mast cell supernatants at various time points will need to be performed to see whether infectious progeny are being generated. Interestingly, similar experiments have been performed by Shirato and Taguchi, who demonstrated that RSV-inoculated HMC-1 cells are not capable of sustaining productive viral infection or expressing viral antigens (Shirato and Taguchi, 2009). Furthermore, we observed that CBMCs inoculated with infectious RSV express higher levels of CD117 as compared to CBMCs inoculated with UV-inactivated RSV or

medium control (Figure 5). It can be speculated that this could be the result of the direct killing by RSV of $CD117^{lo}$-expressing cells in CBMC cultures. Alternatively, infectious, but not UV-inactivated, RSV could directly up-regulate the expression of CD117 on infected cells, or indirectly through the production of cytokines, chemokines and other mediators. The mechanisms underlying the expression of CD117 by CBMCs in response to RSV warrants further investigation.

We have demonstrated that mast cells produce significant amounts of various chemokines, such as CXCL10 and CCL4, in response to RSV. Several studies have reported the potency of these chemokines in recruiting various subsets of effector cells. Future experiments should aim to delineate which subsets of cells migrate to RSV-inoculated CBMC supernatants using trans-well migration/chemotaxis assays that are well established in our laboratory. The effect of particular chemokines on cell recruitment can then be studied by depleting specific chemokines in RSV-CBMC cell supernatants prior to use in transmigration assays. Furthermore, we were able to show that CBMC cultures supported a very low percentage of infected/viral antigen-expressing cells, yet generated substantial amounts of CCL4 and CXCL10 protein in the supernatant. We will therefore investigate whether viral antigen-expressing, or uninfected cells, are producing CXCL10 and CCL4. This can be measured using flow cytometry where CBMC cultures can be double-stained for intracellular RSV antigen and chemokine expression.

Although our work has demonstrated that mast cells are capable of producing various cytokines and chemokines following stimulation with RSV, the mechanisms involved in mediator production remain elusive. Mast cells express TLR3, TLR4, TLR7 and TLR8, all of which are implicated in the response to RSV (Brzezinska-Blaszczyk and Wierzbicki, 2010; Kurt-Jones *et al.*, 2000; Phipps *et al.*, 2007; Rudd *et al.*, 2005). Future studies will aim to address the role for TLRs in cytokine and chemokine expression during RSV disease. The intracellular RNA sensors RIG-I and PKR are implicated in viral clearance and mediator production during *in vitro* and *in vivo* RSV infections (Bhoj *et al.*, 2008; Liu *et al.*, 2007; Minor *et al.*, 2010). Previous work from our laboratory has demonstrated that RSV-inoculated CBMC and HMC-1 cells up-regulate the expression of RIG-I (personal communication, Dr. Michael Brown, December 2010). The role of these sensors in mediating mast cell chemokine and cytokine responses in response to RSV

warrants further investigation. The use of specific siRNA, shRNA and chemical inhibitors to down-regulate the expression and function of these receptors constitutes a useful tool to assess their role in RSV-induced mediator production by mast cells.

Mast cells are potent sources of histamine and lipid mediators such as leukotrienes and prostaglandins, which are highly implicated in airway inflammation (Galli *et al.*, 2005; Wedde-Beer *et al.*, 2002; Welliver *et al.*, 1981). A significantly elevated number of mast cells are found in lungs of guinea pigs infected with RSV as compared with controls. Fascinatingly, the mast cells were found in close proximity to nerve endings, suggesting a potential interaction between mast cells and the nervous system (Wedde-Beer *et al.*, 2002). The production of such mediators in response to RSV by mast cells should be evaluated in future work. Further evaluation of mast cell degranulation in response to RSV and the effect of such products on disease severity is another important aspect of mast cell involvement in RSV inflammation. Elevated tryptase levels have been reported in BAL samples of infants with RSV disease. Tryptase, amongst other mast cell proteases, may be involved in inflammation and tissue remodeling (Everard *et al.*, 1995). Interestingly, mast cell degranulation has been observed in HMC-1 cells co-cultured with RSV-infected A549 cells (Shirato and Taguchi, 2009). Mast cell granules also produce GAGs and other proteoglycans that bind chemokines to generate a stable gradient which is essential for facilitating leukocyte chemotaxis (Lortat-Jacob, 2009) .

While *in vitro* assays are important in understanding mast cell responses, the biological and functional roles for mast cells in RSV inflammation must be studied *in vivo*. No published studies to date have examined the role for mast cells in RSV disease in $Kit^{W\text{-}v}$ or $Kit^{W\text{-}sh}$ mast cell-deficient mice. Future experiments will investigate the *in vivo* role for mast cells using $Kit^{W\text{-}sh}$ mast-cell deficient mice once this defect is backcrossed onto an appropriate background for infection studies, such as BALB/c. Reconstitution of mast cell deficient mice with wild type or mediator deficient mast cells will be an informative tool in deciphering the role for mast cells in the immune response against RSV.

### 4.4 Clinical implications

The findings presented in this thesis are important first steps in deciphering a role for mast cells in the immune response to RSV. These data show that mast cells respond to RSV stimulation by secreting various pro-inflammatory mediators that are capable of inducing an antiviral state and recruiting NK cells, macrophages, $CD4^+$ and $CD8^+$ T-lymphocytes that may help to resolve viral infection. Therefore, based on these preliminary findings it seems logical that preserving mast cell numbers and their capacity to produce cytokines and chemokines would be beneficial during RSV infection. Hence, future clinical applications should potentially avoid the use of therapeutic agents that may reduce mast cell numbers. Such agents may include glucocorticoids, such as dexamethasone, which have been shown to decrease mast cell numbers in skin tissue by down-regulating the expression of SCF mRNA (Finotto *et al.*, 1997).

