SUPPRESSOR OF CYTOKINE SIGNALING PROTEIN REGULATION OF RESPIRATORY SYNCYTIAL VIRUS INFECTION AND EVALUATING AVIAN INFLUENZA INFECTION OF HUMAN BRONCHIAL EPITHELIAL CELLS

by

CHRISTINE M. OSHANSKY-WEILNAU

(Under the Direction of Ralph A. Tripp)

ABSTRACT

Respiratory syncytial virus (RSV) is the most common cause of hospitalization in infants due to any viral respiratory tract agent. Repeat infections are common throughout life and underscore mechanisms by which RSV can evade the immune response. Several RSV proteins have been shown to contribute to immune evasion by modification of cytokine responses, particularly the type I interferon (IFN) response. RSV nonstructural proteins are expressed early during infection and are known to contribute to type I IFN antagonism. Type I IFN signaling is negatively regulated by suppressor of cytokine signaling (SOCS) proteins, which are induced following Toll-like receptor (TLR) activation. Aspects of experimental studies described here were performed to test the hypothesis that RSV mediates TLR induction of the host SOCS pathway to inhibit the antiviral type I IFN response as a means to facilitate virus replication. The role of SOCS1 and SOCS3 regulation of the type I IFN response is examined in fully differentiated normal human bronchial epithelial (NHBE) cells infected with RSV or with a recombinant RSV mutant virus. The results show that RSV G protein modulates SOCS

expression to inhibit type I IFN signaling early in infection, and increased SOCS expression is linked to TLR signaling by RSV F protein. In addition, the studies revealed that RSV surface proteins induce apical and basolateral secretion of proinflammatory chemokines early in infection. These findings suggest that RSV surface proteins signal through a TLR pathway, and indicate an important viral evasion mechanism to reduce the antiviral response to aid virus replication. Influenza virus, like RSV, is an important human respiratory pathogen. Avian influenza viruses (AIV) cause significant morbidity and mortality in poultry, and are considered an important emerging human health threat. The experiments described herein were performed to test the hypothesis that AIV can infect NHBE cells independent of sialic acid linkages present on the cell surface. The results show that AIV productively infect and replicate in NHBE cells independent of the sialic acids on the cell surface. These findings indicate that AIV may not need to acquire changes in hemagglutinin receptor binding specificity to efficiently transmit from human-to-human.

INDEX WORDS: Respiratory syncytial virus, RSV, type I interferon, type I IFN, suppressor of cytokine signaling, SOCS, avian influenza, normal human bronchial epithelial cells, NHBE

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DEDICATION

This dissertation is dedicated to my best friend and husband Mark, who never ceases in his optimism and enthusiasm, and to Thomas and Barbara, my parents who always provide encouragement and are filled with kindness.

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CHAPTER 1

INTRODUCTION

Respiratory syncytial virus (RSV) is the most common cause of serious lower respiratory tract disease in infants and young children worldwide, and is also an important pathogen of the elderly and immune compromised (12, 27, 37, 41). RSV infection does not induce long-term immunity, thus re-infection with the same and different strains may occur throughout life. RSV is a non-segmented, enveloped, negative-strand RNA virus belonging to the *Paramyxoviridae* family. The virus was first isolated from a chimpanzee in 1956 and subsequently from two children in 1957 (4, 6). Peak incidence of disease occurs in the first six months of life when maternal antibodies are present, and infection results in bronchiolitis, pneumonia and often hospitalization. In addition, RSV infection is frequently associated with secondary infections such as otitis media and may predispose for asthma-related illness later in life.

A goal of this study is to understand the mechanisms by which RSV evades the host response to infection. The specific hypothesis of the research described is that RSV mediates Toll-like receptor (TLR) induction of the host suppressor of cytokine signaling (SOCS) pathway to inhibit the antiviral type I interferon (IFN) response to facilitate RSV replication. We utilize an *in vitro* cell culture model consisting of normal human bronchial epithelial (NHBE) cells which has been used to examine various aspects related to infectious diseases including cell tropism, innate immune responses and

histopathology (3, 23, 24, 40). NHBE cells are cultured in an air-liquid interface (ALI) and fully differentiate into an epithelial model of the human airway (16). This culture system emulates classic respiratory epithelium as it consists of polarized, ciliated pseudostratified columnar epithelium with goblet cells. Importantly, the polarized primary epithelial cells develop an apical surface representing the lumen of the airway and nourished via a separate basolateral compartment.

Type I IFNs (IFNα/β) are important in the innate immune response to viral pathogens. Secretion of type I IFN results in the induction of a cellular antiviral response involving the transcriptional up regulation of potentially hundreds of genes resulting in inhibition of viral replication by several mechanisms including translation inhibition, RNA degradation and apoptosis induction. Importantly, secreted IFNα/β also promotes the generation of cellular and humoral immunity (10, 18). Numerous studies have shown that RSV can regulate type I IFN expression during viral replication and inhibit the antiviral state of a cell. For example, a RSV deletion mutant virus lacking the nonstructural (NS)1 and NS2 genes has been shown to induce high levels of type I IFN in a lung epithelial cell line, A549 (30), and the NS2 protein has been shown to be the primary inhibitor of signal transducer and activator of transcription-2 (STAT2) signaling in the Janus kinase (JAK)/STAT pathway (19, 25). Furthermore, this double deletion mutant induces high levels of activated interferon regulatory factor-3 (IRF3) within the nucleus of A549 cells, indicating that either one or both of the NS proteins act to inhibit IRF3 nuclear translocation during RSV infection (31). In the case of bovine RSV, the NS proteins prevent IRF3 phosphorylation and consequent activation (5).

Although the NS1 and NS2 proteins do have a role in modulating the type I IFN response to infection, we hypothesized that the RSV surface proteins, i.e. G (attachment) and/or F (fusion) proteins contribute to immune evasion before viral proteins are expressed during infection. We proposed that immune evasion may be mediated by RSV F and/or G protein interaction with TLRs and induction of SOCS proteins that act to negatively regulate antiviral cytokine responses. This hypothesis is based on the following observations. First, the RSV F protein is able to signal through the TLR4 pathway (17). Second, mice inoculated with RSV show activation of the nuclear factor (NF)-κB pathway early (30 minutes - 1.5 hour) following infection and prior to viral protein expression, while mutation in the *Tlr4* gene region results in reduced RSV-induced NFκB binding activity (14). Finally, SOCS proteins can be induced through TLR stimulation independent of type I IFN (2, 8, 9, 22).

The studies reported here investigate the role of RSV F and G proteins in modulating the host cell response and provide the framework for RSV disease intervention strategies that center on managing SOCS regulation of the antiviral cytokine response. The proposal examines the following specific aims:

Specific Aim 1. To determine the pattern and tempo of SOCS1 and SOCS3 expression linked to regulation of type I IFN following RSV infection in NHBE cells. The *working hypothesis* is that SOCS1 and SOCS3 are the major class of SOCS proteins that negatively regulate antiviral cytokine responses, particularly the type I IFN (α/β) response.

Specific Aim 2. To determine the RSV surface proteins that induce TLR signaling in normal human bronchoepithelial (NHBE) cells. The *working hypothesis* is that RSV surface proteins mediate TLR signaling and expression of SOCS proteins, an effect that can be linked to phosphorylation of IRF3.

Specific Aim 3. To determine the effect of RSV surface protein mediated SOCS regulation on the antiviral chemokine response. The *working hypothesis* is that RSV G and/or F proteins, through TLR activation of SOCS1 and/or SOCS3, modify the expression of chemokines by NHBE cells used to recruit immune cells to sites of infection.

A second goal of these studies was to elucidate the potential mechanisms by which avian influenza viruses (AIV) infect NHBE cells. The specific hypothesis of the studies described is that AIV infect human lung cells independent of sialic acid linkages present on the cell surface. Influenza A viruses are important pathogens that present a significant public health risk and economic burden due to localized outbreaks, seasonal epidemics and pandemic potential. Influenza viruses are segmented, enveloped, negative-strand RNA viruses belonging to the *Orthomyxoviridae* family. They comprise a diverse array of subtypes due to their propensity to change their antigenic profiles and are subtyped based on the antigenic properties of 2 surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). There are currently 16 known HAs and 9 known types of NA. The seasonal epidemics in particular cause more than 200,000 hospitalizations and more than 41,000 deaths each year in the United States (11). To date there have been three pandemic influenza strains that affected the human population which occurred in 1918, 1957 and 1968. The 1918 influenza pandemic was the most severe of these,

resulting in unusually high mortality among the healthy young adult population (29). It remains unclear whether the high rate of mortality due to infection with the 1918 influenza was due to an exaggerated host immune response, increased virulence, existing immunity in the older population infected, or a combination thereof (20). AIVs in particular cause significant morbidity and mortality in poultry and wild birds and are an important emerging human health threat. Since 2003, increased incidence of highly pathogenic influenza virus outbreaks in poultry have resulted in 424 confirmed human infections and 261 deaths as of May 2009 (42).

Influenza HA binds to host cell sialic acid residues coating the host cell surface (32) and mediates viral entry via its receptor binding domain. Sialic acids (sias) are nine-carbon monosaccharides found at the ends of glycan chains that coat all cell surfaces and many secreted proteins, and have a role in physiological and pathological interactions (1, 26, 38, 39). Sias are transferred to terminal sugars of glycoproteins and glycolipids by sialyltransferases, and sias can be added to the galactose carbon-6 forming an α2,6 linkage or to galactose carbon-3 forming an α2,3 linkage. Influenza HA has a strong preference to bind to certain sia moieties and is believed to be an important host range determinant (33-36). Human-adapted influenza viruses preferentially recognize α2,6 linkages, while AIV preferentially recognize α2,3 linkages expressed in the gastrointestinal tracts of birds (7, 15, 21, 28). It is believed that the previous influenza pandemics resulted after acquiring mutations affecting the HA binding specificity from avian-like, α2,3, to human-like, α2,6 (7, 13, 21), thus it is hypothesized that a change in receptor binding specificity is necessary for efficient human-to-human transmission (21).

The studies reported here examined if low pathogenic avian influenza viruses (LPAI) could infect and replicate in NHBE cells independent of sialic acids present on the cell surface. The final specific aim examined was:

Specific Aim 4: To determine if LPAI H5N1, H5N2 and H5N3 isolated from a wild bird or from poultry could infect and replicate within NHBE cells independent of sialic acids present on the cell surface. The *working hypothesis* is that AIV expressing HA molecules that preferentially bind to α2,3-linked sialic acids can infect and replicate in human epithelial cells independent of the sialic acid moiety present.

These specific aims designed to evaluate RSV-mediated immune evasion will aid in understanding the mechanism by which RSV replicates following infection, and likely how RSV may cause repeat infections with relative low immunogenicity associated with the virus. A better understanding of the host cell response to RSV infection is also critical for development of safe and efficacious RSV vaccines and therapeutic treatment modalities. Moreover, understanding important host cell pathways affected by RSV proteins can provide an avenue to ameliorate aspects of pathogenesis linked to infection. Finally, the specific aim designed to investigate the ability of AIV to productively replicate in NHBE cells independent of cell surface sialic acid expression is critical for the advent of improved drugs and vaccines to control infection. Although the mouse models of RSV and influenza infection have provided considerable insight into many of the mechanisms of immunity and disease pathogenesis associated with RSV and influenza infection, studies in NHBE cells will undoubtedly provide answers to questions that remain regarding the human diseases.

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CHAPTER 2

LITERATURE REVIEW

Overview of Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is an enveloped, nonsegmented, negative sense, single-stranded RNA virus belonging to the *Mononegavirales* order, genus *Pneumovirus*, subfamily *Pneumovirinae*, Family *Paramyxoviridae* (297). Viruses of the *Mononegavirales* order share several characteristics. Their genomes are tightly associated with a nucleoprotein and are therefore RNase-resistant. Transcription of the genome occurs 3' to 5' by stopping and reinitiating at each gene junction, and during replication, synthesis of a complementary antigenomic RNA strand occurs (297). The virions themselves are surrounded by a host-acquired lipid bilayer consisting of viral glycoproteins, and during infection, entry of the viral nucleocapsid into the host cell involves virus-specific membrane fusion.

RSV was first isolated in 1956 from a chimpanzee during an outbreak of respiratory illness in a colony of chimpanzees (27) and a year later from two children diagnosed with bronchitis and bronchopneumonia (50). The isolated virus was subsequently named respiratory syncytial virus because of its ability to promote syncytium formation in cell lines *in vitro*. RSV infects virtually all infants before the age of 2 years causing illness manifested by mild upper respiratory tract symptoms to severe

lower respiratory tract disease (2, 104, 115). Each year RSV causes seasonal epidemics during the winter months in temperate climates and during the rainy season in tropical climates. RSV circulates in the population as two major subtypes, A and B. Each subtype differs from its counterpart at the nucleotide level and can be distinguished from one another with antibodies (7, 101, 136, 137, 140, 203), and each year new lineages of RSV subtype A or B emerge in the population.

RSV is considered a ubiquitous virus and is the single most important cause of serious lower respiratory tract illness in infants and young children under the age of 2 years, peaking within the first six months of life when maternal antibodies are still present (1, 114, 269, 280). Furthermore, it is an important pathogen in elderly and immune compromised individuals (24, 60, 83, 84, 90). RSV infection is a primary cause for hospital admissions due to respiratory tract illness in children with infection rates approaching 70% in the first year of life (103). In the United States, over 120,000 infants are hospitalized annually due to RSV infection, and over 200 deaths are associated with RSV-mediated severe lower respiratory tract disease (268). Another study that examined children in the United States demonstrated that lower respiratory tract disease may develop in 20% - 30% of those infected with RSV (263).

RSV has two major surface proteins, the attachment (G) and fusion (F) proteins, and while the small hydrophobic (SH) protein is a minor surface protein on the virion, it is expressed abundantly on the infected host cell (55, 89). RSV primarily infects respiratory epithelial cells by attaching to highly sulfated cell surface iduronic acid-containing glycosaminoglycans (GAGs) such as heparin, heparan sulfate and chondroitin sulfate B (31, 87, 116, 163). Following attachment, the F protein mediates entry and

fusion with the host cell membrane which is thought to occur similarly to influenza hemagglutinin-mediated fusion (86, 87, 273). Internalization of the nucleocapsid into the host cell cytoplasm results in transcription, and later replication, of viral genes via cell ribosomes. The resulting viral antigenome complexes with newly manufactured nucleocapsids and viral particles are manufactured near the cell surface. RSV acquires its lipid envelope from the host cell upon budding from the apical surface. Importantly, it has been shown in fully differentiated bronchial epithelial cells that RSV infection primarily involves ciliated cells and does not lead to its characteristic syncytium formation as it does in immortalized cell lines (329).

RSV Replication

Following RSV attachment through the G protein (31, 87, 116, 163), cell fusion mediated by the F protein is thought to occur in the same manner as influenza virus hemagglutinin which utilizes a fusion peptide at its amino-terminus (273). The RSV hydrophobic fusion peptide inserts into the host cell membrane leading to an unstable intermediate that quickly brings the viral envelope and cell membrane adjacent to each other. Subsequently virus penetration occurs and the nucleocapsid is released into the cytoplasm (11, 127, 170, 249) where the polymerase L protein initiates viral transcription and replication (85). Viral gene transcription occurs 3' to 5' from a single promoter within a leader region at the 3' end and terminates at gene-end sequences at the 5' end of each gene resulting in the production of a series of subgenomic mRNAs (72, 120, 130, 161, 165). mRNAs can be detected as early as 4h post-infection with peak mRNA

synthesis and protein expression occurring between 12h and 20h post-infection. Each time the viral polymerase reaches a gene-end signal, there is a chance that it will not begin transcribing the next gene downstream. This phenomenon results as a major mechanism of controlling viral gene expression that creates a mRNA gradient within the cell. Therefore, genes closer to the promoter at the 3' end of the viral genome are transcribed more frequently than those more distant to the promoter (56). Moreover, during RSV protein synthesis, the level of protein expressed is related to mRNA abundance (72). The matrix (M) protein is purported to play a role in the assembly of mature virions at the cell surface by interacting with both the nucleocapsid and viral envelope proteins. In addition to viral proteins, recent studies have shown that cellular proteins such as Rab11 family interacting protein 2 (FIP2) and actin are involved in the virus assembly and budding process (35, 144, 304). The virus particles mature in clusters at the apical surface of the cell in a filamentous form associated with caveolin-1, and extend from the plasma membrane producing progeny virus particles (36).

Host Immune Response to RSV Infection

Innate Immunity

RSV infection does not induce long-lasting protective immunity, and re-infection with the same and different strains of RSV occurs yearly (296, 319). Innate immunity during viral infection is an evolutionarily conserved defense strategy that is important for mediating non-specific anti-microbial effects and augmenting the adaptive virus-specific immune response. Critical in this regard is host recognition of virus infection. For innate

immunity, Toll-like receptors (TLRs) and other members of the pattern recognition and response (PRR) family of receptors recognize conserved pathogen-associated molecular patterns (PAMPs) (190). Upon activation, TLRs initiate a complex signaling cascade mediated by the toll/interleukin-1 receptor (TIR) domain in the cytoplasmic portion of the TLR, leading to signaling through NF-κB and expression of genes important in the initial response to infection (150). The innate response to viral infection is also dependent upon the expression of type I interferon (IFN). As IFNα/β are secreted, they bind in an autocrine or paracrine fashion to IFN receptors leading to the induction of an antiviral state. RSV has been shown to be a poor inducer of type I IFN (IFNα/β) and cells infected with RSV are resistant to the antiviral effects of IFNα/β (260) suggesting RSV modulates the antiviral state.

Several TLRs are constitutively expressed, but upon infection TLR expression can be up regulated in a variety of cell types including lung epithelial cells, alveolar macrophages and dendritic cells (223). TLRs and PRRs detect specific pathogen components and therefore different pathogens. For example, retinoic acid-inducible gene I (RIG-I)-like helicases including RIG-I and melanoma differentiation-associated gene-5 (MDA5) recognize double-stranded RNA during viral replication and can initiate type I IFN production and an antiviral response (289). Several TLRs and RIG-I are necessary for the initiation of innate immune responses during RSV infection (174, 257, 265). Thus PRRs are often targeted by viruses to evade the host immune response (157).

In recent years, studies have shown that PRRs play a critical role during RSV infection. For instance, RIG-I, but not MDA5, is important in initiating an innate immune response (177). The RSV F protein is able to induce an innate immune response in

monocytes by signaling through CD14 and TLR4 (166), while intracellular TLR3, which detects double stranded RNA, is up regulated by RSV infection independent of the adaptor protein, MyD88 (250). To date, there is no evidence suggesting that TLR3 is necessary for RSV clearance, but it does seem to be important in the maintenance of an appropriate immune environment by promoting a Th1 response and preventing the development of Th2-mediated pathology in the lungs (251). Thus, PRRs have important roles in signaling the host of RSV infection.

RSV infection of many cell types including respiratory epithelial cells results in the modulation of cytokine and chemokine expression patterns, and it has been suggested that the certain patterns of cytokine or chemokine expression in RSV-infected individuals may be an indicator of disease severity (128). RSV infection of respiratory epithelial cells induces a cascade of signaling events mediated by NFκB resulting in expression of many proinflammatory cytokines and chemokines including RANTES, MCP, eotaxin, IL-9, TNFα, IL-6, IL-1 and fractalkine (CX3CL1) (26, 112, 113, 121, 189, 194, 211, 330). These chemokines and cytokines can act directly or via an autocrine or paracrine feedback mechanism to regulate the adaptive immune response to infection. Recent reports have indicated that RSV-infected patients display patterns of increased MIP-1α, RANTES and IL-8 levels within the upper and lower respiratory tract (121). In vitro, RSV infection of bronchial epithelial cells express elevated levels of IL-6, IL-8 and RANTES (326), and the removal of these cytokines or chemokines by neutralizing antibodies results in a less severe form of RSV disease. In particular, depletion of RANTES or eotaxin results in reduced airway hyper-reactivity (AHR) and eosinophilia in RSV-infected mice (188, 293). The critical role cytokines and chemokines play has also

been linked to age as mice infected as neonates display higher illness scores, greater cell recruitment to the lungs and increased IL-4 production (63). It was demonstrated that adult mice re-infected with RSV developed severe disease associated with a Th2-type cytokine expression (63). Clara cell secretory protein (CCSP), also known as CC10, is expressed in abundance from Clara cells which are non-ciliated bronchiolar epithelial cells (272). Importantly, CCSP is implicated as being an immunomodulatory protein that plays a role in inhibition of IFNγ synthesis and secretion from peripheral blood mononuclear cells and Th1 vs. Th2 cytokine profiles within the lung (42, 73, 193, 204, 316).

Discussed in detail later in this chapter, it is likely that RSV proteins modify both the magnitude and cadence of cytokine and chemokine expression. For instance, the RSV G protein contains a CX3C chemokine motif and modifies the chemotaxis of $CX3CR1^{+}$ cells including cytotoxic T cells and NK cells during infection (119, 299). Furthermore, if the RSV G protein is present during an acute RSV infection in mice, modified CC and CXC chemokine mRNA expression profiles and Th1/Th2-type cytokine responses by bronchoalveolar leukocytes is readily observed (298, 300). The G protein also appears to inhibit early chemokine expression including MIP-1α, MIP-1β, MIP-2, MCP-1, and interferon-inducible protein of 10kD (IP-10) resulting in defective immune cell chemotaxis to sites of inflammation (298).

Innate immune cells have an important role in the recognition of pathogen invasion and in orchestrating a robust adaptive immune response. During RSV infection dendritic cells (DCs), natural killer (NK) cells, macrophages, and NK T cells each affect viral clearance. DCs are the major antigen presenting cells during RSV infection (40,

179) and are important immune cells that play a role in NK cell activation and cytotoxicity (201, 202). On their cell surface, DCs display co-stimulatory and inhibitory molecules that determine whether T cells are activated or become tolerant, and the cytokines secreted by DCs influence T cell polarization to Th1, Th2 or T regulatory capacity (70). Respiratory DCs are located within intra-epithelial sites where they conveniently sample the airway for antigen. There are two major subsets of DCs: myeloid or conventional DCs (cDCs) expressing CD11b and CD11c, and plasmacytoid DCs (pDCs) expressing little or no CD11b and B220 (19). The quality of antigen presentation by cDC and pDC in the lung and draining lymph nodes is essential for establishing an effective immune response to RSV infection (274). Depletion of pDCs from the lungs of RSV-infected mice results in greater pathology and drives a Th2 cytokine response, while increased relative pDC numbers provide a more protective effect (274, 275, 314).

Natural killer (NK) cells are an important component of the innate immune system and function in clearance of tumors and virus-infected cells by releasing perforin and granzymes that cause the target cell to die by apoptosis or necrosis. NK cells are recruited to sites of infection and inflammation by chemokines such as MIP-1α (290). During RSV infection, NK cells are found in the lungs very early after infection and reach peak levels between day 3 and 4 post-infection (296, 301).

Macrophages are also important effector cells of the innate immune response. Alveolar macrophages, present primarily within the lower respiratory tract, are substantial sources of pro-inflammatory cytokines including TNFα, IL-6 and IL-8 during RSV infection (17). Alveolar macrophages are required to recruit and activate NK cells in response to RSV infection, and depletion of macrophages reduces the activation and

recruitment of NK cells leading to a limited pro-inflammatory cytokine response and high lung virus titers (236). However, depletion of macrophages has a limited effect on the numbers of T cells recruited and activated, as well as overall lung disease, suggesting that macrophages may be more important in the early responses to RSV infection and contribute less to the adaptive response (236). Moreover, depletion of alveolar macrophages in BALB/c mice prior to RSV exposure results in airway occlusion with accumulations of infected, apoptotic cellular debris (243). However, additional studies are needed in this area to fully elucidate the role of alveolar macrophages during RSV infection.

NK T cells are a subpopulation of CD1d-restricted T cells that co-express an α/β T cell receptor and a variety of NK cell markers (38). NK T cells recognize glycosphingolipids in the context of CD1d, an antigen presenting molecule evolutionarily related to the classical major histocompatibility complex (MHC) class I molecules (20, 34). These cells can produce both Th1- and Th2-type cytokines and thus have the potential to drive the adaptive immune response. During RSV infection, NK T cells have been shown to produce IFNγ early leading to induction of $CD8^+$ T cell responses (142).

Adaptive immunity

The innate immune response directly affects aspects of the adaptive response including Th1- or Th2-type polarization and immune cell recruitment to sites of infection and inflammation. RSV infection induces a robust antibody response against several of its antigens including F, G, M2 and P proteins, but only the F protein induces the development of neutralizing antibodies and is considered the main correlate of immune

protection (58, 109, 318, 323). The RSV F protein has two forms, both of which are capable of inducing humoral immunity: a mature form, found in virions, and an immature folded form found within the infected cell (169, 178, 255). The RSV G protein is the more divergent protein relative to the F protein (54). The two main subgroups of RSV, i.e. strain A and B, have 53% sequence homology within the G protein, but the F protein has 90% sequence similarity. Because of the relatively high sequence diversity in the G protein, few G protein-specific monoclonal antibodies are cross-reactive, and RSV G protein neutralization requires multiple antibodies targeting different epitopes (192). Conversely, most F protein-specific monoclonal antibodies are cross-reactive (54). Interestingly, a majority of the antibodies generated during RSV infection primarily recognize epitopes within the C-terminal region of the G protein (185, 252), but protective monoclonal antibodies target the central conserved cysteine-rich region of the G protein (303). The RSV G protein is heavily glycosylated, and the glycosylation pattern changes depending on the specific cell type infected (95, 96, 221, 222). Thus, the altered glycosylation patterns likely reflect the ability of RSV to change its antigenic profile in order to more effectively evade the immune response (43, 44). Studies in the cotton rat have demonstrated that antibodies are important in reducing virus replication in the lungs while only a limited reduction in nasal virus titer is observed (237). Because RSV infection begins at a mucosal surface, secretory IgA antibody may be involved in protection. For instance, challenge with RSV results in increased levels of serum IgG and mucosal IgA in nasal secretions; here it was found that viral replication in the upper respiratory tract was unrelated to the serum concentration of IgG (195).

While the humoral response has aspects that are protective in RSV infection, T cell mediated immune responses are important in virus clearance. During natural infection of humans, $CD8^+$ T cells recognize F, M, M2 and NS2 proteins, but there is little response to G, P or NS1 proteins (52). In the BALB/c mouse model, $CD8^+$ T cells primarily recognize F, N and M2 proteins (217). Highlighting the difference between the human infection and the murine model of RSV infection, it has been shown that $CD4^+$ T cells recognize epitopes within the non-glycosylated ectodomain of G protein, but these same epitopes are poorly recognized by human $CD4^+$ T cells (118, 292). However, there is an immunodominant peptide within the G protein that can be recognized by both Th1 and Th2 $CD4^+$ T cells in humans (69, 71).

Following RSV infection, virus-specific $CD4^+$ and $CD8^+$ T cells are found both in the lungs and in peripheral tissues, and the clearance of virus is associated with increasing numbers of $CD8^+$ T cells in the lungs (291). While T cell responses to infection occur in the lungs, RSV-specific $CD8^+$ T cells can be found in the peripheral blood of infants. Additionally, RSV-specific $CD4^+$ and $CD8^+$ memory T cells within the lungs are important for providing protection during RSV re-infection (68). In primed BALB/c mice challenged with RSV, the memory $CD4^+$ T cell response to RSV G protein in the lungs is primarily composed of T cells expressing T cell receptor (TCR) Vβ-14 which proliferate and expand into effector T cells (307, 321). RSV infection also leads to the induction of memory $CD4^+$ T cells specific for the RSV F protein. In contrast, mice immunized with RSV F protein results in a broad repertoire of RSV F-specific $CD4^+$ T cells that predominantly express Th1-type responses (45).

RSV Manipulation of the Host Immune Response

Despite the immune response generated to infection, RSV does not mediate durable immunity, thus children and adults are repeatedly infected with the same and different antigenic subtypes of RSV every year (296, 319). It is likely that RSV manipulation of the host immune response contributes to the lack of durable immunity. Laboratory studies have shown that RSV infection results in modified expression patterns of various genes related to protein metabolism, cell growth and proliferation, cytoskeleton organization, regulation of nucleotides and nucleic acid synthesis, and cytokine/chemokine genes associated with inflammation (186, 331). Airway epithelium has an important role as the boundary between the outside environment and the host and acts as a critical first-line defense against pathogens. Because airway epithelial cells are situated at the host-pathogen interface, they also provide a means to which various immune components can sample the outside environment including mucosal dendritic cells (DCs) and intraepithelial lymphocytes (117). RSV has evolved immune evasion strategies to overcome host barriers to promote virus infection and replication. Although the primary function of the RSV G protein is in the attachment of RSV particles to the host cell surface (171), the RSV G protein contains a conserved cysteine-rich region (GCRR) homologous to the fourth subdomain of the tumor necrosis factor (TNF) receptor which has been shown to modify the innate immune response to infection (10, 168, 233, 296, 299). There are two forms of G protein manufactured within infected cells: an integral type II membrane-bound form (Gm) and a secreted soluble form (Gs) (124, 125). Gs protein, produced and secreted from infected cells early after infection (125), may sequester TNFα or other homologues and inhibit proinflammatory effects (168).

TNFα is an important proinflammatory cytokine which has been implicated in several inflammatory conditions (32) as well as in the immune response to RSV (216). The GCRR also contains a CX3C chemokine motif found at amino acid positions 182-186 that interacts with the CX3C chemokine receptor CX3CR1 (299). CX3CR1 mimicry by the G protein has been shown to facilitate RSV infection and alter CX3CL1 (fractalkine) chemotaxis of human and murine leukocytes (299). Expression of the G protein during RSV infection of mice has also been shown to decrease the number of NK cells and activated RSV-specific pulmonary $CX3CR1^{+}$ T cells (119).

Infection of mice with a mutant RSV lacking the G and SH genes results in greater numbers of NK cells in the lungs as well as increased IFNγ and TNFα production suggesting that the G and/or SH surface proteins regulate trafficking of NK cells to the lungs and pro-inflammatory cytokine production (301). Additional studies have shown that the G and/or SH proteins inhibit early macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, monocyte chemoattractant protein (MCP)-1, and interferon-inducible protein of 10kD (IP-10) mRNA expression, all important chemokines that attract NK cells to the lungs in mice (298). Together these data suggest that RSV modulates both the innate and adaptive immune responses to infection.

