Respiratory Syncytial Virus F and G Proteins Induce Interleukin 1α, CC, and CXC Chemokine Responses by Normal Human Bronchoepithelial Cells

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Human respiratory syncytial virus (RSV) is the leading cause of serious lower respiratory tract disease mediated by virus infection in infants and young children and produces serious illness in the elderly and in immunocompromised individuals. RSV infection of host respiratory cells results in the expression and modulation of both cytokine and chemokine expression patterns [1, 2]; certain patterns of cytokine or chemokine expression in RSV-infected individuals may be an indicator of disease severity [3–6]. Dysregulation or inappropriate expression of cytokines or chemokines can modify the early innate response and negatively affect the development and magnitude of the adaptive immune response to RSV infection.

RSV primarily infects ciliated respiratory epithelial cells [7]. During this process, viral proteins are detected by pathogen-sensing pattern recognition receptors associated with the epithelium [8–10], evoking a complex signaling pathway that results in cell activation and regulation of expression of proinflammatory cytokines and chemokines [11–13]. Representative of prominent cytokines and chemokines induced in humans and animal models of RSV infection are CC chemokines (eg, RANTES [regulated on activation, normal T cell expressed and secreted], monocyte chemotactic protein 1 [MCP-1], macrophage inflammatory protein 1α [MIP-1α], macrophage inflammatory protein 1β [MIP-1β]), CXC chemokines (eg, interferon-inducible protein 10 [IP-10], interleukin 8 [IL-8]), and Th2-type cytokines [2, 13–15]. Evidence suggests that these chemokines are important in pathogenesis. Studies that examined neutralizing antibody depletion of RANTES or eotaxin in mice showed that depletion was associated with less severe RSV disease and pulmonary eosinophilia [16, 17].
The presence of the G protein during RSV infection has been shown to inhibit early chemokine messenger RNA expression of MIP-1α, MIP-1β, macrophage inflammatory protein 2 (MIP-2), MCP-1, and IP-10 by bronchoalveolar leukocytes in BALB/c mice [18]. Chemokines are important in recruiting immune cells to sites of infection and inflammation; thus, RSV-mediated changes in the chemokine response may be a strategy to facilitate virus replication. A consequence of modified chemokine expression may be recruitment of inappropriate immune cells. Thymus- and activation-regulated chemokine (TARC) recruits Th2-type immune cells to sites of inflammation [19]. Recent studies have shown that RSV infection of BALB/c mice results in increased TARC expression in the lung, and that mice infected with a recombinant vaccinia virus expressing RSV G protein expressed significantly higher levels of TARC levels, suggesting a feedback loop for TARC production and Th2-type cytokine expression [20].

Because the bronchial epithelium is recognized as an important source of chemokines fundamental to driving inflammation and the immune response to infection, and to better understand the chemokine response to RSV F or G proteins, we examined RSV infection and RSV F or G protein treatment of primary normal human bronchial epithelial (NHBE) cells grown at air-liquid interface. We show that RSV F and G proteins can induce apical and basolateral secretion of interleukin 1α (IL-1α), IL-8, IP-10, and RANTES early in infection. These findings are important for understanding the viral mechanisms that affect immunity and RSV disease pathogenesis.

MATERIALS AND METHODS

Cells, viruses, and viral proteins. NHBE cells (Lonza) from a single healthy 17-year-old male donor were expanded, cryopreserved, and cultured in an air-liquid interface system as described elsewhere [21]. The cells form a pseudo-stratified, highly differentiated model and exhibit a mucociliary phenotype that closely resembles the epithelial tissue of the human respiratory tract. The cells from the same donor were used in all assays, and for culture, the apical surface of the cells was exposed to a humidified 95% air/5% carbon dioxide environment, and the basolateral medium was changed every 2 days. Recombinant RSV/A2 (6340WT) and recombinant RSV/A2 lacking the G gene (6340ΔG) were kindly provided by Dr Peter Collins (National Institutes of Health, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland). Viruses were propagated in VeroE6 cells maintained in Dulbecco modified Eagle medium (Sigma-Aldrich), supplemented with 5% heat-inactivated (60°C) fetal bovine serum (HyClone Laboratories) as described elsewhere [22]. Virus titers were determined by immunostaining plaque assay on VeroE6 cells with anti-RSV F protein monoclonal antibody (clone 131–2A) as described elsewhere [23]. The 6340WT was ultraviolet (UV)–inactivated (>170 J/m²) by exposure to UV light [24] on ice, and inactivated virus assayed by immunostaining plaque assay [25] to confirm inactivation. RSV F and G proteins were isolated and purified from RSV/A2-infected VeroE6 cells as described elsewhere [26]. Purification was confirmed by Western blot analysis with anti-G protein (131–2G) or anti-F protein (131–2A) monoclonal antibody, as described elsewhere [26].

