RESPIRATORY SYNCYTIAL VIRUS ATTACHMENT G AND NONSTRUCTURAL PROTEINS MODULATE THE EARLY ANTIVIRAL HOST RESPONSE TO INFECTION

by

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(Under the Direction of Ralph A. Tripp)

ABSTRACT

Respiratory syncytial virus (RSV) is an important cause of serious lower respiratory tract illness in infants and young children worldwide causing repeat infections throughout life with serious complications occurring in elderly and immune compromised populations. Little is understood about how RSV modulates the host immune response and currently there is no safe effective vaccine available. In the present study, we focused on the early antiviral host immune response to RSV infection, with emphasis on patterns of SOCS and interferon expression. Examination of mouse lung epithelial cells infected with wtRSV, RSVΔG or RSVΔNS1/2 revealed differential induction of SOCS and interferons respective of G/NS gene deletions. RSVΔG infection upregulated secreted IFNβ and ISG15 while RSVΔNS1/2 induced significant SOCS1 and interferon mRNA expression. These data provide additional information regarding the various roles of RSV proteins and highlights RSV G protein as a potent modulator of the early antiviral host response to RSV infection.

INDEX WORDS: Respiratory syncytial virus, SOCS, interferon, ISG15

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CHAPTER 1

INTRODUCTION

Respiratory syncytial virus (RSV) is an important cause of serious lower respiratory tract illness in infants and young children worldwide causing repeat infections throughout life with serious complications occurring in elderly and immune compromised populations (Sorvillo et al., 1984; Graham 1995; Graham 1996; Graham et al., 2000; Falsey et al., 2005a; Olson and Varga 2008). Clinically, RSV infection may be associated with bronchiolitis, pneumonia, and otitis media, and in some cases may predispose for the development of long term wheezing, asthma and allergies (Sarkkinen et al., 1985; Heikkinen et al., 1999; Anderson 2000; Ogra 2004; Falsey et al., 2005b; Falsey 2007; Mohapatra and Boyapalle 2008). It is not fully understood how RSV modulates the host immune response to infection, and despite decades of research, there is no safe and effective vaccine available. Insufficient knowledge of the host immune response to RSV infection contributed to vaccine-enhanced illness in the 1960s following clinical trials of a formalin-inactivated RSV vaccine where 80% of vaccinees subsequently naturally challenged with RSV required hospitalization and resulted in two deaths (Chin et al., 1969; Kim et al., 1969; Openshaw et al., 2001; Tripp 2004).

To better understand the host response to RSV infection, and potentially provide an avenue for RSV disease intervention strategies, we focused on the early antiviral host immune response to RSV infection with emphasis on understanding the role of suppressor of cytokine signaling (SOCS) negative regulation of type I interferon (IFN) expression (Greenhalgh and Hilton 2001; Alexander 2002; Larsen and Ropke 2002; Kubo et al., 2003; Elliott and Johnston

2004; Wormald and Hilton 2004; Tripp et al., 2005; Yoshimura et al., 2007). RSV infection is known to be a poor inducer of antiviral type I IFN cytokines (IFNα and IFNβ) (McIntosh 1978; Hall et al., 1978; Loveys et al., 2000; Bossert et al., 2003), an event believed to be mediated by the two nonstructural (NS) proteins of RSV, i.e. NS1 and NS2 (Schlender et al., 2000; Gotoh et al., 2001; Bossert and Conzelmann 2002; Bossert et al., 2003; Spann et al., 2004; Zhang et al., 2005; Ramaswamy et al., 2006; Elliott et al., 2007). SOCS are intracellular signaling molecules induced upon cytokine receptor activation and function as negative regulators of Janus kinase (JAK)-signal transduction and activator of transcription (STAT) cytokine signaling which in turn induce type I IFN expression (Starr et al., 1997; Endo et al., 1997; Starr and Hilton 1998; Starr and Hilton 1999; Greenhalgh and Hilton 2001; Alexander 2002; Kubo et al., 2003; Vlotides et al., 2004; Wormald and Hilton 2004; Yoshimura et al., 2007).

Based on the governing role of SOCS regulation of JAK-STAT signaling and type I interferon expression, and the fact that RSV nonstructural proteins are type I IFN expression antagonists, we hypothesized that RSV proteins may suppress type I IFN expression through SOCS expression. In these studies, we examined the host SOCS response to RSV infection using a mouse lung epithelial cell line (MLE-15) consisting of type II pneumocytes. These cells represent the distal bronchiolar and alveolar respiratory epithelium which are the primary target for RSV infection and replication. (Wikenheiser et al., 1993; Collins and Crowe 2007).

To determine if individual proteins of RSV are involved in SOCS modulation, examination of MLE-15 cells at 24 or 48 hours following infection with wild-type (wt) RSV or RSV recombinant viruses lacking either the G gene (RSVΔG) or the NS1 and NS2 genes (RSVΔNS1/2) revealed differential SOCS1 and SOCS3 and type I IFN mRNA expression between wtRSV and RSVΔG or RSVΔNS1/2 infections - features linked to G and/or NS1 and

NS2 genes (Moore et al., 2008). Intracellular SOCS1 and SOCS3 protein analysis was consistent with differential SOCS mRNA expression, however little to no intracellular type I IFN proteins were detected among all RSV infections (Moore et al., 2008). While low levels of secreted IFNα protein were detected in the supernatants of wtRSV and RSVΔG-infected cells, secreted IFNβ was detected for all RSV infections, with substantially elevated levels associated with RSVΔG infection. To evaluate downstream effects of modified SOCS1 and SOCS3 and type I IFN expression by RSV proteins, both mRNA and protein levels of interferon stimulated gene-15 (ISG15) were examined. The studies showed that RSV induces ISG15 expression with highly elevated levels associated with RSVΔG infection (Moore et al., 2008), suggesting G protein inhibits ISG15 expression during infection.

Collectively, the results show that SOCS1 and SOCS3 expression are differentially expressed by specific RSV proteins, an effect that changes SOCS governance and negative regulation of type I IFN expression in response to RSV infection. Importantly, the results also show RSV G protein modulates SOCS3, IFNβ and ISG15 expression beyond the type I IFN antagonist effects attributed to NS1 and NS2. Together, these data describe novel and various roles for individual RSV proteins in the modulation of the host immune response to infection, and highlight the RSV G protein as a potent modulator of the host antiviral response to RSV infection. The findings also suggest that SOCS proteins may be candidate therapeutic targets for modulating the host response, and that specific targeting may be useful to restore immune balance and appropriate responses (Hashimoto et al., 2008). The results from this study contribute to the growing understanding of the immune response to RSV infection, and may contribute to the development of a therapeutic approach to treat RSV disease pathogenesis.

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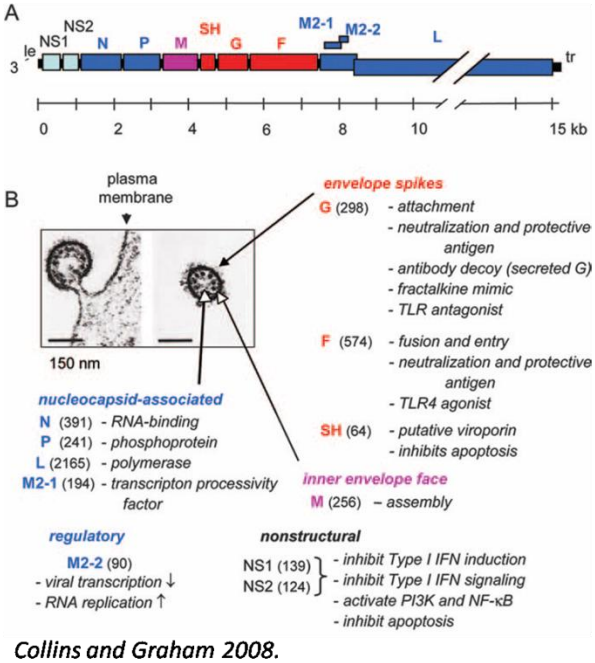
CHAPTER 2

LITERATURE REVIEW

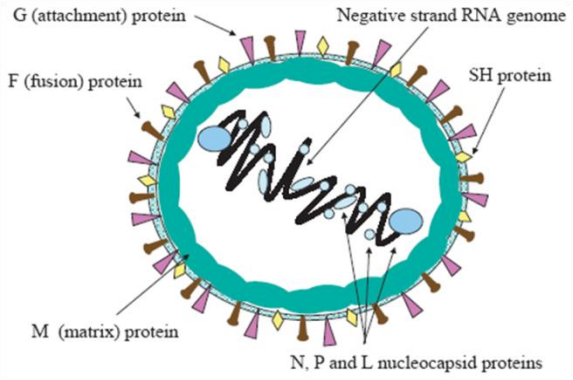
Respiratory Syncytial Virus (RSV)

Overview. Respiratory syncytial virus (RSV) is a single-stranded, non-segmented, negative-sense enveloped RNA virus of the *Paramyxoviridae* family having a 15.2 kb genome that consists of 10 genes in the order of (3'-NS1-NS2-N-P-M-SH-G-F-M2-1/M2-2-L-5') that encode for 11 proteins (Huang et al., 1982; Collins et al., 1983; Collins et al., 1984a; Dickens et al., 1984; Collins et al., 1986). These proteins include nonstructural 1 (NS1), nonstructural 2 (NS2), nucleocapsid (N), phosphoprotein (P), matrix (M), small hydrophobic (SH), attachment glycoprotein (G), fusion glycoprotein (F), M2 ORF1 protein (M2-1), M2 ORF2 protein (M2-2), and RNA-dependent RNA polymerase (L). The lipid envelope that surrounds the nucleocapsid is derived from the host cell plasma membrane during viral budding and contains three virally encoded surface transmembrane glycoproteins F, G and SH. The surface fusion glycoprotein (F), in combination with the surface attachment glycoprotein (G), is responsible for attachment, and the F glycoprotein responsible for virus penetration and fusion of the viral envelope with host cell membranes, and formation of



Collins and Graham 2008.

syncytia (Tripp 2007). The majority of the humoral response is directed against the F and G proteins and is associated with production of neutralizing antibodies while cell-mediated CTL responses are primarily directed to the NS2, N, M, SH, F and M2 proteins (Cherrie et al., 1992).



Hacking and Hull. 2002.

RSV has two major subgroups (A and B) distinguished by differences within the G protein. Both subgroups circulate and cause disease in humans with no established disease variability between them (Anderson et al., 1985; Mufson et al., 1985; Collins et al., 2007; Collins et al., 2008).

RSV Surface Fusion (F) Glycoprotein

The envelope-associated surface fusion (F) glycoprotein is structurally and functionally related to other paramyxoviruses involved in fusion of the virion envelope to the host cell plasma membrane (Collins et al., 1984b; Spriggs et al., 1986; Tripp 2007). It is a type I membrane glycoprotein of approximately 70kDa that contains a cleaved N terminal signal sequence anchored in the membrane by a C terminal membrane anchor sequence allowing for >90% of the N terminal molecule to be extracellular (Arumugham et al., 1989; Paradiso et al., 1989). The F glycoprotein is synthesized as a precursor protein (F_0) in the endoplasmic reticulum consisting of a F_2 domain at amino acid positions 1-130, a cleavage site at amino acid positions 131-136, and an F_1 domain at amino acid positions 137-574. The F_0 protein precursor is cleaved by a trypsin-like protease into disulfide-linked F_1 and F_2 subunits in the trans-Golgi, constituting the biologically active form of the molecule. The F_2 domain is modified by the addition of N-linked sugars, and the F glycoprotein is expressed as a trimer or tetramer (Collins et al., 1984b; Johnson et al., 1988).

RSV Surface Attachment (G) Glycoprotein

The envelope-associated surface attachment G glycoprotein has a number of genetic, structural and functional differences that are unique among the *Paramyxoviridae* viruses, and most virus attachment proteins in general (Satake et al., 1985; Wertz et al., 1985; Wertz et al., 1989; Tripp 2007). The G glycoprotein forms from a polypeptide precursor (32 kDa) that ranges in amino acid length (292-299 amino acids) depending on the virus strain (Sullender et al., 1991; Martinez et al., 1999). The precursor is co-translationally modified by the addition of high mannose N-linked sugars to form an intermediate of 45 kDa, which is followed by addition of O-linked sugars in the trans-Golgi to achieve a mature form of approximately 90 kDa (Wertz et al., 1989; Collins et al., 1992). Most of the carbohydrate is of the O-linked variety, therefore the G glycoprotein has high serine, threonine and proline content (Collins et al., 1992; Collins 1990a). The high proline content may reduce highly folded secondary structure contributing to an extended structure. The G glycoprotein is also palmitylated (Collins et al., 1992). The G glycoprotein is a type II glycoprotein with a single N-terminal hydrophobic region (amino acids 38-66) that serves as a signal peptide and membrane anchor (Wertz et al., 1985; Vijaya et al., 1988; Roberts et al., 1994, Lichtenstein et al., 1996). Proximal to the membrane anchor region is an extracellular ectodomain. In the middle of the ectodomain is a short protein segment and four cysteine residues (173, 176, 182 and 186) that are highly conserved in all RSV isolates (Sullender et al., 1991; Teng et al., 2002). This region contains a CX3C chemokine motif (amino acids 182-186) that may facilitate virus attachment to cells expressing the CX3C chemokine receptor (Tripp et al., 2001). Flanking this region are two glycosylation sites that are highly divergent among all RSV isolates (Sullender et al., 1991; Cane et al., 1991). The G glycoprotein is expressed both membrane-bound and secreted by initiation of translation at an alternate in-

frame AUG codon located in the middle of the hydrophobic transmembrane region or by cleavage mediated by TACE (Roberts et al., 1994). Approximately 15% of the G glycoprotein synthesized in infected cells is secreted in a soluble form lacking the cytoplasmic domain and part of the signal-anchor sequence, but retaining the same characteristics as the membrane bound form, e.g. glycosylation and antibody reactivity (Hendricks et al., 1987; Hendricks et al., 1988).

RSV Surface Small Hydrophobic (SH) Glycoprotein

The small hydrophobic (SH) protein is a short (64 amino acids) envelope-associated surface protein which may be involved in formation of cation-selective channels or viriporins (Tripp 2007; Gan et al., 2008). The SH protein is membrane-anchored at a centrally located anchor sequence such that only a third of the molecule is extracellular. SH accumulates in the infected cell in different forms (SHo, SHg, SHp, SHt), but the SHo unglycosylated species is the most abundant form (Olmsted et al., 1989; Collins et al., 1990b). All forms of SH, except for SHt, are transported to the cell surface, and only SHo and SHp are incorporated into the virion (Collins et al., 1993). The significance of the different forms is unknown, but conservation among human and bovine strains of RSV suggests that they may be involved in virus attachment, penetration or viral uncoating (Collins et al., 1990b; Anderson et al., 1992).

RSV Nonstructural Proteins NS1 and NS2

NS1 and NS2 nonstructural proteins exist as only trace amounts on the the virion (Collins et al., 1990a; Huang et al., 1985; Evans et al., 1996). NS1 and NS2 are abundantly expressed and readily detected in RSV-infected A549 cells by 5 hours post-infection (pi) with maximal intracellular levels reached between 8 and 18 hours pi (Collins and Wertz 1983; Cowton et al., 2006; Bitko et al., 2007). Unique to pneumoviruses, the nonstructural proteins are encoded by separate mRNAs. NS1 and NS2 may act cooperatively to antagonize IFNα/β-induced antiviral

responses, inhibit apoptosis, and the NS2 protein appears to be rapidly processed and secreted (Gotoh et al., 2001; Schlender et al., 2000; Bitko et al., 2007). Studies with recombinant respiratory syncytial viruses with deletions in the NS1 and NS2 have shown that these genes are dispensable for virus replication *in vitro*, however they provide auxiliary functions for efficient RSV replication *in vitro* and *in vivo* (Jin et al., 2000).

RSV Nucleocapsid-Associated Proteins M2-1, N, P and L

The RSV genome encodes for four nucleocapsid-associated proteins, M2-1, N, P and L. The M2 gene contains two overlapping ORFs leading to translation of two proteins M2-1 and M2-2 (Ahmadian et al., 2000; Gould and Easton 2007). The M2-1 protein appears to function as a transcription factor to increase polymerase processivity, and enhance read-through of intergenic junctions during virus transcription (Fearns and Collins 1999b; Hardy et al., 1999; Hardy et al., 2000). It has a Cys3His1 zinc binding motif which is essential for its function, and has been shown to interact with the N protein (Hardy et al., 2000).The RNA binding protein nucleocapsid (N) is a multifunctional protein of 391 amino acids that has a central role in transcription and replication of viral genomic RNA (Collins et al., 1984a; Collins et al., 1985). The phosphoprotein (P) is a key component of the viral RNA-dependent RNA polymerase complex, having two general functions as a transcription and replication factor, and as a chaperone for soluble L protein (Horikami et al., 1992). The P protein is smaller (241 amino acids) than most paramyxovirus P proteins (391-602 amino acids) and is dispensable for virus replication *in vitro* (Villanueva et al., 2000). However, phosphorylation of P protein is required for efficient virus replication *in vitro* and *in vivo* (Villanueva et al., 2000; Khattar et al., 2001). The large viral polymerase (L) is the least abundant of the structural proteins. The L protein is similar in length to other paramyxovirus counterparts (approximately 2,200 amino acids), but

there is limited sequence homology within the paramyxoviruses (Stec et al., 1991). The N, P, and L proteins are the minimal trans-acting proteins required for RNA replication (Yu et al., 1995), however unlike other viruses, RSV transcription and replication appears to lack the requirement that template length be an even multiple of an integer such as six, which is obligatory for nucleocapsid function of other paramyxoviruses, such as measles and Sendai virus (Samal et al., 1996).

RSV Matrix Proteins M and M2-2

Unlike other negative-strand RNA viruses, pneumoviruses such as RSV have two matrix proteins, M and M2 (Collins et al., 1984a; Huang et al., 1985). The M protein is smaller (256 amino acids) than similar paramyxovirus counterparts (335-375 amino acids), and has limited sequence homology (Satake et al., 1984). The M2-2 protein (90 amino acids) is unique to pneumoviruses and is involved in regulating the balance between viral transcription and RNA replication (Bermingham and Collins 1999; Collins and Graham 2008). M and M2 are nonglycosylated proteins that appear to virion-associate. The M protein appears to functionally inactivate nucleocapsid transcription prior to packaging, and to mediate nucleocapsid association with the nascent envelope.

RSV Attachment and Replication within Host Cells

RSV virions primarily attach to cells through the G glycoprotein (Tripp 2007). Attachment appears to be principally mediated by interaction of heparin-binding domains on the G glycoprotein with apical cell surface glycosaminoglycans (Krusat et al., 1997; Bourgeois et al., 1998; Feldman et al., 1999). Moreover, evidence indicates that a CX3C chemokine motif located in the conserved region of the G glycoprotein (amino acids 182-186) may facilitate attachment through interaction with the CX3C chemokine receptor (Tripp et al., 2001), although

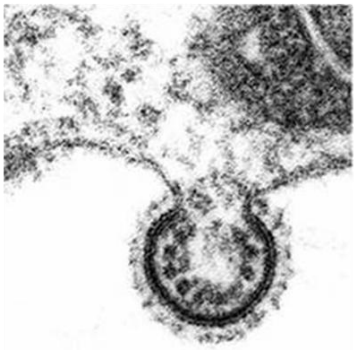
it has been shown that this region is not required for attachment (Teng et al., 2002). The CX3C chemokine motif may also be linked to the recently discovered ability of RSV to infect neuronal cells thereby evading host detection (Li et al., 2006). However, the G glycoprotein itself may not be required for virion attachment to cells (Tripp 2007). Mutant RSV lacking SH and/or G genes have been shown to infect cells, likely through interaction with the F glycoprotein (Karron et al., 1997; Teng et al., 2001; Techaarpornkul et al., 2002).

RSV penetrates cells by fusion with the plasma membrane, a process associated with the F glycoprotein (Bachi 1988). Penetration involves incorporation of the viral envelope into the cell membrane, and nucleocapsid release into the cytoplasm (Levine et al., 1969; Hierholzer et al., 1986; Routledge et al., 1987; Arslanagic et al., 1996). This event may be facilitated by clathrin-mediated endocytosis (Kolokoltsov et al., 2007; Gutierrez-Ortega et al., 2008). The L protein initiates viral transcription and replication proceeds in the cytoplasm without nuclear involvement (Fearns et al., 1999).