RSV nonstructural (NS) proteins have been shown to regulate the type I interferon (IFN) response during infection as well as suppressing DC maturation (30, 176, 205, 241, 242, 260, 276, 277, 305). In particular NS2 protein is the responsible type I IFN antagonist linked to specific down regulation of signal transducer and activator of transcription (STAT)-2 (176, 241, 242). However, the NS1 protein contains elongin C and cullin 2 binding sequences and can potentially act as an ubiquitin E3 ligase to target

STAT2 to the proteasome (80, 242). Likewise, bovine RSV nonstructural proteins have been shown to antagonize type I IFN signaling by inhibiting interferon regulatory factor (IRF3) activation (30). A recent study examining RSV infection of mouse lung epithelial (MLE-15) cells showed that by 24 hours post-infection type I IFN mRNA and IFNβ protein expression were suppressed in the absence of NS1 and NS2 proteins (200). Moreover a role for RSV G protein in inhibiting IFNβ was associated with the induction of suppressor of cytokine signaling (SOCS)-1 and SOCS3 expression. Described in detail later, SOCS proteins function as regulatory proteins to inhibit cytokine signaling pathways (62, 282, 327). For both human and bovine RSV, NS antagonism of the antiviral response coincides with expression of RSV proteins that occur as early as five hours post-infection and may ultimately result in inhibition of the cytotoxic T lymphocyte (CTL) response (25, 158). Type I IFN has an important role in DC maturation, activation of NK cells, differentiation and function of T cells and enhancing primary antibody responses (18, 37), thus RSV-mediated inhibition of IFN production impacts both the innate and adaptive immune responses. Evidence supporting this is provided in part from a study showing that infection of mice with RSV lacking NS1 and NS2 genes results in substantially higher levels of IFNα/β (305). RSV can also interfere with JAK/STAT signaling and chemokine transcription by inducing B-cell lymphoma protein (Bcl)-3 which complexes with STATs in the nucleus resulting in enhanced infection (134).

In addition to antagonism of the antiviral response, RSV nonstructural and small hydrophobic proteins delay premature apoptosis, a feature that results in higher viral titers (25, 93). RSV-infected cells exhibit higher expression levels of the anti-apoptosis gene IEX-1L and increased expression of several B-cell leukemia/lymphoma 2 (Bcl-2)

family members including myeloid cell leukemia-1 (Mcl-1) and Bcl-XL (74, 159, 173, 197). Recent studies suggest other contributing factors include RSV-mediated inhibition of tumor suppressor p53 and Akt activation leading to p53 proteasome degradation (110). The delay of apoptosis has also been linked to the phosphatidylinositol 3-kinase (PI3K)-dependent pathway (294), and to increased ceramidase and sphingosine kinases which lead to increased levels of anti-apoptotic proteins within cells (196). Moreover, it is well established that while RSV infection induces substantial syncytium formation in immortalized cell lines, RSV infection of human airway epithelial (HAE) cell models does not induce extensive cytopathology (322, 329), a feature associated with the ability of RSV to delay apoptosis of epithelial cells.

Respiratory epithelial cells express pattern recognition receptors (PRRs), or Toll-like receptors (TLRs), whose primary function is to recognize common microbial patterns to protect against infection. Several TLRs and RIG-I have been shown to be necessary for generating effective innate immune responses against RSV infection (174, 257, 265). RSV infection of respiratory epithelial cells results in enhanced levels of TLR4 expression on the cell surface within 24h of infection (198, 324). The increased levels of TLR4 results in increased sensitivity to endotoxin, and upon stimulation with lipopolysaccharide (LPS), increased IL-6 and IL-8 production occurs (324). Furthermore, peripheral monocytes isolated from infants with severe RSV bronchiolitis also show increased levels of TLR4 expression (94). TLR4 expression in infants infected with RSV was recently examined. It was shown that infants who possessed two single nucleotide polymorphisms encoding Asp299Gly and Thr399Ile substitutions in the TLR4 ectodomain were more associated with severe RSV disease, suggesting that extracellular

TLR4 polymorphisms are associated with symptomatic RSV disease in high-risk infants (13). Moreover, during a vaccine delivery study using a mouse model of RSV infection, it was shown that greatest protection against RSV challenge depends on intact TLR4-MyD88 signaling pathways (64).

The RSV F protein has been shown to interact with TLR4 and CD14 in human monocytes leading to the activation of nuclear factor (NF)-κB and production of proinflammatory cytokines TNFα, interleukin (IL)-6 and IL-12 (167). In mice deficient in TLR4, RSV infection results in aberrant NK cell trafficking, deficient NK cell function, impaired IL-12 expression and lower virus clearance compared to normal mice (123). While these results could be due to a defect in IL-12R in TLR4-deficient mice (77), our results suggest that RSV surface proteins can initiate a TLR signaling cascade resulting in inhibition of the JAK/STAT pathway. Furthermore, while the mechanism is not yet clear, the RSV G protein may also suppress TLR3/4-mediated cytokine production by interfering with TLR adaptor TNF receptor associated factor (TRIF)/Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule (TICAM)-1 or NFκB activation resulting in decreased proinflammatory cytokine production (233, 265). Another recent study demonstrated that RSV is able to increase TNFα, IL-6, MCP-1 and RANTES production via interaction with TLR2 and TLR6 (206).

Prostaglandins (PG) are implicated in many regulatory mechanisms including immune cell differentiation and inflammatory responses. During RSV infection, increased levels of PG secretion, particularly PGE2 via increased cyclooxygenase (COX)-2 expression is observed (39, 175, 245). COXs are rate-limiting enzymes that convert arachidonic acid to prostanoids, and COX-2 is generally up regulated during

inflammatory processes. Interestingly, TLR4 stimulation also results in up regulation of genes involved the inflammatory response including COX-2 (227, 244). Therefore, it is likely that RSV surface proteins induce COX-2 via TLR4 signaling. Furthermore, extracellular matrix (ECM) components are regulated in part by the action of matrix metalloproteinases (MMP). MMPs play a role in the digestion of gelatin, collagens (types IV,V, XI, XVII) and elastin, all important components of the ECM (12). It was recently found that RSV infection enhances the expression of MMP-9 leading to an increased rate of syncytium formation and more efficient viral replication (325).

One important consequence of respiratory illnesses including severe RSV disease is fluid extravasation into the air spaces of the lung (271). RSV infection of murine and human airway cells results in impaired alveolar fluid clearance (AFC). This is proposed to result from decreased sodium transport across epithelial cells leading to reduced alveolar fluid clearance in mice resulting in increased lung fluid and hypoxemia (66, 67). One study has suggested that the RSV F protein and TLR4 play a role in this AFC impairment (164), and another recent study found that RSV infection of primary bronchial cells resulted in a loss of plasma membrane integrity and cytoskeletal reorganization which was dependent on MAPK signaling (271).

RSV infection of the respiratory epithelium also results in reduced secretion of surfactant proteins (SP), particularly SP-A and SP-D, detected in bronchoalveolar lavage (BAL) (152, 306). SP-A and SP-D, produced by non-ciliated cells, are important in promoting opsonization (224), and SP-A can bind to the RSV F protein thereby enhancing the uptake of RSV-infected cells by macrophages (16, 100). Likewise, SP-D is able to bind to RSV G protein to inhibit infection (126). Thus, decreased lung function in

infants with severe RSV disease may be associated with decreased concentrations of SP-A and SP-D (152).

It is well established that RSV is associated with the exacerbation of asthma and bronchiolitis (267), and that during infection enhanced $CD4^+$ T cell responses and inappropriate cytokine expression results in reduced immune regulation and increased inflammation (99, 143, 267). One recent study showed that murine type II alveolar epithelial cells infected with RSV are unable to inhibit T cell activation suggesting that the tolerogenic state within the lung is at least partly controlled by lung epithelial cells (315). DCs infected with RSV can still differentiate and mature, but they display impaired T cell activation, an effect linked to altered IFNα or IL-1 receptor-alpha expression (235, 256). It has also been shown that direct contact of T cells with RSV F protein expressed on cells inhibits T cell activation (259). Moreover a recent study demonstrated that while murine DCs can undergo normal maturation upon RSV infection, these same RSV-infected DCs were not able to stimulate antigen-specific T cells following TCR engagement indicating a possible defect in the immunological synapse (105). Thus, RSV-infected DCs expressing F protein may also inhibit T cell activation by a related mechanism. While the mechanism of T cell inhibition remains to be elucidated in these models, it is possible that RSV infection promotes tolerance providing further evidence linking severe RSV disease and asthma.

RSV Pathogenesis

Disease severity, genetic factors, environmental risk factors and the success of viral immune evasion all have a role in the immune response and disease pathogenesis associated with RSV infection. RSV infection can be asymptomatic or result in a severe disease including bronchitis and bronchiolitis, and infection may be associated with the development of asthma or chronic illness later in life (267). RSV is the most common causative agent of bronchiolitis in children under the age of 2 years, and this virus is the most important respiratory virus afflicting young children worldwide.

During infection with RSV, the virus interacts with pattern recognition receptors on the surface and within respiratory epithelial and immune cells. Accumulating evidence has shown that RSV surface proteins modify TLR and PRR signaling and downstream signaling processes (123, 167, 174, 206, 250, 264) ultimately affecting patterns of cytokine expression. Negative regulators of cytokine expression, e.g. SOCS proteins are also affected, a feature that may polarize to alter the Th1- or Th2-type cytokine response (160, 200, 327, 332). Therefore, modification of early immune responses can affect the adaptive immune response and contribute to disease pathogenesis. While some studies have shown that children infected with RSV develop a Th1-type response, it is generally thought that young children are more prone to develop a Th2-type cytokine biased response, which is associated with RSV-mediated pathology (29, 247). RSV-specific $CD4^+$ memory T cells have been shown to play a major role in RSV-induced immunopathology, a feature linked to a Th2-type cytokine response and pulmonary eosinophilia (40, 46, 106, 108, 296). This Th2-biasing phenomenon is most apparent in studies examining vaccine-enhanced RSV disease. Cotton rats vaccinated with formalin-

inactivated RSV vaccine or vaccinia vectors (VV) expressing RSV G protein results in a Th2-type $CD4^+$ T cell response and lung eosinophilia upon RSV challenge (46, 59, 75, 215, 238, 317). Interestingly, mice immunized with VV expressing RSV F protein results in the activation of a Th1-type $CD4^+$ T cell response (6, 141). Depletion of $CD4^+$ or $CD8^+$ T cells actually decreases RSV disease severity and illness in small animal models (75, 107). In the absence of IFNγ, mice develop RSV F-specific memory $CD4^+$ T cells that secrete IL-5 and pulmonary eosinophilia following RSV challenge suggesting that IFNγ can modulate the memory $CD4^+$ T cell response to secondary RSV infection (45). Furthermore, the presence of RSV-specific $CD8^+$ memory T cells in the lungs of infected animals may help to regulate the Th2-type $CD4^+$ T cell response and result in reduced vaccine enhanced disease (131, 214, 279). It remains unclear whether $CD8^+$ T cells play such a role in other allergy-related diseases such as airway hyper-responsiveness to RSV infection (261). It was recently shown that RSV-specific memory $CD8^+$ T cells are able to inhibit Th2-associated chemokines, CCL17 and CCL22, and may alter the trafficking of Th2-type cells and eosinophils into the lung (214).

Peripheral blood of neonates infected with RSV has a lower proportion of RSV-specific $CD8^+$ T cells compared to older infants. It is believed that this might be a feature of an immature immune response, the presence of maternal antibodies, or a Th2-type lung environment. It is unknown whether recruited or lung-resident RSV-specific $CD8^+$ T cells are important in the prevention of RSV re-infection. While there is a higher fraction of pulmonary $CD8^+$ memory T cells that secrete IFNγ upon peptide stimulation compared to the draining lymph node, the recall response following RSV challenge is more effective in the lymph node resulting in a rapid export of $CD8^+$ T cells to the lung (218).

In contrast, mice challenged with influenza virus results in more efficient reactivation of resident pulmonary $CD8^+$ T cells (129). Therefore, RSV-specific T cells might become impaired or tolerized within the lung, and evidence suggests that $CD8^+$ T cells can be inactivated in the lung during RSV infection but not within the lymph node or spleen (48). In other studies it has been shown that inactivated $CD8^+$ T cells could be rescued by increased IL-2 expression within the lungs (49), and other studies have shown that inactivation of effector T cells was associated with their location and generally restricted to cells in the lung parenchyma as opposed to the airway (9). It has also been suggested that lung epithelial cells can mediate inhibition of T cell activation (315).

In conclusion, there are many mechanisms involved in the innate and adaptive immune response to RSV infection that contribute to immunity and disease, and it is clear that RSV has co-opted many host responses to infection to facilitate replication. Because there is a deficiency in long-term immunity to subsequent infection, one interpretation of the studies discussed here is that immune modulation may be a feature to allow for RSV persistence. Indeed, it has been shown that RSV can persist within immune-privileged neuronal cells (172, 191, 262, 267, 295). Thus, it is likely that vaccination of infants will not provide sufficient protection and that other disease intervention strategies should be further explored.

Overview of Influenza A Virus

Influenza A viruses are important pathogens that present a significant public health threat and economic burden, particularly avian influenza viruses (AIV). AIV

infections in poultry range from subclinical to acute disease having 100% mortality which contributes to the economic burden. Contemporary seasonal epidemics caused by human influenza viruses also contribute to economic burden as they have been attributed to more than 200,000 hospitalizations each year and more than 41,000 deaths each year in the United States (76). AIV have a large host range that include mammals; thus there is a substantial concern that this reservoir may, particularly in aquatic birds, contribute to the potential for human infection. Over the past century there have been three major novel influenza virus strains afflicting the human population, i.e. viruses that originated in 1918, 1957 and 1968 respectively. The 1918 influenza pandemic was the most severe resulting in unusually high mortality rates (270). It remains unclear whether the high rate of mortality was due to an exaggerated host response to infection, increased virulence, or a combination thereof (182). Beginning in 2003, a looming pandemic threat emerged from a highly pathogenic H5N1 AIV causing 424 confirmed human infections and 261 deaths as of May 2009 (320).

Influenza viruses are enveloped, negative-strand RNA viruses that have eight gene segments, belonging to the *Orthomyxoviridae* family. They comprise a diverse array of subtypes due to their propensity to change antigenic profiles and are therefore subtyped based on the antigenic properties of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). There are currently 16 known HAs and 9 known types of NA. Influenza HA binds to host cell sialic acid residues coating the host cell surface (278) and mediates viral entry via its receptor binding domain. Sialic acids (sias) are nine-carbon monosaccharides typically found at the ends of glycan chains. Sias coat all cell surfaces and many secreted proteins, and have a role in many physiological and

pathological interactions (8, 258, 308, 309). Sias are transferred to terminal sugars of glycoproteins and glycolipids by sialyltransferases, and can be added to the galactose carbon-6 forming an α2,6 linkage or to galactose carbon-3 forming an α2,3 linkage. Influenza HA has a strong preference to bind to certain sia moieties and is an important host range determinant (284-287). Human-adapted influenza viruses preferentially recognize α2,6 linkages, while influenza viruses of avian origin preferentially recognize α2,3 linkages highly expressed in the gastrointestinal tracts of aquatic birds (57, 133, 187, 266). Because previous influenza pandemics resulted after acquiring mutations affecting the HA binding specificity from avian-like, α2,3, to human-like, α2,6 (57, 102, 187), it is hypothesized that a change in receptor binding specificity is necessary for efficient human-to-human transmission (187).

Influenza A Virus Replication

Influenza infection occurs following attachment of the HA receptor binding domain (RBD) to sialic acid-coated glycans on the cell surface. Following attachment, the virus can enter the host cell by clathrin-mediated and clathrin- and caveolin-independent endocytic pathways (253). Once within the endosome, the pH decreases due to M2 ion channel activity causing HA to change conformation and thereby exposing the fusion peptide (230, 273). The hydrophobic fusion peptide inserts into the host cell membrane and results in fusion of the viral and cellular membranes. Following fusion, viral ribonucleoproteins (RNP) are released into the cytoplasm and transported to the nucleus to initiate transcription. Interestingly, influenza employs the method of cap-

snatching whereby the virus uses cellular 5'-methylated cap structures within the nucleus as primers for transcription initiation while NS1 blocks cellular mRNA transport to the cytoplasm (232). While virion assembly occurs in the cytoplasm, the structural proteins HA, NA and M2 are transported to the cell surface where budding ensues (184, 213).

Host Immune Response to Influenza Virus Infection

Influenza virus infection in humans can cause a wide range of disease symptoms from subclinical disease to acute illness characterized by fever, myalgia, and respiratory symptoms such as a nonproductive cough, sore throat and rhinitis. In addition, children infected with influenza may present with otitis media, nausea, and vomiting (47). Those individuals with chronic diseases such as pulmonary or cardiac disease, or diabetes mellitus are at even higher risk for developing hemorrhagic bronchitis, pneumonia and death when infected with influenza. The host immune response to infection contributes to influenza-mediated pathogenesis. Influenza infection primarily infects epithelial cells and is detected by several PRR including TLR3 and RIG-I, which recognize double-stranded RNA, and TLR7, which recognizes single-stranded RNA (15, 111, 180). Stimulation of these pathways leads to the induction of pro-inflammatory cytokines by epithelial cells and immune cells early following infection, an effect paralleling the peak of viral replication and associated with the onset of disease symptoms (122). The influenza NS1 protein may act as an interferon antagonist to modify the antiviral response to infection (97), and likely facilitates virus replication and the associated disease pathogenesis.

The humoral immune response to influenza virus infection is an important aspect of influenza vaccine efficacy. The main correlate of protection against influenza infection is strain-specific virus-neutralizing antibodies directed against the HA, and antibodies specific for the NA have been shown to reduce disease severity by restricting virus release from infected cells and enhancing viral clearance (98, 153). Vaccine studies in which children or adults were given live-attenuated or inactivated influenza vaccines have shown that protection is also correlated with the development of mucosal IgA antibody (53, 138, 139). In addition to HA and NA, antibodies are produced to NP and M2 proteins during infection, and cellular immune responses are important for virus clearance (88, 283). For example, $CD4^+$ T cell-depleted mice display delayed virus clearance, while $CD8^+$ T cell-depleted or deficient mice are still able to clear infection, indicating that $CD4^+$ T cells have a significant role in adaptive immunity (78, 79).

Suppressor of Cytokine Signaling Family of Proteins

The innate and adaptive immune responses are coupled to each other by way of antigen presenting cells such as DCs that process and present antigen in the context of MHC to T cells. Cells of the innate (monocytes/macrophages, NK cells, neutrophils) and the adaptive (T cells and B cells) immune responses secrete cytokines and chemokines to mediate an appropriate response to infection. Cytokine signaling is critical to coordinate the initiation, maintenance and resolution of an inflammatory response as well as evolution of Th1- or Th2-polarized responses. Because cytokines and chemokines play such a large role in instigating an immune response, their expression is tightly regulated

to prevent an imbalance of magnitude and duration. Suppressor of cytokine signaling (SOCS) proteins are an important family of proteins that act to negatively regulate cytokine signaling pathways. They were first identified as inhibitors of the JAK/STAT pathway, but studies have shown that SOCS proteins have a role in regulating not only components of the innate and adaptive immune system but also in developmental functions [reviewed in (327)]. SOCS proteins are a class of proteins that include eight members, i.e. SOCS1 to SOCS7 and cytokine inducible Src homology 2 domain (SH2)-containing protein (CIS). Each member has a variable N-terminal region followed by a central SH2 domain critical for inhibiting Janus kinase (JAK) activity and conserved C-terminal SOCS box motifs that can interact with Elongin B/C complex, a component of E3 ligase (147). The SOCS box also contains a Cullin5 (Cul5) box that directs the SOCS/Elongin B/C complex to bind to Cul5, which in turn binds to an E2 ubiquitin-conjugating enzyme (146, 147, 181). Together this complex forms a functional E3 ubiquitin ligase. SOCS1 and SOCS3 contain an additional kinase inhibitory region (KIR) containing a conserved tyrosine residue thought to be important in inhibiting JAK2 activity (91, 311). SOCS proteins are also orthologs of those found in *Drosophila melanogaster* (41) and *Caenorhabditis elegans* (154). In mammals, SOCS proteins function to negatively regulate various cytokine signaling pathways through one of at least three mechanisms. SOCS proteins can bind to and inhibit tyrosine kinases including JAK and insulin receptor (82, 199, 207). SOCS proteins can bind to cytoplasmic portions of receptors and occupy docking sites for STATs and other proteins (240), and SOCS proteins can target other substrates for proteasomal degradation via the C-terminal SOCS box (92).

SOCS1 was originally identified as a negative regulator of IFNα/β, IFNγ, IL-4, and IL-6 signaling via the JAK/STAT pathway (82, 207, 282). IFNα/β bind to their cell surface receptors and activate the IFN signaling pathway leading to SOCS expression. Mice deficient in SOCS1 are normal at birth but rapidly develop severe immunopathology within three weeks characterized by uncontrolled cytokine signaling (5, 183, 281), and mice antibody-depleted of IFNγ show increased rates of survival (5). Likewise, $Rag1^{-/-}$ mice deficient in SOCS1 also show increased survival due to decreased IFNγ production and signaling (183). SOCS1 and SOCS3 proteins have been shown to inhibit IFNα- and type III IFN-, IFNλ (IL-28A/B and IL-29), induced activation of the JAK/STAT pathway and expression of the antiviral proteins 2',5'-oligoadenylate synthetase (OAS) and MxA (33, 310). Other cytokines also induce SOCS expression. For example, granulocyte-macrophage colony-stimulating factor (GM-CSF) induces expression of CIS and SOCS3 transcripts and antagonizes IFN-mediated signals by blocking STAT1 function (148). Moreover, activation of TLRs by PAMPs is essential for the initiation of the innate and adaptive immune responses. Inadequate or delayed signaling events and subsequent gene expression can lead to inappropriate immune responses, and excessive activation can result in autoimmunity or chronic inflammation. Therefore, TLR signaling is also regulated to maintain a balance between positive and negative inflammatory effects. SOCS1 and SOCS3 proteins are induced upon TLR ligation and can inhibit type I IFN signaling and therefore mediate cross-talk inhibition (14, 65).

SOCS proteins are potent negative regulators of both MyD88-dependent and MyD88-independent TLR signaling. The MyD88-dependent signaling pathway is shared

by all TLRs, except TLR3, which exclusively uses a MyD88-independent pathway (151, 288). LPS induces pro-inflammatory cytokines as well as reactive oxygen and nitrogen species following binding to TLR4 on the surface of macrophages and monocytes eliciting MyD88-dependent and MyD88-independent signaling pathways (23). SOCS1 has been shown to negatively regulate both pathways and over-expression results in defective NFκB activation (155, 208). Furthermore, over-expression of SOCS3 can inhibit inflammation following LPS stimulation (135).

The immune modulation mediated by RSV proteins, particularly cytokine and chemokine responses (298, 301, 302) suggest that RSV may modulate the suppressor of cytokine signaling (SOCS) family of proteins. RSV infection of macrophage-like U937 cells has been shown to increase expression of SOCS1, SOCS3, and CIS mRNA while phosphorylation of STAT1 and STAT2 was decreased (332). Furthermore, expression of the RSV G protein during infection of MLE-15 cells has been associated with decreased IFNβ levels and enhanced SOCS1 and SOCS3 expression (200). Many other viruses are known to modify SOCS expression in order to manipulate the host immune response. Hepatitis C virus (HCV) core protein up regulates SOCS3 and induces ubiquitination of insulin receptor substrate (IRS) in a HCV-associated resistance model (149). HCV core protein is thought to interfere with the JAK/STAT pathway by inducing SOCS3 expression by an unknown mechanism leading to a reduced antiviral state (28, 312). Furthermore, HCV core protein of genotype 3a upregulates SOCS7, interfering with the insulin signaling pathway (226). Influenza A virus infection has also been shown to increase both SOCS1 and SOCS3 and can regulate the initial antiviral response to infection leading to decreased viral titers (225, 234). In severe measles virus infection,

rabies encephalitis or human immunodeficiency virus (HIV)/human papilloma virus (HPV) co-infection, viral infection has been associated with an increase in the number of cells secreting TNFα and a concomitant reduction in SOCS expression (209, 212, 231), while in a chronic hepatitis B model, a positive correlation between the level of SOCS1 expression and inflammation has been shown (333). SOCS1 mRNA has been shown to be significantly increased in patients with human T-lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (210), and negative factor (Nef) protein, an immunosuppressive HIV-1 protein, has been shown to up regulate SOCS proteins in B cells (239). Interestingly, SOCS1 has been shown to directly bind the Gag structural protein in HIV-1 infection (254), and HIV-1 transactivator protein (Tat) has been implicated in immune evasion and interference of IFNγ signaling pathway via SOCS2 induction and altered levels of STAT2 in human monocytes (51).

At least one virus contains its own SOCS box motif; the HIV-1 virion infectivity factor (Vif) acts as an E3 ubiquitin ligase via its SOCS box to overcome the antiviral activity of APOBEC3G to protect HIV-1 DNA from G-to-A hypermutation (156, 328). It is generally thought that poxviruses induce overproduction of cytokines leading to immunopathology. One recent study showed in mice showed that a SOCS1-KIR mimetic provided protection from lethal vaccinia virus challenge, representing a novel therapeutic method (4). Finally, there is at least one instance showing that SOCS proteins also display antiviral properties. During HPV infection of cancer cell lines, it was demonstrated that SOCS1 interacted with HPV E7 protein in a SOCS-box-dependent manner and induced its ubiquitination and subsequent degradation (145).

In addition, protozoan and bacterial infections have been shown to modify SOCS expression. For example, *Toxoplasma gondii* and *Leishmania donovani* induce SOCS1 and SOCS3 expression respectively, both of which interfere with IFNγ signaling pathways (22, 334), and for bacteria, *Mycobacterium bovis bacillus Calmette-Guerin* infection has been shown to increase both SOCS1 and SOCS3 expression to aid in immune evasion (132, 220). Therefore, diverse pathogens have evolved mechanisms to regulate SOCS protein expression and to reduce or inhibit the immune response to infection.

Normal Human Bronchial Epithelial Cells as a Model System

Normal human bronchial epithelial (NHBE) cells (or human airway cells, HAE) have been recognized as a very good in vitro model to emulate aspects of the human airway response to infectious diseases including cell tropism, innate immune responses and histopathology (21, 228, 229, 313). Highlighting their utility as an *in vitro* cell model, NHBE cell cultures have aided in defining three physiological barriers to infection including mucociliary clearance, formation and constitution of the glycocalyx and intercellular junctional complexes. The mammalian airway epithelium is composed of multiple cell types [reviewed in (81, 219)] including ciliated cells, mucus-secreting goblet cells, serous cells, Clara cells and basal cells. Most of these important cell types are found in the NHBE cell model. Basal cells are thought to be stem cell precursors for ciliated and mucus-producing cells, especially within the upper respiratory tract. Within the alveolar regions of the lung, type I and II pneumocytes are abundant and are

important in gas exchange. In a normal human airway, the lumen of the nasal, tracheal and bronchial regions are lined with a ciliated pseudostratified columnar epithelium with goblet cells that lies atop a basal epithelial layer. The lower respiratory tract (LRT) lumen, specifically the bronchiolar region, is lined with simple cuboidal epithelium containing ciliated cells with fewer goblet cells and no basal layer present. While NHBE cell cultures do not contain submucosal glands because they are a simple epithelial cell model, they do contain goblet and other mucin-producing cells.

Epithelial cells lining the human respiratory tract are coated in a thin layer of mucus secreted by resident goblet cells as well as by serous- and other mucus-producing cells within the underlying submucosal glands. In addition to protecting the epithelial lining from pathogen assault or other irritants, mucus aids in preventing dehydration in the airways and alveoli (248). Goblet cells secrete mucins that mix with other secreted proteins to form mucus. Beating cilia on the apical surfaces of epithelial cells induce the flow of mucus up and out of the respiratory tract and into the throat where it is subsequently swallowed, a process termed mucociliary clearance. Therefore, many pathogens, particulates and other irritants are quickly removed prior to engaging the host immune response (246).

An important physiological barrier of the lung in addition to mucin secretion is the glycocalyx. The glycocalyx is formed as an organized meshwork of glycoproteins, particularly those of the mucin family, glycolipids and proteoglycans that are normally heavily sialated and sulfonated and are secreted from the apical surface of airway epithelial cells (3). While the glycocalyx of NHBE cells is not yet fully characterized, it does include moieties such as sialic acid, keratin sulfate, type V collagen and mucins, and

has been found to be an important limiting factor in gene transfer systems using viral vectors (21, 228, 229, 313). Thus, the glycocalyx functions as an effective barrier to not only protect the apical epithelial surface from environmental insult, but also to shield potential host receptors from pathogens entering the airway.

Airway epithelial cells also contain intercellular junctional complexes composed of anchoring and occluding junctions. In addition to the glycocalyceal barrier, these junctional complexes act as effective barriers against pathogens that may pass basolaterally from the apical surface, i.e. the lumen of the airway. As the glycocalyceal barrier is a limiting a factor in gene transfer systems, intercellular junctions have been found to limit transfer efficiency. In these studies the tight junctions can be disrupted resulting in a more efficient gene transfer (61). Because many viral and bacterial pathogens utilize host cell surface receptors to infect, the junctions can function to prevent access to many receptors located on the basolateral surface of the cells.

NHBE cells are cultured at an air-liquid interface (ALI) that fully differentiates into an epithelial model of the human airway (162). The model system consists of polarized, ciliated pseudostratified columnar epithelium containing goblet cells and Clara cells, and it provides an important means to study infectious diseases, particularly viruses, that initially or primarily infect the outermost layer of epithelial cells of the respiratory tract.

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CHAPTER 3

RESPIRATORY SYNCYTIAL VIRUS PROTEINS MODULATE SUPPRESSORS OF CYTOKINE SIGNALING 1 AND 3 AND THE TYPE I INTERFERON RESPONSE TO INFECTION BY A TOLL-LIKE RECEPTOR PATHWAY[1]

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Abstract

Respiratory syncytial virus (RSV) is a common cause of repeat infections throughout life and potentially severe lower respiratory tract illness in infants, young children and the elderly. RSV proteins have been shown to contribute to immune evasion by several means including modification of cytokine and chemokine responses whose expression is negatively regulated by suppressor of cytokine signaling (SOCS) proteins. In this study, we examine the role of SOCS1 and SOCS3 regulation of the type I interferon (IFN) response in normal fully differentiated human bronchial epithelial cells infected with RSV or with a RSV mutant virus lacking the G gene. The results show that RSV G protein modulates SOCS expression to inhibit type I IFN and interferon stimulated gene (ISG)-15 expression very early as well as late in infection, and that SOCS induction is linked to Toll-like receptor (TLR) signaling by RSV F protein indicated by interferon regulatory factor (IRF)-3 activation and nuclear translocation. These findings indicate that RSV surface proteins signal through the TLR pathway suggesting that this may be an important mechanism to reduce type I IFN expression to aid virus replication.