Several studies have demonstrated that mast cells are rather abundant at tumor sites (Takanami *et al.*, 2000). Our laboratory has previously demonstrated that NK cells migrate to reovirus-infected CBMC supernatants in a CXCL8-dependent manner (Burke *et al.*, 2008). NK cells are highly implicated in the targeted killing of cancer cells and constitute an important arm of defense against tumors (Zwirner and Domaica, 2010). Such observations warrant further studies to dissect whether mast cell activation with viruses such as reovirus or RSV and/or viral products could augment tumor killing through the recruitment of NK cells to tumor sites. Theoretically, such studies would have potential in cancer immunotherapy.

### 4.5 Concluding remarks

Mast cells are most classically viewed as the harmful effector cells mediating the undesirable symptoms of allergic inflammation and anaphylaxis. Shockingly, despite their location at sites that closely sample the environment and their immense range of PRRs, their sentinel role at the forefront of host defense and pathogen recognition have been overlooked. The findings described in this thesis stress the importance of studying mast cells in their sentinel roles in host defense against viral infection. By enhancing our understanding of mast cell responses to RSV, this body of work has elucidated the capacity of these tissue granulocytes in mediating antiviral responses and recruiting

effector cells that could clear infection. Such exciting observations enrich our understanding of mast cell immunobiology, and warrant further efforts into deciphering a role for these cells in immunosurveillance during viral infection.

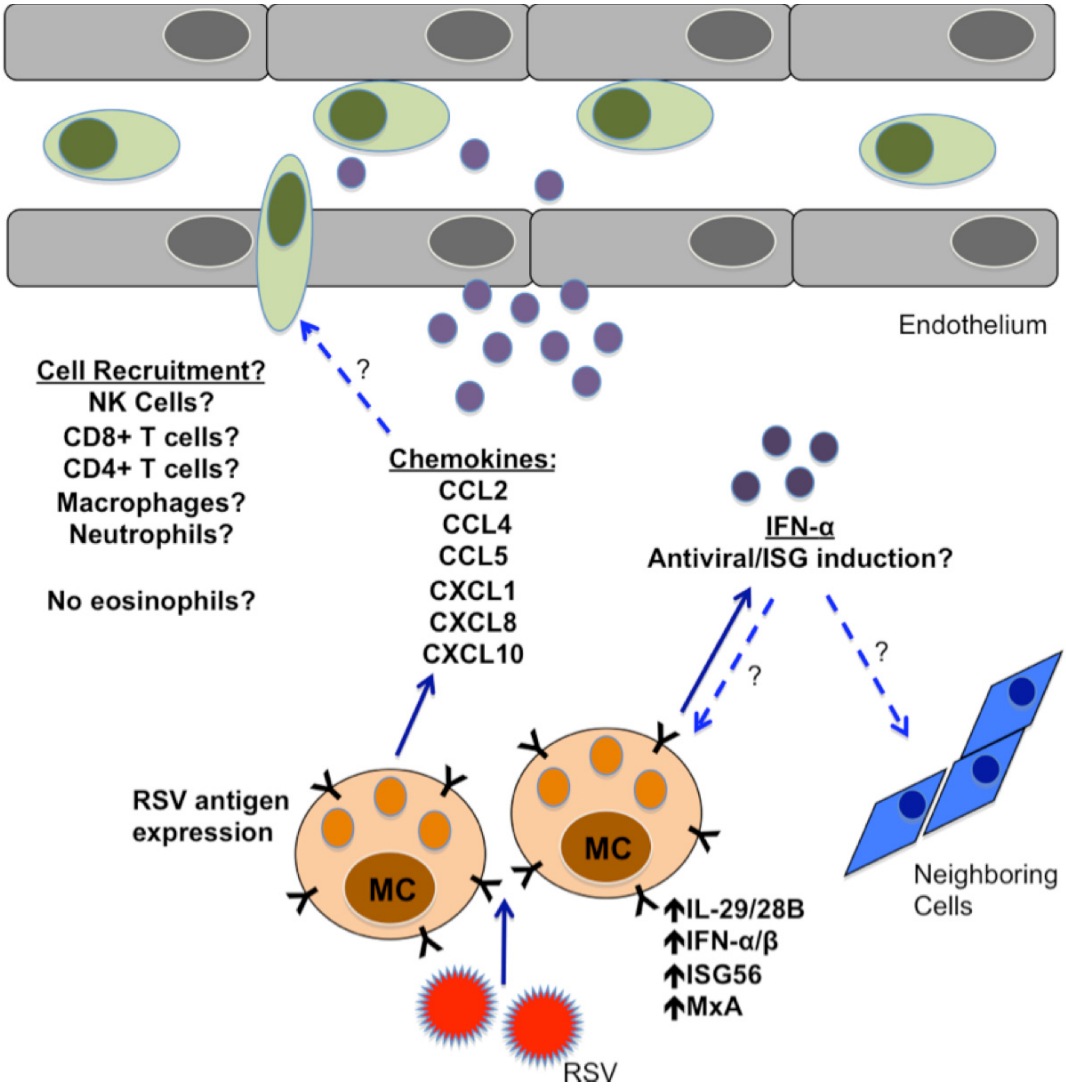



**Figure 18. Proposed model for mast cell involvement in RSV inflammation.**

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