Transcription of RSV mRNAs occurs in a 3' to 5' order from a single promoter near the 3' end (Tripp 2007). Polymerase-mediated transcription results in a series of subgenomic mRNAs that are co-linear copies of the genes with no evidence of mRNA editing or splicing (Dickens et al., 1984; Huang et al., 1985; Kuo et al., 1996; Harmon et al., 2001; Krempl et al., 2002). Sequence and transcriptional mapping analysis of RSV mRNAs indicates that each transcript begins at the first nucleotide of the gene-start signal, is capped at the 5' end, and polyadenylated at the 3' end. mRNAs can be detected by 4h post-infection with peak mRNA synthesis and protein expression occurring 16- 20h post-infection. The level of protein expressed is relative to mRNA abundance (Dickens et al., 1984). In general, there is decreasing amounts of mRNAs with increasing gene distance from promoter sequence. RNA replication is dependent

on active protein synthesis and like other viruses RSV depends on host cell ribosomes for viral protein synthesis.

Virion assembly occurs at the plasma membrane (Tripp 2007). Inclusion bodies containing viral ribonucleoprotein cores appear immediately below the plasma membrane. Nucleocapsids localize with cell membrane containing membrane-bound viral glycoproteins. Virions mature in clusters at the apical surface in a filamentous form associated with caveolin-1, and extend from the plasma membrane (Brown et al., 2002). The released virions may be pleomorphic where theycan be found as spheres or long filaments (Collins and Graham 2008). Experiments using human primary airway epithelial cell cultures have shown that ciliated epithelial cells are targeted by RSV, that infection and shedding occurs via the apical membrane, and that virus is spread to neighboring ciliated cells by the motion of the cilia (Zhang et al., 2002).

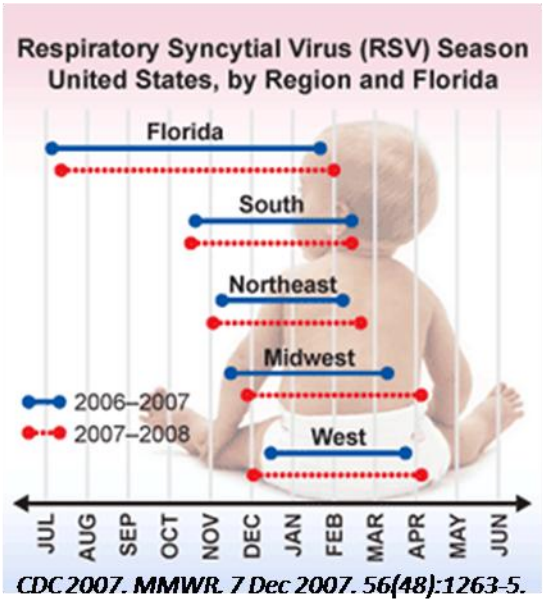


Electron micrograph of respiratory syncytial virus (RSV) virion at a cell membrane. Roberto Garofalo, M.D., Professor and Vice Chair for Research, Department of Pediatrics and Microbiology & Immunology, Scientist, Sealy Center for Vaccine Development, The University of Texas Medical Branch, Galveston, TX . www.wadsworth.org/images/virology/RSV.jpg

RSV Epidemiology

RSV is the single most important respiratory virus infecting infants and young children worldwide and can lead to serious lower respiratory tract illness requiring hospitalization (Crowe 1995; Shay et al., 2001). Approximately 70% of infants are infected within their first year of life and most are infected by 2 years of age (Glezen et al., 1986). Hospitalization rates vary geographically and socioeconomically with industrialized countries having lower rates of 1 in 100 to 1 in 200 during the first year of life (Fisher et al., 1997; Kim et al., 1973; Collins et al., 2007). RSV infections are generally seasonal with serious complications occurring in the elderly and in populations with compromised cardiac, pulmonary, and immune systems (Sorvillo et al.,

1984; Handforth et al., 2000; Falsey et al., 2005). The World Health Organization estimates global annual infection and mortality rates to be 64 million and 160,000 respectively, with U.S. estimates of 18,000 to 75,000 hospitalizations and 90 to 1900 deaths annually (See Appendix A). Naturally acquired immunity to RSV is neither complete nor durable and recurrent infections occur throughout life. Regrettably, to date there is no safe and effective vaccine and only limited prophylactic and therapeutic treatments available.



RSV Disease Pathogenesis

Overview. In 1956, a viral pathogen was isolated from a chimpanzee suffering from an upper respiratory tract infection and was subsequently named chimpanzee coryza agent (CCA) (Blount et al., 1956). In the same year, Chanock and colleagues isolated a viral pathogen related to CCA in 2 infants and suggested the name respiratory syncytial virus (RSV) based on its characteristic formation of large multinucleated cells or syncytia in cell culture (Chanock et al., 1957a; Chanock et al 1957b; Chanock et al., 1962). The virus is spread from person to person via close contact, large aerosolized droplets or fomite contact (Hall et al., 1978a; Hall et al., 1980). Viral replication begins in the superficial respiratory epithelium of the nasopharynx and though mostly associated with mild upper respiratory tract infections related to the common cold, RSV infection can cause serious lower respiratory tract disease to include bronchiolitis and pneumonia and can lead to predisposition for development of long term wheezing, asthma and allergies (Fisher et al., 1997; Kim et al., 1973; Parrott et al., 1973; Sigurs et al., 2005).

According to the Centers for Disease Control and Prevention (CDC), 25-40% of young children will have signs of bronchiolitis and pneumonia during their first RSV infection and 0.5-2% will require hospitalization. Moreover, RSV is the most frequently isolated viral pathogen in middle ear fluid of children with acute otitis media, and may account for as many as 15% of all cases (Sarkkinen et al., 1985; Heikkinen et al., 1999; Anderson et al., 2000).

RSV infection leads to a wide spectrum of respiratory illnesses (Tripp 2007), and is a leading cause of severe respiratory infection in infants and children (Simoes 1999; Krilov et al., 2001; Hall 2001; Weismann 2002; McNamara et al., 2002; Law et al., 2002). RSV infection is most common during infancy and early childhood (CDC 2000), but may also occur in adults, the immune compromised, and the elderly (Englund et al., 1991; Couch et al., 1997; Han et al., 1999; Hall 1999; Simoes 1999; Falsey et al., 2000). In infants, 25 to 40% of infections result in lower respiratory tract involvement, including pneumonia and bronchiolitis (Shay et al., 1999; Hall 2001). Viral replication initiates in the upper respiratory tract, however it can spread to the lower airways by aspiration of secretions or via the respiratory epithelium involving the bronchi, bronchioles, and alveoli, and may continue to macrophages and monocytes (Domurat et al., 1985; Midulla et al., 1989; Becker et al., 1992). Viral infection is associated with necrosis of the bronchiolar epithelium, destruction of the ciliated epithelial cells, and a peribronchiolar infiltrate of lymphocytes and mononuclear cells (Price 1990; Lugo et al., 1993). Interalveolar thickening and filling of alveolar spaces with fluid released from edematous submucosal and adventitial

tissues may lead to airway obstruction (Price 1990; Panitch et al., 1993). The characteristics of the immune response to RSV are not fully elucidated, and it is possible that damage of the epithelium and the bronchiolar ciliary cells may be associated with RSV-specific $CD8^+$ cytotoxic T cells (Garofalo et al., 1996).

Clinical signs of RSV illness frequently begin with rhinorrhea and low-grade fever that is often accompanied by cough and wheezing (Price 1990; Lugo et al., 1993). At the beginning of illness, RSV replicates in the nasopharynx reaching titers between 10^4- 10^6 $TCID_{50}$/ml of nasal secretion in infants (Hall et al., 1975; Hall et al., 1976; Hall 1977). The titer deceases over time, and most patients recover within 1 to 2 weeks post-infection. Some infants continue to shed virus for 3 weeks after hospitalization, and disease severity may correlate with the duration of virus shedding (Hall et al., 1976). Approximately 60% of primary RSV infections are confined to the upper airways; however during a period of 2 -5 days, infection may progress to lower respiratory tract involvement. The mechanism for viral spread to the lower airways is not known, but may occur by epithelial ciliary action, or through aspirated secretions, however RSV can spread cell-to-cell without emerging into the extracellular space.

Severe disease generally involves lower respiratory tract infection, and physical examination may reveal otitis media, rales and diffuse wheezing (Anderson 2000; Greenberg 2001; Panitch 2001; West 2002). The severity of the disease is directly related to the age of the patient. Infants under six month of age are the most severely affected due to smaller, more easily obstructed airways, and decreased ability to clear secretions. Illness may be particularly severe in premature infants, and in those with congenital cardiac or pulmonary disease (Krilov 2001; Shay et al., 2001; Aujard et al., 2002; Law et al., 2002). Apnea occurs in approximately 20% of infants hospitalized with RSV bronchiolitis (Bruhn et al., 1977; Church et al., 1984) with the frequency

of apnea increasing significantly in premature infants, occurring early in the course of RSV disease. Apnea rarely lasts more than a few days, but about 10% of these patients require intubation and mechanical ventilation.

In adults, the most common symptoms of RSV infection are those associated with the common cold, with rhinorrhea, sore throat, and cough (Tripp 2007). In the elderly, RSV may cause significant lower respiratory tract disease including pneumonia and some mortality (Hall et al., 1999; Falsey et al., 2000). RSV is also a significant cause of morbidity and mortality in patients undergoing bone marrow or organ transplantation (Wendt 1997; Small et al., 2002; Billings et al., 2002; Ison et al., 2002). RSV re-infections occur frequently and are often associated with illness (Henderson et al., 1979), however the cumulative effect of multiple re-infections appears to temper subsequent disease, an effect that may be related developing immunity. The severe disease observed in immune compromised or suppressed patients, and in experimental animal models, indicates that cell-mediated immunity is an important mechanism of host defense against RSV (Fishaut et al., 1980; Graham et al., 1991).

RSV Disease Intervention

Overview. In response to the high morbidity and mortality rates associated with RSV disease, a formalin-inactivated (FI) RSV vaccine was developed and clinically tested in the mid 1960s. Participants, aged 2 months to 9 years of age received three intramuscular injections of 100X concentrate plus alum (lot 100). Upon subsequent and natural RSV infection, 80% of the recipients experienced exacerbated vaccine-enhanced disease requiring hospitalization in which two deaths occurred (Kim et al., 1969; Openshaw et al., 2002). Subsequent studies of vaccinees showed the presence of nonfunctional antibodies to RSV F and G glycoproteins leading to poor protection in the presence of natural RSV infection (Murphy et al., 1986). Studies of the

prototypical FI-RSV vaccine in mice have revealed that treatment of RSV with formaldehyde modifies the RSV proteins with carbonyl groups, an effect that preferentially induces Th2-type responses and leads to enhanced disease (Moghaddam et al., 2006). These formalin-modified RSV proteins also likely contributed to the development of circulating non-neutralizing antibodies in vaccinees, and with RSV challenge, subsequent immune complex formation resulting in aberrant compliment activation (Polack et al., 2002). The disastrous results of this vaccine trial served to emphasize the need to better understand the host immune response to RSV infection when designing future vaccines and therapeutics.

RSV infection may cause severe lower respiratory tract infections early and late in life, and repeat infections with same, or different strains of RSV are common (Tripp 2007). These indications suggest a lack of durable immunity. For a RSV vaccine to be effective, it must confer protection better than that associated with natural RSV infection, and for infants, must do so in the first weeks of life. These obstacles may require different vaccines and vaccination strategies.

Early attempts to develop a safe and effective RSV vaccine failed. A formalin-inactivated, alum-precipitated RSV (FI-RSV) vaccine preparation that was intramuscularly administered to young children in RSV vaccine trial in the 1960s was not protective, and when vaccinated children were subsequently exposed to natural RSV infection, they developed enhanced pulmonary disease with several fatalities (Kim et al., 1969; Kapikian et al., 1969). The enhanced pulmonary disease has been attributed to altered immunity to the FI-RSV vaccine, however the exact mechanism is not completely understood but may be linked to chemical modification of the surface glycoproteins by formalin (Moghaddam et al., 2006). These disastrous results were unexpected since natural RSV infection induces a protective, although incomplete, immune response to infection.

Subsequent RSV vaccine efforts were focused on development of attenuated vaccines. Development of temperature-sensitive or cold-passaged vaccine candidates and subsequent examination in adults indicated some effectiveness, but the attenuated vaccine candidates proved too unstable or virulent in children, and often reverted back to wild-type virus (Pringle et al., 1993; Karron et al., 1997; Crowe et al., 1999; Gonzalez et al., 2000; Wright et al., 2000; Crowe 2001; Krilov et al., 2001). Clinical trials with candidate subunit vaccines have also been explored. A series of trial studies examined the effectiveness of a full-length F glycoprotein vaccine (PFP) purified from RSV-infected cells (Paradiso et al., 1994; Tristram et al., 1994; Falsey et al., 1996; Dudas et al., 1998; Groothuis et al., 1998; Gonzalez et al., 2000). An indication from the studies suggested that PFP was safe, but was only moderately immunogenic. A different series of trial studies examined the effectiveness of a recombinant protein (BBG2Na) consisting of amino acids 13-230 of the RSV G glycoprotein fused to the C-terminal domain of streptococcal G protein (Libon et al., 1999; Kneyber et al., 2000; Goetsch et al., 2001; Power et al., 2001). These studies showed that BBG2Na was moderately immunogenic in adults, but did not offer significant protection. Investigation of other candidate subunit vaccines is proceeding, including development of DNA vaccines. Effective subunit vaccines may be useful in RSV seropositive groups at high risk, or immunizing pregnant women to enhance protection of their newborns (Crowe 2001; Kneyber et al., 2002).

Reverse genetics employing full-length RSV complementary DNA to produce transcripts of infectious RNA offers many advantages in RSV vaccine design (Tripp 2007). The entire RSV genome can be precisely manipulated to offer optimal immunogenicity and attenuation. Reverse genetics has been used to delete non-essential genes in the RSV genome with the hope of restricting replication *in vivo*, but not *in vitro*, thus permitting efficient vaccine production

(Collins et al., 2002). Studies with recombinant RSV have shown that five RSV genes, NS1, NS2, SH, G and M2-2 can be deleted or silenced individually, or in certain combinations, without significantly altering viral replication *in vitro* (Collins et al., 2002). Attenuation studies in chimpanzees have shown that recombinant RSV absent of the SH gene is least attenuated, whereas recombinant RSV absent of the M2-2 gene is most attenuated (Teng et al., 2000).

Attenuation of RSV vaccine candidates may also be achieved by taking advantage of the natural host restriction of RSV (Buchholz et al., 2000). Bovine RSV is over-attenuated and poorly immunogenic in seronegative chimpanzees (Buchholz et al., 2000), however bovine RSV replication and immunogenicity can be improved by replacing the F and G genes with human RSV F and G genes (Schmidt et al., 2002). Strategies that focus on systemic replacement or deletion of RSV or bovine RSV genes in chimeric recombinant RSV are anticipated to yield useful vaccine candidates. Another advantage of recombinant RSV is the ability to alter or silence regions that may be associated with altered immunity or pathogenesis. For example, the RSV G glycoprotein has been shown to contain a CX3C chemokine motif (amino acids 182-186) that can bind to the CX3C chemokine receptor, CX3CR1, and mediate leukocyte chemotaxis in a fashion similar to fractalkine (Tripp et al., 2001). Thus, the G glycoprotein CX3C motif may contribute to enhancement of pulmonary disease associated with FI-RSV vaccination in BALB/c mice, and interaction of G glycoprotein CX3C with CX3CR1 may affect respiratory rates in BALB/c mice intravenously treated with G glycoprotein. Therefore, ablation of expression of this region by mutation may enhance the efficacy of recombinant RSV vaccine candidates.

Several therapeutic drugs are currently undergoing clinical trials to address RSV disease. One example is BTA9881, a small molecule fusion inhibitor that targets the RSV F protein and inhibits viral fusion to the cell membrane undergoing phase I safety trials (See Appendix B).

Another drug being tested is a novel RNA interference (RNAi) drug, ALN-RSV01, currently in phase II clinical trials in transplant patients. ALN-RSV01 is administered by nebulization once daily over 3 days that targets and silences the RSV N gene to inhibit viral replication (See Appendix C). These disease intervention strategies may allow medical providers to tailor treatment regiments based on a patient's individual needs and may also allow for use of combinatorial therapies when necessary. However, despite these efforts that target RSV, there are no ongoing clinical trials addressing measures that would counter RSV disease pathogenesis linked to the host immune response to infection.

Although limited, prophylactic treatments are available to address severe RSV disease. Two antibody-based therapeutics are currently approved by the FDA for treatment of RSV disease, i.e. RSV-IGIV (RespiGam®), composed of pooled human immunoglobulin of donors prescreened for high human RSV-neutralizing activity, and palivizumab (Synagis®) which is a humanized monoclonal antibody reactive to the F protein. In cotton rats, Synagis® is 50- to 100-fold more effective on a weight basis in neutralization than RespiGam® (Johnson et al., 1997). However, Synagis® has limited benefit in that it only decreases hospitalization by one day (CDC 1998). The prophylactic effects of Synagis® and RespiGam® wane by one month requiring dosage scheduling every 30 days during the RSV season, i.e. from November to April in the United States. Another monoclonal antibody treatment, motavizumab (Numax™) has completed the last stage of clinical trials but has been abandoned as a drug due to off-target effects. Numax™ was proposed to be ten-times more effective than Synagis® when administered as an aerosol, and was

http://www.medimmune.com/products/synagis/worksheet.asp

indicated for use in children and the elderly (Wu et al., 2007). An additional prophylactic available though not widely used is Ribavirin. Ribavirin was developed in 1970, and in 1980, became available as an inhalant for treatment of RSV disease. It is an aerosolized broad-spectrum antiviral prophylactic that inhibits the 5' cap formation of mRNA and inhibits viral polymerase but its use is associated with negative side effects such as toxicity (Sidwell et al., 1972).

RSV Immunology and Host Factors.

Innate immunity is the first line of defense against RSV infection (Tripp 2007). The agents of innate immunity provide early host resistance to infection. For respiratory viruses, innate immunity may be first mediated by mucus secreted by the membranes lining the respiratory tract. Mucus acts as a protective barrier to block the adherence of respiratory viruses to epithelial cells, and ciliary movement, coughing and sneezing, removes virus particles trapped within mucus. In addition, a family of proteins called collectins has the ability to recognize foreign carbohydrate patterns, interact with phagocytic cells, and induce opsonization of the particles containing foreign carbohydrates (Lu et al., 2002; Shepherd et al., 2002). The collectins, especially mannose-binding protein, and alveolar surfactant molecules SP-A and SP-D, are important agents in innate immunity (McCormack et al., 2002). SP-A is important in innate immunity to RSV infection (LeVine et al., 1999). SP-A binds to the RSV F glycoprotein, but not to the G glycoprotein, and neutralizes RSV infection in a calcium-dependent fashion (Ghildyal et al., 1999).

If viruses penetrate to the respiratory epithelium, destructive soluble factors including enzymes and cytokines are released by the infected cells, or by resident phagocytic cells. Infected epithelial cells and alveolar macrophages release chemokines and pro-inflammatory

cytokines that include tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and CC and CXC chemokines (Arnold et al., 1995; Saito et al., 1997; Becker et al., 2001; Zhang et al., 2001). Expression of these soluble mediators of innate immunity contributes to airway inflammation, bronchial hyperresponsiveness, and exacerbates mucus production. The CC chemokines, such as the macrophage inflammatory proteins (MIPs), regulated upon activation normal T cell expressed and secreted (RANTES) and eotaxin recruit a variety of granular cells to the site of infection or inflammation (Saito et al., 1997; Olszewska-Pazdrak et al., 1998). RANTES, eotaxin and MIP-1α are potent chemokines for eosinophils and increased levels, and/or mRNA, in airway secretions or peripheral blood leukocytes have been found in infants with RSV bronchiolitis (Hornsleth et al., 2001; Noah et al., 2002; Smyth et al., 2002; Tripp et al., 2002). The CXC chemokine IL-8, a potent chemoattractant of neutrophils, has been found in secretions of RSV infected children (Sheeran et al., 1999; Noah et al., 2002; Smyth et al., 2002), and experimentally infected adults (Noah et al., 2000).

Eosinophils are part of the non-specific innate immune response and participate in inflammatory reactions. They contain cationic molecules that are useful for destroying infectious agents, especially helminthes. Neutrophils are non-dividing, short-lived cells containing primary azurophilic granules and secondary granules. The primary azurophilic granules contain myeloperoxidase together with other anti-microbial agents including defensins and cathepsin G. The secondary granules contain lactoferrin and other enzymes. Alveolar macrophages are long-lived, tissue-resident cells that have an important role in phagocytosis. In this role they recognize and remove virally infected epithelial cells and debris, and present viral antigen to T cells, thereby initiating the adaptive immune response. Contact with virally infected cells, and the process of phagocytosis, activates macrophages to secrete soluble mediators including

interferons, lysozyme and other factors that inhibit viral replication and upregulate the inflammatory response (Krilov et al., 1987; Lewis et al., 1989; Tsutsumi et al., 1996). Dendritic cells (DC) are antigen presenting cells uniquely positioned to link innate and adaptive immune responses by responding to sites of inflammation and presenting antigen to T cells (Schwarze 2008). Plasmacytoid DCs are recruited to the lung early in infection and limit viral replication while resident myeloid DCs expand in number late in infection and likely contribute to inflammation (Schwarze 2008).