Introduction

Human respiratory syncytial virus (RSV) is a common cause of serious lower respiratory tract disease in infants and young children and may cause serious illness and some mortality in the elderly and immune compromised. RSV is a non-segmented, enveloped, negative-strand RNA virus belonging to the *Paramyxoviridae* family having two major surface proteins, i.e. the attachment (G) and fusion (F) proteins, and a minor surface protein, the small hydrophobic (SH) protein (19). RSV is known to cause repeat infections throughout life, a feature that may be linked to immune modulation by RSV proteins (53, 57, 58, 61, 63, 64, 66). Several studies have shown that RSV nonstructural NS1 and NS2 proteins can coordinately regulate the type I interferon (IFN) response during infection, and NS2 specifically inhibits signal transducer and activator of transcription (STAT)-2 signaling (37, 52, 53, 56-58). Likewise, for bovine RSV, NS1/NS2 proteins have also been shown to antagonize type I IFN through a mechanism involving interferon regulatory factor (IRF)-3 phosphorylation (9). For human and bovine RSV, NS1/NS2 antagonism of the type I IFN response coincides with expression of these non-structural proteins as early as 5h post-infection (pi) (7).

The RSV F protein has been shown to stimulate the TLR-dependent pathway through activation of CD14 and TLR4 (34). TLR4-deficient mice infected with RSV exhibit impaired pulmonary natural killer (NK) and $CD14^{+}$ cell trafficking, deficient NK cell function, impaired interleukin (IL)-12 expression and impaired virus clearance (24). Infection of airway epithelial cells with RSV increases TLR4 mRNA, protein expression, and TLR4 membrane localization (40). In studies of severe RSV disease in infants, genotyping of two common TLR4 amino acid variants (D259G and T359I) revealed an

association between TLR4 polymorphisms and genetic predisposition to severe viral infection (50). Since the TLR4/CD14 complex is important for the initiation of innate immune responses, it is not surprising that severe RSV disease in infants has been associated with these polymorphic changes.

The central conserved region of the RSV G protein may also modulate the innate immune response to infection (61, 64). This region contains a CX3C chemokine motif at amino acid positions 182-186 which is capable of interacting with the fractalkine receptor, CX3CR1 (64). G protein CX3C-CX3CR1 interaction has been shown to inhibit fractalkine-mediated responses, alter trafficking of $CX3CR1^{+}$ cells, including T lymphocytes and NK cells, as well as modify their functional activity (23). In addition, RSV G protein expression during acute infection has been associated with altered CC and CXC chemokine mRNA expression and Th1/Th2-type cytokine responses by bronchoalveolar leukocytes (63, 65). Furthermore, BALB/c mice infected with RSV show increased and prolonged production of Th2-type associated CCL2, CCL3, CXCL10, and CCL11 expression in total-lung RNA (13).

Because RSV surface proteins have been shown to modulate the innate and adaptive immune responses, particularly for cytokine and chemokine expression, we hypothesized that RSV proteins may impact the suppressor of cytokine signaling (SOCS) family of proteins whose function is to negatively regulate cytokine and chemokine responses (59). SOCS proteins include eight members, i.e. SOCS1 to SOCS7 and CIS. Each SOCS protein contains a central *Src*-homology 2 (SH2) domain that is critical for inhibiting Janus kinase (JAK) activity, as well as a unique carboxy-terminal motif (SOCS box), that has a role in targeting proteins for ubiquitination and proteasomal degradation.

SOCS1 and SOCS3 are the most closely related and best characterized members acting through the JAK/STAT pathway to regulate cytokine expression via a kinase inhibitory region (KIR). Importantly, SOCS proteins can also be induced through TLR stimulation independent of type I IFN (3, 14, 16, 44), and SOCS1 and SOCS3 expression is induced in macrophages by LPS (29, 60). It has recently been shown that SOCS1 contains a nuclear localization signal (NLS) that may have a role in inhibiting STAT-dependent transcriptional activity within the nucleus (4).

Several viruses have been shown to modify SOCS expression to manipulate the host response to infection. Influenza A virus and the hepatitis C virus core protein have been shown to induce SOCS expression and inhibit IFN signaling leading to a reduced anti-viral state (8, 39). Epstein-Barr virus latent infection induces SOCS1 and SOCS3 mRNA in nasopharyngeal epithelial cells (36), and for chronic hepatitis B virus infection, there is a positive correlation between the level of SOCS1 expression and inflammation (72). Negative factor (Nef) protein, an immunosuppressive HIV-1 protein, has been shown to up regulate SOCS proteins in B cells (51), and SOCS1 has been shown to directly bind the HIV structural protein, Gag, during infection to assist in viral trafficking and stability (55). Notably, RSV infection of macrophage-like U937 cells results in increased expression of SOCS1, SOCS3 and CIS mRNA while phosphorylation of STAT1 and STAT2 was shown to be decreased (71).

Thus, multiple families of viruses have adapted strategies to evade or inhibit key elements of the host anti-viral response, and accumulating evidence suggests that a primary mechanism may be linked to the regulation of SOCS expression.

In these studies, we show that RSV infection of fully differentiated primary NHBE cells up regulates SOCS1 and SOCS3 expression, an effect associated with decreased type I IFN and ISG15 expression. Using an RSV mutant virus lacking the G gene, we show that the G protein has an important role in modulation of SOCS1 and SOCS3 to down regulate type I IFN protein expression, and that modulation of SOCS expression may occur through a pathway linked through TLR signaling. We show that purified RSV F or G proteins increase SOCS1 and SOCS3 and decrease ISG15 expression following treatment of NHBE cells, an effect linked to rapid IRF3 activation and translocation into the nucleus following RSV F or G protein treatment. These studies have important implications for understanding anti-viral immunity in normal primary human bronchial epithelial cells and in prospective disease intervention strategies.

Materials and Methods

Cell Culture.

Expansion, cryopreservation, and culturing of normal human bronchial epithelial (NHBE) cells in an air-liquid interface (ALI) system were performed as previously described (33). Briefly, NHBE cells (Lonza, Walkersville, MD) from a single healthy 19-year-old donor were used throughout the study and seeded into vented T75 tissue culture flasks (500 cells/cm^2) until the cells reached 75-80% confluence. Cultures were dissociated with trypsin/EDTA and cultured in an ALI system initiated by seeding NHBE cells (passage-2, $4x10^4$ cells/cm^2) onto Transwell-clear culture inserts (24.5 mm, 0.45 mm pore size; Corning, Lowell, MA) that were thin-coated with rat tail collagen, type I (Collaborative

Res., Bedford, MA). Cells were submerged for the first 5-7 days in medium (BEGM BulletKit, Lonza) supplemented with 25 ng/mL epidermal growth factor (Upstate, Temecula, CA). After cells reached > 95% confluence, the ALI was created by removing the apical medium and feeding cells with BEGM BulletKit medium on their basolateral surface only. The apical surface of the cells was exposed to a humidified 95% air/5% CO_2 environment. Medium beneath the cells was changed every two days. Cells were cultured for 28 days in ALI for at least 35 days in culture.

Virus and virus proteins.

RSV strain A2 (RSV/A2), recombinant RSV/A2 (6340WT) and recombinant RSV/A2 lacking the G gene (6340ΔG) were propagated in VeroE6 cells (African green monkey kidney fibroblasts, American Type Culture Collection CCL 81) maintained in DMEM (Sigma-Aldrich Corp., St. Louis, MO, USA.) supplemented with 5% heat-inactivated (60°C) fetal bovine serum (FBS; Hyclone Laboratories, Salt Lake City, Utah) as previously described (62). 6340WT and 6340ΔG viruses were kindly provided by P. Collins (11). At detectable cytopathic effect, i.e. day 3-4 post-infection, the medium was decanted and cells were sonicated with an Ultrasonic Dismembrator (Fisher Scientific, Pittsburgh, PA) for 5 seconds at maximum output voltage/frequency. The cell lysate was centrifuged at $3{,}000 \times g$ for 7 min at 4°C. Virus titers were determined by immunostaining plaque assay on VeroE6 cells with anti-RSV F protein monoclonal antibody (clone 131-2A) as previously described (43). 6340WT was UV-inactivated by exposure to ultraviolet light for one hour on ice. Subsequent immunostaining plaque assay (66) confirmed inactivation (data not shown). RSV F and G proteins were isolated and purified from RSV/A2-infected VeroE6 cells as previously described (64).

Purification was confirmed by immunoblot with anti-G protein (131-2G) or anti-F protein (131-2A) monoclonal antibody as previously described (64).

NHBE cell infection.

NHBE cells were apically mock-infected or infected with RSV/A2, 6340WT or 6340ΔG at a multiplicity of infection (MOI) of 1 or 3. Cells were harvested in triplicate at the times indicated pi. As appropriate, NHBE cells were apically treated with the following conditions: purified RSV G protein (10μg/mL), purified RSV F protein (10 μg/mL), purified RSV G protein (10 μg/mL) + anti-RSV G protein monoclonal antibody (131-2G; 10 μg/mL), purified RSV F protein (10 μg/mL) + anti-RSV F protein monoclonal antibody (131-2A; 10 μg/mL), or LPS (1 μg/mL; Sigma, St. Louis, MO). RSV and mutant virus infection of NHBE cells was confirmed by immunostaining, immunoblot and qRT-PCR for RSV N gene expression as described here and previously reported (66).

Quantitative RT-PCR.

Total RNA was isolated 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, IFNa1 and IFNb1 gene expression assays (Applied Biosystems, Foster City, CA) and analyzed using MXPro software by Stratagene (La Jolla, CA). Amplifications were done following a 10-min hot start at 95°C in a three-step protocol with 15 s of denaturation (95°C), 30 s of annealing (60°C) and extension at 72°C for 15 s. 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 NHBE cells. Data is presented as fold-differences in gene expression relative to mock-infected or mock-stimulated NHBE cells. Values above 1.0 are considered indicative of up regulation, and values below 1.0 are considered indicative of down regulation. To confirm RSV infection of NHBE cells, RSV N gene expression was determined using the above cycling parameters and the following forward primer, probe and reverse primer sequences: TGACTCTCCTGATTGTGGGATGATA, CAGCATTAGTAATAACTAAATTAG, CGGCTGTAAGACCAGATCTGT.

Flow cytometric analysis of protein expression.

The percent positive NHBE cells staining positive or negative for SOCS1, SOCS3, ISG15, IFNα or IFNβ were determined by flow cytometry using similar methods as previously described (66). Briefly, for intracellular staining, NHBE cells were washed once with PBS and trypsinized for 10 min at 37°C. Cells were collected and centrifuged at 220 x *g* for 5 min and resuspended in BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (San Jose, CA) for 20 min at 4°C and washed with 0.5% saponin (Acro Organics, Morris Plains, NJ) diluted in flow buffer (3% FBS (heat-treated, 1h at 56^{o}C) in PBS). Intracellular protein expression was determined by primary staining with anti-SOCS1 (Abcam, Cambridge, MA), anti-SOCS3 (Novus Biologicals, Littleton, CO) and anti-ISG15 (Cell Signaling, Danvers, MA), and secondary staining with Cy5-conjugated donkey anti-rabbit IgG (Millipore, MA) antibodies. Intracellular IFNα and IFNβ protein expression was determined by APC-conjugated anti-IFNα (clone LT27:295, Miltenyi Biotec, Auburn, CA) and FITC-conjugated anti-IFNβ (clone MMHB-3, PBL

InterferonSource, Piscataway, NJ) monoclonal antibodies diluted in flow buffer for 1 hour at 4°C. Cells were then washed with flow buffer and analyzed on a LSRII flow cytometer using FACSDiva software (Becton-Dickinson, Mountain View, CA). Additional analysis was performed using FlowJo software (TreeStar, Ashland, OR).

Immunoblot analysis.

NHBE cells were infected or apically treated as indicated above. Cells were lysed (50mM Tris-HCl, pH 7.4, 1% Ipegal, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM Na_3VO_4, 1mM NaF, 1mM PMSF, 1µg/mL pepstatin A, 1µg/mL leupeptin HCl, 1µg/mL aprotinin). Proteins were separated by SDS/10.5-14% PAGE and transferred to polyvinylidene diflouride membranes in transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol). Membranes were blocked in Tris-buffered saline (TBS) containing 0.05% (wt/vol) TWEEN20 and 5% (wt/vol) dry skim milk for 1 h and immunoblotted with anti-IRF3 antibody (sc-9082, Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were visualized by incubation with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody with signal development using ECF (Pierce, Rockford, IL).

Confocal Microscopy.

NHBE cells were mock-infected or infected as described above. At 1 h pi or stimulation, the cells were washed with PBS and fixed with 3.7% formaldehyde. Cells were permeabilized with 1% Triton X-100 in PBS for 10 min. The apical surfaces of cultures were exposed to flow buffer to block nonspecific binding prior to addition of one

of the following primary antibodies diluted in flow buffer: anti-TLR-4 antibody (Zymed, Carlsbad, California), anti-RSV F antibody (131.2A), or anti-IRF3 (Santa Cruz). After primary-antibody incubation, the cultures were washed 3 times with PBS containing 0.05% TWEEN20 and incubated with appropriate secondary antibodies: goat anti-rabbit IgG-conjugated to AlexaFluor 680 (Molecular Probes, Carlsbad, California) for TLR4, goat anti-mouse IgG-conjugated to AlexaFluor 488 (Molecular Probes) for RSV F, and donkey anti-rabbit IgG-conjugated to Cy5 (Chemicon). Cells were washed 3 times with PBS containing 0.05% TWEEN20 and stained with 1μg/mL DAPI (4', 6'-diamindino-2-phenylindole) in PBS. Fluorescence was recorded by optical sections using confocal laser scanning microscopy (LSM510Meta, Carl Zeiss).

Statistical analysis of data.

Differences in gene-fold expression in qRT-PCR analysis were evaluated by Student *t* test and considered significant when the *p* value was < 0.05. Data are shown as means ± standard deviation (SD). Differences in protein expression were evaluated by determining the percent (%) median fluorescent intensity (MFI) relative to that of mock-treated cells. Differences in % of mock control were evaluated by unpaired *t* test and considered significant when the *p* value was < 0.05.

Results

RSV modifies SOCS and type I IFN gene expression.

To determine if RSV infection of NHBE cells affected SOCS regulation of the type I IFN response, NHBE cells were infected with wild-type RSV/A2 or recombinant

wild type 6340WT (MOI = 1) (Fig. 3.1). The results show that both SOCS1 and SOCS3 mRNA expression were induced within 15 minutes of 6340WT infection, i.e. the adsorption phase of virus infection (Fig. 3.1A). By 2h post-infection (pi), SOCS1 and SOCS3 mRNA declined but increased again between 6h and 12h pi, the period coinciding with initiation of viral protein expression (7, 35). IFNα and IFNβ mRNA expression increased early in 6340WT infection, peaked by 2-6 hours pi, and declined by 12h pi to background levels (Fig. 3.1B). As predicted, type I IFN mRNA expression generally declined as SOCS mRNA expression increased. Concomitant with early (within 15 minutes of infection) induction of SOCS1 and SOCS3 mRNA expression, there was little induction of type I IFN mRNA expression. Notably, IFNα/β mRNA expression increased as SOCS1 and SOCS3 decreased at 2 h pi. This may be related to the expression of type I IFN antagonists, i.e. NS1/NS2 proteins that have been shown to accumulate in the cell at 5 h pi (20, 58).

To determine the role of the RSV G surface protein in SOCS regulation of the type I IFN response to infection, NHBE cells were infected (MOI = 1) with a recombinant RSV/A2 mutant virus lacking only the G gene (6340ΔG; Fig. 3.1). It is important to note that it is not possible to produce a RSV mutant lacking the F gene, as this gene is essential for virus replication. Examination of RSV N gene expression in NHBE cells infected (MOI = 1) with 6340WT or 6340ΔG was equivalent for the time-points examined (data not shown). The results show that in the absence of G protein expression (6340ΔG), there was a significant ($p < 0.05 – 0.001$) decrease in SOCS1, SOCS3, IFNα and IFNβ mRNA expression detected at 15 min pi (Fig. 3.1). Compared to 6340WT infection, lower levels of SOCS1 and SOCS3 mRNA expression were detected

at 6h and 18h pi, respectively, and lower levels IFNα mRNA expression was detected at 6h pi following 6340ΔG infection. These results suggest that RSV G protein contributes to early induction of SOCS1 and SOCS3 mRNA, and the concomitant decrease of type I IFN mRNA expression.

One interpretation of these findings is that RSV surface proteins may induce a very early SOCS response to alter the tempo and magnitude of the type I IFN response to infection. This feature may be important in facilitating virus replication before NS1/NS2 protein expression. One mechanism linked to SOCS1 and SOCS3 expression is activation through the Toll-like receptor (TLR) pathway (3, 14, 16, 44, 60). As RSV F protein has been shown to bind to TLR4 (34), and NHBE cells have been shown to express TLR4 mRNA (2), it is possible that RSV surface proteins interact through TLR4 and/or other TLR or pattern recognition receptors (PRRs) to initiate these effects.

RSV modifies the type I IFN response.

To further elucidate the mechanisms of RSV modulation of the type I IFN response, NHBE cells were mock infected or infected (MOI = 1) with 6340WT or 6340ΔG to determine SOCS1, SOCS3, IFNα or IFNβ protein expression by flow cytometry (Fig. 3.2). Similar to gene expression detected in Figure 3.1, SOCS1 and SOCS3 proteins were increased over mock-infected cells early (2h pi) after infection (Fig. 3.2A). It is known that SOCS genes are constitutively expressed at low levels in unstimulated cells, but the rate of transcription rapidly increases following cytokine stimulation (1, 15, 32, 46). This feature is related to utilization of constitutively expressed SOCS proteins and the need for rapid re-expression to regulate external and internal

stimuli. Consistent with this, SOCS1 and SOCS3 protein expression was lower between 6h and 18h pi, but subsequently increased by 24h pi. While the data in Figure 3.1 indicates that expression of SOCS message increases between 6h and 12h pi, there is an expected lag in translation into detectable protein levels. The expression of SOCS1 and SOCS3 proteins at 2h pi was associated with low IFNα protein expression until 24h pi (Fig. 3.2B). Although IFNα gene expression was increased (Fig. 3.1B), there was 25-30% less IFNα protein detected compared to the mock-treatment (Figure 3.2B). In contrast, IFNβ protein expression increased by 2h pi, decreased from 6h to 18h pi, and increased again by 24h pi (Fig. 3.2B). Similar to IFNα transcript levels, a higher number of IFNβ transcripts were detected 2h to 6h pi (Fig. 3.1B) and less protein expression indicating RSV modulation of the type I IFN response as anticipated.

To help clarify the role of the RSV G protein in SOCS regulation of the type I IFN response to RSV infection, the levels of SOCS1, SOCS3, IFNα or IFNβ protein expression were compared following 6340WT or 6340ΔG infection (MOI = 1) of NHBE cells (Fig. 3.2). In the absence of G protein expression, there was a significant ($p < 0.01$) increase in SOCS1 and SOCS3 protein expression at 2h and 6h pi (Fig. 3.2A). SOCS1 and SOCS3 protein expression subsequently decreased between 12h and 18h pi, and again increased at 24h pi to levels similar to 6340WT infection. IFNα and IFNβ were expressed at higher levels following 6340ΔG infection compared to 6340WT infection between 2h to 12h pi, with a significant ($p < 0.05 – 0.001$) increase in protein expression between 6h and 12h pi (Fig. 3.2B).

These results suggest that the RSV G protein has an important role in the temporal regulation of SOCS and type I IFN. The results show that SOCS1 and SOCS3 protein

expression remain high 2h - 6h post-6340WT infection while type I IFN protein expression decreases until 24h pi. Absence of the G protein, while linked to greater SOCS expression, also is associated with increased type I IFN protein expression compared to wild type virus infection. It is possible that the regulatory role of the G protein may be masked in this recombinant virus because in the absence of the G gene, it is possible that more F protein is being produced due to a higher gradient of F gene transcription (12). It may also be possible that although G protein reduces SOCS transcription (Fig. 3.1), it may increase SOCS protein stability as a more rapid SOCS protein response is observed during 6340ΔG infection.

Immediate induction of SOCS mRNA by RSV G and F proteins.

Evidence from the RSV deletion mutant virus studies (Fig. 3.1 and 3.2) suggested that one or more RSV surface proteins modulate SOCS1, SOCS3 and type I IFN expression. To support these findings, NHBE cells were apically treated with purified RSV F or G proteins, or purified F or G proteins blocked with anti-F protein or anti-G protein monoclonal antibodies, respectively (Fig. 3.3). Addition of 10 μg/mL RSV F protein to the apical surface of NHBE cells induced a > 3-fold increase in SOCS3 message relative to mock-treated cells within 15 min of treatment; however, treatment with anti-RSV F protein monoclonal antibody resulted in reduced SOCS3 mRNA expression (Fig. 3.3A), as well as IFNα mRNA expression (Fig. 3.3B). Addition of 10 μg/mL of purified RSV G protein to the apical surface of NHBE cells induced a > 2.5-fold increase in SOCS3 mRNA expression relative to mock-treated cells within 15 min of treatment with little change in IFNα and IFNβ mRNA expression (Fig. 3.3). In contrast, treatment with anti-RSV G protein monoclonal antibody resulted in decreased SOCS3

mRNA expression (Fig. 3.3A), while IFNα mRNA expression increased considerably (> 4-fold). As expected from previously published studies (3, 29, 60), treatment with LPS induced SOCS3 mRNA expression with no change in type I IFN gene expression. These results show that RSV F and G proteins induce SOCS3 mRNA expression by NHBE cells, which is consistent with the findings following 6340ΔG infection (Fig. 3.1).

RSV induces SOCS protein expression without infection.

To determine if SOCS regulation of type I IFN was dependent on RSV infection, NHBE cells were apically treated with UV-inactivated 6340WT (UV-6340WT). As shown in Figure 3.4A, treatment of NHBE cells with inactivated virus induced a 45% increase in SOCS1 protein expression at 6h post-treatment, where expression increased 105% relative to mock-treated cells at 24h post-treatment. SOCS3 protein expression increased 20% within 2h of treatment and increased 50% relative to mock-treated cells at 24h post-treatment. Although UV-inactivated 6340WT induced SOCS protein expression, IFNα and IFNβ protein expression also increased at 6h post-treatment, then decreased between 12h and 18h, and subsequently increased again 24h post-treatment (Fig. 3.4B). These results indicate that RSV can induce SOCS protein expression independent of replication, an effect linked to decreased type I IFN expression.

To dissect the response to UV-inactivated virus treatment, NHBE cells were treated with purified RSV F or G protein in studies similar to those in Figure 3.3. Figure 3.4A shows that from 2h to 24h post-treatment, SOCS1 protein expression increases in NHBE cells treated with purified RSV F protein, while SOCS3 protein expression increases beginning 12h post-treatment and peaks by 24h post-treatment. Paralleling the

changes in SOCS proteins, IFNα and IFNβ protein expression was decreased until 12h post-treatment (Fig. 3.4B). Interestingly, a similar response was observed when NHBE cells were treated with purified RSV G protein. SOCS1 protein expression increased relative to mock-treated cells by 2h post-treatment, and increased 140% by 12h, while SOCS3 protein expression increased at 12h, and peaked 70% relative to mock-treated cells by 18h post-treatment (Fig. 3.4A). RSV G protein treatment resulted in a slight induction of IFNα at all time points tested and a robust IFNβ response by 12h post-treatment which quickly declined by 24h post-treatment. Collectively, these results demonstrate that RSV surface proteins alone can induce SOCS1 and SOCS3 protein expression and suppress the type I IFN response.

RSV F and G proteins modify ISG15 protein expression.

The interferon-induced JAK-STAT pathway leads to the induction of hundreds of interferon-stimulated genes (ISGs) including ISG15 (17, 18, 31, 68). ISG15 contains significant sequence homology to ubiquitin and is a member of the ubiquitin-like (UBL) protein family (54). In addition to being induced by type I IFN, ISG15 is also produced following stimulation by LPS and dsRNA in cells (54). Therefore, the induction of ISG15 is an excellent indicator of type I IFN or TLR4 pathway activation.

ISG15 expression was determined in NHBE cells infected (MOI = 1) with 6340WT or 6340ΔG virus, or treated with UV-inactivated 6340WT virus or purified RSV F or G proteins similar to previous SOCS and type I IFN studies (Figures 3.1-3.4). Based on these findings showing that RSV surface proteins modify SOCS1 and SOCS3 expression, and possibly the type I IFN response, it was expected that ISG15 protein

expression would be decreased in NHBE cells infected with RSV or treated with purified RSV proteins. Figure 3.5A shows that while 6340WT infection inhibited ISG15 expression until 24h pi, 6340ΔG infection resulted in increased ISG15 protein expression early after infection, i.e. from 2h to 6h pi, as well as at 24h pi. Although it is possible that RSV infection may induce type I IFNs via the RIG-I pathway, comparison of Figure 3.5A to Figure 3.2A shows a similar pattern of SOCS1 and SOCS3 expression suggesting that RSV G protein may be interfering with the JAK-STAT pathway early during infection.

Treatment of NHBE cells with UV-6340WT or purified RSV F or G surface proteins resulted in decreased ISG15 protein expression 2h post-treatment (Fig. 3.5B). However, UV-6340WT treatment increased ISG15 protein expression by 6h post-treatment, expression subsequently declined from 12h to 18h, and then increased again at 24h post-treatment. These results closely correlate with IFNα and IFNβ protein expression (Fig. 3.4B), and interestingly as SOCS1 expression increased, type I IFN and ISG15 protein expression decreased (Fig. 3.4 and Fig. 3.5B). Treatment of NHBE cells with RSV F protein induced a steady increase in ISG15 protein expression through the duration of study, and the level of ISG15 protein expression was similar to IFNα protein expression (Fig. 3.4B). Likewise, as IFNβ protein expression increased by 12h post-treatment, ISG15 protein expression also increased by 18h post-treatment. RSV G protein stimulated a similar ISG15 response as RSV F protein treatment where ISG15 protein expression increased by 6h and remained steady through the duration of the study. Taken together, these results indicate that as SOCS proteins accumulate within the cell, type I IFN and downstream ISG15 signaling pathways are inhibited.

SOCS1 and SOCS3 are induced via a TLR signaling pathway.

Because the RSV F protein has previously been shown to activate CD14 and TLR4 and stimulate the TLR-dependent pathway (34) and our results here show that RSV surface proteins modulate SOCS regulation of the type I IFN response (Figs. 3.2-3.5), we evaluated whether this mechanism may be linked to signaling through a TLR signaling pathway. The level of TLR4 expression was determined on NHBE cells that were mock-treated or infected with 6340WT (MOI = 1) (Fig. 3.6A). The results show that TLR4 is expressed on the surface of uninfected and RSV-infected NHBE cells, a finding that strengthens the possibility of RSV F protein interaction with TLR4 as a feature that may induce SOCS protein expression resulting in negative regulation of the type I IFN antiviral response. To further address this possibility, NHBE cells were mock infected or infected with RSV (MOI = 1) and analyzed for interferon regulatory factor 3 (IRF3) activation, a feature that would indicate TLR signaling via the TRIF/TRAM pathway (47, 69). As shown in Figure 3.6B, infection with RSV induced IRF3 hyperphosphorylation that is readily detectable between 8 and 12 hours pi demonstrating that IRF3 was activated in the presence of replicating virus, possibly via the MDA5/RIG-I pathway(s). IRF3 hyperphosphorylation (Fig. 3.6B) was also detected in NHBE cells following treatment with purified RSV F protein indicating that RSV F protein alone can induce PRR signaling. Infection of undifferentiated NHBE cells with 6340WT (MOI = 3) induced nuclear translocation of IRF3 within 1 h of addition of virus (Fig. 3.6C). Strikingly, purified RSV F protein alone induces a similar translocation of IRF3 to the nuclei of NHBE cells in the absence of replicating virus (Fig. 3.6C) indicating that the RSV F surface protein is stimulating a TLR pathway as predicted. This early response is

comparable to NHBE cells stimulated with LPS, a known TLR4 agonist and IRF3 activator (26, 49).

Discussion

Interaction of RSV with airway epithelium may induce a variety of intracellular signaling pathways, which result in the activation of anti-viral mechanisms that include the elaboration of cytokines, chemokines, and induction of intrinsic elements that regulate cell survival. Studies in humans and animal models suggest that the host cell response to RSV infection is influenced by RSV protein expression (6, 42, 67), particularly the cytokine and chemokine response which can affect the magnitude, cadence and outcome of the RSV immune response. In this study, the human respiratory epithelial cell response to RSV infection was investigated using an ALI culture system consisting of primary fully differentiated primary NHBE cell cultures. The ALI culture system emulates human respiratory epithelium as it consists of polarized, ciliated pseudostratified columnar epithelium with goblet cells (33, 38, 48). Importantly, the polarized primary epithelial cells are fully differentiated and provide an apical compartment, which mimics the lumen of the airway, as well as a separate basolateral compartment, which provides nourishment to the culture over time.

RSV F protein has been shown to trigger the innate immune response in mice via interaction with TLR4 (24, 34). RSV infection has also been shown to activate the NF-κB pathway in alveolar cells within 30 - 90 minutes pi or following treatment with UV-inactivated virus through a TLR4-dependent mechanism (22), indicating that NF-κB

signaling is independent of viral replication. The data presented in this study are consistent with previous findings for RSV F protein induction of host cell response genes, and show that RSV F and G proteins affect SOCS negative regulation of the type I IFN response, an attribute associated with cross-talk between the TLR and JAK/STAT pathways. In this study, we use a RSV mutant virus lacking the G gene to show a role for G protein induction of SOCS1 and SOCS3 and negative regulation of the type I IFN response to RSV infection. We also show that purified RSV F or G proteins primarily induce SOCS3 negative regulation of type I IFN response very early after treatment. Despite inducing early SOCS3 message expression, RSV F or G protein treatment is associated with low SOCS1 and SOCS3 protein expression early after treatment. This seemingly contrary finding likely relates to the lag period linked to SOCS protein expression, but may indicate that RSV G protein may have an additional role in stabilizing the SOCS proteins, particularly since SOCS proteins have an extremely short half-life and are only stable within cells for 4-8 hours (21). Studies have shown that SOCS1 phosphorylation by the serine/threonine kinase Pim results in stabilization while tyrosine phosphorylation can decrease the half-life of SOCS3 due to a disruption between SOCS3 and Elongin C (10, 21). We also show additional evidence for RSV F and G protein inhibition of the early immune response by the expression pattern of ISG15 following infection or viral protein stimulation. In one aspect of these studies, treatment of NHBE cells with purified F or G proteins induced SOCS protein expression leading to reduced type I IFN and downstream ISG15 protein expression.

In these studies, we show that the RSV F surface protein can signal via a TLR pathway linked to IRF3 activation. Given the precedent for RSV F protein-TLR4

interaction (24, 34), it is likely that RSV F protein interacts with TLR4 expressed on NHBE cells, an effect that activates the IRF3 signaling pathway leading to SOCS expression. Supported by our studies, it also appears that RSV G protein has features of PRR activation of the SOCS signaling cascade and subsequent negative regulation of type I IFN response. Furthermore, the data from our studies indicate that RSV F and G proteins, like LPS that is a TLR agonist, preferentially induce SOCS3 over SOCS1 expression to negatively regulate IFNα (5).