NHBE cell infection. NHBE cells were washed 3 times with phosphate-buffered saline to remove excess mucous secretion on the apical surface before infection and were infected with 6340WT or 6340ΔG at a multiplicity of infection, or MOI, of 1 or apically mock infected with VeroE6 cell lysate. Viruses were allowed to adsorb for 1 h at 37°C; the virus dilutions were removed by aspiration and washed again with phosphate-buffered saline 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 lipopolysaccharide (1 μg/mL; Sigma). Bronchial epithelial basal medium, or BEBM (Lonza), was added to the apical surface of differentiated NHBE cells and were incubated for the indicated times after either infection or treatment at 37°C. RSV and mutant virus infection of NHBE cells was confirmed by immunostaining, immunoblot, and qualitative reverse-transcription polymerase chain reaction for RSV N gene expression, as reported elsewhere [25].

Luminex-based quantification of chemokines. Milliplex MAP human chemokine immunoassay (Millipore) was used for the detection of secreted chemokines from apical and basolateral NHBE cell supernatants using the Luminex xMAP system according to the manufacturer protocol. All experiments were analyzed 3–6 times for accuracy. Briefly, beads coupled with biotinylated anti-IL-1α, anti-IL-8, anti-MCP-1, anti-MIP1α, anti-MIP1β, anti-IP-10, and anti-RANTES monoclonal antibodies were sonicated, mixed, and diluted in phosphate-buffered saline bead diluent. For the assay, beads were diluted 1:4 in bead diluent and incubated overnight at 4°C with apical or basolateral NHBE supernatant. After washing, beads were incubated with streptavidin-phycocerythrin for 1 h at room temperature, washed, and resuspended in wash buffer. The assay was analyzed on a Luminex 200 instrument (Luminex Corporation) using Luminex xPONENT software (version 3.1). Additional analysis was performed using Milliplex Analyst (Millipore).

Statistical analysis of data. Differences in chemokine expression in Luminex analysis were evaluated with a Student t test and considered significant when P < .05. Data are shown as means ± standard deviation.

RESULTS AND DISCUSSION

Accumulated evidence shows that during the acute phase of RSV infection several notable chemokines are expressed, including IL-8, which attracts neutrophils, and RANTES, MCP-
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1, MIP-1α, MIP-1β, and IP-10, which attract monocytes and leukocytes to sites of inflammation [1, 13, 15, 19]. CC chemokines that include MCP-1, MIPs, RANTES, and TARC but neither Th1- or Th2-type cytokines correlate with severity of illness during RSV infection [27], and there is evidence that the expression patterns of these and other chemokines are affected by RSV G protein expression [1, 2, 13]. The RSV components that trigger innate immunity are being discovered, and many of these features are linked to activation of early cell signaling events through pattern recognition receptors (eg, Toll-like receptors [12, 28–30]). In this regard, the 2 major RSV surface proteins, namely, F and G protein, have been shown to interact with the Toll-like receptor pathway [9, 12, 28, 31] and modify suppressor of cytokine signaling, or SOCS, protein-negative regulation of cytokine and chemokine expression [12, 31]. The RSV G protein appears to have a major role in modifying innate and adaptive immune responses at the level of cytokine and chemokine expression [1, 2, 13], and the G protein exhibits CX3C chemokine mimicry linked to G protein interaction with the fractalkine receptor (CX3CR1) [26], an effect that in part mediates immune modulation of CX3CR1+ immune cells that include natural killer and virus-specific T cells [32]. The immune modulatory features attributed to the G protein are likely important in facilitating RSV replication, but these features are also connected to disease pathogenesis. Studies examining the role of the G protein in enhanced pulmonary disease have shown that a formalin-inactivated RSV (FI-RSV) mutant virus lacking the G protein or lacking only the G protein CX3C motif does not prime for enhanced pulmonary disease after RSV challenge [33], and that BAL cells from mice vaccinated with FI-RSV G protein mutant viruses have modified chemokine transcription profiles showing increased expression of MIP-1α, MIP-1β, and MIP-2 messenger RNA expression early after infection [33].