Recognition of pathogens by phagocytic cells is achieved by pattern recognition receptors encoded in the germline (Hallman et al., 2001; Armant et al., 2002). The pattern recognition receptors (PRRs) have broad specificity and recognize pathogen-associated molecular patterns (PAMPs) that differ from pathogen to pathogen, but are not found in the host (Underhill et al., 2002). Thus, PAMPs are perceived as molecular signatures of infection by PRRs resulting in activation of innate and adaptive immune responses (Barton et al., 2002; Sabroe et al., 2002). There are two major groups of PRRs - those that are secreted in the blood and lymph, and those on the surface of cells. Secreted PRRs include components of the complement fixation pathway, e.g. C2, C3, and C4, and cell surface PRR include the toll-like receptors (TLRs) (Akira et al., 2001).

There are >10 known TLRs that specialize in recognition of different PAMPs. For example, TLR2 generally recognizes peptidoglycan of Gram-positive bacteria, while TLR4 generally recognizes lipopolysaccharide in the outer membrane of Gram-negative bacteria (Akira et al., 2001). Interestingly, the RSV F glycoprotein has been shown to stimulate innate immunity through activation of the shared components of CD14 and TLR4 (Kurt-Jones et al., 2000), and TLR4 appears to be important in the innate immune response to RSV infection (Haynes et al.,

2001). TLR-4-deficient mice challenged with RSV exhibit impaired NK cell and $CD14^+$ cell pulmonary trafficking, deficient NK cell function, impaired interleukin (IL)-12 expression and impaired virus clearance. Recent studies suggest that TLR2 is involved in RSV recognition and subsequent innate immune activation with elevated production of TNF-α, IL-6, RANTES and MCP-1 (Murawski et al., 2008). It is possible to hypothesize that TLR2 recognition of RSV may be provoked by interaction with the RSV G protein based on previous studies that associate Gk protein expression with elevated levels of MCP-1 and Th2-type cytokine responses. Activation of TLRs on phagocytic cells can trigger adaptive immune responses by upregulation of T cell co-stimulatory molecules and cytokines required for T cell activation. In this role, phagocytic cells, particularly dendritic cells, have a key role in coupling innate and adaptive immune responses.

The adaptive immune response includes humoral and cell-mediated immunity (Tripp 2007). Antibodies located in the serum, lymphatics, and in secretory pathways mediate humoral immunity. There are five different classes of antibodies or immunoglobulins (Ig) known as IgD, IgA, IgM, IgE and IgG, four subclasses of IgG, and two subclasses of IgA. Soluble antibodies and those on the surface of B cells recognize antigens in the native form. Resistance to respiratory virus infection in the upper airways is mediated by local, secretory IgA, and is transitory. More durable humoral resistance to infection seems to be associated with IgM, and especially IgG antibodies.

In the newborn, passively acquired maternal IgG antibodies provide some protection from infection, a factor that may contribute to the reason why children under 8 weeks of age are rarely infected with RSV (Crowe 2001). In RSV-naïve infants, the maternal serum antibody titer is positively correlated with a reduced level of severe RSV disease (Collins and Graham 2008). Maternal antibody levels diminish during the first 6 months of life (Brandenburg et al., 1997)

leaving infants unprotected against RSV infection, however serum and secretory antibodies appear within days of a primary RSV infection (McIntosh et al., 1978; Welliver et al., 1980). The serum and secretory antibody titers produced in infants are significantly lower than those of older subjects (Murphy et al., 1986; de Sierra et al., 1993), and wane several months after primary infection of infants (Welliver et al., 1980). The diminished serum and neutralizing antibody response has been attributed to suppressive effects of maternally transferred antibodies (Crowe 2001; Wright et al., 2002), however immunological immaturity may also be important. Post-infection, serum antibody levels generally wane, however higher levels are maintained over a longer period after repeated RSV infection (Murphy et al., 1986; Wagner et al., 1989). As the infant matures, the presence of neutralizing IgA in respiratory secretions produced by local mucosal B cells may become more important. Studies of experimentally induced RSV disease in healthy adult volunteers indicate that the presence of nasal IgA neutralizing antibody correlates more closely with protection than does the presence of serum antibody (Mills et al., 1971).

Complement is an important component of innate immunity and the humoral response to RSV infection. RSV infected epithelial cells can activate complement by both the classical and alternative complement pathways (Smith et al., 1981; Edwards et al., 1986), and complement is important for enhanced antibody-mediated virus neutralization. However, complement activation is also associated with enhanced RSV disease mediated by activation of C3a resulting in airway hyperreactivity (Polack et al., 2002; Melendi et al., 2007).

Cell-mediated immunity to respiratory virus infection involves both adaptive immune cells, i.e. $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells, and innate immune cells, i.e. natural killer (NK) cells (Tripp 2007). The role of cell-mediated immunity in virus clearance is difficult to assess in humans, however studies in patients with deficiencies in cellular immunity have

shown that cellular immunity has a pivotal role in protection from severe RSV disease and limiting virus shedding (Fishaut et al., 1980; Chandwani et al., 1990). I*n vitro* studies of human peripheral blood mononuclear cells (PBMC) have shown that NK cells and $CD4^+$ and $CD8^+$ T cells have potent antiviral responses. Exposure of adult PBMCs to RSV immediately upregulates NK activity, a feature dependent upon IL-15 induction (Fawaz et al., 1999). Infants hospitalized because of RSV infection have increased numbers of $CD16^+/CD56^+$ NK cells, and express T helper-1 (Th1) and T helper-2 (Th2) cytokines, and enhanced CC chemokine messenger RNA (Tripp et al., 2002). Antibody-dependent cellular cytotoxicity (ADCC), a feature associated with NK cells, has been detected in human PBMC from RSV infected individuals using serum antibodies and antibodies from nasopharyngeal secretions (Scott et al., 1977; Meguro et al., 1979; Cranage et al., 1981; Kaul et al., 1982). RSV-specific cytotoxic T cell responses in PBMC from infants with acute RSV infection can be detected rapidly after infection (Isaacs et al., 1987; Chiba et al., 1989). The response is age-dependent, as >65% of infants 6-24 months of age, and approximately 35% of infants under 5 months of age exhibit RSV-specific cellular cytotoxicity.

Examination of the cytotoxic T cell (CTL) repertoire of adults to RSV proteins showed that N, SH, F, M, M2 and NS2 proteins are targeted by CTL with no predilection toward a particular MHC phenotype (Bangham et al., 1986; Cherrie et al., 1992). Most data concerning RSV-specific $CD4^+$ or $CD8^+$ T cell responses are derived from experimental animal studies, as it is difficult to assess responses in the lungs of infected patients, particularly infants and young children. Examining alterations of $CD4^+$ or $CD8^+$ T cell responses in PBMC may be useful as a measure of immune status, however studies that examine bronchial lavage cells from infants infected with RSV may provide better information. For example, a study that examined bronchial lavage cells from infants infected with RSV identified large numbers of neutrophils in

the upper airway (93%) and lower airway (76%), but few $CD4^+$ and $CD8^+$ T cells with median $CD4^+/CD8^+$ ratios being 22:1 in the upper airway, and 15:1 for the lower and upper airways (Everard et al., 1994). These results suggested that neutrophils probably have a major role in RSV disease, and discounted the concept that excessive lymphocyte cytotoxicity is associated with RSV bronchiolitis.

The role of $CD4^+$ and $CD8^+$ T lymphocytes in terminating RSV infection, causing disease, and protecting from re-infection have been investigated using experimental animal models, particularly BALB/c mice. In BALB/c mice, the M2, F and N proteins are the major targets for CTL (Openshaw et al., 1990; Nicholas et al., 1990; Openshaw 1995; Tripp 2007). Both $CD4^+$ and $CD8^+$ T cell subsets have been shown to be involved in terminating RSV replication, as antibody depletion of both T lymphocyte subsets markedly prolongs RSV replication (Graham et al., 1991). In addition, both $CD4^+$ and $CD8^+$ T cells have been shown to contribute to disease pathogenesis, although $CD8^+$ T cells appeared to have a dominant role. Investigation of the RSV proteins associated with host resistance has shown that sensitization with F or G glycoproteins induces almost complete resistance to RSV challenge despite depletion of both $CD4^+$ and $CD8^+$ T cells prior to challenge, indicating that host resistance is mediated by the humoral response to F or G glycoproteins (Connors et al., 1992). However, host resistance may also be mediated by sensitization with the M2 protein, and depletion of $CD8^+$ T cells abrogates M2-associated protection, while depletion of $CD4^+$ T cells has an intermediate effect (Connors et al., 1992). Although both $CD4^+$ and $CD8^+$ T cells contribute to disease pathogenesis, passive transfer of RSV-specific $CD4^+$ T cells into RSV-immune mice mediates more severe immunopathology and pulmonary eosinophilia compared to transfer of $CD8^+$ T cells (Alwan et al., 1992).

Distinct types of pulmonary disease are mediated by functionally different T cell subsets reactive to different RSV proteins. Adoptive transfer of T cell lines recognizing G glycoprotein into RSV infected mice induces severe illness, characterized by pulmonary eosinophilia and hemorrhaging, compared to transfer of M2-specific, or F glycoprotein-specific T cell lines (Alwan et al., 1994). It is likely that Th2-type cytokine expression by the G glycoprotein-specific cell line contributed to the disease severity, since these cells were $CD4^+$ and expressed IL-4 and IL-5, whereas M2-specific or F glycoprotein-specific T cell lines were $CD4^+$ or $CD8^+$ and expressed Th1-type cytokines. The relationship of Th1- to Th2-type cytokine expression is critical in the outcome of RSV immunity or disease pathogenesis (Lemanske 1998; Welliver 2000; Graham et al., 2002). Primary RSV infection induces a mixed Th1- and Th2-type cytokine response with limited disease pathogenesis (Openshaw et al., 2002), and from animal studies, it appears that early interferon-gamma (IFNγ) expression is key in controlling the Th1/Th2 cytokine balance (Spender et al., 1998; Boelen et al., 2002; Openshaw et al., 2002). Absence of IFNγ early after primary infection or during subsequent RSV infections has been shown to result in a predominant Th2-type cytokine response and increased disease severity (Hussell et al., 1997; Boelen et al., 2002; Durbin et al., 2002). The relationship between activation antigen and cytokine expression on leukocytes responding to RSV infection has been investigated (Tripp et al., 2000). This study showed that $CD54^+$ and $CD102^+$ lymphocytes express high levels of IL-2, IL-4, IL-5 and IFNγ, whereas lymphocytes expressing $CD44^+$, $CD49d^+$ or $CD62L^{lo}$ also express these cytokines but to a lesser extent. DNA analysis of lymphocytes expressing IL-2 or IFN-gamma revealed higher G2'M levels compared to lymphocytes expressing IL-4 or IL-5, suggesting greater activation of Th1-type lymphocytes in the lung.

RSV Proteins and Host Immune Modulation

Overview. Although our current understanding of how RSV modulates the host immune response to infection is incomplete, mounting evidence has attributed certain aspects of host immune modulation to specific RSV proteins. For example, the surface attachment glycoprotein (G) protein has been suggested to modify the pattern, magnitude and tempo of cytokine expression, the surface fusion (F) protein to induce innate immunity through TLR4 signaling, the surface small hydrophobic (SH) protein is believed to confer an anti-apoptotic effect and the nonstructural proteins (NS1 and NS2) have been shown to be potent type I interferon antagonists. Collectively, these studies suggest that RSV may utilize many immune modulating strategies in order to promote enhanced viral replication and facilitate immune evasion.

The RSV surface (F) fusion protein has been shown to play a role in modulating different aspects of host immune responses to RSV infection. Robust neutralizing antibody responses are directed against the F protein following infection and are the antigen of choice for development of passive immunization strategies for RSV disease intervention such as Synagis®. RSV infection upregulates TLR4 expression in respiratory epithelium and *in vitro* studies show that the F protein interacts with TLR4 in a CD14-dependent manner thereby stimulating innate immunity (Kurt-Jones et al., 2000). *In vivo* studies in RSV-infected TLR4-deficient mice show decreased IL-12 expression, reduced trafficking of pulmonary natural killer (NK) cells and $CD14^{+}$ cells, functionally impaired NK cells and decreased viral clearance (Haynes et al., 2001). Subsequent studies in patients with severe RSV disease have shown high frequencies of two TLR4 polymorphisms (Monick et al., 2003; Awomoyi et al., 2007; Mailaparambil et al., 2008), suggesting that defects in TLR4 predispose for exacerbated disease. Indeed, TLR4 polymorphisms are associated with development of other diseases to include periodontitis,

Crohn's disease and Alzheimer's disease (Emingil et al., 2007; Hong et al., 2007; Balistreri et al., 2008). Vaccination studies in mice scarified with recombinant vaccinia virus (vv) expressing RSV F protein (rVV-F) demonstrated production of neutralizing antibodies with enhanced IL-2 production and functional CTL responses demonstrating its role in Th1-type cytokine responses (Openshaw et al., 1992; Alwan et al., 1993).

The RSV surface attachment glycoprotein G has been implicated as a potent immune modulating viral glycoprotein that may function to facilitate immune evasion thereby enhancing viral replication. Very few RSV G-specific monoclonal antibodies effectively neutralize RSV due to antigenic strain variations and single amino acid changes that result in escape mutations (Melero et al., 1997; Collins et al., 2008). In mice, the RSV G protein is associated with reduced CD11+ and NK cell trafficking to the lung, reduced CC and CXC chemokine mRNA expression by pulmonary leukocytes, and reduced Th1- and increased Th2-type intracellular chemokine expression by pulmonary CD3+ T cells (Tripp et al., 1999; Tripp et al., 2000; Tripp 2004). Research suggests that although primary RSV infection in humans and mice is generally characterized by a mixed Th1/Th2 cytokine response (Durbin et al., 2004; Krishnan et al., 2004; Tripp et al., 2002; Tripp et al., 2005), studies in mice have revealed a predominant Th1-type response after primary infection with an RSV deletion mutant lacking both the G and SH genes when compared to infection with wild-type RSV (Tripp et al 1999) suggesting that RSV G and/or SH proteins prime Th2-type cytokine responses. Vaccination studies in mice scarified with recombinant vaccinia virus expressing RSV G protein (rVV-G) demonstrated production of neutralizing antibodies with accompanying exacerbated disease characterized by skewed Th2-type cytokine responses (IL-4 and IL-5), pulmonary eosinophilia and lack of functional CTL responses (Openshaw et al., 1992; Alwan et al., 1993). RSV G protein also contains a CX3C

chemokine motif (Tripp et al., 2001). The CX3C chemokine motif allows RSV G protein to bind to the cellular fractalkine chemokine receptor CX3CR1 thereby modulating immune responses, facilitating infection and altering leukocyte chemotaxis. Approximately 15% of the G protein synthesized in infected cells is secreted and may be associated with fractalkine mimicry, reduced antiviral T cell responses and can act as a decoy to shield RSV from neutralization by host RSV G-specific antibodies (Tripp et al., 2001; Harcourt et al., 2006; Bukreyev et al., 2008; Collins et al., 2008). Recently, studies in HEK293 cells and mDC cultures demonstrated that secreted RSV G protein inhibits IFNβ promoter activation even in the presence of IFNβ stimulators such as LPS, polyI:C and purified RSV F protein and may serve to facilitate viral replication (Shingai et al., 2008). Studies in primary neuronal cell cultures show that RSV can infect these immune privileged cells by a mechanism linked to expression of the RSV G protein and the G protein CX3C motif (Li et al., 2006) which may serve to evade host detection thereby facilitating viral persistence. Together, these data demonstrate that RSV G protein is a potent immune modulator that functions to facilitate immune evasion and enhance viral replication.

The third surface protein of RSV is the small hydrophobic (SH) protein which remains to be clearly defined. *In vitro* studies in L929 and A549 cells show that the SH protein inhibits apoptosis and TNF-α-induced NF-κB activation (Fuentes et al., 2007). Previous analysis of RSV-infected cells has shown that the cell membrane, Golgi complex, and the endoplasmic reticulum are major sites of SH accumulation with little incorporation of SH into the viral envelope (Rixon et al., 2004). More recent studies have shown that the transmembrane domains of SH proteins can form pentamers that form cation-selective ion channels in planar lipid bilayers (Gan et al., 2008). These pentameric channels are indicative of viroporins which may play roles in virus assembly and release, apoptosis, pathogenesis and cytotoxicity (Gan et al.,

2008; Collins et al., 2008). However, more studies will need to be conducted to further elucidate the functions of the SH protein.

The first two genes in the RSV genome encode for the two nonstructural proteins, i.e. NS1 and NS2. They are found in abundance in RSV-infected cells due to their promoter proximity but are not associated with the virion itself and have been shown to function as potent type I IFN antagonists. Wild-type RSV is a poor inducer of IFNα and IFNβ (McIntosh et al., 1978; Hall et al., 1978b), however infection with recombinant RSV deletion mutant virus lacking NS1/2 genes was shown to induce type I IFNs in A549 cells and human macrophages, suggesting that RSV NS proteins have an important role in inhibiting IFN gene expression (Spann et al., 2004). The mechanism for NS1/2 IFN antagonism appears to be linked to NS proteins suppressing the activation and nuclear translocation of the IFN-regulatory factor (IRF)-3 (Spann et al., 2005), and by degrading the signal transducer and activator of transcription 2 (STAT2) with Elongin-Cullin E3 ligase (Elliott et al., 2007) thereby interfering with the type I IFN JAK-STAT signaling pathway in HEp-2 cells. Small interfering RNA studies targeting the NS1 protein show increased type I IFN expression accompanied by decreased viral replication in RSV-infected A549 cells and decreased inflammation and lung viral titers in mice (Zhang et al., 2005). Early expression of both nonstructural proteins is shown to have an anti-apoptotic effect on RSV-infected A549 cells in an IFN-signaling-independent manner (Bitko et al., 2007) and is hypothesized to be mediated via activation of NF-κB and the cellular prosurvival P13K/AKT pathways which lead to induction of Bcl family members and inactivation of downstream promoters of apoptosis. Suppression of type I IFNs and apoptosis are clear examples of immune evasion strategies employed by RSV. In addition to suppressing type I IFNs and apoptosis, it has

recently been shown that NS proteins suppress dendritic cell (DC) maturation which may result in decreased antigen presentation (Munir et al., 2008).

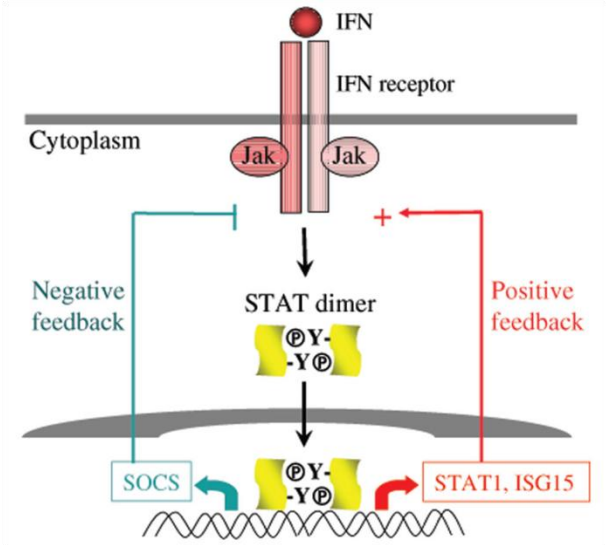
Type I Interferons (IFN)

An agent that was released from Influenza A-infected chorio-allantoic membranes into media that could then be used to interfere with primary infection of naïve membranes was termed 'interferon' in 1957 (Isaacs et al., 1957). These interferon molecules are now classified into two distinct types: type I and type II (Tsang et al., 2007). Type I interferons (IFNα and IFNβ) are potent host antiviral molecules that are rapidly induced in response to viral infection. IFNα and IFNβ signal through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signal transduction pathway and interfere with viral replication and gene transcription. Interferons are secreted by infected cells in an autocrine and paracrine manner and bind to the surface expressed IFNα/β receptors to act on neighboring cells to prevent further viral dissemination. The initial response to infection results primarily in IFNβ production but switches to IFNα during the subsequent amplification phase of the IFN response (Marie et al., 1998). As such, beyond the discussion of RSV IFN modulation, there are studies demonstrating that many viruses have developed similar evasion strategies to subvert the antiviral type I interferon response. For example, porcine reproductive and respiratory syndrome virus (PRRSV) suppresses IFNβ production in MARC-145 cells by interfering with the activation of IFNβ promoter stimulator (IPS)-1 in the RIG-I signaling pathway (Luo et al., 2008).