Several studies have shown that human RSV NS1/NS2 proteins can regulate the type I IFN response during infection (37, 56-58), and studies examining bovine RSV have shown that NS1/NS2 proteins antagonize type I IFN through a mechanism involving IRF3 phosphorylation (9). It is important to note that, although IRF3 is constitutively expressed in many cell types, it is activated and translocated into the nucleus in response to DNA-damaging and stress-inducing agents, as well as to external signals that activate PRR such as LPS (27, 28, 45). In this study, purified RSV F protein alone was shown to activate IRF3 and mediate nuclear translocation (Figure 3.6C), strongly suggesting that these signaling events are mediated through TLR signaling. RIG-I, an intracellular receptor that binds to dsRNA in the cytosol and can induce type I IFN independent of TLR signaling (70) may be associated with signaling following RSV infection; however, RIG-I signaling cannot be the case for the findings using purified RSV F or G proteins, thus emphasizing the likelihood of signaling events mediated through cell surface PRR.

RSV NS1/NS2 antagonism of the type I IFN response (9, 37, 53, 57, 58), although important in facilitating RSV replication, likely occurs downstream of the early type I IFN antagonism mediated by RSV F and G proteins. Following infection of cells,

NS1/NS2 protein synthesis is not detectable until 5 hours pi, and does not accumulate in the cell until 12 hours pi (7, 20, 35). Thus, the early (15 minutes - 2 hours) inhibition of the type I IFN response reported here is not likely mediated by nonstructural proteins. Studies from our laboratory examining murine type II respiratory epithelial cells (MLE-15 cells) infected with wild type or RSV mutant viruses lacking the NS1, NS2 or NS1/NS2 genes show a similar early tempo and pattern of increased SOCS1 and SOCS3 expression associated with decreased type I IFN expression which supports the results presented showing negative regulation of the type I IFN response occurring in the absence of NS1 or NS2 (41). Following RSV infection and endocytosis via a clathrin-mediated pathway (30), it is likely that RSV surface and non-structural proteins interact with other TLRs, including those within early endosomes (25), to facilitate and exacerbate inhibition of the host cell anti-viral response.

In summary, we propose a model (Fig. 3.7) for RSV modulation of the anti-viral response in which RSV F and G proteins interact with PRRs (e.g. TLRs) to induce SOCS negative regulation of the type I IFN response through a mechanism linked to IRF3 activation. The consequence is a reduction, not inhibition, of type I IFN expression. In the model, a low level of type I IFN remains to maintain autocrine and paracrine surface receptor signaling of the JAK/STAT pathway, a feature that further enhances SOCS protein negative regulation of type I IFN expression. The net result is an effect that lowers the anti-viral threshold to facilitate virus replication. Working from this model, it should be possible to develop new disease intervention strategies for RSV that have been previously elusive.

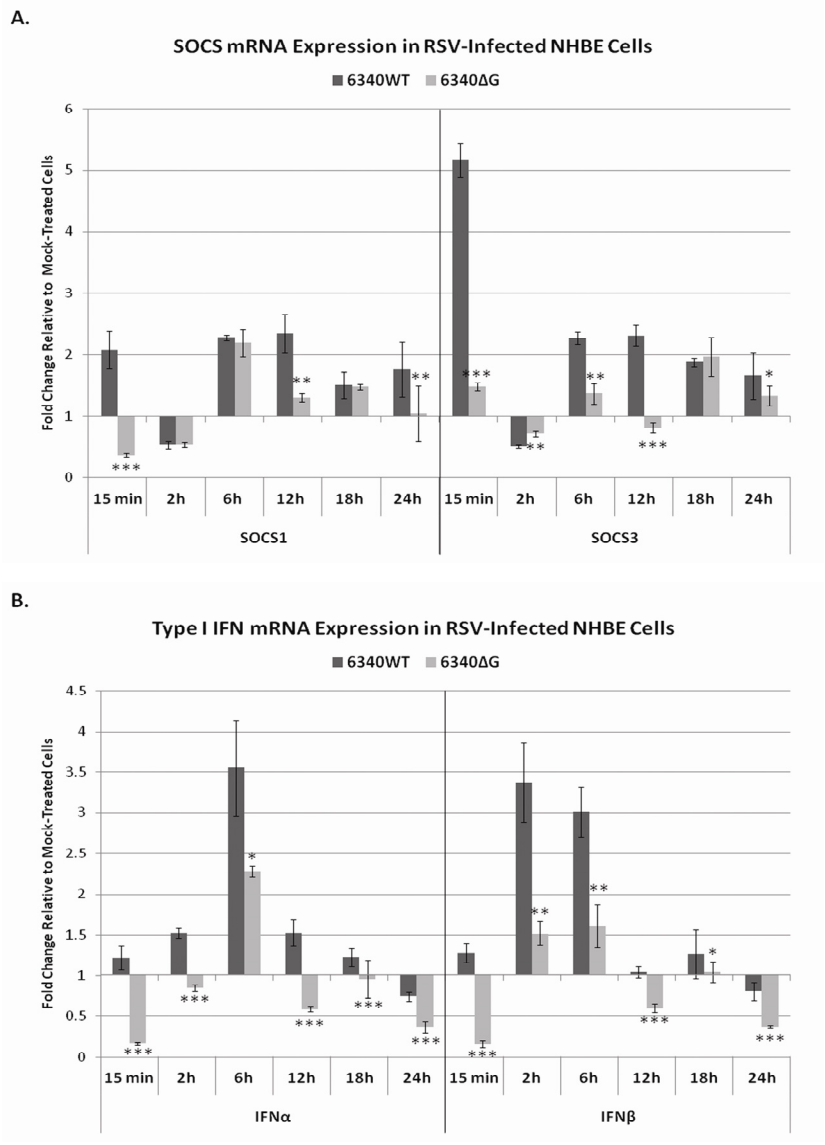


Figure 3.1. RSV G protein induction of SOCS1 and SOCS3 mRNA expression is associated with a concomitant decrease in type I IFN mRNA expression. Normal human bronchial epithelial (NHBE) cells were mock infected or infected with 6340WT or 6340_G at a multiplicity of infection (MOI) of 1. Cells were harvested at the times indicated. SOCS1, SOCS3 (A), and IFNα and IFNβ (B), mRNA expression were measured by comparative quantitative real-time PCR. Transcript levels were first normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) expression and then calibrated to the mock-infected controls. Data is presented as fold-differences in gene expression. Values above 1.0 are considered up regulation, and values below 1.0 are considered down regulation relative to mock-infected NHBE cells. Differences in gene fold expression were evaluated by Student t test and considered significant when the p value was < 0.05 (*), < 0.01 (**) or < 0.001 (***). Data are shown as means with standard deviation (SD).

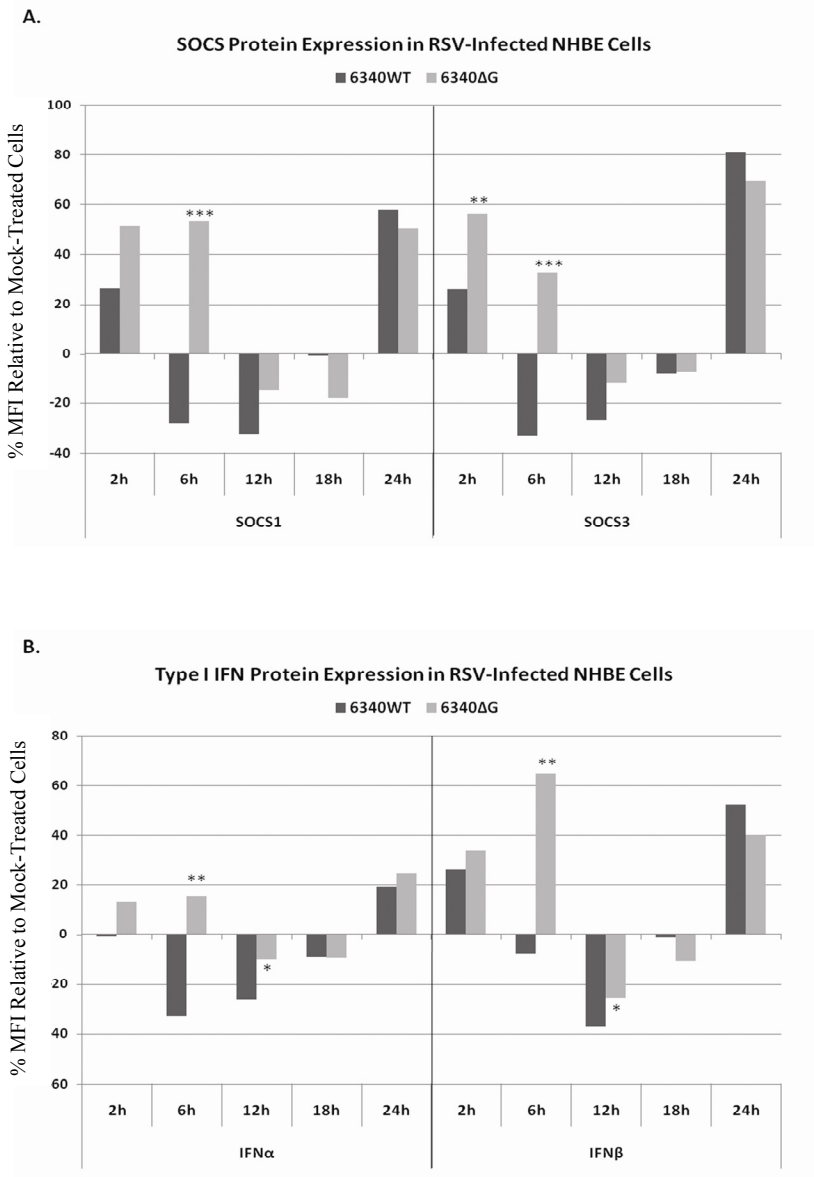


Figure 3.2. RSV G protein modifies the type I IFN anti-viral protein response. Normal human bronchial epithelial (NHBE) cells were mock infected or infected with 6340WT or 6340ΔG at a multiplicity of infection (MOI) of 1. Cells were harvested at the times indicated. SOCS1, SOCS3 (A), and IFNα and IFNβ (B), protein expression were measured by flow cytometric analysis. Experiments were performed in triplicate, and results are shown as percent median fluorescent intensity (MFI) relative to the mock-infected condition. Differences in % of mock control were evaluated by unpaired *t* test and considered significant when the *p* value was < 0.05 (*), < 0.01 (**) or < 0.001 (***).

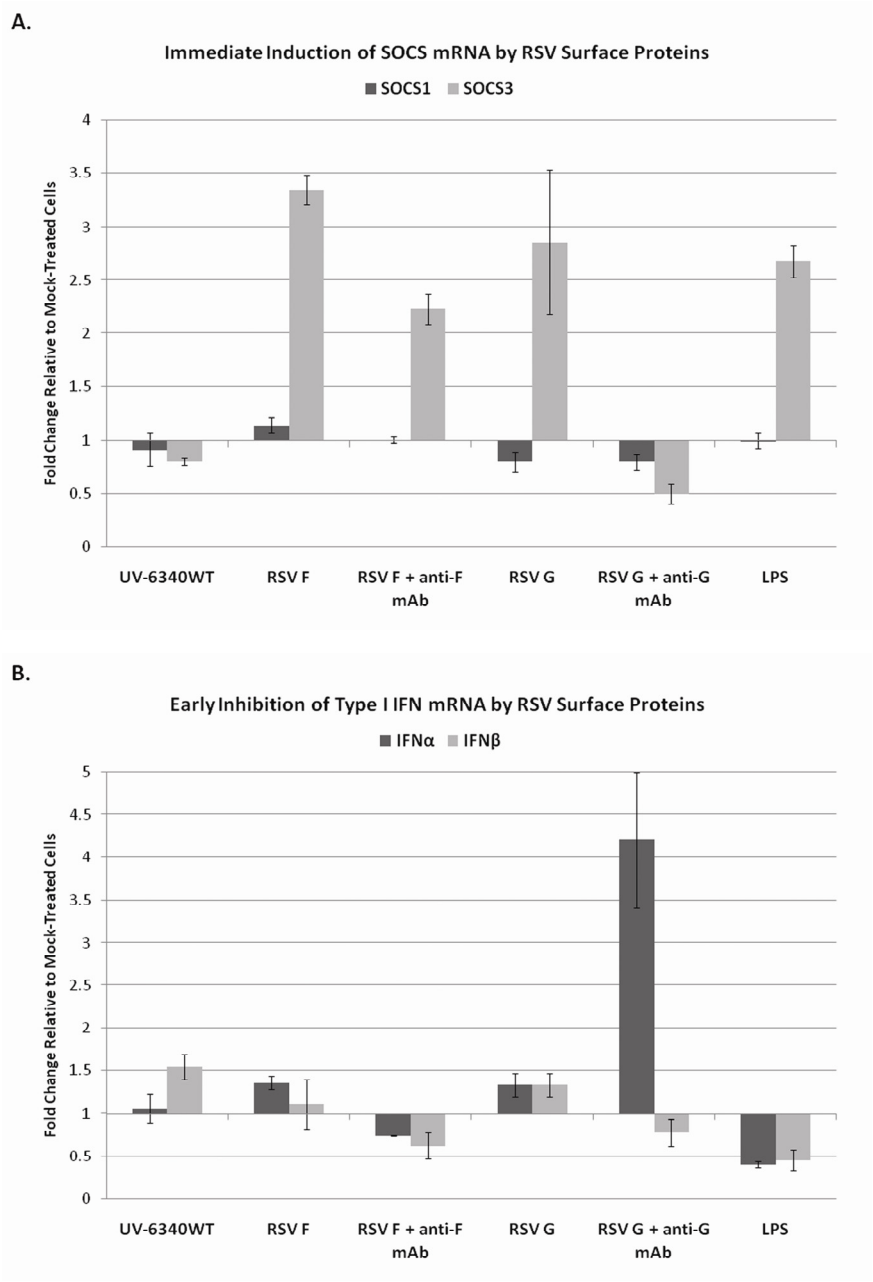


Figure 3.3. RSV G and F proteins stimulate SOCS3 mRNA expression, an effect associated with decreased type I IFN mRNA expression. Normal human bronchial epithelial (NHBE) cells were mock-treated or treated with UV-inactivated 6340WT (MOI = 1), purified RSV G or F proteins (each 10μg/mL), purified RSV G or F proteins pre-incubated with anti-G or anti-F monoclonal antibodies (Ab), respectively, or LPS (1μg/mL). Cells were harvested at 15 minutes post-treatment. SOCS1 and SOCS3 (A) and IFNα and IFNβ (B) message expression were measured by real-time comparative qRT-PCR. Transcript levels were normalized to HPRT expression and calibrated to the mock condition. Data is presented as fold-differences in gene expression relative to mock-stimulated NHBE cells. Values above 1.0 are considered up regulation, and values below 1.0 are considered down regulation.

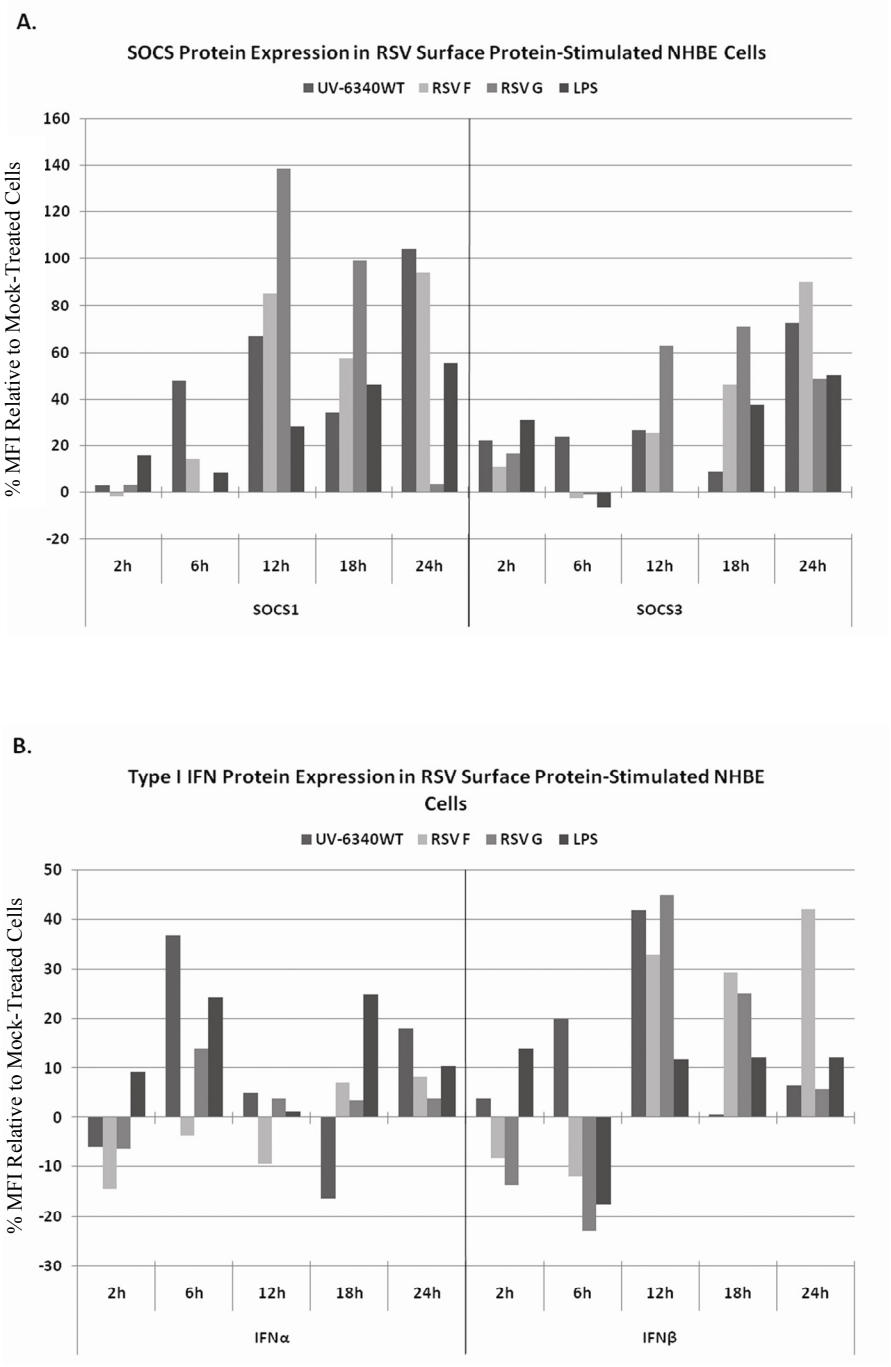


Figure 3.4. RSV surface proteins modify the type I IFN anti-viral protein response. Normal human bronchial epithelial (NHBE) cells were mock-treated or treated with UV-inactivated 6340WT (MOI = 1), purified RSV G or F proteins (each 10μg/mL), or LPS (1μg/mL). Cells were harvested at the times indicated. SOCS1, SOCS3 (A), and IFNα and IFNβ (B), protein expression were measured by flow cytometric analysis. Experiments were performed in triplicate, and results are shown as percent median fluorescent intensity (MFI) relative to the mock-infected condition.

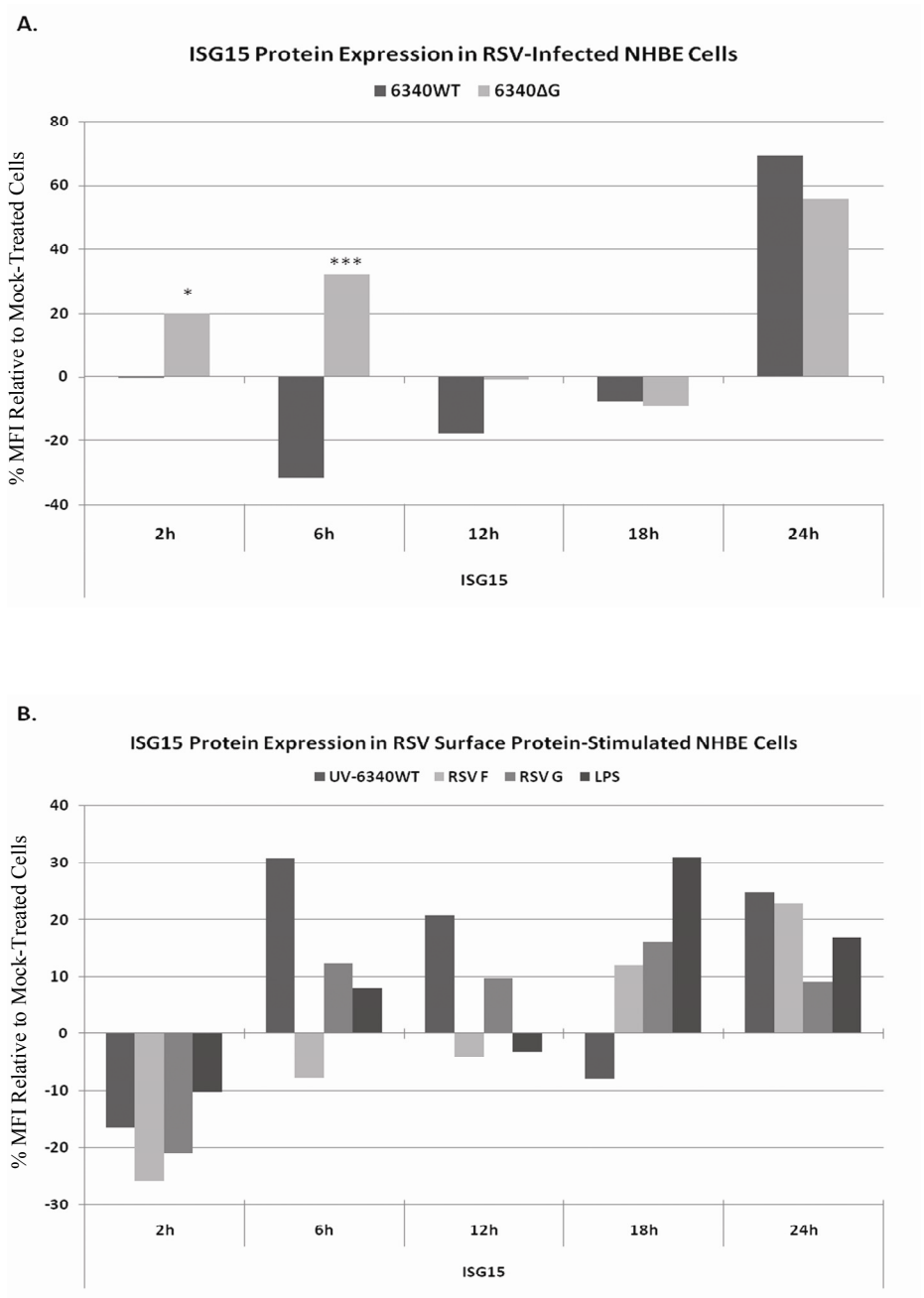
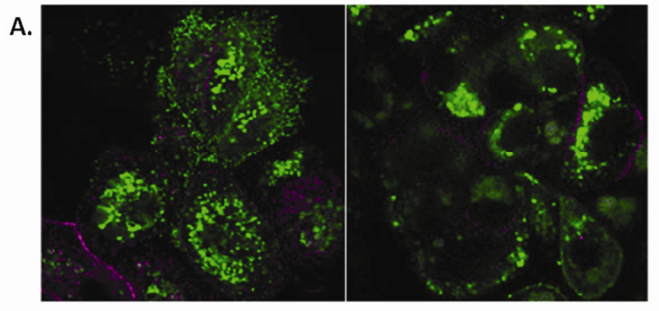


Figure 3.5. RSV surface proteins modify ISG15 protein expression. Normal human bronchial epithelial (NHBE) cells were mock infected or infected with 6340WT or 6340ΔG at a multiplicity of infection (MOI) of 1 (A) or mock-treated or treated with UV-inactivated 6340WT (MOI = 1), purified RSV G or F proteins (each 10μg/mL), or LPS (1μg/mL) (B). Cells were harvested at the times indicated. ISG15 protein expression was measured by flow cytometric analysis. Experiments were performed in triplicate, and results are shown as percent median fluorescent intensity (MFI) relative to the mock-infected condition. Differences in % of mock control were evaluated by unpaired t test and considered significant when the *p* value was < 0.05 (*), < 0.01 (**) or < 0.001 (***).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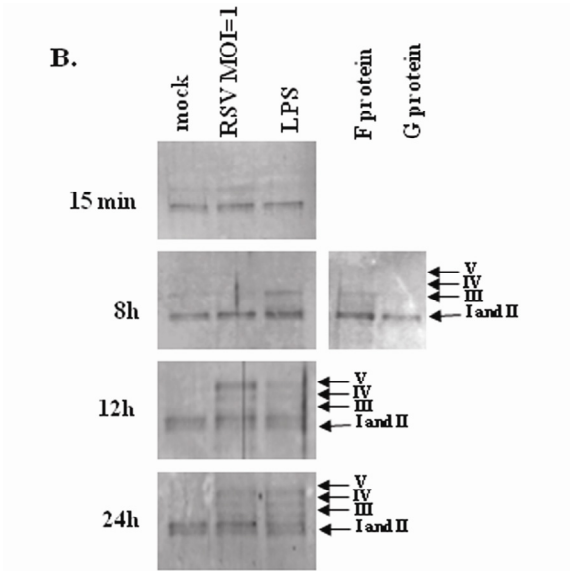
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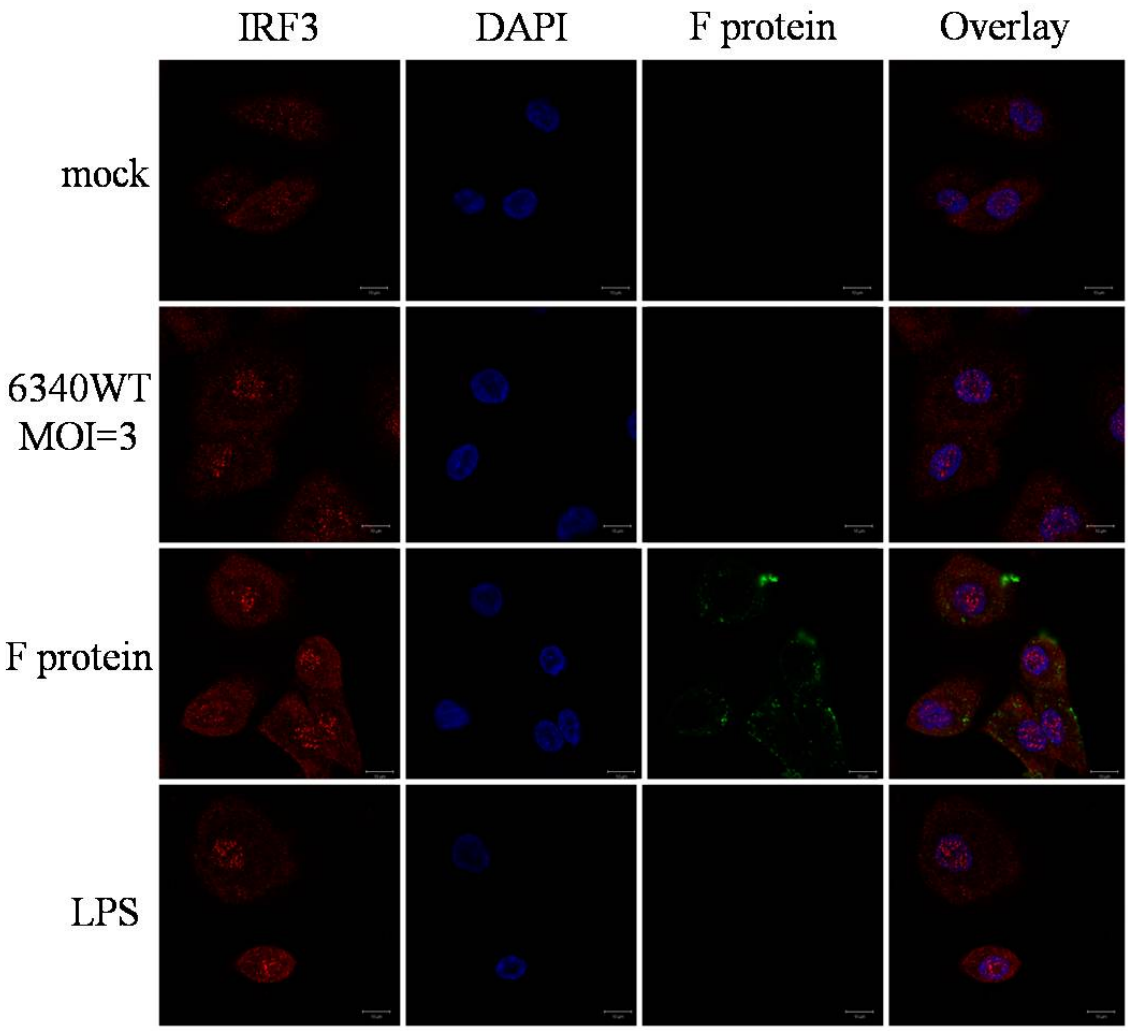

Figure 3.6. Purified RSV F protein and RSV infection induces IRF3 activation and nuclear translocation. (A) NHBE cells were infected with RSV at a MOI = 1. Cells were fixed with 3.7% formaldehyde at day 7 post-infection and stained for TLR4 (AlexaFluor 680, violet) and RSV F protein (AlexaFluor 488, green). Shown are two panels of representative cell areas. (B) NHBE cells were mock infected, infected with RSV at a MOI = 1, stimulated with purified RSV G or F proteins, or LPS for the times indicated. Cells were lysed, the proteins separated by SDS/PAGE, and immunoblotted with anti-IRF3 antibody. Hyperphosphorylation of IRF3 is indicated by the arrows. (C) Representative NHBE cells were infected with 6340WT (MOI = 3), mock-treated, treated with purified RSV F protein (10µg/mL) or treated with LPS (1µg/mL) for 1 hour. Cells were fixed with 3.7% formaldehyde at the times indicated and the nuclei stained (DAPI, blue), or immunostained with anti-IRF3 (Cy5, red) and anti-RSV F protein (AlexaFluor 488, green) antibodies. Magnification, 100x objective.