The role of RSV F or G proteins in apical or basolateral chemokine induction by normal human bronchial epithelial cells has not to our knowledge been investigated; thus, we examined chemokine expression levels from fully differentiated primary NHBE cells after RSV infection or treatment with purified RSV F or G proteins. Contrary to previous studies that used a transformed type II alveolar cell line (A549) [34, 35], there was no increase in IL-1α secretion by NHBE cells during live RSV infection (6340WT) or after infection with a mutant virus lacking the G gene (6340ΔG) (Figures 1 and 2). However, NHBE cells treated apically with purified RSV G protein secreted up to 676 pg/mL of IL-1α from the apical surface (Figure 1B) and 120 pg/mL of IL-1α from the basolateral surface (Figure 2B) by 18 h after treatment. These findings suggest that, during RSV infection, the G protein itself may induce IL-1α expression by human airway cells, a feature that may lead to up-regulated cellular adhesion molecules, for example, intercellular adhesion molecule-1 (ICAM-1, CD54) [36], and immune pathogenesis by enhancing immune cell adherence to infected airway cells.

Several chemokine receptors have been shown to be involved in the host response to RSV infection, particularly CCR1 and CXCRI [37, 38]. The ligands of CCR1 include MIP-1α, RANTES, and MCP-1 [37], and an important ligand for CXCRI is IL-8 [39–41]. RSV infection of NHBE cells was associated with significantly increased levels (P <.05) of apical and basolateral IL-8 secretion relative to mock-infected cells throughout the duration of the study (Figures 1C and 2C), and infection with 6340ΔG produced similar, albeit significantly lower (P <.05), apical IL-8 levels between 18 and 24 h after infection relative to 6340WT-infected cells (Figure 1C). UV-inactivated 6340WT treatment of NHBE cells induced IL-8 levels that were similar to those seen in live infection between 6 and 24 h after infection (Figure 1C). In contrast to IL-1α induction, RSV F or G proteins did not induce substantial apical IL-8 secretion (Figure 1D). However, RSV F protein treatment induced detectable basolateral IL-8 secretion by 24 h after treatment that was not significantly different from mock-treated cells (Figure 2D). These results suggest that F protein may not induce autocrine or paracrine IL-8 signaling among respiratory epithelial cells but may contribute to the inflammatory response linked to neutrophil recruitment by IL-8.

IP-10 appears to be an important chemokine in the response to RSV infection [1, 2]. Nasopharyngeal aspirates from children <2 years of age showed increased levels of IP-10 as well as IL-8, MIP-1α, and MIP-1β during severe RSV infection [15]. In addition, RSV infection in mice leads to increased expression of IP-10 messenger RNA in total lung RNA [42]. In the studies reported here, 6340WT RSV infection of NHBE cells induced low levels of IP-10 secretion both apically and basolaterally (Figures 1 and 2); however, infection with 6340ΔG resulted in significantly (P <.05) higher levels of IP-10 relative to 6340WT infection at 18 h after infection, suggesting that G protein inhibits IP-10 expression. This observation is consistent with other findings in mice, showing that infection with a RSV G protein deletion mutant virus results in increased early MIP-1α, MIP-1β, MIP-2, MCP-1, and IP-10 messenger RNA expression relative to wild-type RSV infection [18]. Consistent with IL-8 secretion induced by infection (Figures 1C and 2C), IP-10 is highly expressed after UV-inactivated 6340WT treatment (Figures 1E and 2E), indicating that UV inactivation may elicit an alternate route of chemokine stimulation compared with 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 (Figures 1F and 2F).