Interferon Stimulated Gene (ISG)-15

Interferon stimulated gene (ISG)-15 is a ubiquitin-like protein which is rapidly induced in response to viral infection, type I interferons, LPS and polyI:C (Lenschow et al., 2007). ISG15 is transcriptionally regulated by type I interferons and is part of a positive feedback loop for

JAK-STAT signaling (Reich et al., 1987; D'Cunha et al., 1996; Ivashkiv et al., 2004). ISG15 exhibits antiviral activity against RNA and DNA viruses (Lenschow et al., 2007) and conjugates to intracellular target proteins following type I interferon stimulation and functions to either stabilize target proteins or mark them for degradation, a process known as ISGylation. Over 100 intracellular target proteins have been identified and include STAT1, JAK1, ERK1, MxA, RIG-I, and IRF3 (Malakhov et al., 2003; Giannakopoulos et al., 2005; Zhao et al., 2005; Lu et al., 2006). UBP43, a protease that removes ISG15 from target proteins, is also upregulated in response to interferons and may function to negatively regulate interferon-induced signaling by disrupting ISGylation (Malakhov et al., 2003). As with type I interferons, viruses have developed immune strategies to circumvent the antiviral effects of ISG15. One such example is the ability of the NS1 protein of the influenza B virus to inhibit conjugation of ISG15 to target proteins (Yuan et al., 2001). However, to date there are no published studies demonstrating RSV modulation of ISG15 expression.

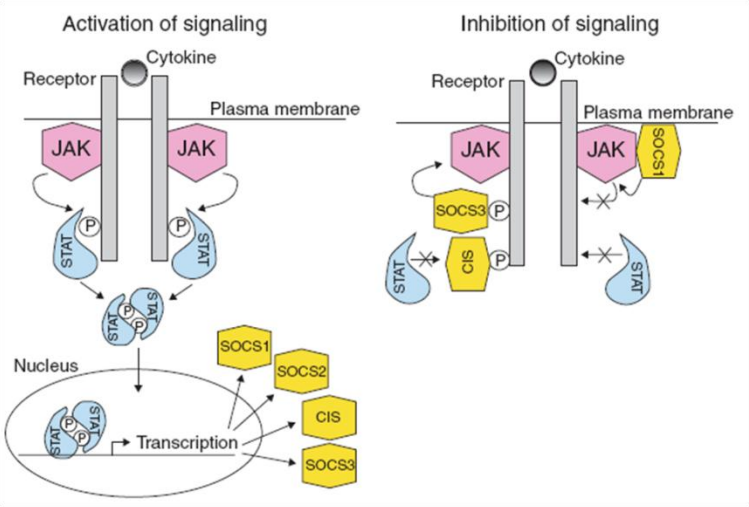


Ivashkiv and Hu 2004. Signaling by STATs.

Suppressor of Cytokine Signaling (SOCS)

Unlike ISG15, suppressor of cytokine signaling (SOCS) proteins are recognized as negative regulators of cytokine signaling. There are eight members of the SOCS family of intracellular proteins (SOCS1 to SOCS7 and CIS) and all share a central SH2 domain and a C-terminal SOCS box (Wormald et al., 2004). SOCS are induced upon cytokine receptor activation via the JAK-STAT signal transduction pathway and inhibit JAK-STAT signaling

activity. SOCS have been shown to regulate JAK/STAT signaling via four mechanisms: (1) binding to JAKs thereby inhibiting phosphorylation, (2) competing with STATs by blocking docking and phosphorylation, (3) promoting degradation of specific signaling proteins, and (4) binding to cytokine receptors. Of all the members, SOCS1 and SOCS3 appear to be the most effective in regulating type I IFN expression. SOCS1 can directly associate with high affinity with all four JAK molecules directly inhibiting their catalytic activity while SOCS3 functions in part by interacting with activated cytokine receptors (Elliott et al., 2007). SOCS1 induction by HIV-1 has been linked to attenuation of antigen presentation and enhanced stability and trafficking of the HIV-1 p55 Gag polyprotein (Ryo et al., 2008; Song et al., 2006). Recent studies in A549 cells demonstrate that Influenza A virus induces SOCS3 expression to inhibit type I IFN signaling via an NF-κB-dependent mechanism that was associated with impaired STAT1 and STAT2 phosphorylation (Pauli et al., 2008). Similar *in vitro* studies in BEAS-2B cells also showed RIG-I/MAVS/IFNAR1-dependent upregulation of SOCS1 and SOCS3 with subsequent reduction in IFNβ expression following influenza A virus infection (Pothlichet et al., 2008). Given the importance of SOCS proteins in governing patterns of cytokine expression, SOCS1 and SOCS3 have been proposed as T helper cell lineage markers since of SOCS1 is expressed 5-fold higher in Th1-type cells compared to Th2-type cells, and SOCS3 proteins expressed 23-fold higher in Th2-type cells compared to Th1-type cells (Egwuagu et al., 2002). Certain bacteria, viruses and parasites have



Krebs and Hilton 2001. SOCS Proteins: Negative Regulators of Cytokine Signaling

co-opted mechanisms to modulate the host cell SOCS response to infection to facilitate evasion (Baetz et al., 2007). Recent studies have shown that RSV infection induces SOCS1, SOCS3 and CIS in U937 cells and HEp-2 cell cultures resulting in inhibition of STAT1 and STAT2 phosphorylation (Zhao et al., 2007; Hashimoto 2008). Introduction of small interfering RNA molecules against these SOCS1 and SOCS3 suppressed SOCS expression, increased expression of 2'-5'OAS1 and decreased RSV replication in HEp-2 cells. Based on the relationship of SOCS1 and SOCS3 with respect to type I IFN regulation, this data serves to introduce SOCS molecules as possible therapeutic targets for immune modulation therapy. Successful targeting of viral upregulation of SOCS molecules may restore appropriate host immune responses leading to viral elimination.

Mouse Lung Epithelial (MLE)-15 Cells

RSV is known to infect cell types found within both the upper and lower respiratory tract to include ciliated cells, as well as type I and type II pneumocytes (Collins et al., 2008). Binding to apical surfaces of cell membranes is mediated primarily by RSV F and G surface protein interactions with cellular membrane glycosaminoglycans (Gags), especially heparin sulfate and chondroitin sulfate B (Martinez et al., 2000; Bourgeois et al., 1998; Feldman et al., 1999; Collins et al., 2007). In addition, the chemokine receptor CX3CR1 has been identified as a receptor for the RSV G protein (Tripp et al., 2001). Mouse lung epithelial (MLE)-15 cells are an immortalized type II pneumocyte cell line representing the distal bronchiolar and alveolar epithelium that maintain their differentiated phenotypes and functional characteristics for up to 30-40 cell culture passages (Wikenheiser et al., 1993). Derived from lung tumors of 4 month-old SP-C/Tag transgenic mice, these cells have microvilli, express SP-A, SP-B and SP-C, form basement membranes, and are capable of expressing MHC class I antigens (Nguyen et al., 2002;

Wikenheiser et al., 1993; Zhao et al., 2001). In general, type II pneumocytes comprise approximately 15% of total lung cells and are found at the air-liquid interface. From this position, type II pneumocyte cells are able to respond to airborne stimuli as well as interact with various immune cells such as CD8+ T cells which are known to be important immune mediators of respiratory viral infections. MLE-15 cells are a relevant choice of *in vitro* model system for our studies as they represent the distal bronchiolar and alveolar respiratory epithelium of mice, the most common animal model used in the study of host responses to RSV infection, and allow the examination of the unique responses of a single cell type found within the lung.

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Appendix A

Acute Respiratory Infections

Respiratory syncytial virus (RSV)

- Disease Burden
- Virology
- Vaccine
- Bibliography [pdf 20kb]

Disease Burden

RSV is the single most important cause of severe LRIs in infants and young children. RSV disease spectrum includes a wide array of respiratory symptoms, from rhinitis and otitis media to pneumonia and bronchiolitis, the latter two diseases being associated with substantial morbidity and mortality. Humans are the only known reservoir for RSV. Spread of the virus from contaminated nasal secretions occurs via large respiratory droplets, so close contact with an infected individual or contaminated surface is required for transmission. RSV can persist for several hours on toys or other objects, which explains the high rate of nosocomial RSV infections, particularly in paediatric wards.

The global annual infection and mortality figures for RSV are estimated to be 64 million and 160 000 respectively. In temperate climates, RSV is well documented as a cause of yearly winter epidemics of acute LRI, including bronchiolitis and pneumonia. In the USA nearly all children, by two years of age, have been infected with RSV, is estimated to be responsible for 18 000 to 75 000 hospitalizations and 90 to 1900 deaths annually. The incidence rate of RSV-associated LRI in otherwise healthy children was calculated as 37 per 1000 child-year in the first two years of life (45 per 1000 child-year in infants less than 6 months old) and the risk of hospitalization as 6 per 1000 child-years (11 per 1000 child-years in the first six months of life). Incidence is higher in children with cardio-pulmonary disease and in those born prematurely, who constitute almost half of RSV-related hospital admissions in the USA. Children who experience a more severe LRI caused by RSV later have an increased incidence of childhood asthma. These studies serve as a basis for anticipating widespread use of RSV vaccines in industrialized countries, where the costs of caring for patients with severe LRI and their sequelae are substantial. RSV also is increasingly recognized as a important cause of morbidity from influenza-like illness in the elderly.

Few population-based estimates of the incidence of RSV disease in developing countries are available, although existing data clearly indicate that, there also, the virus accounts for a high proportion of LRIs in children. Studies in Brazil, Colombia and Thailand show that RSV causes 20–30% of LRI cases in children from 1–4 years of age, a proportion similar to that in industrialized countries. In addition to accurate incidence rates, other important data for developing countries are lacking, such as the severity and case–fatality rates for RSV infection at the community level and the median age of first infection. Preliminary data from community-based studies suggest that the median age of first infection may vary between communities. This information is important for vaccination programme planners, when considering the optimal schedule for vaccination. For example, maternal immunization against RSV would be a desirable strategy to adopt if rates of infection during the first two months of life were found to be high.

Another confusing aspect of the epidemiology of RSV infection that may have an impact on vaccine use is the seasonality of the disease. In Europe and North America, RSV disease occurs as well-defined seasonal outbreaks during the winter and spring months. Studies in developing countries with temperate climates, such as Argentina and Pakistan, have shown a similar seasonal pattern. On the other hand, studies in tropical countries often have reported an increase in RSV in the rainy season but this has not been a constant finding. Indeed, marked differences in the seasonal occurrence of RSV disease have been reported from geographically contiguous regions, e.g. Mozambique and South Africa, or Bangladesh and India. Cultural and behavioral patterns in the community might affect the acquisition and spread of RSV infection. A clear understanding of the local epidemiology of the disease will be critical for the implementation of a successful vaccine development and introduction programme.

Virology

RSV belongs to the family Paramyxoviridae, subfamily Pneumovirinae, genus Pneumovirus. The genome of RSV is a 15,222 nucleotide-long, single-stranded, negative-sense RNA molecule whose tight association with the viral N protein forms a nucleocapsid wrapped inside the viral envelope. The latter contains virally encoded F, G and SH glycoproteins. The F and G glycoproteins are the only two components that induce RSV neutralizing antibody and therefore are of prime importance for vaccine development. The sequence of the F protein, which is responsible for fusion of the virus envelope with the target cell membrane, is highly conserved among RSV isolates. In contrast, that of the G protein, which is responsible for virus attachment, is relatively variable; two groups of RSV strains have been described, the A and B groups, based on differences in the antigenicity of the G glycoprotein. Current efforts are directed towards the development of a vaccine that will incorporate strains in both groups, or will be directed against the F protein.

Vaccine

Development of vaccines to prevent RSV infection have been complicated by the fact that host immune responses appear to play a role in the pathogenesis of the disease. Early studies in the 1960s showed that children vaccinated with a formalin-inactivated RSV vaccine suffered from more severe disease on subsequent exposure to the virus as compared to unvaccinated controls. These early trials resulted in the hospitalization of 80% of vaccinees and two deaths. The

enhanced severity of disease has been reproduced in animal models and is thought to result from inadequate levels of serum-neutralizing antibodies, lack of local immunity, and excessive induction of a type 2 helper T-cell-like (Th2) immune response with pulmonary eosinophilia and increased production of IL-4 and IL-5 cytokines.

In addition, naturally acquired immunity to RSV is neither complete nor durable and recurrent infections occur frequently. In a study performed in Houston, Texas, it was found that 83% of the children who acquired RSV infection during their first year of life were reinfected during their second year, and 46% were reinfected during their third year. At least two thirds of these children also were infected with PIV-3 in their first two years of life. Older children and adults, however, usually are protected against RSV-related LRIs, suggesting that protection against severe disease develops after primary infection.

Passive immunization in the form of RSV-neutralizing immune globulin or humanized monoclonal antibodies given prophylactically has been shown to prevent RSV infection in newborns with underlying cardiopulmonary disease, particularly small, premature infants. This demonstrates that humoral antibody plays a major role in protection against disease. In general, secretory IgAs and serum antibodies appear to protect against infection of the upper and lower respiratory tracts, respectively, while T-cell immunity targeted to internal viral proteins appears to terminate viral infections. Although live attenuated vaccines seem preferable for immunization of naive infants than inactivated or subunit vaccines, the latter may be useful for immunization of the elderly and high-risk children, as well as for maternal immunization. Candidate vaccines based on purified F protein (PFP-1, -2 and -3) have been found safe and immunogenic in healthy adults and in children over 12 months of age, with or without underlying pulmonary disease, as well as in elderly subjects and in pregnant women. A Phase I study of PFP-2 was conducted in 35 women in the 30th to 40th week of pregnancy; the vaccine was well tolerated and induced RSV anti-F antibody titres that were persistently fourfold higher in newborns to vaccinated mothers than to those who had received a placebo. No increase in the frequency or morbidity of respiratory disease was observed in infants from vaccinated mothers. Maternal immunization using a PFP subunit vaccine would be an interesting strategy to protect infants younger than six months of age who respond poorly to vaccination.

The efficacy of a subunit PFP-3 vaccine was tested in a Phase III trial on 298 children 1 to 12 years of age with cystic fibrosis. The vaccine was well tolerated and induced a four-fold increase in RSV neutralizing antibody titres, but this was not associated with significant protection against LRI episodes as compared to placebo recipients.

A subunit vaccine consisting of co-purified F, G, and M proteins from RSV A has been tested in healthy adult volunteers in the presence of either alum or polyphosphazene (PCPP) as an adjuvant. Neutralizing antibody responses to RSV A and RSV B were detected in 76–93% of the vaccinees, but titres waned after one year, suggesting that annual immunization with this vaccine will be necessary.

A subunit approach also was investigated using the conserved central domain of the G protein of an RSV-A strain, whose sequence is relatively conserved among groups A and B viruses. A recombinant vaccine candidate, BBG2Na (Pierre Fabre), was developed by fusing this domain

(G2Na) to the albumin-binding region (BB) of streptococcal protein G. The candidate vaccine elicited a protective immune response in animals, but was moderately immunogenic in adult human volunteers and its clinical development was interrupted due to the appearance of unexpected side effects (purpura) in a few immunized volunteers. Another RSV candidate vaccine is a synthetic peptide of the conserved region of the G protein administered intranasally, either alone or in combination with cholera toxin. Protection was conferred to mice even without the cholera toxin.

Live, attenuated RSV vaccines that could be delivered to the respiratory mucosa through intranasal immunization have been in development for more than a decade, based on temperature-sensitive (ts), cold-adapted (ca) or cold-passaged (cp) mutant strains of the virus. Difficulties for such a vaccine arise from over- or under-attenuation of the virus and limited genetic stability. Most attenuated live RSV strains tested in humans to date caused mild to moderate congestion in the upper respiratory tract of infants one to two months old and, therefore, were considered as insufficiently attenuated for early infancy. Recombinant RSV vaccines with deletion mutations in nonessential genes (SH, NS1 or NS2), and both cp and ts mutations in essential genes, are currently being evaluated.

Recombinant DNA technology also has provided the possibility of engineering a chimeric virus containing the genes of human PIV-3 surface glycoproteins F and NH, together with those of RSV glycoproteins F and G, in a bovine PIV-3 genetic background. A first candidate vaccine was found to be attenuated and to induce an immune response to both human PIV-3 and RSV in rhesus monkeys and should presently enter clinical trials. Similarly, a bovine PIV-3 genome was engineered to express human PIV-3 F and HN proteins and either native or soluble RSV protein F. Resulting recombinants induced RSV neutralizing antibodies and protective immunity against RSV challenge in African Green monkeys. These b/h PIV3/RSV F vaccines will presently be tested for safety and efficacy in human clinivcal trials as bivalent vaccines to protect infants from both RSV and PIV-3 infection and disease.

Finally, a combination of a live-attenuated vaccine with a subunit vaccine also is being considered for protecting adults against RSV illness, although a preliminary test of this strategy in healthy young and elderly adults was inconclusive.

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Appendix B

For Immediate Release

RSV Drug Starts Phase Ia Clinical Trials

MELBOURNE and GAITHERSBURG, Md., U.S.A. – **(July 17, 2007)**

Biota Holdings Limited (ASX:BTA) and MedImmune Inc., today announced the start of a Phase Ia clinical trial for their respiratory syncytial virus (RSV) antiviral drug, BTA9881, with the goal of providing a treatment for RSV infected infants and adults. Developed from original research by Biota, the drug was licensed to MedImmune on December 14, 2005.

The trial is an oral, single dose escalating, double-blinded, placebo-controlled study in 72 healthy adult volunteers. The primary objective of the trial is to assess the safety and tolerability of BTA9881, with a secondary objective to determine its pharmacokinetic properties in adults. It is being conducted with acceptance by an Independent Ethics Committee (IEC) for the clinical trial centre and by notification to the Australian Therapeutic Goods Administration. Results of the study are expected by the end of 2007.

*"We are pleased to begin the clinical testing stage of this promising anti-RSV target,"*said Genevieve Losonsky, M.D., Vice President of Clinical Development, Infectious Diseases at MedImmune.

Under the terms of the licensing agreement, MedImmune is to provide Biota with a US$3 million payment upon the initiation of the trial.

Biota CEO Peter Cook stated, *"We have been working very closely with MedImmune over the last 18 months and are delighted to have progressed BTA9881 to clinical trial stage. Biota now has three products in clinic. We have consistently focused on the delivery of milestones to generate value for our shareholders and to progress our products closer to market."*

BTA9881 is a small molecule fusion inhibitor, designed to specifically inhibit the process by which RSV infects a cell. The drug will be used to stop replication of RSV in an infected patient with the aim of clearing the infection or reducing the clinical impact of the disease.

About respiratory syncytial virus

Respiratory syncytial virus (RSV) infects people of all ages, but particularly infants, causing similar symptoms to influenza. In the northern hemisphere, the RSV season usually starts in the fall and runs through the spring.

The virus is highly contagious, infecting virtually all infants under the age of two and reinfection is common. For example approximately 50 percent of children will experience two RSV infections by the age of two.

RSV is the most common cause of bronchiolitis and pneumonia in infants and, according to the World Health Organisation (WHO), is the single most important cause of severe lower respiratory infections in infants and young children. The Centers for Disease Control and Prevention (CDC) state that 25 to 40 percent of young children will have signs of bronchiolitis and pneumonia during their first RSV infection and 0.5 to 2 percent will require hospitalisation. In particular, RSV can cause severe or lifethreatening illness in infants who are born prematurely or those with chronic lung or heart disease.

RSV can also have serious consequences in the elderly and patients with chronic lung or heart disease or with compromised immune systems.

About clinical trials

There are three phases of clinical trials before a new drug can be marketed. The respective phases are:

Phase I

The new medicine is tested in a small group (20-100) of healthy volunteers, often in a hospital setting, to determine its safety profile, including the safe dose range. Pharmacokinetic studies examine how the drug is absorbed, distributed, metabolized and excreted, as well as the duration of its action. There can be a number of Phase I studies and can take from six months to one year to fully complete.

Phase II

Placebo-controlled trials involve approximately 100 to 500 volunteer patients who have the disease being studied. The goal of this phase is to establish if the new medicine effectively treats the disease. There can be a number of such studies before the full completion of Phase II.

Phase III

The new medicine is tested in placebo-controlled trials with much larger numbers of volunteer patients to generate statistically significant safety and efficacy data, across a variety of age and ethnic groups and other population variables.

About Biota

Biota is a leading antiviral drug development company based in Melbourne Australia, with key expertise in respiratory diseases, particularly influenza. Biota developed the first-in-class neuraminidase inhibitor, zanamivir, subsequently marketed by GlaxoSmithKline as Relenza.