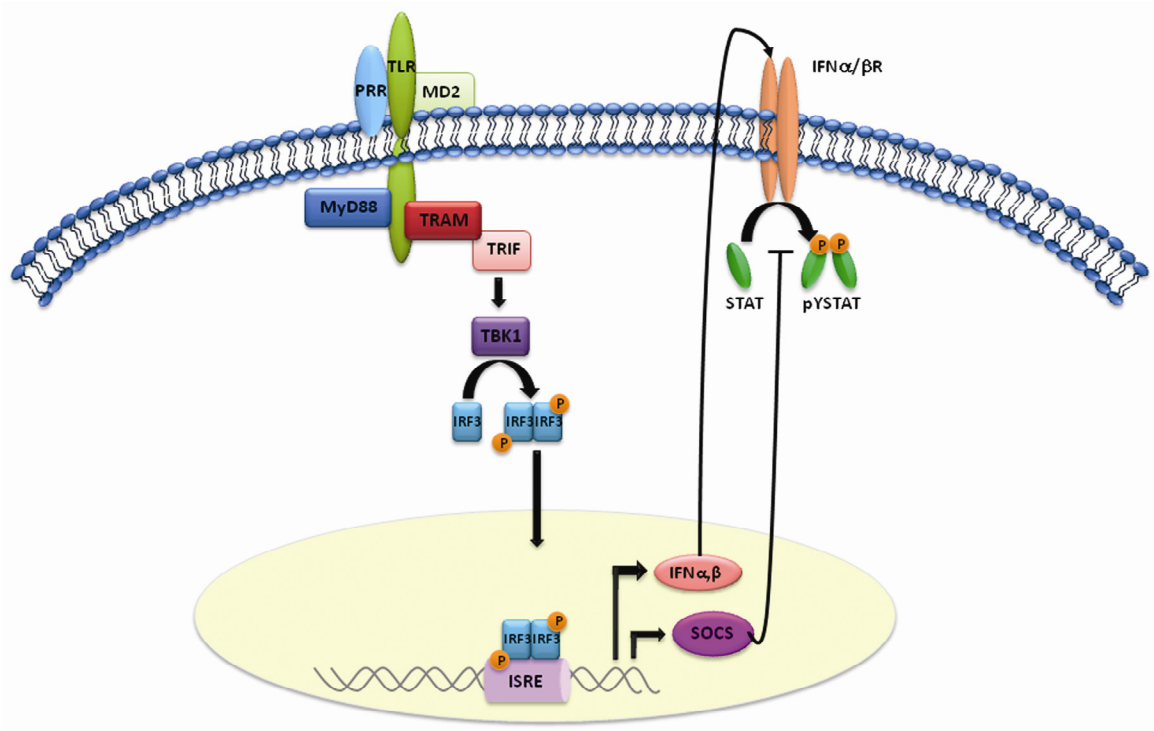

Figure 3.7. Proposed model for RSV modulation of the anti-viral type I IFN response. RSV surface proteins interact with cell surface pattern recognition receptors (PRR), an example being Toll-like receptor (TLR). TLRs mediate recognition of viral structures (pathogen-associated molecular patterns; PAMPs). Upon TLR stimulation, at least some of the receptors dimerism and adaptor molecules are recruited to the receptor complex. Subsequently, signaling molecules among which are TRAM and TRIF, are recruited and activate IRF3 resulting in IRF3 phosphorylation, induction of the ISRE, leading to IFNα/β and SOCS expression. In turn, type I IFNs are secreted and act in an autocrine and paracrine fashion to activate JAK/STAT signaling. SOCS proteins subsequently negatively regulate type I IFN and cytokine expression.

Acknowledgements

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CHAPTER 4

RESPIRATORY SYNCYTIAL VIRUS (RSV) SURFACE GLYCOPROTEINS MODIFY THE CHEMOKINE RESPONSE IN HUMAN BRONCHOEPITHELIAL CELLS[1]

[1]Oshansky, C. M., J. Barber, J. Crabtree, and R. A. Tripp. To be submitted to *Journal of Infectious Diseases.*

Abstract

Human respiratory syncytial virus (RSV) is a ubiquitous virus causing serious lower respiratory tract disease in infants and young children worldwide. Previous studies have shown that RSV modulates cytokine and chemokine expression patterns, and that certain expression profiles may be indicators of disease severity. Here we show that RSV infection of fully differentiated primary human bronchial epithelial (NHBE) cells induce apical and basal secretion of IL-8, IP-10, MCP-1, and RANTES. Purified RSV G (attachment) protein stimulates the secretion of IL-1α, IP-10, and RANTES, while purified F (fusion) protein elicits production of IL-8, IP-10, and RANTES. Moreover, UV-inactivated wild-type RSV treatment of NHBE cells induces apical IL-8, IP-10, and MCP-1 secretion independent of infection, suggesting that RSV proteins alone can modify chemokine responses and affect the early immune response to infection.

Introduction

Human respiratory syncytial virus (RSV) is the most important cause of serious lower respiratory tract disease mediated by virus infection in infants and young children and causes serious illness in the elderly and immune compromised individuals. Annually, 85,000 to 144,000 infants are hospitalized due to severe RSV disease in the United States (46), and of these infections, 20-25% result in pneumonia and 70% result in bronchiolitis (19, 56). Globally, severe RSV disease is estimated at 64 million cases with 160,000 deaths each year (1). RSV is a pneumovirus belonging to the *Paramyxoviridae* family. It is a non-segmented, enveloped, negative-strand RNA virus with two major surface proteins, i.e. the attachment (G) and fusion (F) proteins, and a minor component, the small hydrophobic (SH) protein (13). Currently there is no vaccine available for prevention of RSV infection, and an individual may be repeatedly infected with RSV each season. It is thought that the capacity for RSV to cause repeat infections throughout life may be linked to immune modulation, an effect contributed to by RSV-encoded proteins that include the viral attachment (G) and fusion (F) glycoproteins (20, 23, 28, 29, 39, 50, 52).

RSV infection of host cell types results in the expression and modulation of both cytokine and chemokine expression patterns (49, 55), and it has been suggested that the certain patterns of cytokine or chemokine expression in RSV-infected individuals may be an indicator of disease severity (24). The inappropriate early innate response to RSV infection likely affects the development and magnitude of the adaptive immune response and facilitates virus replication. RSV primarily infects respiratory epithelial cells and as a result induces a cascade of signaling events mediated by nuclear factor (NF)-κB resulting

in expression of many proinflammatory cytokines and chemokines including regulated upon activation, normal T-cell expressed, and secreted (RANTES) or chemokine (C-C motif) ligand (CCL) 5, monocyte chemotactic protein-1 (MCP-1) or CCL2, eotaxin, interleukin (IL)-9, tumor necrosis factor (TNF) α, IL-6, IL-1 and fractalkine (CX3CL1) (5, 17, 18, 21, 31, 33, 41, 60). These chemokines and cytokines can act directly or via autocrine or paracrine feedback mechanisms to regulate the adaptive immune response to infection. Recent reports have indicated that RSV-infected patients display patterns of increased MIP-1α, RANTES and IL-8 (CXCL8) levels within the upper and lower respiratory tract (21), and *in vitro*, RSV infection of BEAS-2B cell (a bronchial epithelial cell line) induces elevated levels of IL-6, IL-8 and RANTES (59). In support of the hypothesis that these chemokines are important in pathogenesis, neutralizing antibody-depletion of RANTES or eotaxin in mice results in a less severe form of RSV disease and eosinophilia (30, 48). Compared to adults, mice infected as neonates display higher illness scores, greater cell recruitment to the lungs and increased IL-4 production, linking immune immaturity to severity of disease (9). Furthermore, adult mice re-infected with RSV developed severe disease associated with Th2-type cytokine expression (9).

RSV proteins have been shown to modify both the magnitude and timing of cytokine and chemokine expression. The RSV G protein contains a conserved CX3C chemokine motif and modifies the recruitment of $CX3CR1^+$ cells including $CD8^+$ T cells and natural killer (NK) cells during infection (20, 52). During the acute phase of RSV infection, the presence of the RSV G protein results in altered CC and CXC chemokine mRNA expression profiles (51), and mixed Th1/Th2-type cytokine responses by bronchoalveolar leukocytes (53). The presence of the G protein during RSV infection

also inhibits early chemokine mRNA expression of MIP-1α, MIP-1β, MIP-2, MCP-1, and interferon-inducible protein of 10kD (IP-10, CXCL10) (51). Furthermore, infection of mice with a mutant RSV lacking the G and SH genes resulted in greater numbers of NK cells in the lungs as well as increased IFNγ and TNFα production suggesting that the G and/or SH surface proteins regulate trafficking of NK cells to the lungs and pro-inflammatory cytokine production (54). BALB/c mice infected with wild type RSV show increased and prolonged production of Th2-type associated monocyte chemotactic protein (MCP)-1 (CCL2), macrophage inflammatory protein (MIP)-1α (CCL3), IP-10 (CXCL10), and eotaxin-1 (CCL11) expression in total-lung RNA (8).

The other major RSV surface protein, i.e. the F protein, also modulates immunity. Infection of human airway epithelial cells with RSV increases TLR4 mRNA, protein expression, and TLR4 membrane localization (35). The RSV F protein has been shown to stimulate the TLR-dependent pathway in monocytes through activation of TLR4 in a CD14-dependent manner (28), and TLR4-deficient mice have been shown to have reduced viral clearance compared to wild-type mice (28). TLR4-deficient mice infected with RSV exhibit impaired pulmonary NK cell and monocyte infiltration, deficient NK cell function, impaired IL-12 expression and impaired virus clearance (23); however the TLR4-null mice also contain a defect in IL-12R and thus have a limited ability to confer IL-12 signaling during acute RSV infection (11).

To better understand the cytokine and chemokine response to infection in human respiratory epithelial cells, we examined RSV infection of primary normal human bronchial epithelial (NHBE) cells. We show that RSV surface proteins, particularly the G

and F proteins, can induce apical and basolateral secretion of IL-1α, IL-8, IP-10, and RANTES early in infection.

Materials and Methods

Cells, viruses and viral proteins

Normal human bronchial epithelial (NHBE) cells (Lonza, Walkersville, MD) were expanded, cryopreserved, and cultured in an air-liquid interface (ALI) system as previously described (27). The apical surface of the cells was exposed to a humidified 95% air/5% CO_2 environment, and the basal medium was changed every two days. Recombinant RSV/A2 (6340WT) and recombinant RSV/A2 lacking the G gene (6340ΔG) were kindly provided by Dr. Peter Collins (NIH, NIAID, Bethesda, MD) (7). Viruses were propagated in VeroE6 cells maintained in DMEM (Sigma-Aldrich Corp., St. Louis, MO, USA.) supplemented with 5% heat-inactivated (60°C) fetal bovine serum (FBS; Hyclone Laboratories, Salt Lake City, UT) as previously described (50). Virus titers were determined by immunostaining plaque assay on VeroE6 cells with anti-RSV F protein monoclonal antibody (clone 131-2A) as previously described (40). 6340WT was UV-inactivated by exposure to ultraviolet light for one hour on ice. Subsequent immunostaining plaque assay (54) confirmed inactivation (data not shown). RSV F and G proteins were isolated and purified from RSV/A2-infected VeroE6 cells as previously described (52). Purification was confirmed by immunoblot with anti-G protein (131-2G) or anti-F protein (131-2A) monoclonal antibody as previously described (52).

NHBE cell infection.

NHBE cells were washed 3 times with PBS to remove excess mucus secretion on the apical surface prior to infection and were apically mock infected with VeroE6 cell lysate (VCL) or infected with 6340WT or 6340ΔG at a multiplicity of infection (MOI) of 1. Viruses were allowed to adsorb for 1h at 37°C, the virus dilutions were removed by aspiration and washed again with PBS 3 times. NHBE cells were apically treated with the following conditions: purified RSV G protein (10 μg/mL), purified RSV F protein (10 μg/mL), or LPS (1 μg/mL; Sigma, St. Louis, MO). Bronchial epithelial basal medium (BEBM) (Lonza) was added to the apical surface of differentiated NHBE cells and incubated for the indicated times post-infection or post-treatment at 37°C. RSV and mutant virus infection of NHBE cells was confirmed by immunostaining, immunoblot and qRT-PCR for RSV N gene expression as previously reported (54).

Luminex-based detection of cytokines and chemokines

MILLIPLEX MAP human cytokine/chemokine immunoassay (Millipore, St. Charles, MO) was used for the detection of secreted cytokines and chemokines from NHBE cell apical and basal cell-free supernatants using the Luminex® xMAP™ system according to the manufacturer protocol. Briefly, beads coupled with biotinylated anti-IL-1α, anti-IL-8, anti-MCP-1, anti-MIP1α, anti-MIP1β, anti-IP-10, anti-RANTES mAbs were sonicated, mixed and diluted in bead diluent. For the assay, beads were diluted 1:4 in bead diluent and incubated overnight at 4°C with NHBE apical or basolateral supernatant. After washing, beads were incubated with streptavidin-phycoerythrin for 1 hour at room temperature, washed, and resuspended in wash buffer. The assay was

analyzed on a Luminex 200 instrument (Luminex Corporation, Austin, TX) using Luminex xPONENT 3.1 software. Additional analysis was performed using MILLIPLEX Analyst (Millipore).

Results and Discussion

Accumulating evidence suggests that RSV infection induces the secretion of several chemokines including IL-8 (CXCL8), which attracts neutrophils, and RANTES (CCL5), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), and IP-10 (CXCL10), which attract monocytes and leukocytes to sites of inflammation (2-4, 32, 42). RSV-mediated bronchiolitis and inflammation are associated with high levels of MCP-1 and MIP-1α in nasal secretions (57), and the immune response to infection contributes to RSV disease. Important to immune pathogenesis are T cells which produce many cytokines that direct the host immune response to Th1-, Th2-, or mixed Th1/Th2-type responses, and T cells are sensitive to local and systemic chemokine levels used to recruit them to sites of infection and inflammation. The RSV G protein has been shown to modify various aspects of the host immune response to infection, and G protein CX3C chemokine mimicry linked to G protein interaction with the fractalkine receptor (CX3CR1) (52) contributes to immune modulation of $CX3CR1^{+}$ immune cells that include NK and virus-specific T cells (20). This interaction leads to a reduction in the overall antiviral T cell response to RSV infection in mice, and inhibition of NK cell trafficking and chemokine transcript expression by pulmonary leukocytes (20, 51). Studies examining the role of the G protein and G protein CX3C motif in enhanced pulmonary disease showed that

formalin-inactivated RSV (FI-RSV) mutant virus lacking the G protein or only the G CX3C motif did not prime for enhanced disease following RSV challenge, and had modified chemokine transcription profiles in bronchoalveolar lavage (BAL)-derived cells with increased expression of MIP-1α, MIP-1β, and MIP-2 mRNAs by 8 hours post-infection (22).

Because RSV has been shown to modify the immune response, in particular the induction of suppressor of cytokine signaling (SOCS) proteins (36, 61), it is likely that RSV surface proteins can modify chemokine expression in lung epithelial cells. As the apical or basolateral chemokine induction in human bronchial epithelial cells has not been investigated, we examined chemokine expression levels from fully differentiated primary NHBE cells following RSV infection or treatment with purified RSV F or G proteins. Contrary to previous studies that used A549 cells (6, 44), a type II alveolar cell, there was no increase in IL-1α secretion in NHBE cells during live RSV infection (6340WT) or with a mutant virus lacking the G gene (6340ΔG) (Figure 4.1). However, NHBE cells treated apically with purified RSV G protein secreted up to 676 pg/mL of IL-1α from the apical surface (Figure 4.1A) and 120 pg/mL of IL-1α from the basolateral surface (Figure 4.1B) by 18h post-treatment. In contrast, little to no IL-1α was secreted in response to medium alone. These findings indicate that during RSV infection, G protein induces IL-1α expression in human airway cells likely leading to enhanced levels of endothelial cellular adhesion molecules, a feature that might contribute to immune pathogenesis by enhancing immune cell adherence to infected airway cells.

Several chemokine receptors been shown to be involved in the host response to RSV infection, particularly CCR1 and CXCR1. The ligands of CCR1 include MIP-1α

(CCL3), RANTES (CCL5) and MCP-3 (CCL7) (10, 12, 25, 34, 37, 38), and an important ligand for CXCR1 is IL-8 (26, 43, 45). RSV infection of NHBE cells was associated with increased levels of apical IL-8 secretion throughout the duration of the experiment (Figure 4.2A), but only a slight increase in basal IL-8 secretion was observed relative to mock-infected cells (Figure 4.2B). Infection of NHBE cells with 6340ΔG resulted in similar, but lower apical IL-8 levels between 18h and 24h p.i. Interestingly, UV-inactivated 6340WT-treatment of NHBE cells led to substantial (1.2×10^5 pg/mL) apical secretion of IL-8 by 2h of treatment, and IL-8 levels were similar to live infection between 6h and 24h pi (Figure 4.2A). UV-inactivation was confirmed by immunostaining plaque assay (results not shown). In contrast to IL-1α induction, RSV surface proteins did not induce substantial apical IL-8 secretion; however, RSV F protein treatment induced greater basolateral IL-8 secretion by 24h post-treatment relative to mock-treated cells (Figure 4.2B).

IP-10 appears to be an important chemokine in the response to RSV infection. Nasopharyngeal aspirates from children less than 2 years of age showed increased levels of IP-10, as well as IL-8, MIP-1α, and MIP-1β during severe RSV infection (4). In addition, RSV infection in mice leads to increased expression of IP-10 (CXCL10) mRNA in total lung RNA (8). In these studies, 6340WT infection of NHBE cells induced IP-10 secretion both apically and basolaterally (Figure 4.3), however data from NHBE cells infected with 6340ΔG suggest that G protein inhibits IP-10 expression between 18h and 24h pi. Consistent with IL-8 secretion induced by infection (Figure 4.2), IP-10 is highly expressed following UV-inactivated 6340WT treatment (Figure 4.3) indicating that UV-inactivation may elicit an alternate route of chemokine stimulation compared to live virus

infection. Purified RSV F or G protein treatment elicited low levels of apical and basolateral IP-10 secretion relative to mock-treated NHBE cells over the course of treatment (Figure 4.3).

While increased MCP-1 levels in nasal secretions are associated with bronchiolitis and inflammation (57), RSV infection of human airway cells has been shown to mediate little MCP-1 secretion, but high levels of RANTES in cell supernatants (2). Contrary to these findings, 6340WT and 6340ΔG infection of NHBE cells resulted in substantially high levels of apical secretion (6×10^4 pg/mL) of MCP-1 that remained high from 2h to 24h post-infection (Figure 4.4A). Furthermore, UV-inactivated 6340WT treatment of NHBE cells also elicited MCP-1 apical secretion to a level similar to that induced by live virus, but treatment with purified RSV F or G proteins had no effect on MCP-1 secretion relative to mock-treated cells (Figure 4.4A and B). These results indicate that the RSV G protein, and likely that the F protein as well, may not be primary mediators of apically-expressed MCP-1. In contrast, basolateral secretion of MCP-1 was substantially higher in 6340ΔG infected NHBE supernatant compared to 6340WT-infected NHBE cell supernatant indicating that the G protein has a role in modifying MCP-1 basal expression (Figure 4.4B).

RANTES (CCL5) is an important chemokine that functions in recruitment of monocytes, T cells and eosinophils to areas of inflammation via chemokine receptors CCR1, CCR3 and CCR5 (15, 16, 58). Increased RANTES and MIP-1α levels have been observed in nasopharyngeal and tracheal secretions, and these levels positively correlated with RSV disease severity (14, 47, 48). RSV infection of NHBE cells induced high levels of RANTES apical secretion (Figure 4.5A), but substantially lower RANTES secretion

from the basolateral surface (Figure 4.5B). RSV F and G both induce RANTES secretion from the apical surface; however the G protein induces greater RANTES production from both apical and basal surfaces (Figure 4.5). These results are somewhat in contrast the results from an earlier study which showed that treatment of HEp-2 cells with a recombinant RANTES (CCL5) inhibited RSV infection, and this inhibition was related to presence of the RSV F protein (12).

In conclusion, RSV surface proteins have a role in the induction of chemokines shown to be important in immune pathogenesis during severe RSV disease. We show for the first time that RSV infection and treatment with purified RSV proteins induces differential chemokine expression from the apical and basolateral NHBE monolayer surfaces, and it remains to be determined which viral and host factors are involved in this process.

Acknowledgments

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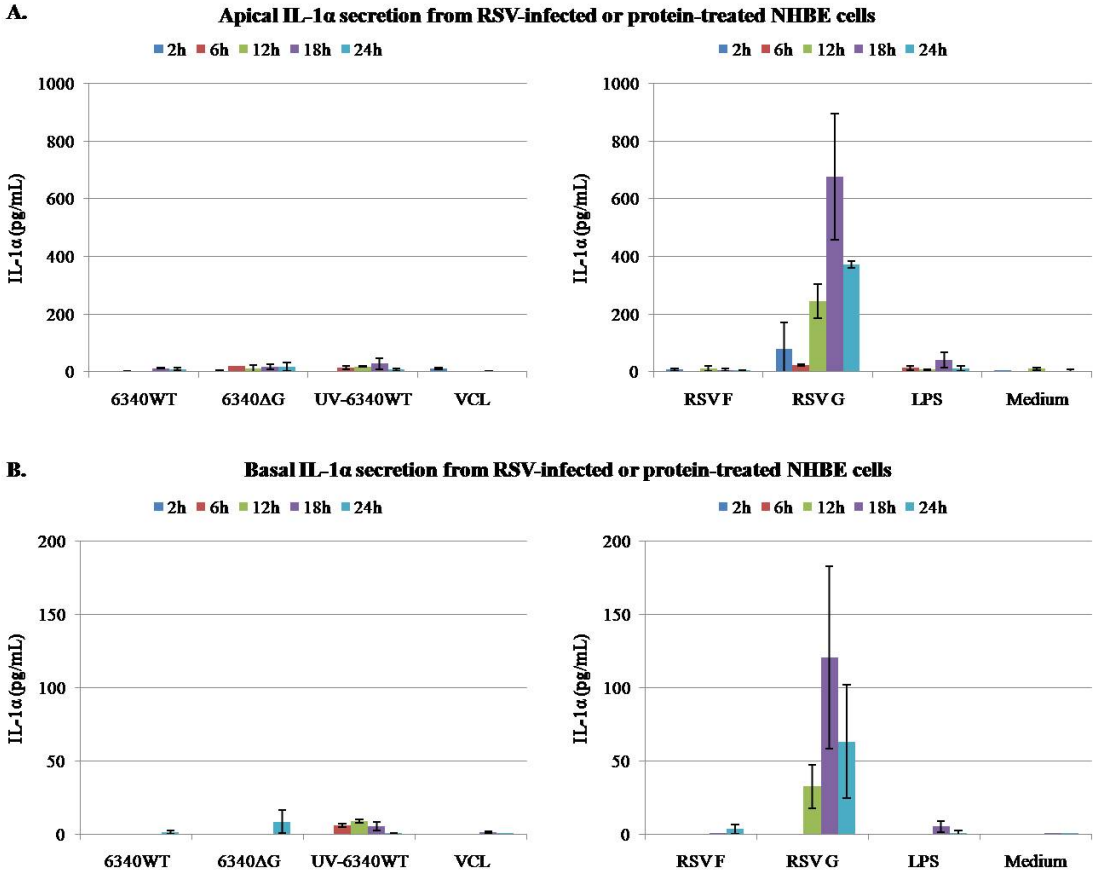

Figure 4.1. The RSV G protein induces apical and basolateral secretion of IL-1α from NHBE cells. NHBE cells were apically mock infected with Vero cell lysate (VCL), UV-inactivated 6340WT, or infected with 6340WT or 6340ΔG at MOI = 1 (A and B; left). NHBE cells were apically treated with purified RSV F or G proteins (10 μg/mL) or LPS (1 μg/mL) (A and B; right). At the indicated times post-treatment or post-infection, apical supernatants (A) or basal media (B) were analyzed for the presence of IL-1α.

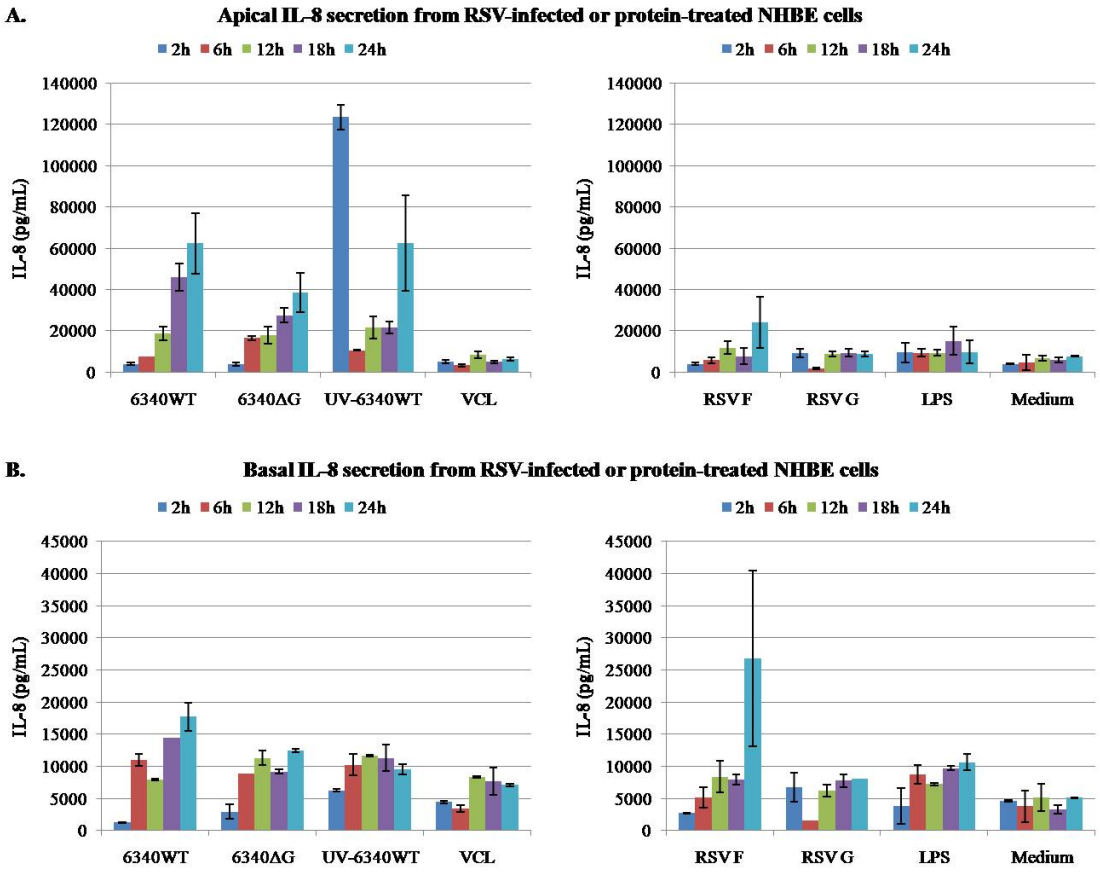

Figure 4.2. The RSV F protein can induce IL-8 secretion from NHBE cells. NHBE cells were apically mock infected with Vero cell lysate (VCL), UV-inactivated 6340WT, or infected with 6340WT or 6340ΔG at MOI = 1 (A and B; left). NHBE cells were apically treated with purified RSV F or G proteins (10 μg/mL) or LPS (1 μg/mL) (A and B; right). At the indicated times post-treatment or post-infection, apical supernatants (A) or basal media (B) were analyzed for the presence of IL-8.

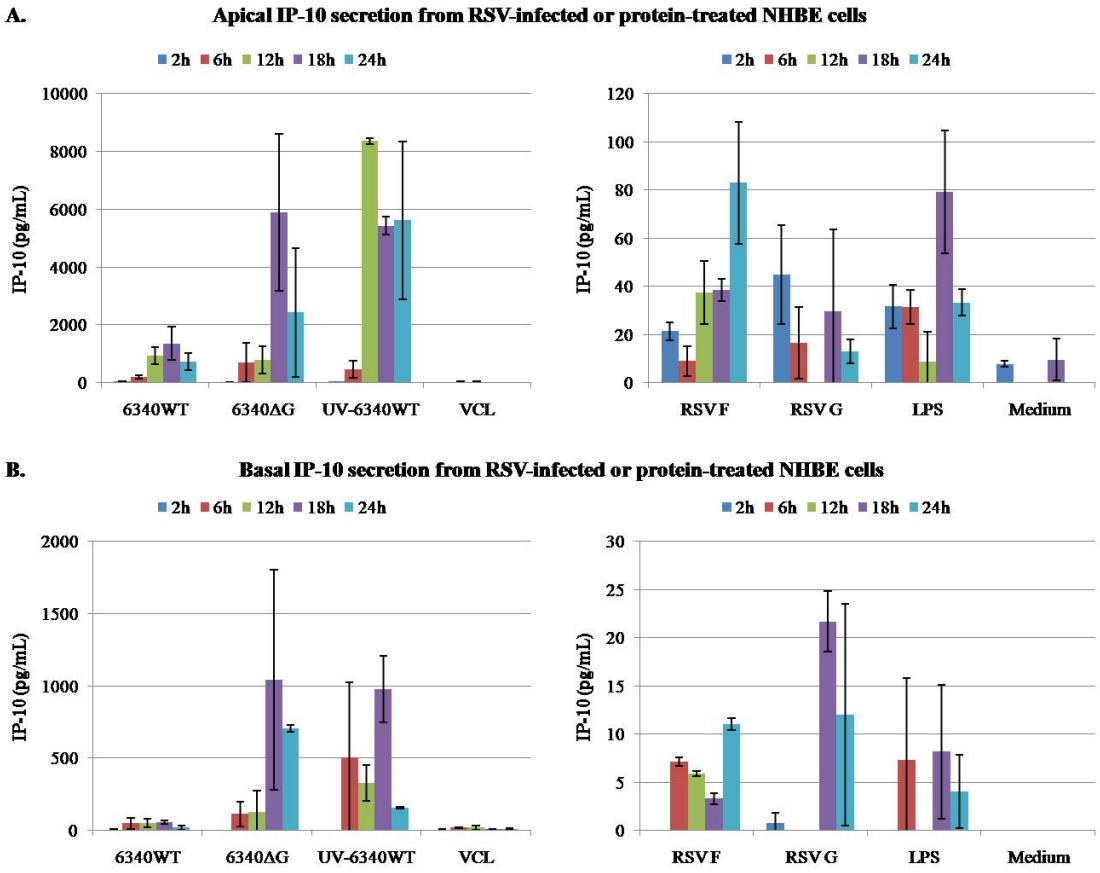

Figure 4.3. The RSV F protein can induce IP-10 secretion from NHBE cells. NHBE cells were apically mock infected with Vero cell lysate (VCL), UV-inactivated 6340WT, or infected with 6340WT or 6340ΔG at MOI = 1 (A and B; left). NHBE cells were apically treated with purified RSV F or G proteins (10 μg/mL) or LPS (1 μg/mL) (A and B; right). At the indicated times post-treatment or post-infection, apical supernatants (A) or basal media (B) were analyzed for the presence of IP-10.