Whereas increased MCP-1 levels in nasal secretions are associated with bronchiolitis and inflammation [27], RSV infection of human airway cells has previously been shown to mediate modest MCP-1 secretion but induce high levels of RAN-
Figure 1. Apical chemokine secretion patterns of respiratory syncytial virus (RSV)–infected or RSV protein-treated normal human bronchial epithelial (NHBE) cells. Cells were apically mock infected with Vero cell lysate (VCL), ultraviolet (UV)–inactivated 6340WT, or infected with 6340WT or 6340ΔG virus at multiplicity of infection of 1. NHBE cells were apically treated with purified RSV F or G proteins (10 μg/mL) or lipopolysaccharide (LPS) (1 μg/mL). At the indicated times after either treatment or infection, apical supernatants were analyzed for the level of interleukin 1α (IL-1α), interleukin 8 (IL-8), interferon-inducible protein 10 (IP-10), monocyte chemotactic protein 1 (MCP-1), or RANTES (regulated on activation, normal T cell expressed and secreted). Data are shown as means ± standard deviation and are representative of 3 independent experiments. Differences in chemokine expression were evaluated with a Student t test and considered significant when \(P<.05\). An asterisk (*) denotes differences from the control (VCL or medium alone), and a dagger (†) denotes differences from 6340WT infection.
Figure 2. Basolateral chemokine secretion patterns of respiratory syncytial virus (RSV)–infected or RSV protein-treated normal human bronchial epithelial (NHBE) cells. NHBE cells were apically mock infected with Vero cell lysate (VCL), ultraviolet (UV)–inactivated 6340WT, or infected with 6340WT or 6340ΔG at multiplicity of infection of 1. NHBE cells were apically treated with purified RSV F or G proteins (10 μg/mL) or lipopolysaccharide (LPS) (1 μg/mL). At the indicated times after either treatment or infection, basolateral media were analyzed for the presence of interleukin 1α (IL-1α), interleukin 8 (IL-8), interferon-inducible protein 10 (IP-10), monocyte chemotactic protein 1 (MCP-1), or RANTES (regulated on activation, normal T cell expressed and secreted). Data are shown as means ± standard deviation and are representative of 3 independent experiments. Differences in chemokine expression were evaluated with a Student t test and considered significant when \( P < .05 \). An asterisk (*) denotes differences from the control (VCL, or medium alone), and a dagger (†) denotes differences from 6340WT infection.
TES expressed in cell culture supernatants [43]. Contrary to these findings, 6340WT and 6340ΔG infection of NHBE cells resulted in high levels of apical MCP-1 secretion (ranging from 5.7 × 10^9 to 9.6 × 10^9 pg/mL for 6340WT and from 6.4 × 10^8 to 1 × 10^8 pg/mL for 6340ΔG) that remained high from 2 to 24 h after infection (Figure 1G). Furthermore, UV-inactivated 6340WT treatment of NHBE cells also elicited apical MCP-1 secretion to a level similar to that induced by live virus infection, but treatment with purified RSV F or G proteins had little effect on MCP-1 secretion relative to mock-treated cells (Figures 1H and 2H). These results suggest that the RSV F and G proteins are not primary mediators of apically expressed MCP-1. In contrast, basolateral secretion of MCP-1 was higher in 6340ΔG-infected NHBE supernatant than that in 6340WT-infected NHBE cell supernatant, indicating that the G protein has a role in modifying basolateral MCP-1 expression (Figure 2G). It is possible that these differential effects may be linked to inducible nitric oxide synthase (iNOS) expression. RSV infection has been shown to induce iNOS gene expression in nasopharyngeal exudate cells obtained from infants during the acute phase of RSV bronchiolitis [44] and in human airway cell lines responding to RSV infection [45, 46]. Thus, it is possible that G protein induces cellular expression of nitrite components (eg, NO), which has been shown to modulate the expression of MCP-1 in cultured human endothelial cells [47]. In addition, iNOS gene expression, initially induced by RSV G protein, may be further enhanced in a paracrine fashion by proinflammatory cytokines released by infection-activated inflammatory cells [44].

RANTES has been detected in nasopharyngeal and tracheal secretions of patients with severe RSV disease [16, 48, 49]. In this study, RSV infection of NHBE cells induced high levels of apical RANTES secretion ranging from 4 × 10^8 to 8 × 10^8 pg/mL for 6340WT, and 6.8 × 10^7 to 8.9 × 10^7 pg/mL for 6340ΔG (Figure 1I), but lower basolateral RANTES secretion (Figure 2I). Both purified RSV F and G protein treatment induced apical RANTES secretion; however, the G protein induced significantly greater RANTES production (P < 0.05) from both apical and basolateral surfaces (Figures 1J and 2J). The finding that 6340WT and 6340ΔG infection induces RANTES expression suggests that the effect is contributed by features other than the G protein; however, this finding contrasts with the finding that purified G protein induces higher RANTES expression than F protein treatment. The data show that F protein is a mediator of RANTES expression, but exogenous treatment with purified G protein may signal RANTES expression through a different pathway (eg, Toll-like receptors [12]) than exogenous F protein treatment, or through that associated with virus infection.

In conclusion, RSV, and specifically RSV F and G surface proteins, mediate induction of important CC and CXC chemokines in NHBE cells. We show for the first time to our knowledge that RSV infection or treatment with purified RSV F or G proteins induces differential chemokine expression from the apical and basolateral surfaces in NHBE cells. The pattern, magnitude, and directional expression of the chemokines induced by RSV F and G proteins suggest a mechanism by which these viral proteins may contribute to immune-mediated pathogenesis associated with infection. These studies provide a better translational view of how chemokines are involved in the process of RSV infection and suggest important targets for RSV disease intervention.

References


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