Biota research breakthroughs have included a series of candidate drugs aimed at treatment of respiratory syncytial virus (RSV) disease, licensed to MedImmune Inc. and novel nucleoside analogues designed to treat hepatitis C virus (HCV) infections, licensed to Boehringer Ingelheim. Biota has clinical trials underway with its lead compound for human rhinovirus (HRV) infection in patients with compromised respiration or immune systems. In addition, Biota has key partnerships with Daiichi-Sankyo for the development of second generation influenza antivirals and with Inverness Medical to market Biota developed FLU OIA influenza diagnostics.

About MedImmune

MedImmune strives to provide better medicines to patients, new medical options for physicians and rewarding careers to employees. Dedicated to advancing science and medicine to help people live better lives, the company is focused on the areas of infectious diseases, cancer and inflammatory diseases. With approximately 3,000 employees worldwide, MedImmune is headquartered in Maryland. For more information visit the company's Web site at www.medimmune.com.

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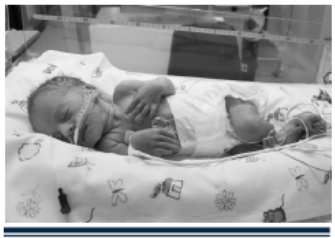
Appendix C

Respiratory Syncytial Virus (RSV)



"These promising new human data demonstrate significant anti-viral activity for an RNAi therapeutic in a major respiratory disease, an accomplishment that is notable in clinical medicine. Moreover, with a positive outcome in GEMINI, ALN-RSV01 represents the first new approach in decades for a drug demonstrated to have human anti-viral efficacy against RSV."

John P. DeVincenzo, M.D.
Associate Professor, Pediatrics and Infectious Diseases,
University of Tennessee Health Science Center

"I am encouraged by the potential for ALN-RSV01 based on its safety, tolerability, and anti-viral activity to date and I look forward to working with Alnylam in developing this RNAi therapeutic for the treatment of RSV infection in lung transplant patients. These patients have very limited treatment options and pulmonologists are in need for an RSV therapy in this critical disease area."

Martin Zamora, M.D.
Professor of Medicine, and Medical Director,
Lung Transplant Program,
University of Colorado Health Sciences Center

About the Disease

- RSV is a highly contagious virus that causes infections in both the upper and lower respiratory tracts
- RSV is the most common respiratory pathogen in infants and young children
- RSV infects nearly every child at least once by the age of two and is a major cause of hospitalization due to respiratory infection in children, people with compromised immune systems, and others
- RSV infection in the pediatric and adult populations account for >300,000 hospitalizations per year in the U.S.
- RSV infection typically results in cold-like symptoms but can lead to more serious respiratory illnesses such as croup, pneumonia, bronchiolitis, and in extreme cases, death

Incidence

According to the National Institutes of Health, RSV infections lead to more than 125,000 hospitalizations and between 1,250 and 2,500 deaths each year. The highest rates of RSV illness occur in infants under 6 months old, with a peak at age 2 to 3 months. Patients with severely compromised cardiac, pulmonary or immune systems, such as premature infants or those undergoing certain transplants, can acquire RSV infection. RSV is a significant cause of disease and hospitalization of elderly adults and those with underlying heart or lung conditions.

RSV infection in lung transplant patients is associated with significant morbidity - up to 15 to 20% of infected patients develop acute or chronic lung rejection. These patients are also at risk for an increase in frequency of bronchiolitis obliterans syndrome, a manifestation of chronic rejection that is associated with a high five-year mortality rate.

Current Treatments

A treatment for RSV infection represents a major unmet medical need in children, the elderly and in patients with compromised immune systems. There is no vaccine for RSV and the only available anti-viral has limited utilization and is not highly effective. Despite the number of RSV infections, there are very few drugs on the market to protect against or treat RSV. Current management of RSV consists of supportive care. An effective prophylactic is available for a small number of high risk infants as a monthly injection. However, even after receiving the prophylactic, patients are still at risk of being infected with RSV. Clinicians agree that there is a significant need for novel therapeutics to effectively treat patients infected with RSV.

RNA Interference (RNAi) as a Therapeutic for RSV

The development of potent and specific anti-viral therapies for prevention and treatment of infection has proven difficult using traditional pharmaceutical approaches. The therapeutic benefit of available vaccines and anti-virals is limited by the ability to interact with existing protein targets usually located on the surface of viruses. RNAi anti-viral therapeutics, on the other hand, are not restricted to targeting the viral surface proteins and can be specifically designed to target other highly conserved internal viral proteins essential for replication and infection that have, up until now, been considered "un-druggable."

Alnylam's Progress to Date

Since initiating the ALN-RSV01 therapeutic program in 2005, Alnylam has made rapid progress. Our RNAi therapeutic was designed to target the nucleocapsid "N" gene of the RSV genome, a gene that is critical for the replication of the virus. ALN-RSV01 silences the N gene, thereby reducing the virus' ability to reproduce. Extensive pre-clinical work in animals demonstrated potent and highly specific anti-viral efficacy with molecular proof of an RNAi mechanism of action. We believe the results we have demonstrated to date underscore not only to the potential to treat RSV, but the broader potential for RNAi therapeutics in human disease.

- Alnylam previously completed Phase I human clinical trials of ALN-RSV01 using both intranasal and inhaled formulations and these trials demonstrated that ALN-RSV01 was safe and well tolerated in healthy volunteers. The inhaled formulation is delivered via a nebulizer,
- In February 2008, Alnylam announced it had achieved human proof-of-concept with an RNAi therapeutic, a first for the industry. Results from the company's Phase II GEMINI study demonstrated that ALN-RSV01 was safe and well tolerated, and demonstrated statistically significant anti-viral efficacy with an approximately 40% reduction in RSV infection rate and 95% increase in infection-free subjects.
- In April 2008, Alnylam initiated a Phase II clinical trial to assess the safety and tolerability of aerosolized ALN-RSV01 versus placebo in adult lung transplant patients naturally infected with RSV. Those receiving ALN-RSV01 will have drug administered by inhalation via nebulizer which is the expected delivery formulation for commercialization. As a secondary objective, this trial will be the first to evaluate the anti-viral activity of ALN-RSV01 in a naturally acquired RSV lower respiratory tract infection.
- ALN-RSV01 is expected to advance into the pediatric patient population by the second half of 2008.

About Alnylam

Alnylam is a biopharmaceutical company developing novel therapeutics based on RNA interference, or RNAi. The company is applying its therapeutic expertise in RNAi to address significant medical needs, many of which cannot effectively be addressed with small molecules or antibodies, the current major classes of drugs. Alnylam is leading the translation of RNAi as a new class of innovative medicines with peer-reviewed research efforts published in the world's top scientific journals including *Nature, Nature Medicine,* and *Cell*. The company is leveraging these capabilities to build a broad pipeline of RNAi therapeutics; its most advanced program is in Phase II human clinical trials for the treatment of respiratory syncytial virus (RSV) infection. In addition, the company is developing RNAi therapeutics for the treatment of a wide range of disease areas, including hypercholesterolemia, liver cancers, and Huntington's disease. The company's leadership position in fundamental patents, technology, and know-how relating to RNAi has enabled it to form major alliances with leading companies including Medtronic, Novartis, Biogen Idec, and Roche. To reflect its outlook for key scientific, clinical, and business initiatives, Alnylam has established *RNAi 2010* which includes the company's plan to significantly expand the scope of delivery solutions for RNAi therapeutics, have four or more programs in clinical development, and to form four or more new major business collaborations, all by the end of 2010. Alnylam is a joint owner of Regulus Therapeutics LLC, a joint venture focused on the discovery, development, and commercialization of microRNA therapeutics. Founded in 2002, Alnylam maintains headquarters in Cambridge, Massachusetts. For more information, visit www.alnylam.com.

Various statements in this document regarding Alnylam Pharmaceuticals' business which are not historical facts are forward-looking statements that involve risks and uncertainties. For a discussion of such risks and uncertainties, which could cause actual results to differ from those contained in the forward-looking statements, see "Risk Factors" in our most recent quarterly report on Form 10-Q.

May 8, 2008

CHAPTER 3

RESPIRATORY SYNCYTIAL VIRUS (RSV) ATTACHMENT AND NONSTRUCTURAL PROTEINS MODIFY THE TYPE I INTERFERON RESPONSE ASSOCIATED WITH SUPPRESSOR OF CYTOKINE SIGNALING (SOCS) PROTEINS AND IFN-STIMULATED GENE-15 (ISG15)[1]

[1] Moore, E.C., J. Barber and R.A. Tripp. Accepted by *Virology Journal*.
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Abstract

Respiratory syncytial virus (RSV) is a major cause of severe lower airway disease in infants and young children, but no safe and effective RSV vaccine is yet available. Factors attributing to this problem are associated with an incomplete understanding of the mechanisms by which RSV modulates the host cell response to infection. In the present study, we investigate suppressor of cytokine signaling (SOCS)-1 and SOCS3 expression associated with the type I IFN and IFN-stimulated gene (ISG)-15 response following infection of mouse lung epithelial (MLE-15) cells with RSV or RSV mutant viruses lacking the G gene, or NS1 and NS2 gene deletions. Studies in MLE-15 cells are important as this cell line represents the distal bronchiolar and alveolar epithelium of mice, the most common animal model used to evaluate the host cell response to RSV infection, and exhibit morphologic characteristics of alveolar type II cells, a primary cell type targeted during RSV infection. These results show an important role for SOCS1 regulation of the antiviral host response to RSV infection, and demonstrate a novel role for RSV G protein manipulation of SOCS3 and modulation of ISG15 and IFNβ mRNA expression.

Background

Respiratory syncytial virus (RSV), a member of the *Pneumovirus* genus within the family *Paramyxoviridae,* is the single most important viral respiratory pathogen infecting infants and young children worldwide, as well as an important cause of respiratory tract illness in the elderly, transplant patients, and immune suppressed (17, 32, 49, 68, 71). The RSV genome (15kb) is single-stranded, negative-sense RNA that contains 10 transcription units which are sequentially transcribed to produce 11 proteins in the following order: NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2, and L (72). The NS1 and NS2 non-structural proteins are not expressed on the

virion but are two of the most abundantly expressed RNAs in RSV-infected cells due to their promoter-proximal location (9, 16, 21) These accessory proteins have been shown to act cooperatively to suppress the activation and nuclear translocation of the IFN-regulatory factor IRF-3 (6, 67), and inhibit the type I IFN signaling cascade by mediating proteosome degradation of signal transducer and activator of transcription 2 (STAT2) with Elongin-Cullin E3 ligase (15, 42). Additionally, constructs of "humanized" NS1 and NS2 recombinant protein expressed in *Escherichia coli* have been shown to decrease STAT2 levels as well as type I IFN responsiveness (42), and recent RNA interference (RNAi) studies in mice targeting NS proteins for silencing by short interfering RNA (siRNA) resulted in inhibition of RSV replication in mice (89). The NS1 and NS2 proteins may also function to facilitate RSV replication outside the interferon arena as they have an anti-apoptotic effect on RSV-infected A549 cells thereby enhancing viral replication (4).

Increasing evidence suggests that other RSV proteins, particularly the surface proteins on the virion, have important roles in facilitating RSV infection and replication (71). The RSV surface attachment protein, i.e. G protein, has been shown to modify pulmonary trafficking of immune cells (75), as well as the pattern and type of cytokine and chemokine expression by bronchoalveolar leukocytes (BAL) and bronchoepithelial cells in RSV-infected mice (73, 75) and in RSV-infected humans (3, 33, 69). The G protein has been shown to have a CX3C chemokine motif in the central conserved region of the protein that can mimic some of the activities of fractalkine, the only known CX3C chemokine, specifically binding to CX3CR1 and mediating CX3C-CX3CR1 leukocyte chemotaxis (26, 74). Importantly, anti-G protein antibody responses after recent RSV infection or vaccination in humans are associated with inhibition of RSV G protein CX3C-CX3CR1 interaction and G protein-mediated leukocyte chemotaxis (27).

The G protein has also been shown to inhibit Toll-like receptor (TLR) 3/4-mediated IFN-beta induction (64), a feature that may facilitate virus replication. Interestingly, the RSV F protein has been shown to induce aspects of innate immunity through TLR4 signaling (40), and TLR4-deficient mice challenged with RSV exhibit impaired NK cell and $CD14^+$ cell pulmonary trafficking, deficient NK cell function, impaired interleukin-12 expression, and impaired virus clearance compared to mice expressing TLR4 (28). In addition, TLR4 polymorphisms in humans are linked to impaired responses to respiratory syncytial virus (79) and the genetic predisposition to severe RSV infection (56). These features appear contradictory to facilitating RSV replication, but F protein activation of TLR signaling may be an important feature to desensitize TLR activation of immunity. For example, RSV has been shown to mediate long-term desensitization of lung alveolar macrophages to TLR ligands (12). This feature may be linked to the lack of durable protective immunity associated with RSV infection (70, 71). Finally, the RSV SH protein is linked to altered Th1-type cytokine and chemokine expression by BAL cells (75), and can inhibit TNFα signaling (19). Taken together, RSV surface proteins have immune modulatory features that appear to facilitate infection and replication.

It is not surprising that TLRs have an important role in the host response to RSV infection. Viral infection has been shown to activate TLRs and retinoic acid inducible gene I (RIG-I) signaling pathways leading to phosphorylation of interferon regulatory factor3 (IRF3) and IRF7 and stimulation of type I interferon (IFN) transcription, a process important for innate antiviral immunity (38). Production of type I IFN depends on activation of IRF3 and IRF7 (30, 51, 63) where type I IFN expression is negatively regulated by suppressor of cytokine signaling (SOCS) proteins (11, 35). SOCS proteins are mainly regulated at the transcriptional level but can be directly induced by stimulation of TLRs where they do not interfere with direct TLR

signaling, but instead regulate paracrine IFN signaling (11). The SOCS protein family is comprised of eight proteins (CIS, cytokine-inducible SH2-containing protein, SOCS1–7) of structural and functional homology (11, 35). Of the family members, SOCS1 and SOCS3 appear to be the most effective in regulating type I IFN expression. SOCS1 can directly associate with high affinity to all Janus kinases (JAKs) directly inhibiting their catalytic activity, while SOCS3 functions in part by interacting with activated cytokine receptors (15).

Numerous studies have established that type I IFN expression regulates hundreds of host genes that include STAT1, JAK1, ERK1, MxA, RIG-I, and IRF3 (13, 20, 39, 44, 46, 90). One important IFN-stimulated gene that encodes an ubiquitin-like protein is IFN-stimulated gene (ISG)-15 (ISG15). ISG15 is one of the earliest ISG induced by type I IFN and has been shown to target several components of the antiviral signaling pathway (39) . Virally-induced ISG15 promotes an antiviral state by subverting proteosome-mediated degradation of IRF3 in infected cells (55). As for type I IFNs, viruses have adapted to circumvent the antiviral effects of ISG15. One example is the ability of the NS1 protein of the influenza B virus to inhibit conjugation of ISG15 to target proteins (87). Since IFN genes are generally transcriptionally silent until induced, for example by binding of TLR-activated transcription factors to their promoters, ISG15 expression can reveal pathogen-TLR activation of the type I IFN response.

RSV infects ciliated airway epithelial cells in the respiratory tract (29, 88) and type II pneumocytes (10, 53, 78, 80, 81). A majority of RSV studies have used the mouse model to evaluate the host response to infection. This model has been useful to understand aspects of the immunobiology of infection. Mouse lung epithelial (MLE)-15 cells offer a good option to emulate the mouse model of RSV infection as these cells are a type II pneumocyte cell line representing the distal bronchiolar and alveolar epithelium that maintain their differentiated

phenotypes and functional characteristics for up to 30-40 cell culture passages (83). MLE-15 cells also express microvilli, SP-A, SP-B and SP-C, form basement membranes, and are capable of expressing MHC class I antigens (50, 83, 91). In general, type II pneumocytes comprise approximately 15% of total lung cells, and are found at the air-liquid interface (54, 86). From this position, type II pneumocyte cells are able to respond to airborne stimuli as well as interact with various immune cells such as $CD8^+$ T cells which are known to be important immune mediators of respiratory viral infections.

The studies reported here focus on the early antiviral host response in MLE-15 cells to RSV infection and the role of RSV surface proteins in modulating this response. The studies center on SOCS1 and SOCS3 negative regulation of the type I IFN response and ISG15 expression following infection with RSV or RSV mutant viruses lacking the G gene, or NS1 and NS2 gene deletions. These results indicate an important role for SOCS1 regulation of the antiviral host response to RSV infection, and reveal a novel role for RSV G protein modulation of SOCS3, ISG15 and IFNβ mRNA expression.

Results

RSV stimulation of SOCS1, SOCS3, IFNα and IFNβ mRNA expression

To determine the relationship between RSV infection, RSV proteins, and SOCS regulation of the type I IFN response, MLE-15 cells were infected with RSV (WT) or RSV mutant viruses lacking both the NS1 and NS2 genes (ΔNS1/2) or the G gene (ΔG). The level of RSV and RSV mutant virus replication in MLE-15 cells infected at a multiplicity of infection (MOI) = 1.0 at 24 and 48h post-infection (pi) was determined by quantitative real-time PCR analysis of RSV nucleocapsid (N) gene expression. At 24h pi, the level of virus replication was similar between RSV and RSV mutant viruses where N gene copies were 2.6×10^5 for WT, 2.1 x

10^5 for ΔNS1/2, and 2.7 x 10^5 for ΔG viruses. However, at 48h pi, the level of ΔNS1/2 virus replication was significantly ($p<0.01$) lower (6.4 x 10^4 N gene copies) compared to RSV (5.5 x 10^5 N gene copies) or ΔG (4.9 x 10^5 N gene copies) virus replication which was not significantly ($p<0.05$) different from each other. Visual examination of RSV and RSV mutant virus infected MLE-15 cells at 48h pi showed higher cytopathic effects for ΔNS1/2 infected cells compared to RSV or ΔG infected MLE-15 cells. These findings are consistent with the report showing RSV nonstructural proteins have an important role in delaying apoptosis linked to infection (4).

RSV and RSV mutant virus infection of MLE-15 cells at 24h pi was associated with IFNα, IFNβ and SOCS1 and SOCS3 mRNA expression. SOCS1 mRNA expression was significantly ($p<0.01$) lower in ΔNS1/2 virus infected MLE-15 cells compared to WT or ΔG virus infected cells (Figure 1A). This finding is in keeping with the findings of NS1/NS2 antagonism of type I IFNs (6, 66, 67) and suggests the possibility that type I IFN antagonism is linked to NS1/NS2 induction of SOCS1 and subsequent negative regulation of type I IFN activity (11, 35). The level of SOCS3 mRNA expression was similar in WT, ΔG or ΔNS1/2 virus infected MLE-15 cells. Since the level of virus replication was similar between RSV and RSV mutant viruses at 24h pi, and SOCS1 mRNA expression was significantly lower in ΔNS1/2 virus infected MLE-15 cells, these results suggest that RSV infection of MLE-15 cells preferentially induces SOCS1 over SOCS3 mRNA expression, an effect associated with NS1/NS2 expression. Despite differences in SOCS1 mRNA expression, the levels of IFNα and IFNβ mRNA expression were similar between RSV and RSV mutant virus infected MLE-15 cells. This is not unexpected because SOCS proteins form part of a classical negative feedback loop that is time-dependent (35), thus RSV and RSV mutant virus infection of MLE-15 cells and IFNα, IFNβ and SOCS1 and SOCS3 mRNA expression was examined at 48h pi.

At 48h pi, ΔNS1/2 virus infected MLE-15 cells had significantly ($p<0.01$) higher IFNα and IFNβ mRNA expression compared to WT or ΔG virus infected cells (Figure 1B), indicating a governing function of NS1/NS2 in type I IFN antagonism. In addition, a higher level of SOCS1 mRNA expression was evident at 48h pi compared to similar infection at 24h pi (Figure 1A) despite a significantly ($p<0.05$) lower N gene copy compared to WT or ΔG virus infected cells. The higher SOCS1 mRNA expression at 48h pi possibly reflects a compensating host cell mechanism to regulate type I IFN expression as SOCS3 mRNA expression also increased. The levels of IFNα and IFNβ and SOCS1 and SOCS3 mRNA expression were similar between WT and ΔG virus infected MLE-15 cells. Comparing time-points post-WT or ΔG virus infection, no significant ($p<0.05$) changes in IFNα, IFNβ or SOCS1 mRNA expression were observed at 24h pi (Figure 1A) or 48h pi (Figure 1B); however, SOCS3 mRNA expression was considerably decreased from 24h pi to 48h pi.