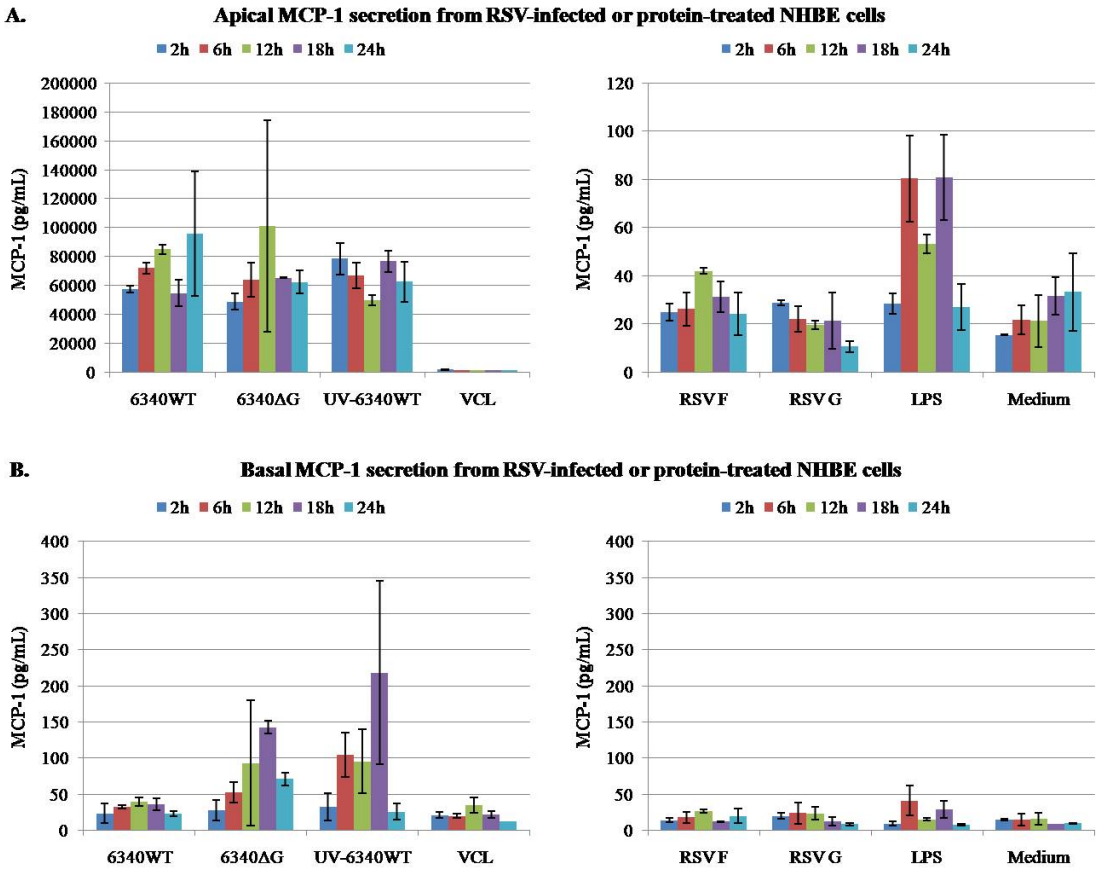

Figure 4.4. UV-inactivated RSV treatment of NHBE cells induces MCP-1 secretion independent of surface F or G proteins. NHBE cells were apically mock infected with Vero cell lysate (VCL), UV-inactivated 6340WT, or infected with 6340WT or 6340ΔG at MOI = 1 (A and B; left). NHBE cells were apically treated with purified RSV F or G proteins (10 μg/mL) or LPS (1 μg/mL) (A and B; right). At the indicated times post-treatment or post-infection, apical supernatants (A) or basal media (B) were analyzed for the presence of MCP-1.

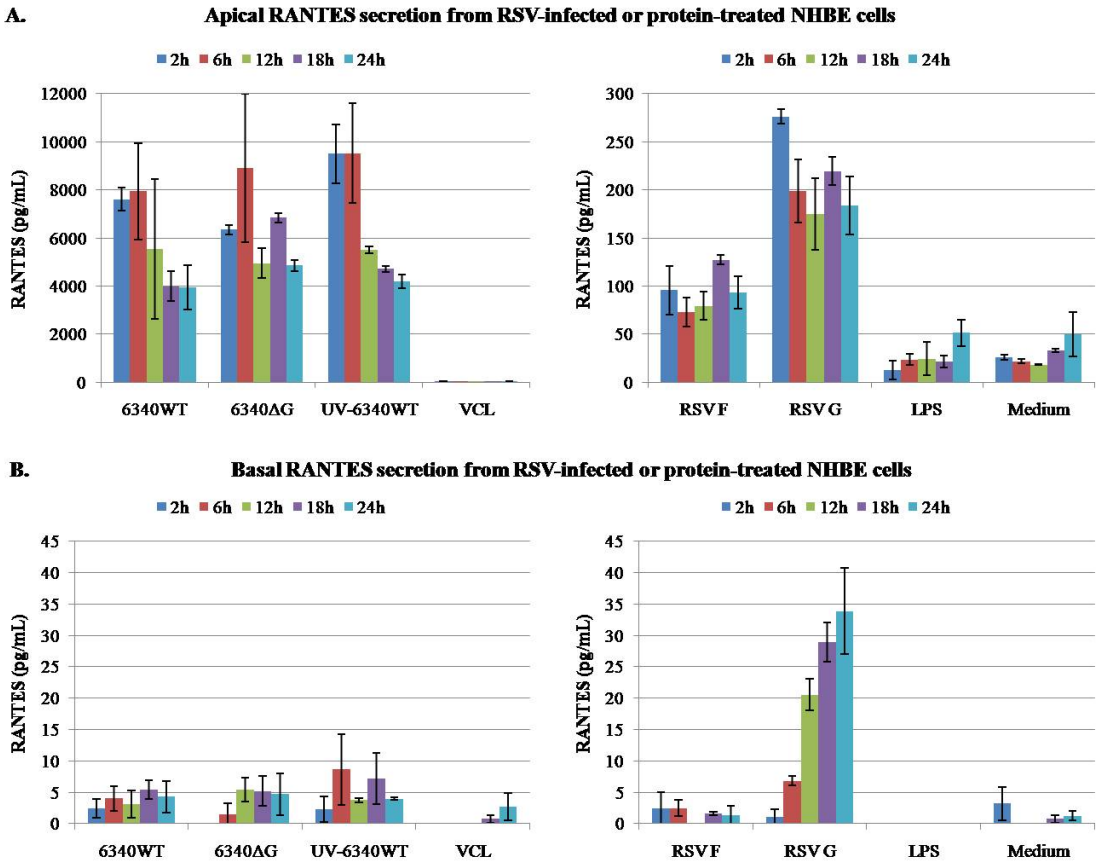

Figure 4.5. RSV G and F proteins induce apical RANTES secretion, but RSV G protein alone induces basolateral RANTES secretion. NHBE cells were apically mock infected with Vero cell lysate (VCL), UV-inactivated 6340WT, or infected with 6340WT or 6340ΔG at MOI = 1 (A and B; left). NHBE cells were apically treated with purified RSV F or G proteins (10 μg/mL) or LPS (1 μg/mL) (A and B; right). At the indicated times post-treatment or post-infection, apical supernatants (A) or basal media (B) were analyzed for the presence of RANTES.

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CHAPTER 5

AVIAN INFLUENZA VIRUS INFECTION OF HUMAN BRONCHIAL EPITHELIAL CELLS IS INDEPENDENT OF SIALIC ACID RECEPTOR EXPRESSION AND MEDIATES A DISTINCTIVE CYTOKINE AND CHEMOKINE RESPONSE

[1]Oshansky, C. M., L. P. Jones, G. M. Saavedra Ebner, J. Barber, J. Crabtree, S. M. Tompkins and R. A. Tripp. To be submitted to *Journal of Experimental Medicine*.

Abstract

Avian influenza A viruses (AIV) are recognized as an important emerging threat to public health. Recently, highly pathogenic avian H5N1 influenza crossed the zoonotic barrier and caused rare but fatal disease in some humans, but fortunately has yet to acquire the ability for efficient human-to-human transmission. The binding preference of AIV and human influenza viruses for α2,3 versus α2,6 sialic acid moieties, respectively, on the cell surface has been proposed as a barrier in cross-species transmission. In this study we show using a fully differentiated, primary normal human bronchial epithelial (NHBE) cell model that low pathogenic H5N1, H5N2 and H5N3 AIV readily infect and replicate in NHBE cells independent of sialic acids present on the cell surface. NHBE cells treated with neuraminidase prior to infection are readily infected despite the loss of conventional sialic acid moieties. These results provide an important shift in view of the requirements for AIV infection of human airway cells.

Introduction

Influenza A viruses are important pathogens that present a significant threat to public health, veterinary health, and can cause an extensive economic burden, particularly avian influenza virus (AIV) infection in poultry. Influenza viruses are segmented, enveloped, negative-strand RNA viruses belonging to the *Orthomyxoviridae* family. They comprise a diverse array of subtypes due to their propensity to change antigenic profiles and are subtyped based on the antigenic properties of 2 surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). There are currently 16 known HAs and 9 known types of NA. Seasonal epidemics cause more than 200,000 hospitalizations and more than 41,000 deaths each year in the United States alone (10). Three novel influenza viruses caused pandemics in 1918, 1957 and 1968, respectively. The 1918 influenza pandemic was the most severe resulting in unusually high mortality among healthy young adults (28). It remains unclear the precise features that contributed to the high rate of mortality due to infection with the 1918 influenza virus, but it has been shown that a single mutation in the PB1-F2 genome of 1918 influenza A viruses (and recently recognized as well for highly H5N1 avian influenza) contributed to increased virulence (2, 6, 22). Moreover, since 2003, there has been an increased incidence of highly pathogenic avian influenza (HPAI) virus outbreaks in poultry, and recently HPAI H5N1 has crossed species barriers to infect >400 humans resulting in a >60% fatality rate (258 deaths) as of May 2009 (41).

It is believed that sialic acid receptors for influenza viruses are important barriers in cross-species transmission. Sialic acids are nine-carbon monosaccharides found at the ends of glycan chains. Sialic acids coat many host cell surfaces and secreted proteins (1,

25, 38, 39). The most common sialic acids found in mammals are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Sialic acids are transferred to terminal sugars of glycoproteins and glycolipids by sialyltransferases, and can be added to the galactose carbon-6 forming an α2,6 linkage or to galactose carbon-3 forming an α2,3 linkage (1, 32, 39). The detection of α2,3 or α2,6 linkages can be readily determined by use of plant lectins that bind to glycolipids and glycoproteins containing sialic acids in α2,6 or α2,3 configuration. For instance, a lectin from the seed of *Maackia amurensis* tree (MAA) is specific for sialic acid α2,3, while a lectin obtained from the elderberry plant *Sambucus nigra* (SNA) is specific for sialic acid α2,6 (26, 40).

Influenza HA binds to host cell sialic acid residues coating the host cell surface (30) and mediates viral entry via its receptor binding domain. Influenza HA has a strong preference to bind to certain sialic acid moieties, and this is believed to be an important host range determinant (32-35). Human influenza viruses preferentially bind α2,6 linkages, while AIV preferentially bind α2,3 linkages highly expressed in the gastrointestinal tracts of aquatic birds (7, 15, 20, 27). Early experiments showed that SNA preferentially bound to the surface of ciliated tracheal epithelial cells indicating the presence of sialic acid α2,6, and MAA bound goblet cells indicating the presence of sialic acid α2,3 (3). These studies suggested that ciliated cells, but not goblet cells, were a primary target for human H3 influenza infection and were subsequently confirmed by using a fluorescently-labeled H3 virus which primarily infected ciliated cells (8). The AIV outbreak that occurred in 1997 in Hong Kong in humans was caused by a H5N1 strain (A/Hong Kong/156/1997) in which all eight genes were of avian origin and therefore preferentially bound to sialic acid α2,3. The currently circulating H5N1 virus

has primarily afflicted birds and fowl and has maintained its sialic acid α2,3 binding preference. However, influenza pandemics have resulted after AIV have acquired mutations changing their HA binding specificity from avian-like, α2,3, to human-like, α2,6 (7, 13, 20), thus a HA mutation in H5N1 allowing for a change in receptor binding specificity could allow for efficient human-to-human transmission (20).

In these studies, we determined if low pathogenic H5N1, H5N2 and H5N3 AIV could bind to, infect, and replicate in fully differentiated, normal human bronchial epithelial (NHBE) cells. We demonstrate that these viruses readily infect, replicate, and are apically released from NHBE cells independent of sialic acids present on the cell surface. Further, we show that low pathogenic AIV can still infect and replicate in NHBE cells that are sialidase-treated prior to infection.

Materials and Methods

Cells and viruses

Normal human bronchial epithelial (NHBE) cells (Lonza, Walkersville, MD) were expanded, cryopreserved, and cultured in an air-liquid interface system as previously described (18). The apical surface of the cells was exposed to a humidified 95% air / 5% CO_2 environment, and the basal medium was changed every two days.

The low pathogenic AIV (LPAI) strains A/mute swan/MI/451072-2/2006 (H5N1), A/chicken/Pennsylvania/13609/1993 (H5N2), and A/chicken/TX/167280-4/02 (H5N3) were kindly provided by Dr. David Suarez, USDA-Southeast Poultry Research Laboratory,

Athens, GA. A/New York/55/2004 (H3N2) was kindly provided by Dr. Richard Webby, St. Jude Children's Research Hospital, Memphis, TN. Stock viruses were prepared by inoculating 9-day old specific pathogen-free (SPF) eggs and harvesting the allantoic fluid 48h post-inoculation. Viral titers were obtained by serial dilution on Madin-Darby canine kidney (MDCK) epithelial cells in the presence of 1 μg/ml trypsin (Sigma), and 50% egg infectious doses (EID_{50}) were performed in 9-day old SPF chicken embryos and calculated according to the method of Reed and Muench (24).

Sequencing of influenza hemagglutinin and neuraminidase genes

The RNeasy Kit (Qiagen, Valencia, CA) was used to extract RNA, and the One-step RT-PCR Kit (Qiagen) was employed to amplify the HA and NA gene segments for direct sequencing of PCR products using gene segment-specific amplification primers (Table 5.1). Full-length amplicons were subjected to purification by agarose gel electrophoresis for cycle sequencing. Cycle sequencing reactions were carried out using an ABI 9700 thermocycler and optimized to produce the maximal length of read while economizing the use of BigDye reagent (Applied Biosystems Inc., Foster City, CA). The resulting 10μl cycle sequencing reaction was comprised of: 2μl template, 1μl ABI BigDye v3.1, 1μl (1pmole) sequencing primer, 2μl ABI 5X sequencing buffer, 4μl distilled water. Each amplicon was subjected to cycle sequencing reactions using both the forward and reverse amplifying primers. Internal primers were employed to fill in gaps and generate sequence at the 5' and 3' termini of each amplicon (Table 5.2). This scheme resulted in at least two reads for each nucleotide of the sequence. Cycle sequencing reactions were purified using Cleanseq reagent (Agencourt, Beverly, MA) and eluted in 40μl of 0.1mM EDTA. Purified cycle sequencing products were loaded onto an ABI

3130XL genetic analyzer and separated by capillary electrophoresis through an 80cm capillary array. The resulting sequence traces were trimmed and assembled using Sequencher software (Genecodes, Ann Arbor, MI).

Table 5.1 Gene Segment Amplification Primers

Gene	Primer	Sequence (5'→3')
NA	*N1 and N2 viruses*	
	Bm-NA-1	TATTCGTCTCAGGGAGCAAAAGCAGGAGT
	Bm-NA-1413R	ATATCGTCTCGTATTAGTAGAAACAAGGAGTTTTTT
	N3 virus	
	Bm-N3-1	TATTCGTCTCAGGGAGCAAAAGCAGGTGC
	Bm-N3-1420R	ATATCGTCTCGTATTAGTAGAAACAAGGTGCTTTTT
HA		
	UGAHA-F	AGCAAAAGCAGGGGTCYAWACTATSAAA
	UGAHA-R	AGTAGAAACAAGGGTGTTTTTAATTATAATCTG

Viral infection of NHBE cells

Human and LPAI viruses were diluted in BEBM (Lonza) to equal titers, as determined by MDCK plaque assay. NHBE cells were washed 3 times with PBS to remove excess mucus secretion on the apical surface prior to infection. Viruses were allowed to adsorb for 1h at 37°C, the virus dilutions were removed by aspiration and washed again with PBS 3 times. NHBE cells were incubated for the indicated times p.i. at 37°C. Viruses released apically were harvested by the apical addition and collection of 300 µl of 0.05% BSA-BEBM allowed to equilibrate at 37°C for 30 min. Samples were stored at –80°C until assayed.

Sialidase Treatment and Influenza Infection of NHBE Cells

To remove sialic acid moieties from the cell surfaces or to confirm the specificity of lectin binding, NHBE cells were apically treated with 25 mU/mL of neuraminidase from *Clostridium perfringens* (Sigma, St. Louis, MO) for 1 hour at 37°C. Following sialidase incubation, cells were washed three times with PBS. As appropriate, NHBE cells were apically mock infected or infected with A/mute swan/MI/451072-2/2006 (H5N1), A/chicken/Pennsylvania/13609/1993 (H5N2), A/chicken/TX/167280-4/02 (H5N3), or A/NY/55/2004 (H3N2) at the indicated multiplicities of infection (MOI). Cells were fixed in 3.7% formaldehyde for 30 min or harvested in triplicate at the times indicated post-infection.

Quantitative RT-PCR

Total RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA) and stored at -80°C until used. Reverse transcription was performed using random hexamers and MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). Influenza M gene expression was measured using a TaqMan real-time quantitative reverse transcriptase PCR (qRT-PCR) assay using previously described primers and probe (29). Transcript levels were determined following a 10-minute hot start at 95°C in a three-step protocol with 15 s of denaturation (95°C), 30 s of annealing (60°C) and extension at 72°C for 15 s and analyzed using MXPro software by Stratagene (La Jolla, CA). Copy numbers were determined by generation of a standard curve using plasmid DNA encoding influenza M gene. Plasmid DNA concentrations were measured by optical density using a spectrophotometer.

Table 5.2 Internal Sequencing Primers

Gene	Primer	Sequence (5'→3')
H5N1		
NA	MINA-425F	TCAAGGGGCCTTATTGAATG
	MINA-898F	GAGACAACTGGCATGGTTCA
	MINA-770R	CTGGCCATTACTTGGTCCAT
	MINA-1269R	AATCCAACCCCGTCAGTTCT
HA	MIHA-295F	TGGTCATACATCGTGGAAAAA
	MIHA-815F	GCTCCTGAATATGCGTACAAAA
	MIHA-633R	TGTTTGTTCAGCTGCATCATT
	MIHA-1146R	ATCCACTTCCCTGCTCATTG
H5N2		
NA	PANA-405F	GTTGGGTGTTCCGTTTCACT
	PANA-776F	TTTGGGTATCGGGGATAACA
	PANA-790R	GATGGGATGCTTGTTGACAG
HA	PAHA-283F	GCCTGTGCTATCCAGGAGAC
	PAHA-1251F	GGAAGATGGGTTTTTGGATG
	PAHA-620R	GACACATAAGTGTTCGAGTTCTGG
	PAHA-1099R	ATCCACTTCCCTGCTCATTG
H5N3		
NA	TXNA-347F	GGGAGCACTGCTAGGGACTA
	TXNA-876F	GGAAACAGGGTATGTTTGCAG
	TXNA-713R	TCCAGTAAATCCTGTGATCTGC
	TXNA-1197R	AGGGCTGAAAACAGTCCTTG
HA	TXHA-287F	GTGCTATCCAGGAGGCTTCA
	TXHA-809F	GCAATCATGAAAAGTGAACTGG
	TXHA-1305F	TGGAAAATGAAAGAACTCTGGA
	TXHA-657R	TGACCGGGATTGACCTCTTA
	TXHA-1140R	TCCCATCAATTGCTTTCTGG

Flow cytometric analysis of surface sialic acid residues

The percent positive NHBE cells staining positive or negative for sialic acids α2,3 or α2,6 were determined by flow cytometry. Briefly, NHBE cells were washed once with PBS and trypsinized for 10 min at 37°C. Cells were collected and centrifuged at 220 x *g* for 5 min and resuspended in 2% formaldehyde for 30 min on ice and washed with flow buffer (1% BSA-0.1% sodium azide in PBS). Surface residue expression was determined

by primary staining with 20 μg/mL biotinylated *Maackia amurensis* lectin-II (MAA-II) (B-1265, Vector Laboratories, Burlingame, CA) for α2,3 sias, or for α2,6 sias 20 μg/mL biotinylated *Sambucus nigra* lectin (SNA) (B-1305, Vector Laboratories) for 1 hour on ice. Secondary staining was performed with APC-conjugated streptavidin (BD, Mountain View, CA) diluted in flow buffer for 1 hour on ice. Cells were washed with flow buffer and analyzed on a LSRII flow cytometer using FACSDiva software (BD). Additional analysis was performed using FlowJo software (TreeStar, Ashland, OR).

Confocal Microscopy

Cells were fixed for 30 minutes in 3.7% formaldehyde at the times indicated post-infection. Sialic acid staining was performed as previously described (42). Briefly, to stain for α2,3 sias, cells were incubated with 20 μg/mL biotinylated MAA-II (Vector Laboratories), or for α2,6 sias 20 μg/mL biotinylated SNA (Vector Laboratories) for 1 hour at room temperature, washed with PBS and incubated with 15 μg/mL Texas Red streptavidin (Vector Laboratories). MAA-II was specifically chosen because it preferentially binds to Siaα2-3Galβ1-3(Siaα2-6)GalNAc and not to non-sia residues as do other isoforms of MAA (16). Following washing, cells were permeabilized in PBS containing 0.5% TX-100, washed in PBS-0.05%TWEEN (PBS-T) and incubated with mouse anti-NP IgG2a diluted in 3% bovine serum albumin (BSA) in PBS-T. The cells were then washed with PBS-T, incubated for one hour with anti-mouse IgG AlexaFluor488 (Molecular Probes, Carlsbad, California) and anti-β-tubulin directly conjugated to FITC (cilia stain). Cells were rapid stained with DAPI (1μg/mL). After washing with PBS-T, membranes were excised from their culture inserts and mounted on glass slides.

Luminex-based detection of cytokines and chemokines

The Luminex® xMAP™ system is a high-throughput microsphere-based suspension array, and a MILLIPLEX MAP human cytokine/chemokine immunoassay (Millipore, St. Charles, MO) was used for the rapid immunological detection of secreted cytokines and chemokines from NHBE cells according to the manufacturer protocol. Briefly, beads coupled with biotinylated anti-IL-1α, anti-IL-1β, anti-IL-8, anti-MCP-1, anti-MIP-1α, anti-MIP1β, anti-IP-10, anti-RANTES mAbs were sonicated, mixed and diluted in bead diluent. For the assay, beads were diluted 1:4 in bead diluent and incubated overnight at 4°C with NHBE apical wash or basolateral supernatant. After washing, beads were incubated with streptavidin-phycoerythrin for 1 hour at room temperature, washed, and resuspended in wash buffer. The assay was analyzed on a Luminex 200 instrument (Luminex Corporation, Austin, TX) using Luminex xPONENT 3.1 software. Additional analysis was performed using MILLIPLEX Analyst (Millipore).

Results

LPAI virus replicates and is shed apically from NHBE cells

To determine if LPAI viruses can infect NHBE cells, the cells were apically infected with A/chicken/Pennsylvania/13609/1993(H5N2) (A/CK/PA) (Figure 5.1A) or A/chicken/TX/167280-4/02(H5N3) (A/CK/TX) (Figure 5.1B) at a MOI of 0.001 (equivalent to $10^{4.38}$ EID_{50}/mL for A/CK/PA and $10^{3.86}$ EID_{50}/mL for A/CK/TX). This low MOI was chosen in order to detect virus replication by apical wash. Figure 5.1A shows that within 24h pi, NHBE cells infected with A/CK/PA had apical wash titers of

$10^{4.99}$ EID_{50}/mL at 24h pi which peaked by 48h pi to $10^{5.27}$ EID_{50}/mL. NHBE cells infected with A/CK/TX had apical wash titers that increased slightly at 24h pi to $10^{3.97}$ EID_{50}/mL, but subsequently increased to $10^{4.25}$ EID_{50}/mL at 48h pi, and peaked at $10^{5.3}$ EID_{50}/mL at 72h pi (Figure 5.1B). As the EID_{50} values were determined from apical washes of infected cells, the results indicate that both A/CK/PA and A/CK/TX replicate and are shed apically from NHBE cells. The results shown here support previous findings in which human tracheobronchial epithelial cells were productively infected with avian influenza A/Duck/Singapore/5/97 (H5N3) (36).

NHBE cells express α2,6 and α2,3 sialic acid receptors

Given the propensity of AIV infection of NHBE cells, the cells were stained with lectins to determine the α2,6 and α2,3 sialic acid residue expression. MAA-II lectin preferentially binds to α2,3 sialic acids, and SNA lectin preferentially binds to α2,6 sialic acids. Figure 5.2A shows that α2,6 sialic acids are abundantly expressed on the NHBE cell surface, while α2,3 sialic acids are expressed at a lower level. Previous reports suggest that AIV infect ciliated cells which primarily express α2,3 sialic acids, while human viruses preferentially infect nonciliated cells expressing α2,6 linked sialic acids (21). The specificity of staining using MAA-II or SNA lectins was confirmed by pre-treating the apical surface of NHBE cells with neuraminidase from *Clostridium perfringens* (image inserts in Figure 5.2A and 5.2B) which shows that sialidase treatment removes detectable sialic acids from the cells.

To determine the relative distribution of α2,3 or α2,6 sialic acids on the surface of NHBE cells, the cells were trypsinized from their culture inserts and lectin-stained. Figure 5.2C shows that α2,6 residues are abundantly expressed on most NHBE cells however staining for α2,3 residues indicates that while most cells do express some level of α2,3 sialic acid, there are two populations, i.e. a population that is dimly positive and a population brightly positive as determined by flow cytometry.

AIVs infect NHBE cells independent of α2,3 sialic acids.

Previous studies suggest that AIV primarily infect ciliated cells expressing α2,3 sialic acids (21). However, infection of NHBE cells with avian A/mute swan/MI, A/CK/PA, A/CK/TX, or human A/NY/55/04 occurred independent of sialic acid surface expression (Figure 5.3). These AIV replicated well (as measured by NP protein expression) between 24h and 48h pi and by 72h pi, A/Mute Swan/MI (H5N1) and A/CK/PA (H5N2) induced severe cytopathic effects indicated by drastic changes in cell morphology and loss of the confluent cell lawn. Human A/NY/55/04 (H3N2) robustly spread throughout the NHBE cell culture and induced substantial cytopathic effects such as cell loss (data not shown). As replication proceeded by AIV or human viruses at 24h, 48h and 72h pi, there was a progressive loss of cell surface expression of α2,6 sialic acids, albeit to a slightly lesser extent during A/CK/TX (H5N3) infection. These effects are likely attributable to influenza neuraminidase expression during replication (Figure 5.3A).

AIV infect sialidase-treated NHBE cells

Since there appeared to be no HA- sialic acid barrier between AIV and human influenza virus infection of NHBE cells, the NHBE cells were treated with neuraminidase from *Clostridium perfringens* to confirm if infection could be inhibited (Figure 5.2A and B; image inserts). Recent findings suggest that sialidase treatment can reduce influenza virus infection, but total inhibition does not occur (31). This is in contrast to parainfluenza virus infection of tracheobronchial epithelial cells in which sialidase pre-treatment abolishes infection (17, 36). Similarly, sialidase treatment of NHBE cells had little effect on the level of influenza infection and replication as measured by NP protein expression at 24h pi (Figure 5.4).

AIVs induces differential chemokine expression patterns by NHBE cells.

Previous research has shown that pro-inflammatory cytokines and chemokines including interferon (IFN) α/β, interleukin (IL)-1α, IL-1β, IL-6, IL-8, tumor necrosis factor alpha (TNFα), macrophage inflammatory protein (MIP)-1α, MIP-1β, and monocyte chemotactic protein (MCP)-1 are detected at elevated levels in the respiratory tracts of individuals during the acute phase of influenza infection (11, 14). Patients infected with H5N1 have been shown to have higher levels of systemic IFNγ, IL-6, interferon-inducible protein of 10 kD (IP-10) and MCP-1 compared to individuals infected with human influenza subtypes (9, 23, 37). Therefore, we examined the apical and basal secretion patterns of IL-1α, IL-1β, IL-8, IP-10, MIP-1α, MIP-1β, MCP-1, and RANTES following infection by AIV and human influenza viruses. In these studies no appreciable IL-1β, MIP-1α or MIP-1β levels were detected from the basal or apical

compartments of AIV or human influenza-infected NHBE cells, however AIVs induced differential cytokine and chemokine secretion patterns over time (Figure 5.5).

Compared to mock-infected NHBE cells, A/CK/TX (H5N3) induced a higher level of IL-1α that could be apically detected at 24h pi (Figure 5.5A), but A/Mute Swan/MI (H5N1) induced a higher degree of basal expression early during infection, i.e. from 2h to 6h pi (Figure 5.5B). Likewise, human A/NY stimulated higher levels of basal expression of IL-1α at 2h pi and 24h pi (Figure 5.5B). IL-8 levels detected following AIV or human influenza virus infected NHBE cells were similar to mock-infected control levels (Figure 5.5C and D). Interestingly, IP-10 was differentially induced following AIV influenza infection. A/Mute Swan/MI infection of NHBE cells induced 2 – 6 fold higher apical secretion of IP-10 compared to A/NY-infected cells, and AIV from chickens (H5N2 or H5N3) displayed a more rapid induction of IP-10 compared to the wild bird viral isolate (A/Mute Swan/MI) over the first 24h of infection (Figure 5.5E). Basal secretion of IP-10 was similar among AIV where, for example, A/Mute Swan/MI stimulated a rapid induction of IP-10 between 18h and 24h pi, while A/NY did not stimulate basal IP-10 expression until 24h pi (Figure 5.5F). Similar to IL-8 secretion, avian and human influenza infection of NHBE cells did not induce an appreciable amount of MCP-1 secretion relative to mock-infected cells (Figure 5.5G and H), however, A/NY infection was associated with an approximate 2-fold increase of MCP-1 above mock-infected cells at 12h and 24h pi (Figure 5.5H).

Interestingly, infection with AIVs inhibits basal MCP-1 secretion relative to mock-infected NHBE cells. This is in contrast to findings *in vivo* where individuals infected with H5N1 showed high serum levels of MCP-1 that appeared to correlate with

disease severity (9, 23). It is likely that a variety of cell types e.g. mast cells, fibroblasts, mononuclear cells and T cells are recruited to the respiratory epithelium during infection and have a role in secreting MCP-1. Avian influenza viruses also induce higher levels of RANTES secretion from both the apical and basal surfaces of NHBE cells (Figure 5.5I and J). These results are similar to *in vivo* findings in which individuals infected with H5N1 had higher systemic levels of RANTES compared to individuals infected with influenza A and B (23). The AIV isolated from chickens, i.e. A/CK/PA (H5N2) and A/CK/TX (H5N3), induced higher RANTES secretion over 24h pi compared to A/Mute Swan/MI (H5N1) (Figure 5.5I and J). This difference may highlight host adaptation within avian influenza virus species and how this may affect pathology during human infection.

Discussion

Influenza viruses primarily infect respiratory epithelial cells that are situated at the interface between the outside environment and the host which acts as a first line of defense against pathogens and other foreign material. Fully differentiated NHBE cells emulate the human respiratory epithelium, thus these cell cultures are a good *in vitro* correlate to evaluate respiratory virus infection and evaluate therapeutics. It has been recently suggested that the use of a sialidase fusion protein that can be inhaled leading to the removal of sialic acids from the airway epithelium is a possible prophylactic and treatment for influenza infection (19). However, as we show here using sialidase-treated NHBE cells (Figure 5.4), NHBE cells are readily infected in the absence of detectable sialic acids, a finding that is consistent with similar studies which demonstrated that

sialidase-treated MDCK cells can still be infected with influenza (31), and that sialidase-treated human airway epithelial cells can be infected with a H3N2 virus (36). Furthermore, infection of ST6Gal I sialyltransferase knockout mice, which lack the enzyme necessary for the attachment of α2,6 sialic acid to N-linked glycoproteins on the cell surface, can be infected with human influenza and produce similar lung virus titers compared to wild-type animals (12). Therefore, it is likely sialic acids provide a relatively low-affinity interaction for influenza viruses while other influenza virus receptors remain to be identified.

Studies of humans infected with H5N1 virus who had severe disease showed that these individuals also had high serum levels of IP-10 and monokine induced by IFNγ (MIG) (23), and H5N1 viruses induced higher levels of TNFα and IP-10 in human macrophages compared to H1N1 viruses (5). Furthermore, H5N1 virus has been shown to induce IP-10, IFNβ, RANTES and IL-6 mRNA in human primary alveolar (type II pneumocytes) cells and NHBE cells (4). Interestingly, studies with Calu-3 cells have shown that H5N1 infection results in a weak anti-viral response characterized by little interferon regulatory factor (IRF)-3 nuclear accumulation, reduced IFNβ production and limited interferon stimulated gene (ISG) induction compared to H3N2 infection (42). We show here that NHBE cells infected with LPAI H5N1, H5N2 and H5N3 viruses induce robust IP-10 and RANTES responses early during infection compared to human H3N2 infection (Figures 5.5E, F, I and J). Moreover, our data strongly suggests that viral species origin has a role in chemokine expression by NHBE cells as H5N2 and H5N3 viruses of chicken origin induce a more potent chemokine response than does H5N1 isolated in the wild from a mute swan. Together, these data indicate that avian influenza

viruses can induce pro-inflammatory cytokines more rapidly and to a greater magnitude than human influenza H1N1 and H3N2 viruses and likely contributes to disease pathogenesis in humans infected with H5N1.

Acknowledgements

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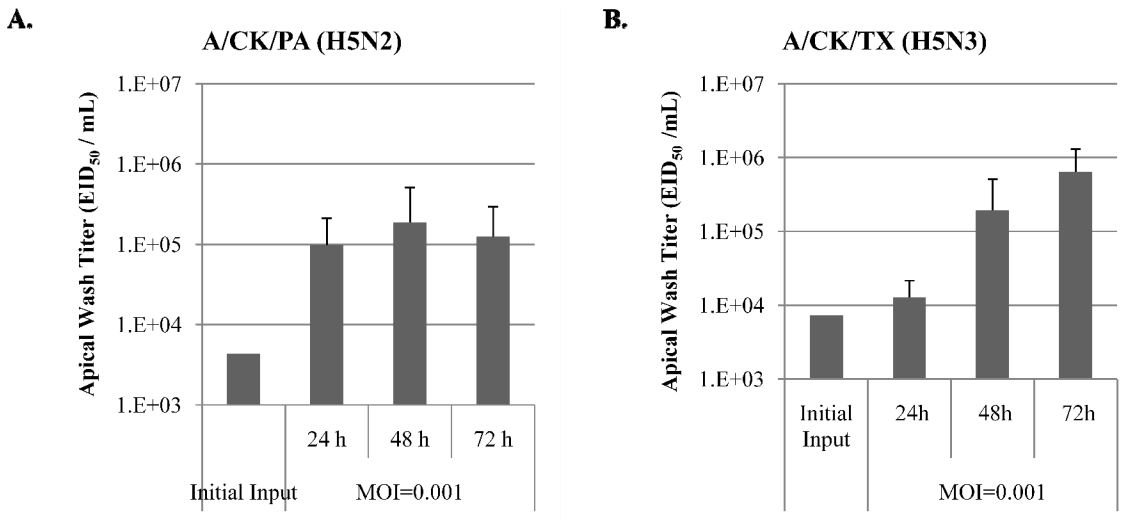


Figure 5.1. Avian influenza viruses obtained from birds replicate and are shed apically from NHBE cells. NHBE cells were infected with A/CK/PA (H5N2) (A) or A/CK/TX (H5N3) (B) at MOI=0.001. At the indicated times post-infection, BEBM-0.05% BSA was added to the apical surface of the cells and incubated for 30 minutes at 37°C. EID_{50} titers were determined according the Reed and Meunch method (24). Results shown are compiled from two independent experiments.

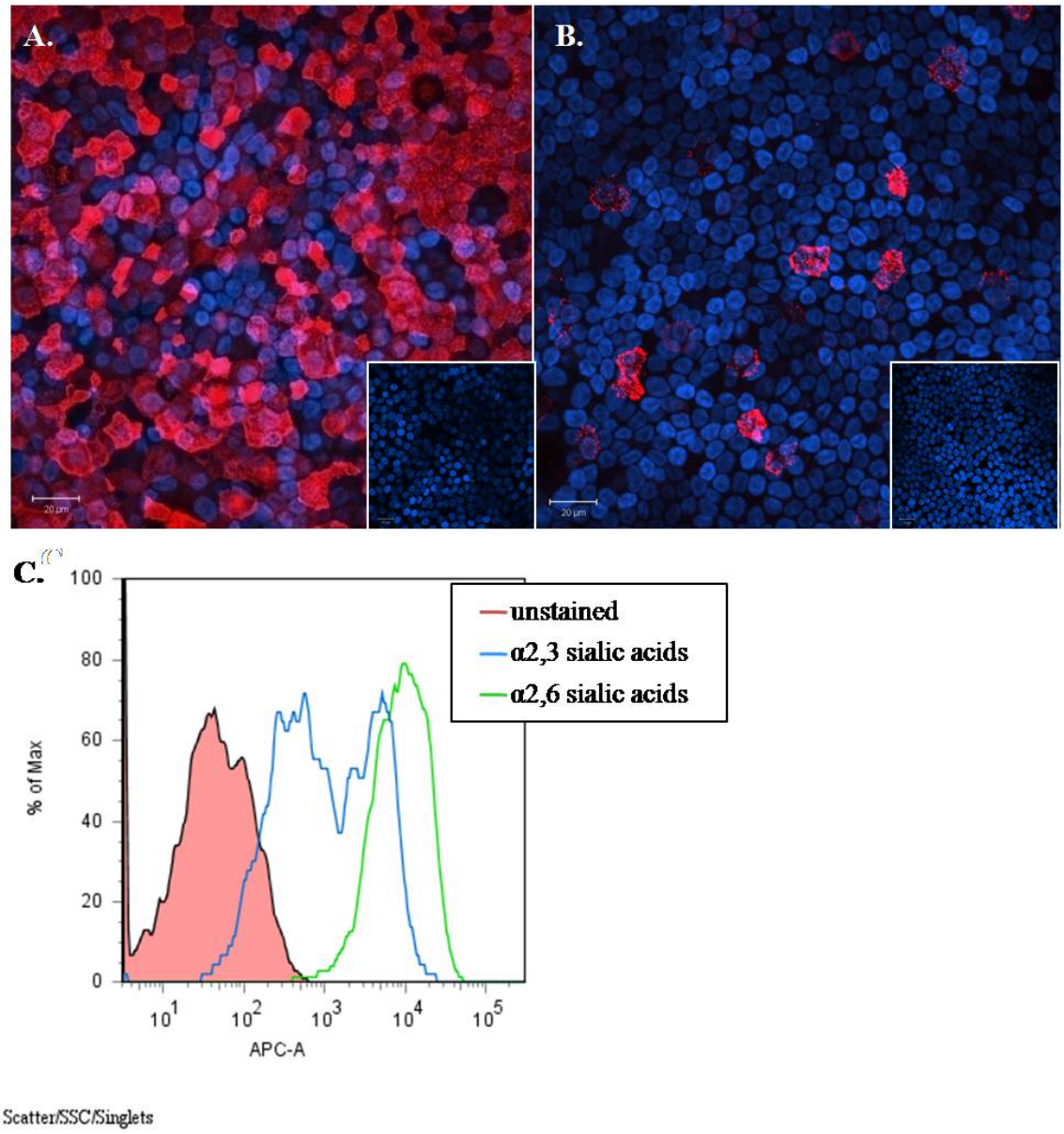


Figure 5.2. Fully differentiated NHBE cells express both α2,6 and α2,3 sialic acid linkages. NHBE cells were stained for α2,6 (A) or α2,3 (B) linked sialic acids shown in red. Cells pre-treated with neuraminidase abolishes sialic acid residue staining (image inserts). (C) NHBE cells were trypsinized, fixed with 2% formaldehyde, and analyzed by flow cytometry to determine relative percentage of cells staining positive for α2,3 (blue), or α2,6 sialic acid moieties (green). Results shown are representative of four independent experiments.

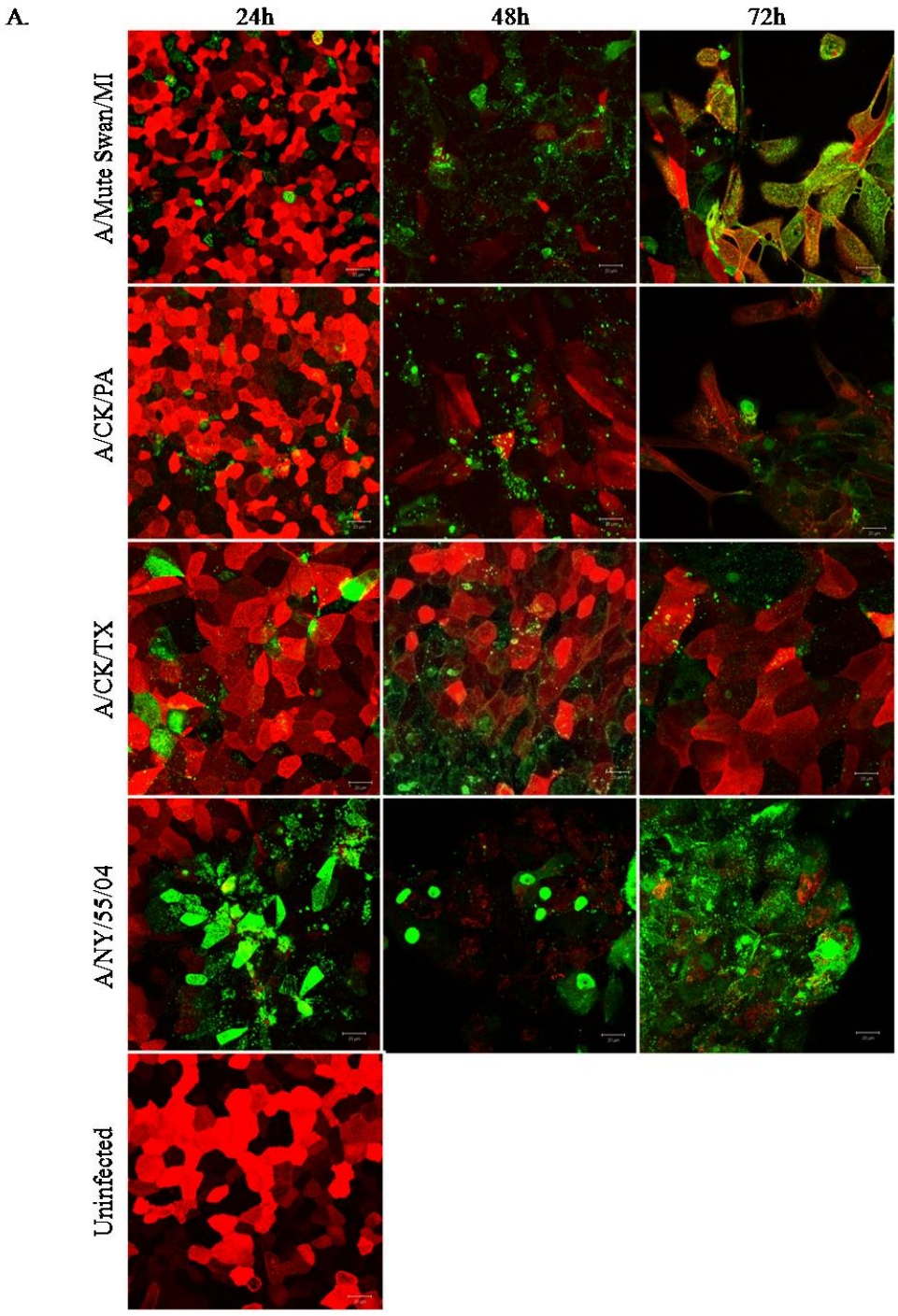
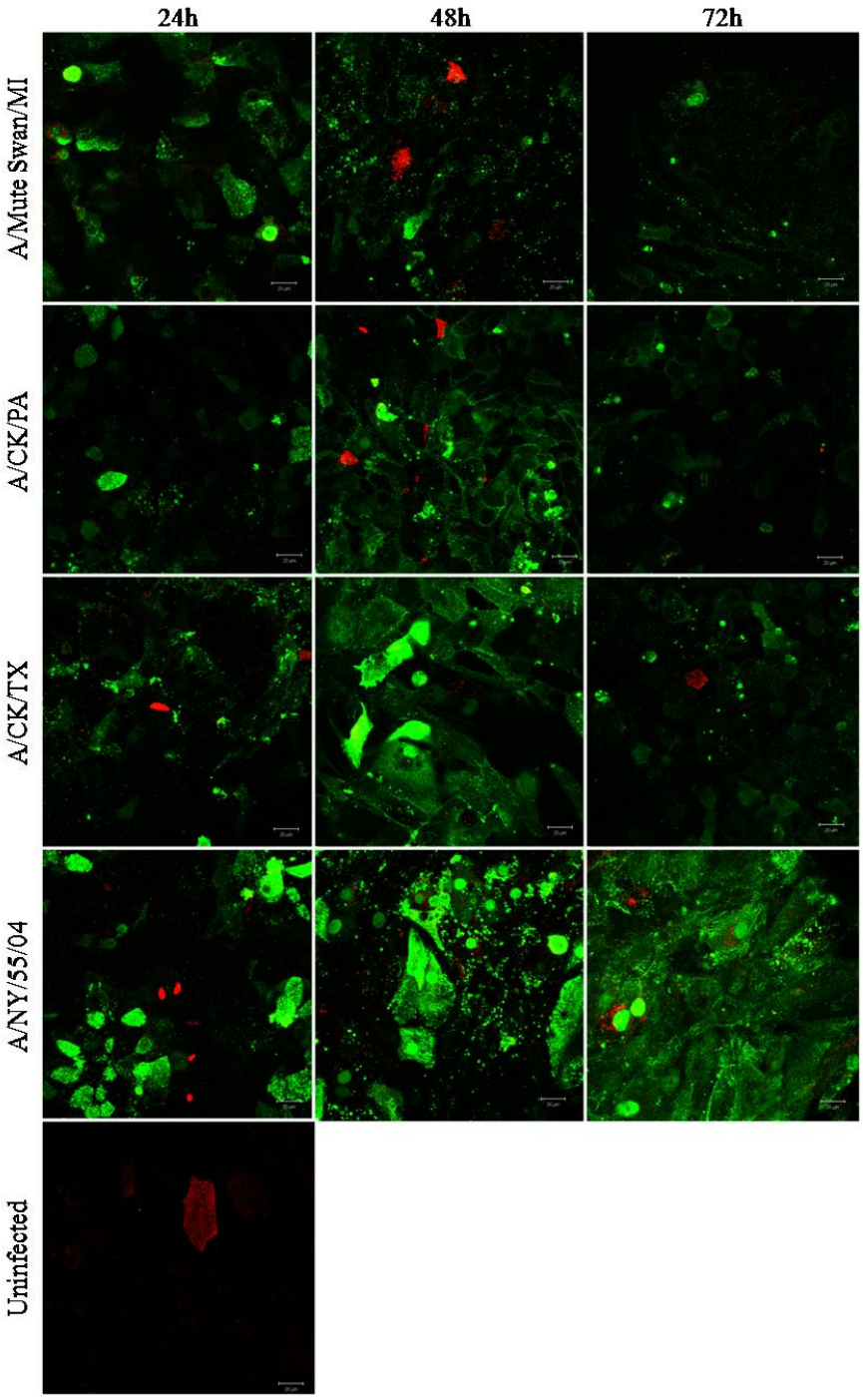


Figure 5.3. AIVs infect NHBE cells independent of α2,3 sia expression. NHBE cells were infected with A/Mute Swan/MI, A/CK/PA, A/CK/TX and A/NY/55/04 at MOI = 0.5. At the indicated times post-infection, cells were fixed with 3.7% formaldehyde in PBS for 30 minutes. Cells were immunostained for α2,6 (A) or α2,3 sias (B; opposite page) (red) and influenza NP (green). Results shown are representative of two independent experiments.

B.



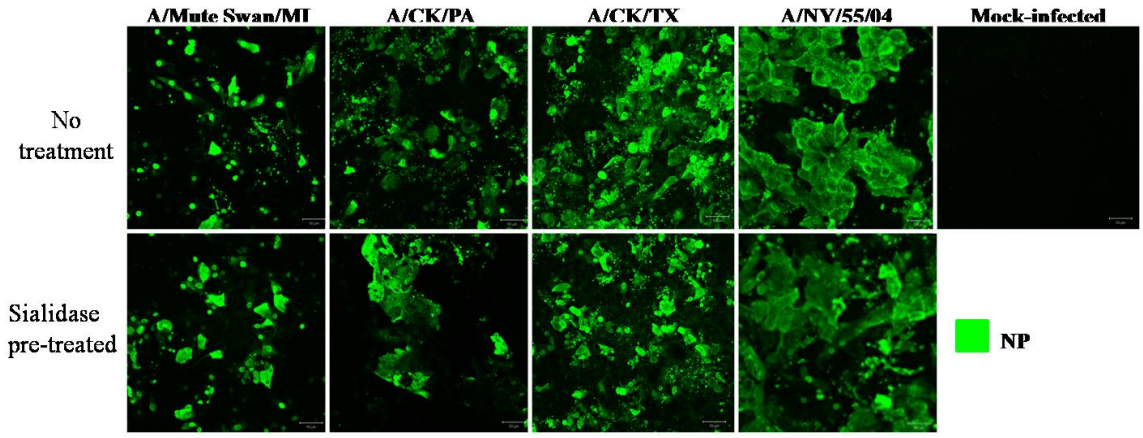


Figure 5.4. Sialidase-treated NHBE cells are robustly infected by AIVs. NHBE cells were mock-treated (top panels) or treated with 25mU/mL neuraminidase (bottom panels) for 1 hour at 37C, washed with PBS, and infected with the indicated viruses at a MOI of 0.5. Cells were fixed in 3.7% formaldehyde at 24h p.i. and immunostained for influenza NP expression. Results shown are representative of two experiments.

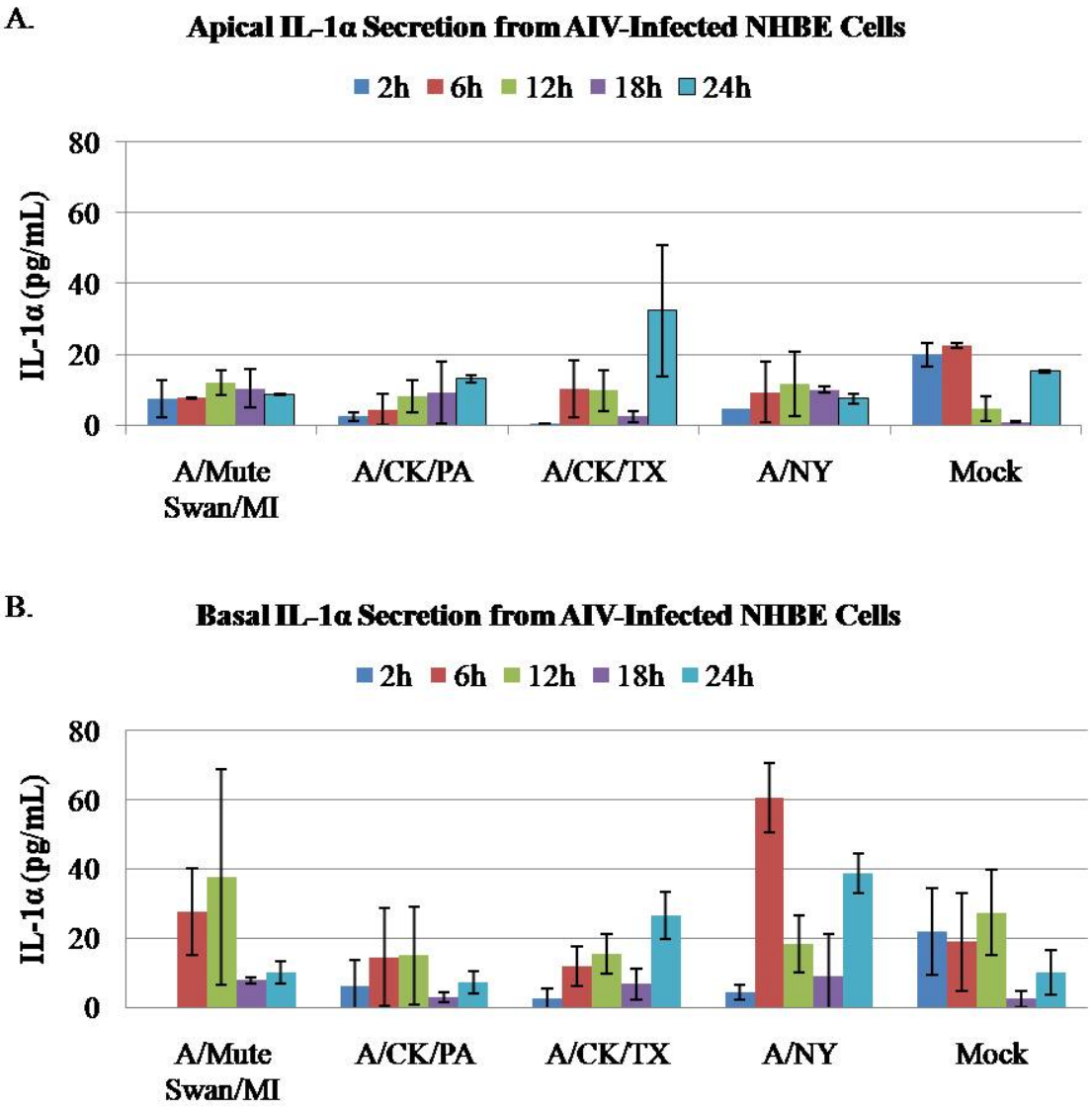
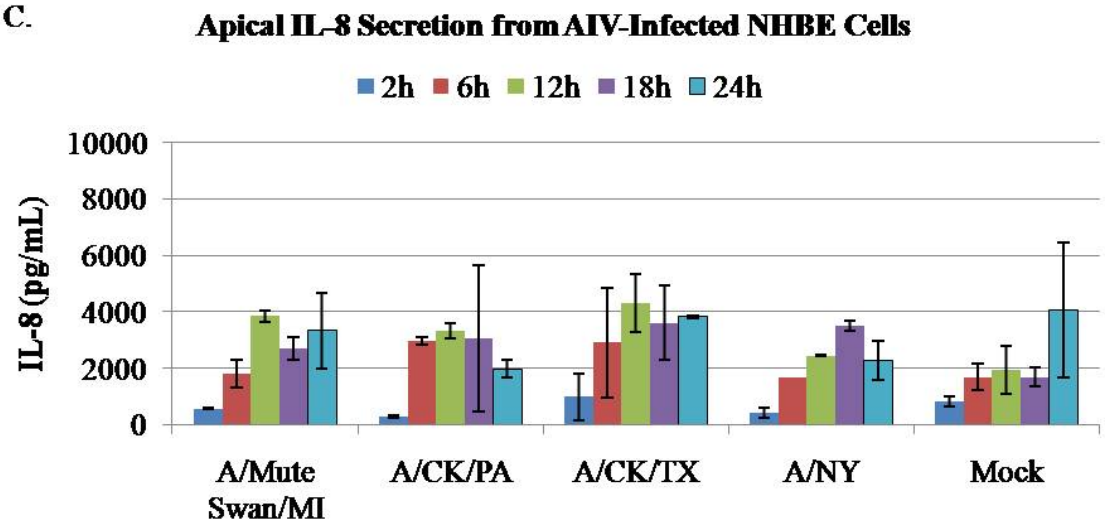
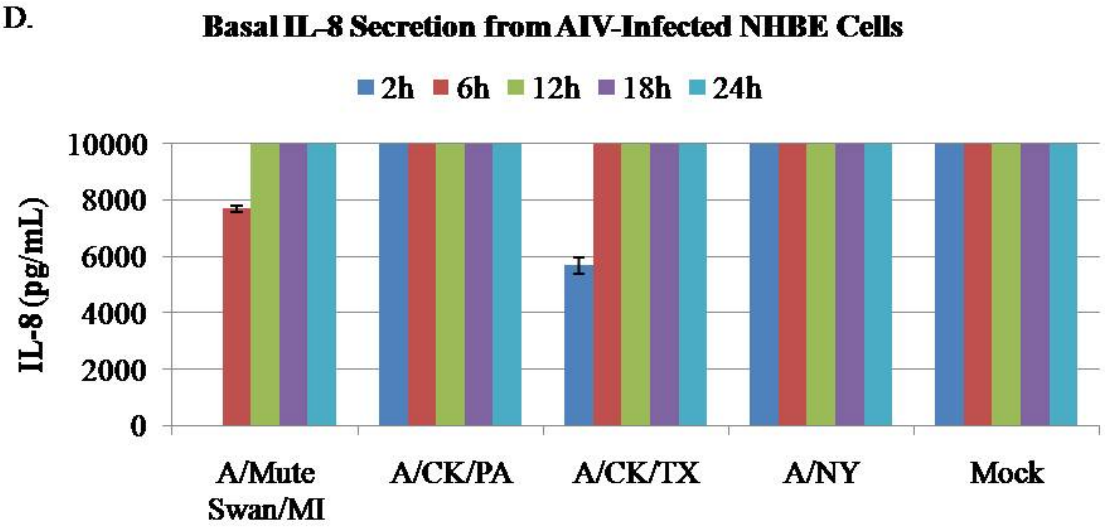


Figure 5.5. Avian influenza viruses elicit differential chemokine secretion patterns from NHBE cells. NHBE cells were infected in triplicate with the indicated viruses at MOI = 0.5. Apical washes (A, C, E, G, I) and basolateral media (B, D, F, H, J) were collected at the indicated times post-infection and analyzed for the presence of IL-1α (A and B), IL-8 (C and D), MCP-1 (E and F), IP-10 (G and H), and RANTES (I and J).

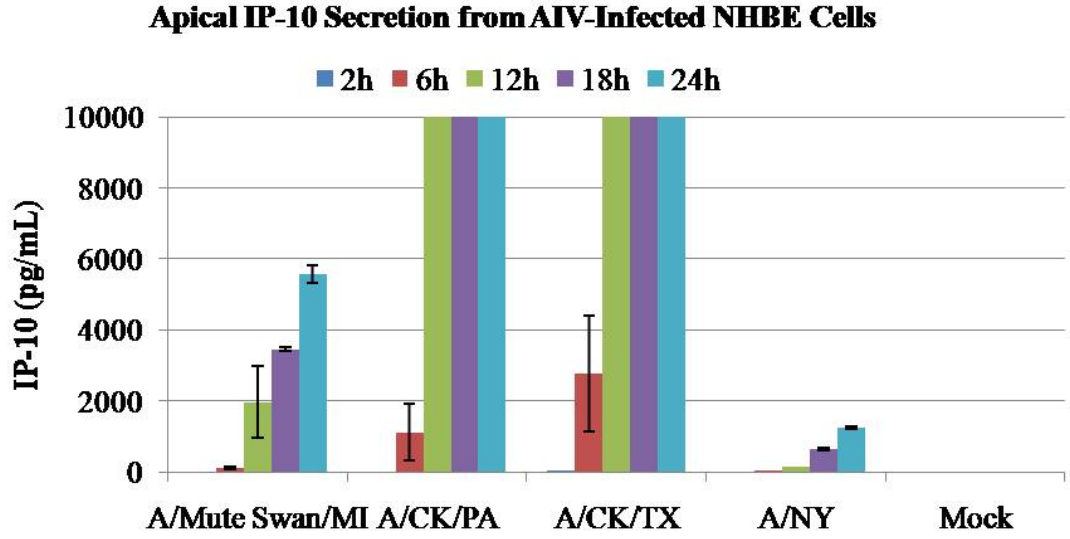
C.


Apical IL-8 Secretion from AIV-Infected NHBE Cells
2h
6h
12h
18h
24h
IL-8 (pg/mL)
10000
8000
6000
4000
2000
0
A/Mute Swan/MI
A/CK/PA
A/CK/TX
A/NY
Mock

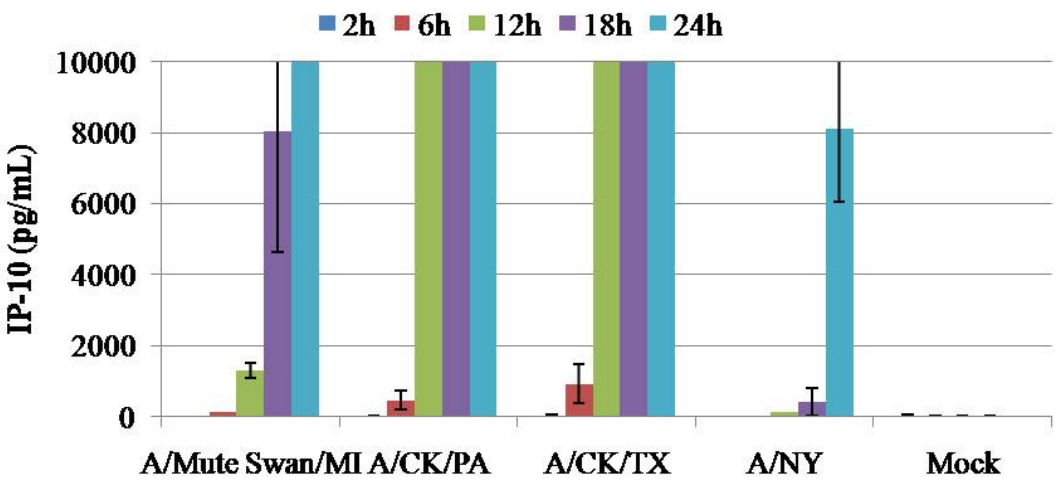
D.