RSV stimulation of SOCS1, SOCS3, IFNα and IFNβ protein expression

To determine if the type I IFN and SOCS mRNA expression profiles in RSV and RSV mutant virus infected cells were reiterated by protein expression, intracellular IFNα, IFNβ and SOCS1 and SOCS3 protein levels were determined at 24h and 48 h pi by flow cytometry (Figure 2). At 24h or 48h pi, IFNα and IFNβ protein expression in RSV and RSV mutant virus infected MLE-15 cells was low and not readily detected. In the mouse, total IFNα is comprised of at least 14 IFNα genes and 3 IFNα pseudogenes (77), and because the quantity of IFNα measured depends on the specificity of the detection antibody for these isoforms, detection of IFNα is limited. Moreover, low levels of type I interferon protein expression would be predicted in part because of the transient nature of these proteins as they are rapidly secreted and their expression is regulated by factors linked to IFN-stimulated genes such as ISG15 which targets several

components of the IFN signaling pathway (39, 82). At 24h pi, SOCS1 protein expression levels were similar following infection with RSV or RSV mutant viruses; however SOCS3 protein expression was significantly ($p<0.05$) higher in ΔG virus infected cells compared to WT infected cells, and substantially higher compared to ΔNS/2 virus infected cells (Figure 2A). The higher SOCS3 protein expression following ΔG virus infection suggests that G protein expression reduces SOCS3 protein expression during RSV infection. This may be important to enhance SOCS-mediated negative regulation of cytokine expression (11, 35) and/or alter the Th1/Th2 cell differentiation process to facilitate virus replication, as SOCS3 has been linked to the development of Th2-type responses (37). At 48h pi, ΔNS1/2 virus infected cells expressed significantly higher ($p<0.05$) SOCS1 protein compared to WT and ΔG virus infected MLE-15 cells (Figure 2B), a finding consistent with SOCS1 mRNA expression at 48h pi (Figure 1B), and the concept that NS1/NS2 proteins mediate IFN antagonism in part by affecting SOCS1 negative regulation of type I IFN activity (11, 35). Similar to the 24h pi finding, at 48h pi ΔG virus infected cells expressed significantly ($p<0.05$) higher SOCS3 protein compared to WT or ΔNS1/2 infected cells (Figure 2B). Since NS1/NS2 in RSV has been shown to act cooperatively to suppress the activation and nuclear translocation of the IFN-regulatory factor IRF-3 (6, 67), and antagonize type I IFN activity by inhibiting the type I IFN and the signaling cascade (15, 42), the results indicate that SOCS3 may not have an essential role governing type I IFN during RSV infection, but may have an ancillary role to facilitate virus replication.

RSVΔG virus infection mediates enhanced IFNβ secretion

Intracellular type I IFN expression in RSV and RSV mutant virus infected MLE-15 cells was not effectively detected above background levels at 24h and 48h pi by flow cytometry. Commercially available mouse IFNα ELISA kits were evaluated but found to have a poor

threshold of detection as expected given the limited specificity of the detection antibody used in the kits for detection of the numerous IFNα isoforms (77). However, IFNβ was detected in all RSV and RSV mutant virus infected MLE-15 cell culture supernatants (Figure 3). MLE-15 cells infected with ΔG virus had significantly ($p<0.01$) higher levels of IFNβ compared to WT or ΔNS1/2 virus infected cells at 24h and 48h pi, indicating that G protein expression inhibits IFNβ protein expression. RSV has been shown to down-regulate STAT2 protein expression (15) and the type I IFN JAK-STAT pathway (57), thus it is possible that G protein inhibits cellular transcription factors involved in IFNβ signaling. IFNβ levels in the supernatant from ΔNS1/2 virus infected cells was slightly but insignificantly lower compared to cell culture supernatant from WT virus infected cells.

ISG15 expression is increased in the absence of G protein expression

Expression of the interferon-stimulated gene, ISG15, was determined in RSV and RSV mutant virus infected MLE-15 cells (Figure 4). ISG15 has been shown to modify several important molecules linked to and affecting type I interferon signal transduction, is released from cells to mediate extracellular cytokine-like activities, and evidence suggests that IFNβ and ISG15 are induced in parallel as a primary response to infection (2, 55, 58, 60). The level of ISG15 mRNA expression (Figure 4A) was similar to the level of ISG15 protein expression at 24h and 48h pi where similar levels were observed following WT or ΔNS1/2 infection of MLE-15 cells. However, ISG15 mRNA (Figure 4A) and protein (Figure 4B) levels were significantly ($p<0.05$) higher in ΔG virus infected cells compared to WT or ΔNS1/2 virus infected cells indicating that G protein expression impedes ISG15 mRNA and protein expression. These findings are consistent with IFNβ governance of ISG15 expression (2, 55, 58, 60), and the finding that G protein expression inhibits IFNβ protein expression (Figure 3).

Discussion

Numerous studies investigating the host cell response associated with RSV infection have shown that RSV proteins can affect the spectrum of the antiviral cytokine response (3, 21, 45, 53, 71, 76), but the mechanisms linked to RSV protein regulation of the associated cell signaling pathway remains unclear. The studies reported here examine the early antiviral host response in MLE-15 cells to RSV infection and the role of RSV surface proteins in modulating this response. Studies in MLE-15 cells are important as this cell line represents the distal bronchiolar and alveolar epithelium of mice (83), and mice are the most common animal model used to evaluate the host cell response to RSV infection. MLE-15 cells exhibit morphologic characteristics of alveolar type II cells that include microvilli, cytoplasmic multi-vesicular bodies, and multi-lamellar inclusion bodies, maintain functional characteristics of distal respiratory epithelial cells including the expression of surfactant proteins (83), thus using MLE-15 cells as a proxy for RSV infection in mice offers several advantages to advance studies examining the host cell response to infection. In these studies, the role of SOCS1 and SOCS3 negative regulation of the type I IFN response and ISG15 expression were evaluated after infection of MLE-15 cells with RSV or RSV mutant viruses lacking the G gene, or having NS1 and NS2 gene deletions. RSV and RSV mutant virus infection of MLE-15 cells induced different type I IFN and SOCS1 and SOCS3 mRNA expression patterns at 24h and 48h pi, a feature that may be linked to sequential RSV gene expression due to their promoter-proximal location in the genome (9, 16, 21). At 24h pi, SOCS1 mRNA expression was significantly lower in ΔNS1/2 virus infected MLE-15 cells compared to WT or ΔG virus infected cells. This finding is consistent with NS1/NS2 antagonism of type I IFN activity (6, 66, 67). These results also indicate that NS1/NS2 may in part mediate type I IFN antagonism through the induction of SOCS1 which negatively regulates type I IFN

expression (11, 35). At 48h pi, SOCS1 mRNA and protein expression was higher in ΔNS1/2 virus infected MLE-15 cells compared to WT or ΔG virus infected cells suggesting a host cell compensating mechanism to negatively regulate an earlier increase in type I IFN expression or cell signaling. Interestingly, SOCS3 protein expression was significantly higher in MLE-15 cells infected with ΔG virus compared to WT or ΔNS1/2 virus infected cells, indicating that G protein expression deters SOCS3 protein expression during RSV infection. Since SOCS3 is predominantly expressed during the Th2-type immune response and reciprocally inhibits Th1-type differentiation processes (37), the results suggest that G protein may induce SOCS3 protein expression to facilitate RSV replication by inhibiting antiviral Th1-type responses.

Several factors negatively regulate IFNβ, and for RSV, it has been recently shown that RSV G proteins mediates down-regulation of IFNβ by inhibiting IFNβ promoter activation (64), demonstrating yet another novel function of the G protein in the regulation of host cell response. In the study reported here, significantly higher levels of IFNβ expression were detected in the cell culture supernatants of ΔG virus infected MLE-15 cells compared to WT or ΔNS1/2 virus infected cells, a finding consistent with the G protein inhibition of IFNβ promoter activation (64). No increase in IFNβ expression was detected in the cell culture supernatant of ΔNS1/2 virus infected MLE-15 cells relative to WT virus infected cells despite the reported finding that NS1 and NS2 act cooperatively to suppress activation and nuclear translocation of IRF3 (67). Since RSV-induced cytokine gene expression occurs through the activation of a subset of transcription factors including IRF3 (31), the ability of RSV to induce expression and catalytic activity IKKε which blocks RSV-induced IRF3 phosphorylation, nuclear translocation and DNA-binding, and leading to inhibition of cytokine gene transcription, mRNA expression and protein synthesis (31) may mask the activities of NS1/NS2.

Interferon stimulated gene (ISG)-15 is a type I interferon-induced molecule that is rapidly upregulated in response to viral infection (55, 58). Expression of ISG15 mRNA and protein expression was significantly upregulated in the absence of the RSV G gene (ΔG virus) at 24h and 48h pi indicating the novel finding that G protein modifies ISG15 expression to limit its role in the antiviral host cell response. ISG15 is one of scores of ISGs which may be induced directly or indirectly by virus proteins or byproducts of virus infection (61); however, as expression of ISG15 mRNA and protein was similar between ΔNS1/2 and WT virus infection of MLE-15 cells, it is unlikely NS1/NS2 has a role in modifying ISG15. The finding in this study that G protein expression inhibits IFNβ and ISG15 protein expression is consistent with evidence suggesting that IFNβ and ISG15 are induced in parallel as a primary response to infection (2, 55, 58, 60), and that this pathway is targeted by RSV G protein.

Conclusions

The findings from this study show an important role for SOCS1 regulation of the early type I IFN response to RSV infection, and allude to the possibility that NS1/NS2 may in part mediate type I IFN antagonism through the induction of SOCS1 negative regulation of type I IFN expression. In addition, the results show that RSV G protein has reduced SOCS3 expression and shows a previously unrecognized role of G protein in regulation of IFNβ and ISG15 expression. Notably, these studies were performed using MLE-15 cells, a type II alveolar cell line that represents the distal bronchiolar and alveolar epithelium of mice, the most common animal model used to evaluate the host cell response to RSV infection. Thus, these findings have important implications in understanding the mechanisms linked to RSV disease pathogenesis and treatment.

Methods

Viruses and cells

Type I IFN-free virus stocks of recombinant RSV strain A2 (6340WT), 6340WT lacking the G protein gene (6340ΔG), and 6340WT lacking NS1 and NS2 genes (ΔNS1/2) (kind gift of Peter Collins, NIH) were propagated in Vero cells (African green monkey kidney fibroblasts, ATCC CCL 81) maintained in DMEM (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 5% heat-inactivated (56°C) fetal bovine serum (FBS; Hyclone Laboratories, Salt Lake City, Utah) as previously described (75). Infectious virus titers were determined on Vero cells by endpoint dilution and counting of infected cell foci stained for indirect immunofluorescence with an RSV F-specific monoclonal antibody (clone 131-2A) as previously described (75).

Mouse lung epithelial (MLE)-15 cells (kind gift from Dr. Jeffrey A. Whitsett, Children's Hospital Medical Center, Cincinnati, Ohio) are an immortalized type II pneumocyte cell line representing the distal bronchiolar and alveolar epithelium that maintain their differentiated phenotypes and functional characteristics for up to 30-40 cell culture passages. MLE-15 cells were propagated in hydrocortisone-insulin-transferrin-β-estradiol-sodium selenite (HITES) medium supplemented with 4% fetal bovine serum as previously described (83).

RNA isolation and quantitative real-time PCR

Total RNA was isolated from uninfected, uninfected Vero cell lysate treated, and RSV and RSV mutant virus infected (MOI = 1) MLE-15 cells at 24h or 48h pi using RNeasy Mini kit (Qiagen, Valencia, CA) and stored at -80°C until used. Reverse transcription of pooled RNA was performed using random hexamers and MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). cDNA diluted 1:4 was used as template using SOCS1, SOCS3, pooled IFNα4 and IFNα9, and IFNβ1 gene expression assays (Applied Biosystems, Foster City, CA) and analyzed

using MX300P software by Stratagene (La Jolla, CA). Each gene of interest was normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) expression and calibrated to its corresponding expression in mock-infected or mock-stimulated MLE-15 cells. Data is presented as fold-differences in gene expression relative to mock-infected or mock-stimulated MLE-15 cells.

To establish a standard curve for the quantitation of RSV N gene present in RSV-infected MLE-15 cells, the RSV N gene was amplified by PCR and inserted into a pcDNA3.1 vector. This vector was then used to transform competent E. coli One Shot® TOP10 cells (Invitrogen, Carlsbad, CA). The colonies were screened for ampicillin resistance and the resulting plasmid containing the RSV N gene was verified by sequence analysis. The standard curve was created using 10-fold serial dilutions of 1ug/ul of RSV N gene plasmid. Samples along with standard curve dilutions were analyzed by real-time PCR with the Stratagene Mx3000P or Mx3005P for 40 cycles with custom RSV N gene primers purchased from Applied BioSystems. Data is expressed as copies of RSV N gene.

Intracellular protein analysis by flow cytometry

MLE-15 cells were infected with WT, ΔG or ΔNS1/2 virus at a MOI = 1.0, mock infected with uninfected Vero cell lysate, or incubated in the presence of media alone. At 24 and 48 hours pi, the cells were treated with 1μg/ml BD GolgiPlug™ (Brefeldin A, BD Pharmingen, San Diego, CA) for 5 hours prior to fixation with 4% formaldehyde and analyzed or stored at 4°C prior to staining. Cells were permeabilized with 1X BD Perm/Wash™ and stained with either rabbit anti-SOCS1 polyclonal antibody or goat anti-SOCS3 polyclonal antibodies (Abcam, Cambridge UK), rabbit anti-ISG15 polyclonal antibody (Cell Signaling Technology, Danvers, MA) or rat anti-mouse IFNα or IFNβ polyclonal antibody (PBL InterferonSource, Piscataway, NJ) using

similar methods as previously described (75). Intracellular protein expression was analyzed using a BD LSR II flow cytometer and evaluating 30,000 gated events. Data is presented as fold increase relative to cells cultured in the presence of media only.

ELISA quantitation in cell supernatants

MLE-15 cells were infected with WT, ΔG or ΔNS1/2 virus at a MOI = 1.0, mock infected with uninfected Vero cell lysate, or incubated in the presence of media alone. At 24 and 48 hours pi, cells supernatants were collected, centrifuged to remove potential cell contamination and debris, and used immediately or stored at -80°C prior to analysis. Levels of IFNβ in cell culture supernatants were measured using the Mouse Interferon Beta ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ) according to the manufacturer's protocol. Absorbance at 450nm was read using the BIO-TEK PowerWave XS microplate reader (Tecan US, Durham, NC) and the data was analyzed using KC junior software (Tecan US, Durham, NC).

Statistics

All experiments in this study were independently performed 5-6 times. For PCR assays, differences in gene fold expression were evaluated by Student *t* test and considered significant when the *P* value was <0.05. Data are shown as means ± standard errors (SE) of the means. Comparison of results between RSV and RSV mutant virus experiments were performed by the Mann-Whitney U test using the InStat 3.05 biostatistics package (GraphPad, San Diego, CA). Unless otherwise indicated, mean ± SEM is shown.

Competing interests

The authors have no financial or non-financial competing interests.

Authors' contributions

EM carried out the molecular studies, cell studies and ELISA assays, participated in the flow cytometry, and drafted the manuscript. JB performed the flow cytometry. RT conceived the study, participated in the design of the study, and with EM performed the statistical analysis. All authors read and approved the final manuscript.

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Figure Legends

Figure 3-1

RSV stimulation of SOCS1, SOCS3, IFNα and IFNβ mRNA expression. MLE-15 cells were mock-infected or infected with WT, ΔG, or ΔNS1/2 virus at a multiplicity of infection (MOI) of 1 for 24h (A) or 48h (B). Cells were harvested at the times indicated. SOCS1, SOCS3, IFNα and IFNβ mRNA expression were measured by real-time PCR. Transcript levels were normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) expression and calibrated to the mock condition. Data is presented as fold-differences in gene expression relative to mock-infected MLE-15 cells. Differences in gene fold expression between virus infection groups were evaluated by Mann-Whitney U test and noted as significant as denoted by an asterisk. Data are shown as means ± standard errors (SE) of the means.

Figure 3-2

RSV stimulation of SOCS1 and SOCS3 protein expression. RSV stimulation of SOCS1 and SOCS3 protein expression was determined in MLE-15 cells that were mock-infected or infected with WT, ΔG, or ΔNS1/2 virus at a multiplicity of infection (MOI) of 1 for 24h (A) or 48h (B). Cells were harvested at the times indicated and intracellular SOCS1 or SOCS3 levels determined by flow cytometry. Data is presented as fold-differences in protein expression relative to mock-infected cells. Differences in fold expression between virus infection groups were evaluated by Mann-Whitney U test and noted as significant as denoted by an asterisk. Data are shown as means ± standard errors (SE) of the means.

Figure 3-3

RSVΔG virus infection mediates enhanced IFNβ secretion. The levels of IFNβ in MLE-15 cell culture supernatant were determined following infection with WT, ΔG, or ΔNS1/2 virus at a

multiplicity of infection (MOI) of 1 for 24h (A) or 48h (B) as indicated. Data are shown as means ± standard errors (SE) of the means.

Figure 3-4

ISG15 expression is increased in the absence of G protein expression. MLE-15 cells were mock-infected or infected with WT, ΔG, or ΔNS1/2 virus at a multiplicity of infection (MOI) of 1 for 24h or 48h as indicated. ISG15 message expression was measured by real-time PCR (A). Transcript levels were normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) expression and calibrated to the mock condition. (B) RSV stimulation of ISG15 protein expression was determined in MLE-15 cells that were mock-infected or infected with WT, ΔG, or ΔNS1/2 virus at a multiplicity of infection (MOI) of 1 for 24h or 48h as indicated. Cells were harvested and ISG15 levels determined by flow cytometry. Data is presented as fold-differences in protein expression relative to mock-infected cells. Differences in fold expression between virus infection groups were evaluated by Mann-Whitney U test and noted as significant as denoted by an asterisk. Data are shown as means ± standard errors (SE) of the means.

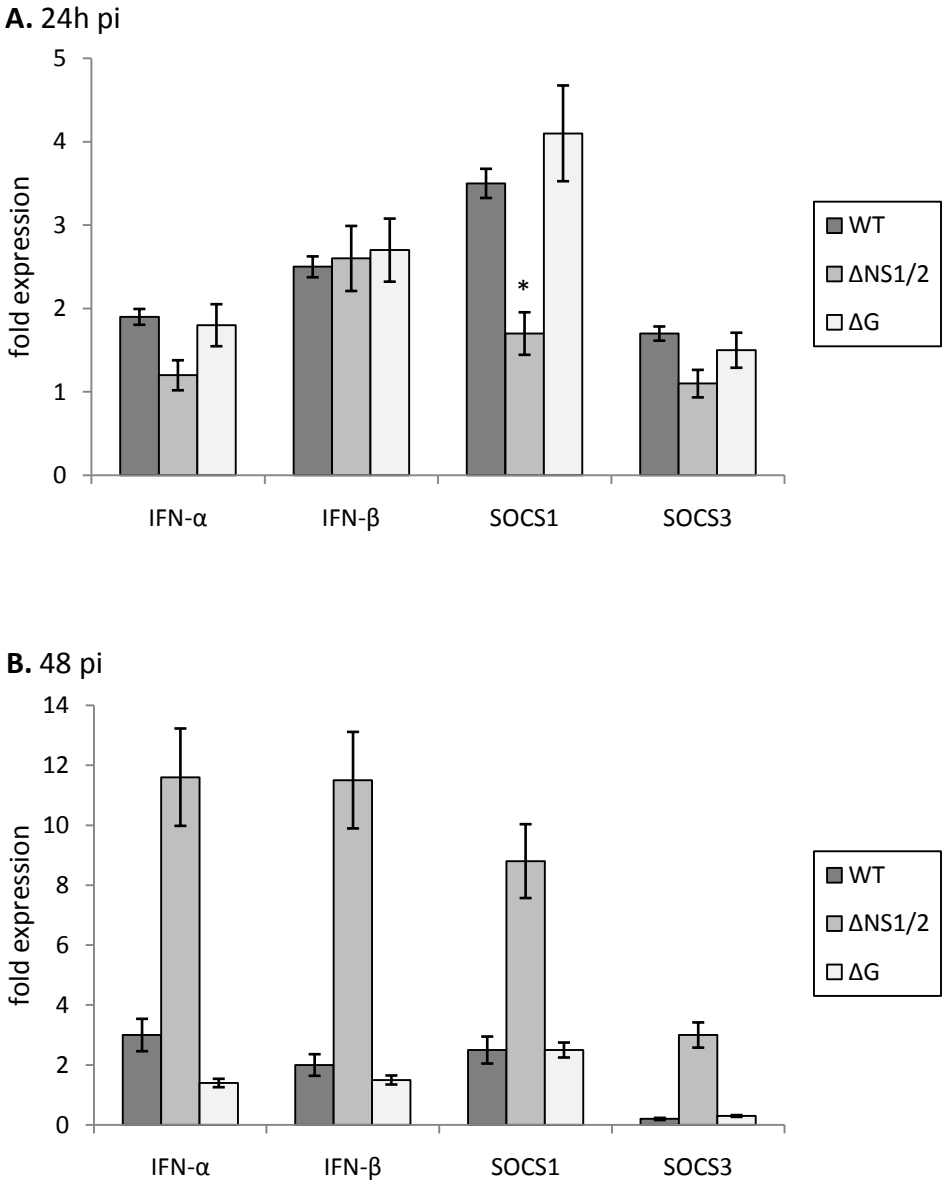
A. 24h pi
fold expression
5
4
3
2
1
0
*
IFN-α
IFN-β
SOCS1
SOCS3
WT
ΔNS1/2
ΔG
B. 48 pi
fold expression
14
12
10
8
6
4
2
0
IFN-α
IFN-β
SOCS1
SOCS3
WT
ΔNS1/2
ΔG

Figure 3-1

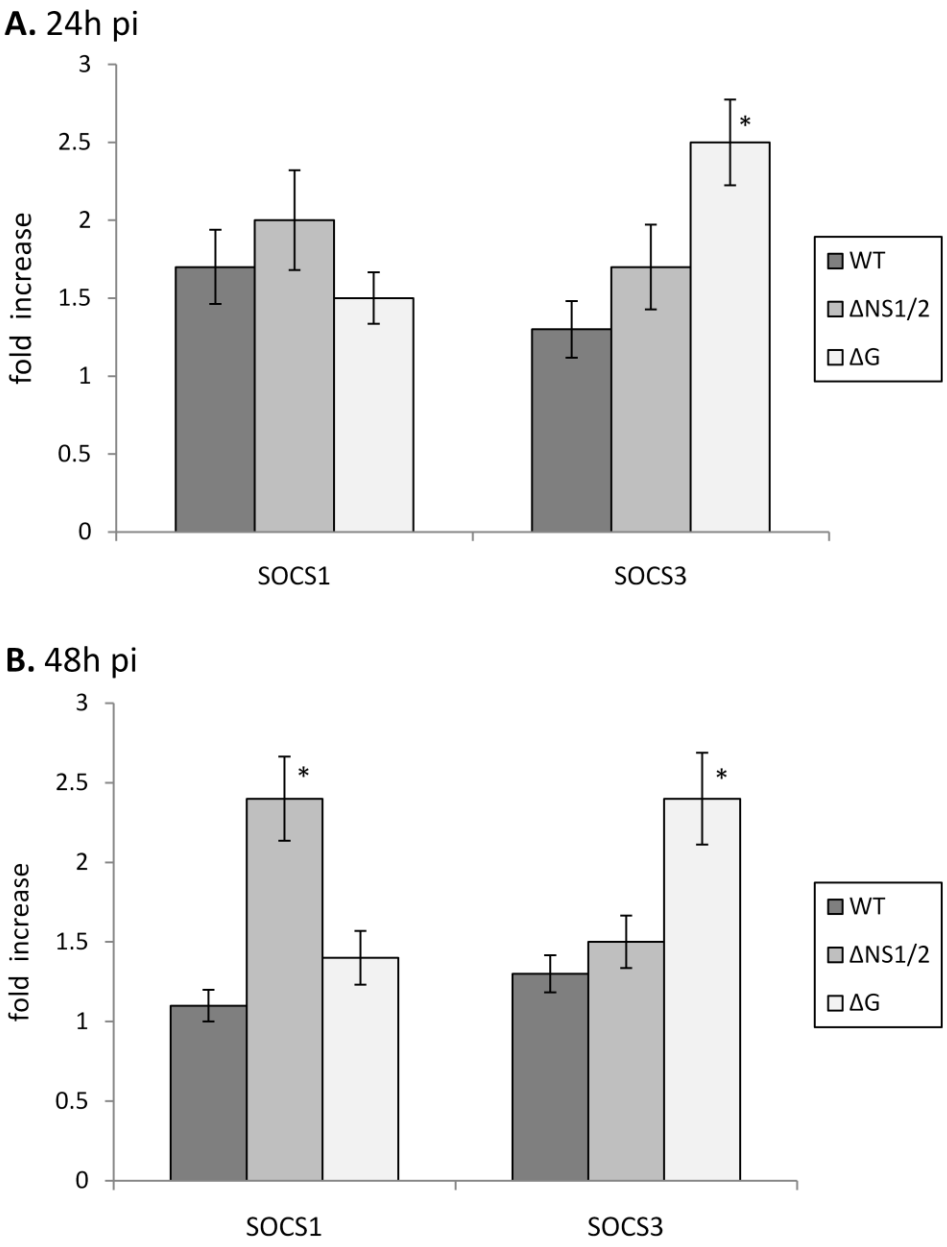
A. 24h pi
fold increase
3
2.5
2
1.5
1
0.5
0
*
SOCS1
SOCS3
WT
ΔNS1/2
ΔG
B. 48h pi
fold increase
3
2.5
2
1.5
1
0.5
0
*
*
SOCS1
SOCS3
WT
ΔNS1/2
ΔG

Figure 3-2

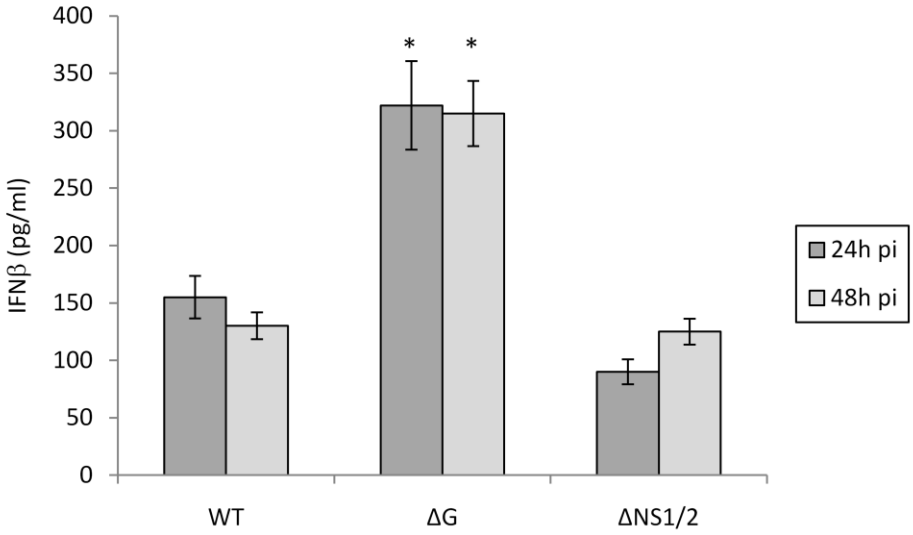
400
350
300
250
200
150
100
50
0
IFNβ (pg/ml)
*
*
24h pi
48h pi
WT
ΔG
ΔNS1/2

Figure 3-3

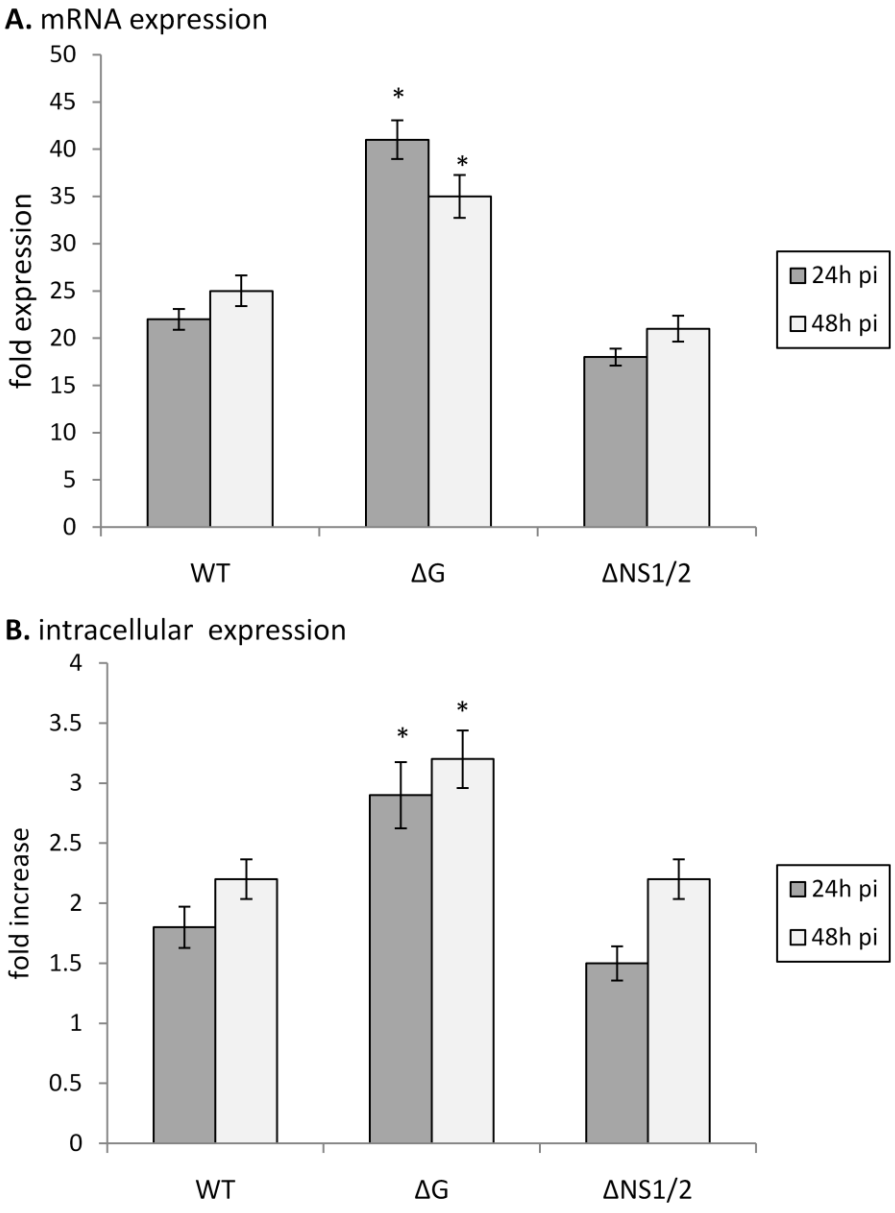
A. mRNA expression
fold expression
50
45
40
35
30
25
20
15
10
5
0
*
*
WT
ΔG
ΔNS1/2
24h pi
48h pi
B. intracellular expression
fold increase
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
WT
ΔG
ΔNS1/2
24h pi
48h pi

Figure 3-4

CHAPTER 4

VESICULAR STOMATITIS VIRUS (VSV) BIOASSAY FOR DETECTION OF INTERFERON SECRETED FROM RSV-INFECTED MLE-15 CELLS

Introduction

Respiratory syncytial virus (RSV) has been shown to be a poor inducer of host antiviral type I interferon, i.e. IFNα and IFNβ (McIntosh 1978; Hall et al., 1978). Our studies examining type I IFNs induced in response to RSV infection of MLE-15 cells showed upregulation of type I interferon mRNA following RSV infection; however, little to no intracellular type I IFN proteins were detected by flow cytometry in MLE-15 cells infected with wild-type (wt) RSV or with recombinant RSV lacking the G or the NS1 and NS2 genes at 24 and 48 hours post-infection (pi) (Moore et al., 2008). This finding could be attributed to the transient nature of these proteins as they are rapidly induced and secreted by infected cells to act in an autocrine and paracrine manner to establish an antiviral environment. Therefore, we evaluated type I IFN protein expression by bioassay and by commercial ELISA kits to quantitate the amount of type I interferons detectable in the cell culture supernatants.

As shown in Chapter 3, IFNβ was readily detected in the supernatants of RSV-infected MLE-15 cells at both 24 and 48 hours pi with the commercially available murine IFNβ ELISA kit (Mouse Interferon Beta ELISA Kit PBL 42400-1, PBL Biomedical Laboratories, Piscataway, NJ) (Moore et al., 2008). Importantly, it is known that during viral infection that the initial host cell response is primarily IFNβ production, but later switches predominance to IFNα during the subsequent amplification phase of the IFN response (Marie et al., 1998). However, levels of

IFNα were not detected in fresh or frozen supernatants by the commercially available IFNα ELISA kit. This inability to detect IFNα could be attributable to lower limits of the threshold of detection provided by the kit (12.5-300 pg/ml) and/or the limited specificity of the monoclonal antibodies employed in the kit as murine IFNα has at least 14 isoforms and three pseudogenes (van Pesch et al., 2004; Tsang et al., 2007).

To determine if the presence of IFNα in the supernatants of RSV-infected MLE-15 cells could be established, a sensitive IFN bioassay was employed. Bioassays are quantitative or qualitative assays that can be used to assess the effect of a substance on a living organism and are known to be substantially more sensitive than ELISA analysis. The bioassay used was based on vesicular stomatitis virus (VSV) and murine L929 cell infection (UNIT 6.9 Measurement of Antiviral Activity Induced by Interferons α, β, and γ, Current Protocols in Immunology, Copyright © 1991 by John Wiley and Sons, Inc.) to quantitatively determine the presence and concentration of bioactive IFNα in the supernatants of RSV-infected MLE-15 cells by measuring the inhibition of VSV cytopathic effect (CPE). The VSV bioassay can be used to determine levels of IFNα, IFNβ, and IFNγ in cell culture supernatants or serum of murine or human sources and plates can be scored either visually with crystal violet staining or spectrophotometrically by absorbance.

Results

A representative 96-well VSV bioassay plate is shown in Figure 4-1. In order to evaluate the bioactivity of IFNα alone, an optimal concentration of polyclonal anti-IFNβ (PBL product # 32400-1) was used with recombinant IFNβ (PBL product # 12400-1) control in order to effectively neutralize IFNβ activity in the samples. It was not necessary to determine the optimal concentration of anti-IFNγ because our previous studies show that MLE-15 cells do not produce

IFNγ in response to RSV infection (unpublished data from our laboratory). Recombinant IFNα (PBL product # 12100-1) was used as a standard and along with the unknown samples was subjected to 2-fold serial dilutions. The endpoint of the assay was determined visually by crystal violet staining for the presence of 100% CPE, or complete cytopathic lysis of the L929 monolayer. In this assay, the first dilution in a series of the recombinant IFNα standard that exhibits 100% CPE is given an arbitrary value of 100U/ml. Concentrations of bioactivity of unknown samples are then subsequently interpolated from the rIFNα standard dilution curve. As determined by the VSV bioassay, MLE-15 cells were shown to secrete bioactive IFNα in response to wtRSV and RSVΔG infection (Figure 4-2); however, no IFNα bioactivity above the negative controls was detected in the supernatants of MLE-15 cells infected with RSVΔNS1/2 at either 24 or 48 hours pi.

Discussion

In support of the real-time PCR data (Chapter 3) which showed upregulation of type I IFN mRNA in response to RSV infection (Moore et al., 2008), we also demonstrated by bioassay that low levels of bioactive IFNα are secreted from MLE-15 cells infected with wtRSV and RSVΔG. Upon reassessment of our raw IFNα ELISA data, absorbance patterns emerged with respect to the wtRSV and RSVΔG samples that corresponded with the bioassay results (data not shown). Moreover, in support of the absence of detection of bioactive IFNα in the supernatants of RSVΔNS1/2-infected MLE-15 cells, ELISA absorbance levels of RSVΔNS1/2 samples were similar to the blank absorbance levels (data not shown). Significant differences in levels of bioactive IFNα were not detected between wtRSV and RSVΔG infections at 24h pi, however, levels were significantly decreased in wtRSV supernatants at 48h pi ($P<0.05$) while levels were higher following ΔG infection at both 24 and 48 hours pi. These findings are consistent with

known patterns of cytokine and chemokine expression during RSV infections (Guerrero-Plata et al., 2005a, Guerrero-Plata et al., 2005b; Tripp 2004).

The inability to detect bioactive IFNα in the supernatants of RSVΔNS1/2-infected MLE-15 cells is unclear as the NS proteins have been shown to be type I IFN antagonists in human models of RSV disease (Spann et al., 2004; Ramaswamy et al., 2006; Elliott et al., 2007). However, several factors may account for this discrepancy including limited thresholds of detection in the bioassay, model system and/or time points chosen for bioactive IFNα examination, or interrupted translation of IFNα mRNA due to rapid uninhibited induction of apoptosis in the absence of suppression by NS1 and NS2 proteins (Bitko et al., 2007). Induction of apoptosis characterized by nuclear condensation and internucleosomal DNA fragmentation leads to amplification of the protease cascade whereby the cell will undergo organized degradation of cellular organelles (Alberts et al., 2002). This event may lead to the accumulation of untranslated mRNA within the apoptotic cell.

Lack of detectable bioactive IFNα in supernatants from RSVΔNS1/2-infected MLE-15 cells may also reflect the state of infection of these cells. Our quantitative real-time PCR for RSV N gene copy number showed a lack of robust replication of RSVΔNS1/2 within MLE-15 cells at both 24 and 48 hours pi (Moore et al., 2008). Active RSV replication has been shown to be required for the initial burst of type I IFN production in BALB/c mice (Hornung et al., 2004; Guerrero-Plata et al., 2006; Jewell et al., 2007), and may reflect poor activation of IRF7, the primary transcription factor for induction of IFNα (Marie et al., 1998; Sato et al., 1998; Sato et al., 2000). MLE-15 cells appear to more effectively control RSVΔNS1/2 replication as compared to similar wtRSV and RSVΔG infections and could explain the observed modulation of IFNα.

Conclusions

In this study we show that MLE-15 cells secrete bioactive IFNα in response to wtRSV and RSVΔG infection, with no detectable levels found in RSVΔNS1/2-infected MLE-15 samples. It remains to be determined if the absence of detectable bioactive IFNα in the supernatants of RSVΔNS1/2-infected MLE-15 cells is due to rapid induction of apoptosis, absence of active robust viral replication, or other undetermined mechanism.

Methods

Virus and cells

Vesicular stomatitis virus (VSV) (kind gift of Kim Klonowski, University of Georgia, USA) was propagated in Vero cells (African green monkey kidney fibroblasts, ATCC CCL 81) maintained in DMEM (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 5% heat-inactivated (56°C) fetal bovine serum (FBS; Hyclone Laboratories, Salt Lake City, Utah, USA) and infectious virus titers were determined on Vero cells as previously described (UNIT 6.9 Measurement of Antiviral Activity Induced by Interferons α, β, and γ, Current Protocols in Immunology, Copyright © 1991 by John Wiley and Sons, Inc.). IFN-sensitive murine L929 fibroblasts (kind gift of Jeff Hogan, University of Georgia, USA) were propagated and maintained as previously described (UNIT 6.9 Measurement of Antiviral Activity Induced by Interferons α, β, and γ, Current Protocols in Immunology, Copyright © 1991 by John Wiley and Sons, Inc.).

VSV Bioassay

The VSV bioassay was performed according to established method (UNIT 6.9 Measurement of Antiviral Activity Induced by Interferons α, β, and γ, Current Protocols in Immunology, Copyright © 1991 by John Wiley and Sons, Inc.) with minor modifications. Briefly, serially

diluted samples, serially diluted recombinant IFNα (PBL product # 12100-1), anti-IFNβ (PBL product # 32400-1), and L929 cells were pipetted into the appropriate wells of a 96-well tissue culture plate and incubated at 37°C in a 5% CO_2 humidified atmosphere for 12 hours. Following incubation, supernatants were removed and VSV was added to achieve an MOI of 0.1 and plates were returned to the incubator for 48 hours. Wells were washed with ice cold Hank's Balanced Salt Solution (HBSS), fixed and stained with 0.05% (wt/vol) crystal violet with 4% formaldehyde for 15 minutes, and allowed to air dry. Plates were scored visually by endpoint determination of 100% cytopathic effect (CPE) which is the absence of the L929 monolayer.

Determination of optimal anti-IFNβ concentration

The optimal concentration of anti-IFNβ to neutralize IFNβ in the unknown samples was determined as previously described with no modifications (UNIT 6.9 Measurement of Antiviral Activity Induced by Interferons α, β, and γ, Current Protocols in Immunology, Copyright © 1991 by John Wiley and Sons, Inc.).

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Figure Legends

Figure 4-1

Results of a typical VSV bioassay plate. Unknown samples, IFNα standard, anti-IFNβ and L929 cells were pipetted into the appropriate wells of a 96-well tissue culture plate and incubated for 12 hours at 37°C/5% CO_2 prior to addition of VSV diluted to an MOI of 0.1 followed by another incubation for 48 hours. After 48 hours, wells were washed, stained and fixed prior to visual scoring by endpoint analysis for 100% CPE. Image of plate was produced using the Typhoon™ 9210 Variable Mode Imager, Amersham Biosciences.