Basal IL-8 Secretion from AIV-Infected NHBE Cells
2h
6h
12h
18h
24h
IL-8 (pg/mL)
10000
8000
6000
4000
2000
0
A/Mute Swan/MI
A/CK/PA
A/CK/TX
A/NY
Mock

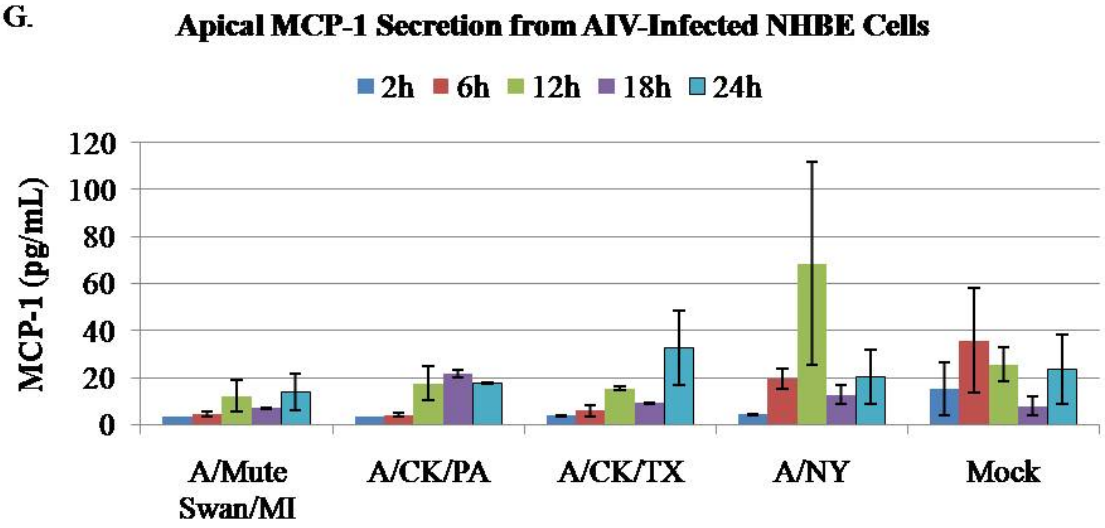
E.


Apical IP-10 Secretion from AIV-Infected NHBE Cells
2h
6h
12h
18h
24h
IP-10 (pg/mL)
10000
8000
6000
4000
2000
0
A/Mute Swan/MI
A/CK/PA
A/CK/TX
A/NY
Mock

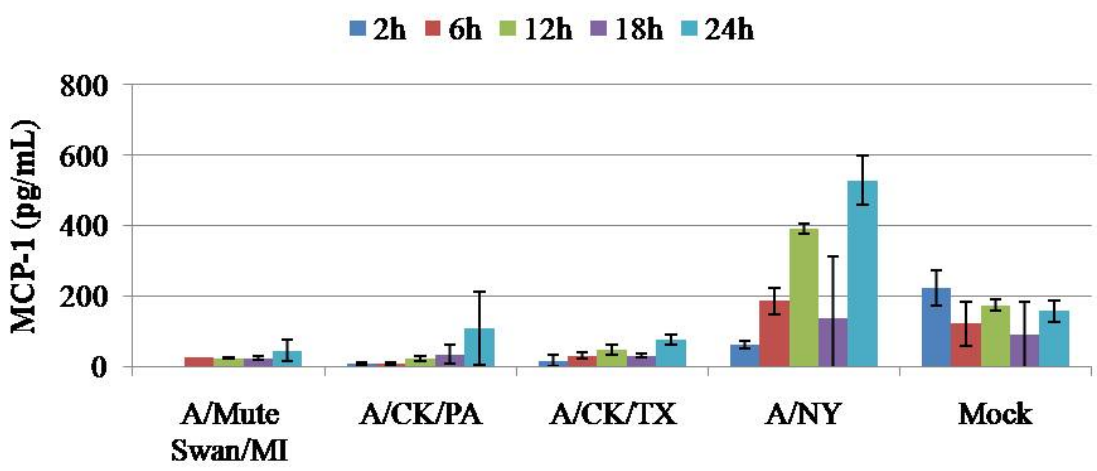
F.


Basal IP-10 Secretion from AIV-Infected NHBE Cells
2h
6h
12h
18h
24h
IP-10 (pg/mL)
10000
8000
6000
4000
2000
0
A/Mute Swan/MI
A/CK/PA
A/CK/TX
A/NY
Mock

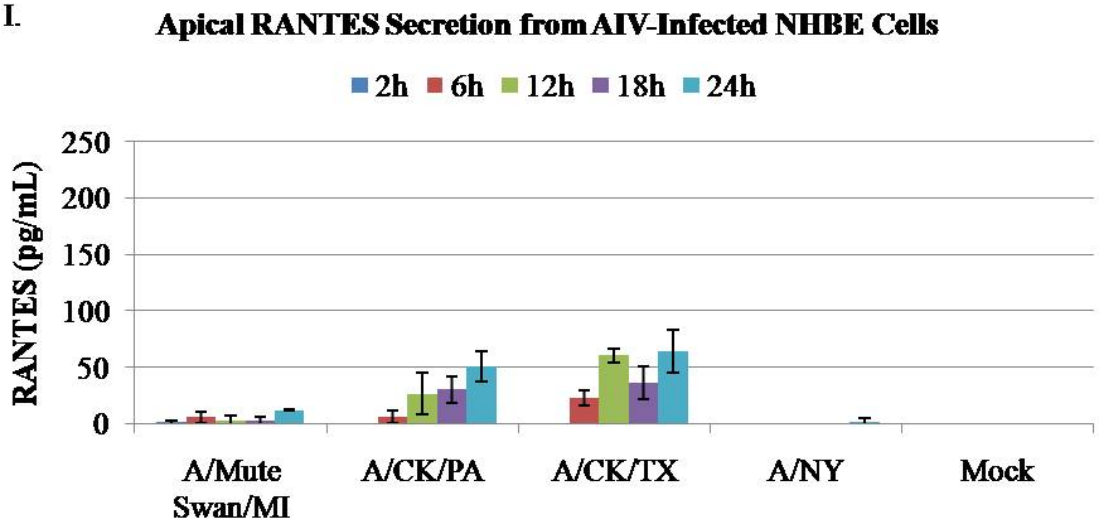
G.


Apical MCP-1 Secretion from AIV-Infected NHBE Cells
2h
6h
12h
18h
24h
MCP-1 (pg/mL)
120
100
80
60
40
20
0
A/Mute Swan/MI
A/CK/PA
A/CK/TX
A/NY
Mock

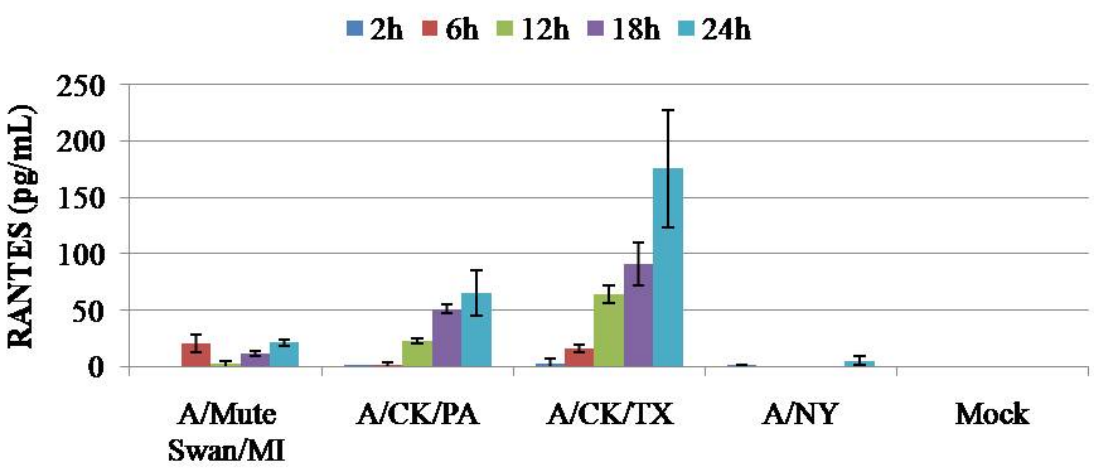
H.


Basal MCP-1 Secretion from AIV-Infected NHBE Cells
2h
6h
12h
18h
24h
MCP-1 (pg/mL)
800
600
400
200
0
A/Mute Swan/MI
A/CK/PA
A/CK/TX
A/NY
Mock

I.


Apical RANTES Secretion from AIV-Infected NHBE Cells
2h
6h
12h
18h
24h
RANTES (pg/mL)
250
200
150
100
50
0
A/Mute Swan/MI
A/CK/PA
A/CK/TX
A/NY
Mock

J.


Basal RANTES Secretion from AIV-Infected NHBE Cells
2h
6h
12h
18h
24h
RANTES (pg/mL)
250
200
150
100
50
0
A/Mute Swan/MI
A/CK/PA
A/CK/TX
A/NY
Mock

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CHAPTER 6

CONCLUSIONS

As noted in the Introduction, viruses have evolved to manipulate host barriers, both those maintained by the host immune system and physical barriers established at mucosal interfaces. RSV is the most common cause of serious lower respiratory tract disease in infants and young children worldwide and is an important pathogen of the elderly and immune compromised. Despite the health importance of RSV, there is currently no vaccine to prevent RSV infection and treatments are limited in efficacy. Understanding the viral and host factors are involved in disease pathogenesis is critically needed to properly implement vaccine design and disease intervention strategies, including antivirals. The experiments completed in this study were designed to determine the mechanisms by which RSV evades the immune response to facilitate replication. The hypothesis addressed was that RSV mediates TLR induction of the host SOCS pathway to inhibit the antiviral type I IFN response to facilitate RSV replication. The specific aims that addressed the hypothesis were:

Specific Aim 1. To determine the pattern of SOCS1 and SOCS3 expression linked to regulation of type I IFN following RSV infection in NHBE cells. The working hypothesis is that SOCS1 and SOCS3 are the major class of SOCS proteins that negatively regulate anti-viral cytokine responses, particularly the type I IFN (α/β) response. The data shown in chapter 3 indicate the RSV G protein has a role in the

induction of SOCS1 and SOCS3 and negative regulation of the type I IFN response to RSV infection. Furthermore, the RSV F and G proteins induce SOCS3 expression and induce negative regulation of type I IFN response very early after treatment of NHBE cells.

Specific Aim 2. To determine the RSV surface proteins which induce TLR signaling in NHBE cells. The working hypothesis is that RSV surface proteins mediate TLR signaling and expression of SOCS proteins, an effect that can be linked to phosphorylation of IRF3. The data shown in chapter 3 demonstrate that the RSV F surface protein can signal via a TLR pathway linked to IRF3 activation, and that the RSV G protein can activate an alternative PRR resulting in initiation of the SOCS signaling cascade and subsequent negative regulation of type I IFN response. There is also evidence that RSV F and G protein inhibit the early immune response by the expression pattern of ISG15 following infection or viral protein stimulation. Here, treatment of NHBE cells with purified F or G proteins induced SOCS protein expression leading to reduced type I IFN and downstream ISG15 protein expression.

Specific Aim 3. To determine the effect of RSV surface protein mediated regulation of the anti-viral chemokine response. The working hypothesis is that RSV G (attachment) and/or F proteins modify the expression of chemokines by NHBE cells used to recruit immune cells to sites of infection. The results shown in chapter 4 demonstrate that RSV surface proteins, particularly the G and F proteins, can induce apical and basolateral secretion of IL-1α, IL-8, IP-10, and RANTES early in infection. This is the first known study to examine apical and basolateral secretion patterns in fully differentiated NHBE cells.

Together, these findings provide compelling evidence for a previously unrecognized immune evasion mechanism by which RSV surface proteins interact with cell surface PRRs to induce the expression of SOCS proteins. SOCS1 and SOCS3 proteins consequently inhibit the JAK/STAT signaling pathway prior to the accumulation of RSV nonstructural proteins within the cell. While the RSV nonstructural proteins, NS2 in particular, are known to antagonize the type I IFN JAK/STAT signaling pathway, the results shown here indicate that RSV can inhibit type I IFN signaling prior to NS protein accumulation. Since vaccine components often include attenuated viruses or purified viral proteins, the results shown here provide evidence that RSV surface proteins alone can modify the early immune response and likely impact the development of an appropriate adaptive immune response. Future vaccine design should consider these factors that may modify vaccine efficacy.

Also, as discussed in the Introduction, avian influenza A viruses can cause significant morbidity and mortality in poultry and are an important emerging human health threat. Since 2003, the World Health Organization has documented an increased incidence of highly pathogenic influenza virus outbreaks in poultry that have resulted in 424 confirmed human infections and 261 deaths as of May 2009, a fatality rate of over 60%. Therefore, a second goal of this proposal is to further elucidate mechanisms by which influenza A viruses infect human bronchial epithelial cells and to determine novel methods of inhibiting virus replication (Appendix). The hypothesis stated that influenza viruses of avian origin infect human lung cells independent of sialic acid linkages present on the cell surface, and was tested through the completion of the following specific aim:

Specific Aim 4. To determine if low pathogenic H5N1, H5N2 and H5N3 avian influenza viruses infect and replicate in human bronchial epithelial cells independent of sialic acids present on the cell surface. The working hypothesis is that avian influenza viruses express HA molecules that preferentially bind to α2,3-linked sialic acids will infect and replicate in human bronchial epithelial cells independent of sialic acids. The results shown in chapter 5 demonstrate that H5N1, H5N2 and H5N3 AIV infect, replicate, and are shed apically from fully differentiated NHBE cells independent of the distribution of α2,3 or α2,6 sialic acids on the cell surface. The studies also show that NHBE cells infected with LPAI H5N1, H5N2 and H5N3 viruses induce a robust IP-10 and RANTES response early during infection compared to human H3N2 infection. Finally, the data strongly suggests that viral species origin has a role in chemokine induction in bronchial epithelial cells as H5N2 and H5N3 of chicken origin induce a more potent chemokine response than does H5N1 of mute swan origin.

Together, these findings demonstrate that avian influenza A viruses can productively infect and replicate in fully differentiated human lung epithelium independent of the presence of known viral sialic acid receptors. Earlier hypotheses indicating that AIV must undergo a change in HA receptor binding specificity in order to efficiently transmit from human-to-human will likely need to be revised, as the data in chapter 5 show that three H5 AIV strains already possess the capacity to infect humans. Furthermore, it is probable that sialic acid linkages are only one of many host factors that determine influenza virus tropism.

APPENDIX

A NOVEL THERAPEUTIC STRATEGY UTILIZING RNA INTERFERENCE TO INHIBIT VIRAL REPLICATION[1]

[1]Oshansky, C.M., J. Barber, S.M. Tompkins, J. Crabtree, A. De Fougerolles, T. Novobrantseva, A. Sprague and R.A. Tripp. Presented at the American Society for Virology, 27th Annual Meeting, Cornell University, Ithaca, New York, 2008.

Introduction

Common methods of gene delivery to cells or tissues include both viral (retroviruses and adenoviruses) and non-viral vectors. While the results are promising in clinical gene therapy, these vectors have the potential to be neutralized by pre-existing immunity or become immunogenic once in the host. In contrast, non-viral vectors, including cationic lipids and polycationic polymers, while relatively non-immunogenic, induce some level of cytotoxicity along with low gene expression. Polyethyleneimine (PEI) is a cationic polymer that can effectively deliver genes to the nucleus both *in vivo* and *in vitro* while providing protection from nucleases (1, 6, 8). Macroaggregated albumin (MAA) is commonly used in humans to measure pulmonary blood flow (2). When injected intravenously, MAA particles, 20 – 50 μm in diameter, are large enough that they will not pass through the pulmonary capillary bed and therefore accumulate in the peripheral tissue for clearance by resident macrophages (3, 11). If MAA particles are radio-labeled, pulmonary blood flow can be imaged due to particle collection in the pulmonary capillary bed proportional to blood flow (4). When PEI is coupled to MAA, plasmid DNA binds avidly to the complex and elicits high gene expression upon transfection (5, 7). Importantly, MAA-PEI is effective at delivering DNA vaccines to mucosal sites and targets pulmonary interstitial macrophages and dendritic cells (9, 10). As a novel therapeutic, we propose that MAA-PEI can be used to transfect small inhibitory RNAs (siRNAs) into primary human bronchial cells indicating that NHBE cells can be used as an *in vitro* model to evaluate optimal siRNA delivery strategies to inhibit influenza replication. We further show that influenza A infection influences siRNA localization within the cell, and that MAA-PEI, a novel transfection tool, is

effective at delivering influenza-specific siRNA to NHBE cells resulting in inhibition of viral gene expression.

Methods

Cells and viruses

A/New Caledonia/20/1999 (H1N1) was kindly provided by Dr. Jacqueline Katz, Centers for Disease Control and Prevention (CDC), Atlanta, GA. Stock viruses were prepared by inoculating 9-day old specific pathogen-free (SPF) eggs and harvesting the allantoic fluid 48h post-inoculation. Viral titers were obtained by serial dilution on Madin-Darby canine kidney (MDCK) epithelial cells in the presence of 1 μg/ml trypsin (Sigma, St. Louis, MO).

Transfection of NHBE Cells

MAA particles were prepared as previously described (9, 10). MAA particles were conjugated to PEI (Sigma) as previously described (9). TransIT TKO (Mirus Bio, Madison, WI) was used according to the manufacturer recommendations. Fully differentiated NHBE cells were transfected apically for 12h, 24h, 36h or 48h with 100nM control Cy3-siRNA (Alnylam Pharmaceuticals, Cambridge, MA), influenza NP-specific siRNA ("A" or "C") (Alnylam Pharmaceuticals), or nonspecific mismatch controls ("B" or "D") (Alnylam Pharmaceuticals) using TransIT TKO (9.2 μg/mL) according to the manufacturer protocol or MAA-PEI (1 μg/mL). Briefly, TransIT-TKO or MAA-PEI was incubated with the siRNA for 20 minutes at room temperature and added to the apical

surface of NHBE cells for the times indicated. Following transfection, NHBE cells were mock infected with uninfected allantoic fluid or infected with A/New Caledonia/20/99 (H1N1) at a multiplicity of infection (MOI) of 1. Cells were fixed in 3.7% formaldehyde in PBS or harvested at the times indicated.

Viral infection of NHBE cells

NHBE cells were washed 3 times with PBS to remove excess mucus secretion on the apical surface prior to infection. A/New Caledonia/20/99 (H1N1) was allowed to adsorb for 1h at 37°C, removed by aspiration and washed again with PBS 3 times. NHBE cells were incubated for the indicated times p.i. at 37°C.

Quantitative RT-PCR

Total RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA) and stored at -80°C until used. Reverse transcription was performed using random hexamers and MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). Influenza NP gene expression was measured using a TaqMan real-time quantitative reverse transcriptase PCR (qRT-PCR) assay. Transcript levels were determined following a 10-minute hot start at 95°C in a three-step protocol with 15 s of denaturation (95°C), 30 s of annealing (60°C) and extension at 72°C for 15 s and analyzed using MXPro software by Stratagene (La Jolla, CA). Copy number was determined by generation of a standard curve using ten-fold serial dilutions of plasmid DNA encoding the influenza NP gene. Plasmid DNA concentrations were measured by optical density using a spectrophotometer.

Confocal Microscopy

Cells were fixed for 30 minutes in 3.7% formaldehyde at the times indicated post-infection. Following washing, cells were permeabilized in PBS containing 0.5% TX-100, washed in PBS-0.05%TWEEN (PBS-T) and incubated with mouse anti-NP IgG2a diluted in 3% bovine serum albumin (BSA) in PBS-T. The cells were then washed with PBS-T, incubated for one hour with anti-mouse IgG AlexaFluor488 (Molecular Probes, Carlsbad, California) and anti-β-tubulin directly conjugated to FITC (cilia stain). Cells were rapid stained with DAPI (1μg/mL). After washing with PBS-T, membranes were excised from their culture inserts and mounted on glass slides.

Results

siRNA is delivered to a perinuclear location during MAA-PEI transfection

NHBE cells were transfected with a control Cy3-labeled siRNA for 12h, 24h, 36h or 48h using a cationic lipid-based transfection reagent, TransIT TKO, or a novel nucleic acid delivery method, MAA-PEI. The particular control siRNA used is not known to have any sequence homology with human sequences. Transfection using TransIT TKO resulted in little Cy3-siRNA localization within the cell when cells were transfected for 12h – 48h as shown in Figure A.1A. However, cells transfected with Cy3-siRNA, shown in yellow, using MAA-PEI resulted in efficient transfection by 12h and obvious perinuclear localization by 48h (Fig. A.1A).

Next, the effect of influenza infection on Cy3-siRNA localization within NHBE cells was determined. As before, cells were transfected with TransIT TKO or MAA-PEI for 12h, 24h, 36h, or 48h, and then infected with A/New Caledonia/20/99 (H1N1) at a MOI of 1 for 48h. Infection of NHBE cells was verified by immunostaining for influenza NP protein shown in green (Fig. A.1B). As in uninfected cells, little or no Cy3-siRNA is evident when cells transfected with TransIT TKO for 12h – 36h, and low levels can be detected when cells are transfected for 48h. In contrast, cells transfected using MAA-PEI exhibit a considerable increase in Cy3-siRNA levels within the cell. Interestingly, siRNA is not only localized to a perinuclear area within the cell, but it is also found in increasing amounts within the cytoplasm as transfection time increases (Fig. A.1B).

Influenza-Specific siRNA Inhibits Viral Replication in NHBE Cells

To determine if delivery of influenza NP-specific siRNA to NHBE cells using a commercial cationic liposome-mediated delivery system or MAA-PEI could result in inhibition of NP gene expression, cells were transfected with NP-specific siRNA (A and C) and sequence mismatch control siRNA (B and D) for 24h. Following transfection, cells were infected with A/New Caledonia/20/1999 (H1N1) at a MOI of 1 for 24h. At 24h post-infection, cells were harvested for RNA isolation and NP gene transcript levels were determined by quantitative RT-PCR. Despite the presence of little to no Cy3-siRNA upon TransIT TKO transfection, Figure A.2A shows that relative to infection alone, NP-specific siRNAs A and C decrease NP expression by 50% and 80% respectively. Mismatch control siRNAs reveal no NP inhibition. Likewise, MAA-PEI delivery of siRNA A reveals a 20% knockdown of NP expression relative to infection alone, and siRNA C induces 90% knockdown of NP expression (Fig. A.2B). These results suggest that MAA-PEI is effective

at delivering influenza-specific siRNA to NHBE cells resulting in inhibition of viral gene expression.

Conclusions

In this study, we showed that NHBE cells can be transfected utilizing a novel transfection method whereby positively charged PEI attracts with negatively charged nucleic acids, and when PEI is conjugated to MAA, it is taken into the cell. Using a control Cy3-labeled siRNA, one may visualize the location of the siRNA within the cell. Our results demonstrated that in contrast to commercially available liposome-mediated transfection method, MAA-PEI transfection leads to a higher Cy3 signal within the cell, and the siRNA is localized to a perinuclear location. Furthermore, MAA-PEI is effective at delivering influenza-specific siRNA to NHBE cells resulting in inhibition of influenza NP gene expression.

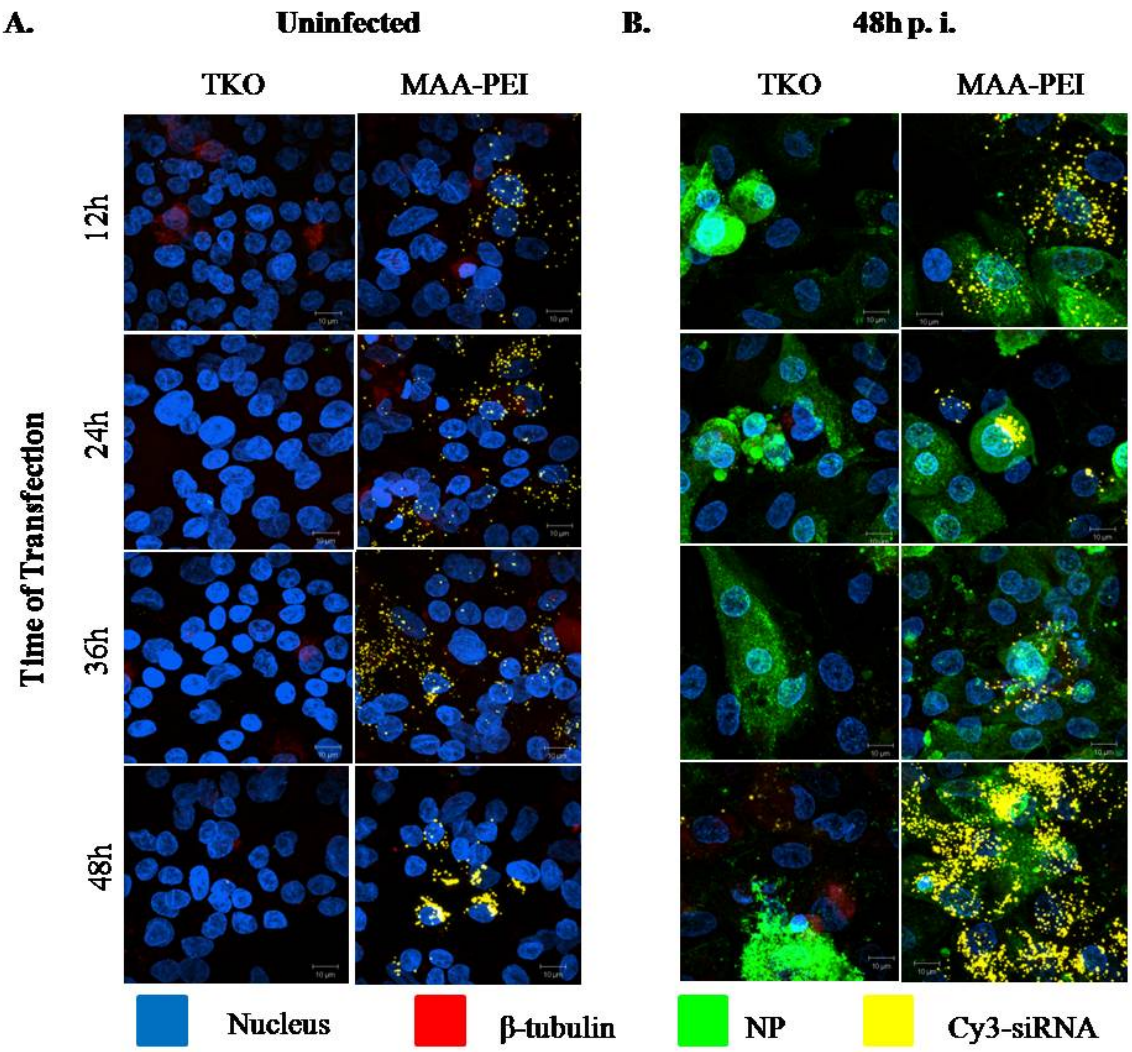

Figure A.1. MAA-PEI delivers siRNA to a perinuclear location during influenza A infection. NHBE cells were transfected with a control Cy3-labeled siRNA using TransIT TKO or MAA-PEI and mock-infected (A) or infected with A/New Caledonia/20/1999 (H1N1) MOI=1 for 24h (B). Cells were fixed 24h p.i. and immunostained for influenza NP expression (green) and β-tubulin indicating ciliated cells (red). DAPI staining indicates cell nuclei (blue), and Cy3-siRNA is depicted in yellow.

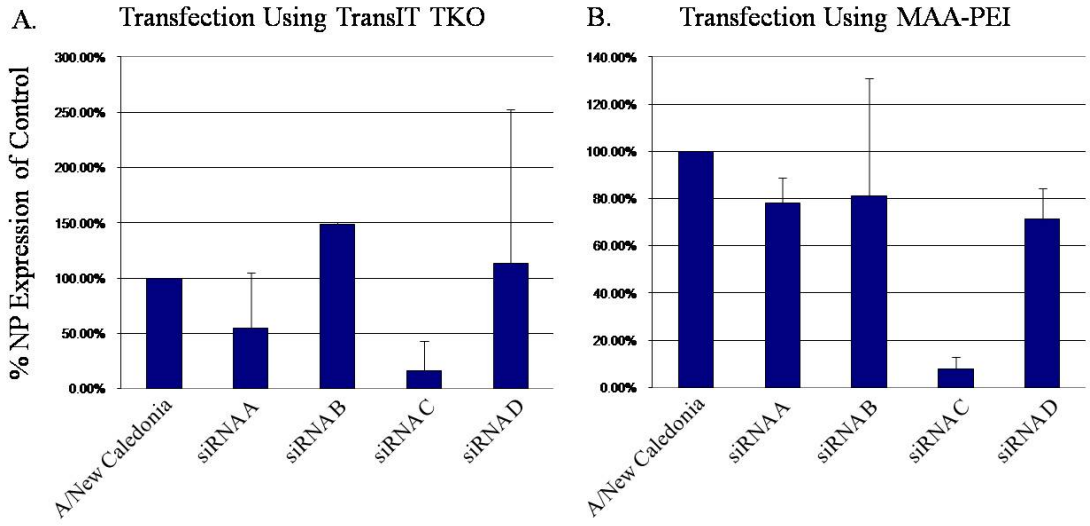

Figure A.2. Influenza NP-specific siRNA inhibits viral replication in NHBE cells. NHBE cells were transfected with NP-specific siRNAs, siRNA A and siRNA C, or their associated mismatch controls, siRNA B and siRNA D, respectively, with TransIT TKO (A) or MAA-PEI (B). Cells were apically transfected for 24h and then infected with A/New Caledonia/20/1999 (H1N1), MOI=1, for 24h. Following infection for 24h, total RNA was harvested and subjected to qRT-PCR. Data are presented as a percentage of NP expression relative to A/New Caledonia-infected cells alone.

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