Figure 4-2

wtRSV and RSVΔG induce secretion of bioactive IFNα by MLE-15 cells. MLE-15 cells were infected at an MOI of 1.0 with wtRSV, RSVΔG, RSVΔNS1/2, mock infected with Vero cell lysate, or cultured in the presence of media as explained in the Methods section in Chapter 3. Data is presented as U/ml relative to recombinant IFNα standard. Differences in protein levels between virus infection groups were evaluated by the Student *t* test and noted as significant as denoted by an asterisk (* $P<0.05$). Data are shown as means ± standard errors (SE) of the means.

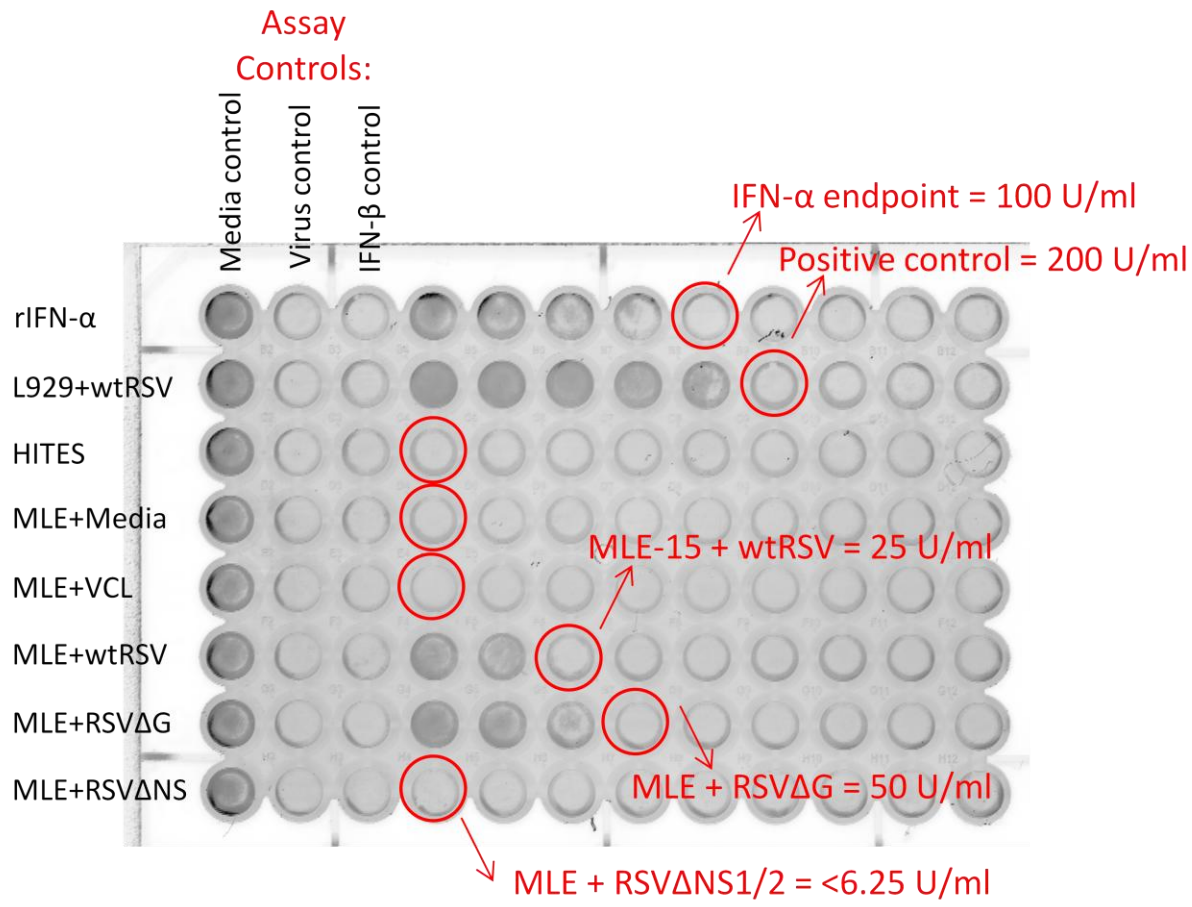
Assay Controls:
Media control
Virus control
IFN-β control
IFN-α endpoint = 100 U/ml
Positive control = 200 U/ml
rIFN-α
L929+wtRSV
HITES
MLE+Media
MLE+VCL
MLE+wtRSV
MLE+RSVΔG
MLE+RSVΔNS
MLE-15 + wtRSV = 25 U/ml
MLE + RSVΔG = 50 U/ml
MLE + RSVΔNS1/2 = <6.25 U/ml

Figure 4-1

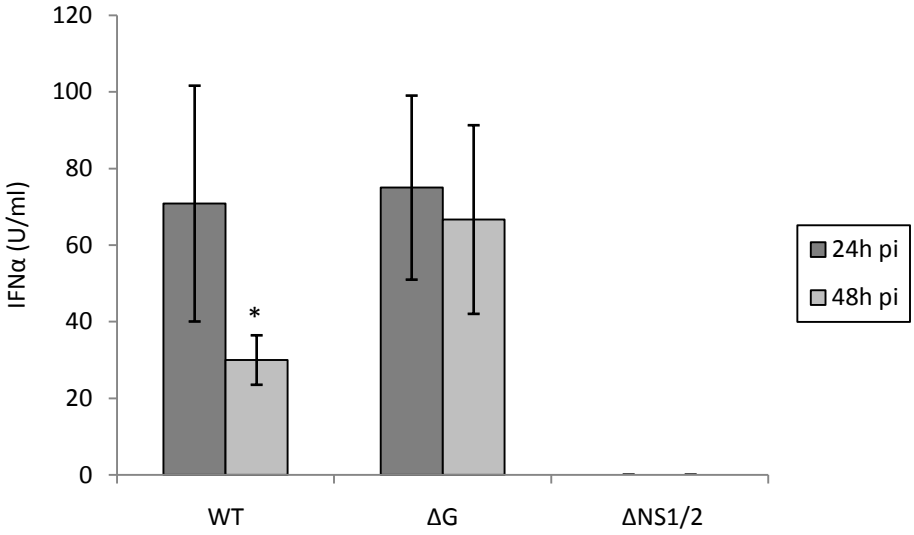

Figure 4-2

CHAPTER 5

CONCLUSIONS

Accumulating evidence indicates that RSV proteins have significant and unique roles in modulating the host immune response to infection thereby contributing to immune evasion, enhanced viral replication and potential viral persistence. For example, previous studies have shown that the RSV surface attachment protein, or G glycoprotein, and nonstructural proteins (NS1 and NS2) modify the host cytokine and chemokine response to viral infection. Specifically, the G protein has been shown to modify the pattern, magnitude and tempo of cytokine expression (Alwan and Openshaw 1993; Konig et al., 1996; Johnson and Graham 1999; Tripp et al., 1999; Tripp et al., 2000; Tripp et al., 2001; Tripp 2004; Shingai et al., 2008), and the two nonstructural proteins, NS1 and NS2, are largely responsible for type I interferon (IFN) antagonism (Bossert et al., 2003; Spann et al., 2004; Spann et al., 2005; Zhang et al., 2005; Ramaswamy et al., 2006; Elliott et al., 2007). Since all cytokines are negatively regulated by suppressors of cytokine signaling (SOCS) proteins, the mechanisms for virus-mediated modulation of the cytokine responses are likely linked to SOCS proteins. Several studies indicate that bacteria, viruses and parasites have adapted mechanisms to modulate the host cell SOCS response to facilitate immune evasion (Baetz et al., 2007).

In these studies, we examined some of the features of the early antiviral host immune response to RSV infection, focusing on the role of SOCS protein negative regulation of type I interferon (IFN) expression. We evaluated the role of three RSV proteins in modulating these responses by comparing responses in mouse lung epithelial-15 (MLE-15) cells infected with

wild-type RSV (wtRSV) or with RSV gene deletion mutant viruses lacking only the G gene (RSVΔG) or the NS1 and NS2 genes (RSVΔNS1/2). We evaluated the responses in MLE-15 cells as these cells represent type II pneumocytes from the mouse lung and are a relevant *in vitro* model for RSV studies. From these studies we showed that RSV and RSV proteins induce SOCS expression and modulate the pattern of SOCS1 and SOCS3 expression, an effect that negatively regulates IFNα and IFNβ expression having downstream effects on ISG15 expression and the antiviral cytokine response.

SOCS proteins not only negatively regulate cytokine responses through interruption of Janus kinase (JAK) – signal transducer and activator of transcription (STAT) signaling (Starr et al., 1997; Endo et al., 1997; Starr and Hilton 1998; Starr and Hilton 1999; Kubo et al., 2003; Vlotides et al., 2004), or suppressing JAK-STAT signaling by binding to and blocking the activity of JAKs and competing with STATs for phosphorylated binding sites on receptors, but SOCS proteins may also be involved in proteasomal degradation of specific target molecules such as VAV, JAK2 kinase and NF-κB by stimulating ubiquitin-dependent degradation of these molecules via formation of E3 ubiquitin ligase complexes (De Sepulveda et al., 2000; Monni et al., 2001; Frantsve et al., 2001; Kile et al., 2002; Ryo et al., 2003; Wormald and Hilton 2004).

Moreover, SOCS proteins may also indirectly contribute to RSV pathogenicity. For example, SOCS1 has been shown to be induced following HIV-1 infection of human T cell line MOLT-4 cells and to directly bind to and enhance the stability and trafficking of HIV-1 Gag polyprotein, a feature that attenuates dendritic cell (DC) antigen presentation and thus control of both HIV-1-specific humoral and cellular responses (Song et al., 2006; Ryo et al., 2008). RSV has also been shown to attenuate the antigen-presenting properties of monocyte-derived dendritic cells (moDCs), an event that may likely be linked to RSV NS protein suppression of moDC

maturation (Guerrero-Plata et al., 2006; Munir et al., 2008). Although thorough understanding of the mechanism(s) underlying this effect remains to be determined, a potential mechanism is possibly linked to RSV regulation of SOCS protein expression. *In vitro* and *in vivo* silencing of SOCS1 in antigen-presenting DCs strongly enhances antigen presentation and antigen-specific anti-tumor immunity (Shen et al., 2004; Kobayashi and Yoshimura 2005).

Recent studies that have shown that RSV infection induces SOCS1, SOCS3 and CIS in U937 cells and HEp-2 cell cultures which resulted in inhibition of STAT1 and STAT2 phosphorylation (Zhao et al., 2007; Moore et al., 2008; Hashimoto et al., 2008). Other siRNA studies designed to determine the effects of SOCS1 and SOCS3 silencing on antiviral host responses showed that silencing SOCS1 and SOCS3 induced STAT1 and STAT2 phosphorylation and increased expression of 2'-5'OAS1, an intracellular antiviral protein that activates RNase L, an effect that tied to decreased RSV replication in HEp-2 cells (Hashimoto et al., 2008).

As RSV is known to be a poor inducer of antiviral type I IFNα and IFNβ (McIntosh 1978; Hall et al., 1978), several clinical trials in the late 1980s and early 1990s determined the effects of treatment of infants and adults with RSV-mediated respiratory disease with intranasal or intramuscular administration of recombinant alpha-2A-IFN. Unfortunately, both infants and adults receiving treatment failed to show a significant benefit in disease outcomes (Chiba et al., 1988; Portnoy et al., 1988; Higgins et al., 1990; Chipps et al., 1993; Sung et al., 1993; Hodge and Chetcuti 2000). Consistent with these findings, in *vitro* and *in vivo* small animal studies have shown minimal efficacy in reducing RSV replication following exogenous application of type I IFNs (Loveys et al., 2000; Guerrero-Plata et al., 2005). A conclusion that can be interpreted from

these studies is that it is likely that the timing of and route of IFN delivery is important in efficacy.

In the studies presented here, the detection of low levels of intracellular type I interferons was not surprising as these molecules are rapidly synthesized and secreted by virally infected cells to act in an autocrine and paracrine manner on adjacent cells to establish an antiviral environment. The studies showed that higher levels of secreted IFNβ were associated with RSVΔG infection while no detectable levels of IFNα were evident in the supernatant of RSVΔNS1/2-infected cells. These findings are supported by recent studies that suggest the RSV G protein may inhibit IFNβ promoter activation via modulation of the TICAM-1 signaling pathway leading to reduced activation of IFNβ transcription factors IRF3 and IRF7 (Shingai et al., 2008). In addition, recent studies have shown that the presence of active viral replication may be required to induce the initial burst of IFNα lacking in RSVΔNS1/2-infected samples (Jewell et al., 2007). Curiously, we did not see the same type I IFN protein expression patterns in RSV-infected murine MLE-15 cells as previously described in the literature with respect to RSVΔNS1/2-infections. Previous studies have shown that RSVΔNS1/2 infection of A549 cells and primary human peripheral blood monocytes results in significant increases in type I IFN protein expression as compared to similar infections with wtRSV (Spann et al., 2004). Differences seen in our studies may be attributable to choice of time points examined post-infection and/or differences between model systems as previous studies were performed in cell lines of human origin (Spann et al., 2004; Ramaswamy et al., 2006; Elliott et al., 2007). Species-specific differences attributable to model systems have been shown to occur at many levels. For example, it has been shown that the RSV NS1 protein degrades human but not mouse STAT2 (Lo et al., 2005; Elliott et al., 2007). Side-by-side comparisons of type I IFN production in

RSVΔNS1/2-infected MLE-15 and A549 cells may serve to clarify any discrepancies and may further validate the use of MLE-15 cells as a relevant *in vitro* model system for future RSV studies.

Of particular interest in the studies described herein is the novel finding that RSV G protein negatively modulates both IFNβ and ISG15 expression. This data is also consistent with findings suggesting that IFNβ and ISG15 are induced in parallel as a primary response to infection (Andersen and Hassel 2006). IFNβ and ISG15 are both transcriptionally activated by IRF3 and NF-κB and have been classified as TLR3/TLR4 primary response genes (Doyle et al., 2002). Thus, the increase of IFNβ and ISG15 detected in RSVΔG-infected MLE-15 cells suggests a modulatory role for RSV G protein in the direct and indirect regulation of IFNβ and ISG15 through linked signaling molecules such as IRF3, NF-κB or RIG-I (Liu et al., 2007). This hypothesis is supported by recent studies in HEK293 cells and moDC cultures that demonstrate that secreted RSV G protein inhibits IFNβ promoter activation by modulation of the TICAM-1 signaling pathway even in the presence of IFNβ stimulators such as LPS, polyI:C and purified RSV F protein and may serve to facilitate viral replication (Shingai et al., 2008). Interruption of TICAM-1 signaling leads to reduced activation of IFNβ transcription factors IRF3 and IRF7. These findings further highlight the role of the RSV G protein as a potent immune modulator that functions to subvert the host antiviral immune response to infection thereby enhancing viral replication.

Recent evidence suggests that RSV NS1 and NS2 proteins act independently and cooperatively to modulate the host IFN response by suppressing the activation and nuclear translocation of the IFN-regulatory factor (IRF)-3 (Spann et al., 2005) and by degrading the signal transducer and activator of transcription 2 (STAT2) with Elongin-Cullin E3 ligase (Elliott

et al., 2007) thereby interfering with the type I IFN JAK-STAT signaling pathway in RSV-infected HEp-2 cells. siRNA studies targeting the NS1 protein show increased type I IFN expression accompanied by decreased viral replication in RSV-infected A549 cells and decreased inflammation and viral titers in the lungs of mice (Zhang et al., 2005). Subsequent studies have highlighted an important role for IRF3 activation and nuclear translocation in promoting proper antiviral host immune responses to RSV infection and have linked subversion of this response to modulation of IRF3 activation by RSV NS proteins and by the RSV G protein (Spann et al., 2005; Liu et al., 2007; Shingai et al., 2008).

RSV G protein expression has been linked to modulation of cytokine and chemokine expression and Th2-driven cytokine responses (Durbin and Durbin 2004; Krishnan et al., 2004; Tripp et al., 2002). This feature is unique to the G protein. For example, mice primed with recombinant vaccinia virus expressing the RSV F protein develop an enhanced Th1-type cytokine response upon subsequent RSV challenge with accompanying recruitment of IFN-gamma-secreting CD4+ and CD8+ T cells to the lung in equal proportions (Openshaw et al., 1992; Alwan and Openshaw 1993; Culley et al., 2006). Conversely, mice primed with recombinant vaccinia virus expressing the RSV G protein develop Th2-type cytokine responses following RSV challenge with recruitment of predominately Th2- CD4+ T cells and eosinophils to the lung (Openshaw et al., 1992; Alwan and Openshaw 1993; Hussel et al., 1997; Culley et al., 2006). These studies offer valuable insight and provide further evidence of the potent immunomodulatory roles of individual RSV proteins on the host response to viral infection.

The RSV G and F surface proteins mediate aspects of immune modulation through interaction with pattern recognition receptors expressed on the surface of host cells. Recent studies suggest that cell membrane associated toll-like receptor 2 (TLR2) is involved in RSV

recognition and subsequent innate immune activation with elevated production of TNF-α, IL-6, RANTES and the macrophage chemoattractant protein-1 (MCP-1) (Murawski et al., 2008). These studies did not identify RSV surface protein(s) involved in TLR2 recognition; however, they elucidated an important aspect of the host response to RSV infection. For example MCP-1 is a CC cytokine that attracts monocytes, dendritic cells, T cells and natural killer (NK) cells to sites of inflammation, is involved in Th2-type cytokine polarization, is induced by TLR2 stimulation, and is linked to RSV pathogenesis (Carr et al., 1994; Allavena et al., 1994; Maghazachi et al., 1994; Xu et al., 1996; Gu et al., 2000; Ogra 2004; Culley et al., 2006; Murawski et al., 2008). Interestingly, Epstein-Barr virus (EBV) has recently been shown to induce MCP-1 in human monocytes via TLR2 activation (Gaudreault et al., 2007). It is possible to hypothesize that TLR2 recognition of RSV may be provoked by interaction with the RSV G protein based on previous studies that associated G protein expression with elevated levels of MCP-1, and Th2-type cytokine responses. Accumulating evidence indicates that RSV G protein is important in immune evasion to promote virus replication and potentially persistence. The G protein has been linked to suppression of IFN-β promoter activity, increased Th2-type cytokine and modified chemokine expression, infection of immune privileged neuronal cells and processes, antigenic variability that reduces the efficiency of neutralizing antibodies, suppression of the antiviral molecule ISG15 and altered innate immune responses (Alwan and Openshaw 1993; Konig et al., 1996; Melero et al., 1997; Johnson and Graham 1999; Tripp et al., 1999; Tripp et al., 2000; Tripp et al., 2002; Tripp 2004; Durbin and Durbin 2004; Krishnan et al., 2004; Li et al., 2006; Moore et al., 2008; Shingai et al., 2008). The secreted form of the G protein, which is shed from infected cells as early as 6 hours post-infection, is linked with altered lymphocyte trafficking associated with fractalkine mimicry and has been shown to act as a decoy

to shield RSV from neutralizing antibodies (Hendricks et al., 1988; Tripp et al., 2001; Bukreyev et al., 2008).

Collectively, the data presented in these studies contributes to and supports the growing knowledge base of virus-mediated mechanisms associated with immune evasion and RSV pathogenesis. We also noted increased induction of apoptosis in RSVΔNS1/2-infected MLE-15 cells, a feature also previously reported in the current literature (Bitko et al., 2007). This is a potentially important evasion strategy as delay of early onset of apoptosis may promote viral replication. Moreover, we show the novel finding that expression of the potent antiviral molecule ISG15 is attenuated by G protein expression (Moore et al., 2008). In addition to its potent antiviral properties, ISG15 is a positive regulator of JAK-STAT signaling and has been shown to subvert ubiquitin-mediated degradation of IRF3 thereby enhancing activation and nuclear translocation of IRF3, an important transcription factor for both IFNβ and ISG15 (Malakhova et al., 2003; Ivashkiv and Hu 2004; Lu et al., 2006). It is likely that down-modulation of antiviral IFNβ and ISG15 expression serve to facilitate viral replication. Our results also show a role for SOCS regulation in the early antiviral host immune response to RSV infection. Our findings reported here reveal that RSV infection stimulates differential SOCS1 and SOCS3 expression, a feature linked to specific RSV proteins. Our findings provide rationale to support the initiation of studies that would focus on therapeutic targeting of SOCS molecules to control RSV disease. Although prophylactic drugs are currently available, until a safe and effective vaccine also becomes available to prevent serious RSV illness, there is a need to explore other avenues of disease intervention. This may require non-traditional approaches involving development and utilization of combinatorial therapeutics that not only target RSV, but also aid in restoring proper immune responses to infection.

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